Synthetic Analogues of Polynucleotides. Part V.[1] Analogues of Trinucleoside Diphosphates containing Carboxymethylthymidine

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Analogues of trinucleoside diphosphates in which the nucleoside units are linked by acetate ester linkages (·O·CH_2·CO_2·), instead of the phosphodiester linkages of the natural compounds, have been synthesised. 3′-*O*-Carboxymethyl-5′-*O*-tritylthymidine was condensed with the 2-cyanoethyl ester of 3′-*O*-carboxymethylthymidine to give a compound in which two thymidine residues were linked by an acetate ester linkage (II; R^1 = trityl, R^2 = CH_2·CH_2·CN). The cyanoethyl group was selectively removed with potassium t-butoxide in dimethylformamide and the resulting carboxylic acid was condensed with 2′,3′-*O*-isopropylidene- or 2′,3′-*O*-anisylidene-ribonucleosides to give, after removal of the acid-labile protecting groups, thymidinylacetyl-(3′ → 5′)-thymidinylacetyl-(3′ → 5′)-ribonucleosides (III; R^1 = R^2 = R^3 = H), where the ribonucleosides were adenosine, uridine, guanosine, inosine, and cytidine. Evidence for base stacking in these analogues was obtained by u.v. and n.m.r. spectroscopy.

In previous papers[1,2] the synthesis of an analogue of polythymidylic acid in which the thymidine residues were linked by means of an acetate group (·O·CH_2·CO_2·) was described. This analogue showed a hypochromic effect when mixed with polyadenylic acid both in salt solutions and in water, indicating that some interaction was occurring between the two polymers. Molecular models of the synthetic polymer showed that the spacing of the nucleoside groups was similar to that in the natural polynucleotides, so it seemed probable that the interaction was similar to that occurring between polyuridylic acid and polyadenylic acid. We have therefore attempted to synthesise oligomers of a similar type containing other nucleosides in addition to thymidine. We hoped that, by use of a stepwise procedure, oligomers of any desired sequence might be obtained. Such oligomers might be expected to take part in, or interfere with, the process of protein biosynthesis in living systems.

The problems involved in the synthesis of oligomers of this type resemble those encountered in the chemical synthesis of oligonucleotides,[3] and involve the use of appropriate blocking groups and reaction conditions. Because of the relative stability of the phosphodiester

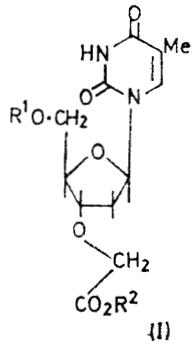
[1] Part IV, M. H. Halford and A. S. Jones, *J. Chem. Soc.* (*C*), 1968, 2667.
[2] M. H. Halford and A. S. Jones, *Nature*, 1968, **217**, 638.
[3] H. G. Khorana, *Biochem. J.*, 1968, **109**, 709; B. E. Griffin and C. B. Reese, *Tetrahedron*, 1969, **25**, 4057.

linkages in alkali, alkali-labile blocking groups could be used for oligonucleotide synthesis. In the present case, however, the greater lability of the internucleoside linkages in alkali necessitated the use of blocking groups that could be removed under non-alkaline or very mild alkaline conditions. The 5′-hydroxy-group was blocked with the acid-labile trityl group, the 2′- and 3′-hydroxy-groups of the ribonucleosides were blocked with the isopropylidene or anisylidene group, and the carboxy-group was blocked with the 2-cyanoethyl group, which could be removed by a base-catalysed elimination, under conditions which did not hydrolyse the internucleoside linkages. By use of these procedures, analogues of trinucleoside diphosphates have been synthesised.

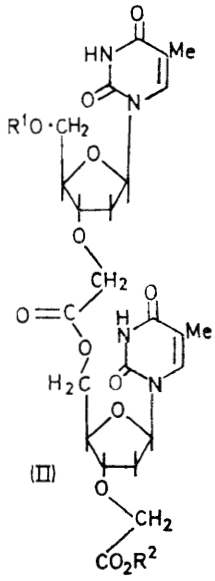
The starting material was 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine (I; R^1 = trityl, R^2 = H), the synthesis of which from 5′-*O*-tritylthymidine has already been described.[1] In the present work much higher yields (*ca.* 90%) were obtained by using an excess (2·4 mol.) of sodium hydride in the reaction with sodium chloroacetate. This compound was then converted into its 2-cyanoethyl ester (I; R^1 = trityl, R^2 = CH_2·CH_2·CN) by condensation with 3-hydroxypropiononitrile in the presence of dicyclohexylcarbodi-imide. This cyanoethyl ester reacted slowly with alcohols at room temperature. The precise nature of this reaction was not determined but it appeared to be ester exchange. Use of alcohol-containing solvents in the chromatography of this substance was therefore avoided. The trityl group was removed by mild acid hydrolysis to give 3′-*O*-(2-cyanoethoxycarbonylmethyl)thymidine (I; R^1 = H, R^2 = CH_2·CH_2·CN). The 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine in the form of its pyridinium salt was then condensed with this in the presence of dicyclohexylcarbodi-imide to give the fully protected dimer (II; R^1 = trityl, R^2 = CH_2·CH_2·CN), 2-cyanoethyl 5′-*O*-tritylthymidinylacetyl-(3′ → 5′)-thymidin-3′-ylacetate. The compound was characterised by means of its i.r. and n.m.r. spectra and elemental analysis, and by the fact that upon alkaline hydrolysis it gave equimolar amounts of the ether (I; R^1 = trityl, R^2 = H) and the alcohol (I; R^1 = H, R^2 = H). The cyanoethyl group was removed selectively from the dimer (II; R^1 = trityl, R^2 = CH_2·CH_2·CN) by treatment with potassium t-butoxide in anhydrous dimethylformamide, to give the required 5′-*O*-tritylthymidinylacetyl-(3′ → 5′)-thymidin-3′-ylacetic acid (II; R^1 = trityl, R^2 = H).

A small amount of hydrolysis (*ca.* 12%) of the internucleoside linkage occurred, to give 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine and 3′-*O*-carboxymethylthymidine. These contaminated the product, but as the mixture was difficult to separate on a preparative scale it was used unmodified in subsequent stages of the synthesis. The impurities could be readily removed by chromatography at the final stage.

To characterise compound (II) (R^1 = trityl, R^2 = H) a sample was purified by paper chromatography. Hydrolysis of this material with dilute alkali gave equimolar amounts of 3′-*O*-carboxymethyl-5′-tritylthymidine and 3′-*O*-carboxymethylthymidine. Colorimetric determination showed the presence of one trityl group for every two thymine residues. Hydrolysis with acetic acid under conditions which removed the trityl group, but not the 2-cyanoethyl group, from the 2-cyanoethyl ester of 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine, gave a product which moved as an acid on paper electrophoresis at pH 6·8 and was not 3′-*O*-carboxymethylthymidine.



(I)

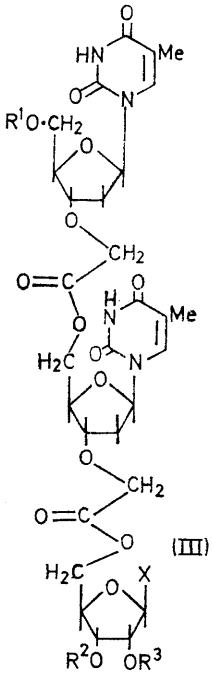


(II)

The fact that selective removal of the terminal cyanoethyl group was achieved to an acceptable degree was partly due to the fact that it proceeded by an elimination reaction which was favoured by the conditions used. The relative stability of the internucleoside carboxylic ester linkage may be partly attributable to steric hindrance. Molecular models show that there would be some, but not extreme, steric hindrance to the formation of the reaction intermediate carrying the t-butoxide group and the two nucleoside residues on the carboxylic ester carbon atom. Another factor is that a limited amount of base was used (2 mol.), part of which would have been rapidly consumed by the elimination reaction. No t-butyl ester was detected in the products, so it appeared that the small amount of fission of the internucleoside linkage was due to the presence of hydroxide ion formed from small amounts of water in the solvent.

For the synthesis of oligomers containing three nucleoside units it is convenient to use a ribonucleoside derivative as the terminal unit. This is because an acid-labile 2′,3′-cyclic acetal can be used as the terminal blocking group. The dimer (II; R^1 = trityl, R^2 = H) as the pyridinium salt was condensed with 2′,3′-*O*-isopropylideneadenosine in the presence of dicyclohexylcarbodi-imide to give a trimer (III; R^1 = trityl, R^2R^3 = isopropylidene, X = adenine). The u.v. spectrum of the product showed that the adenine residue had not been acylated, and upon alkaline hydrolysis the

compound gave equimolar amounts of 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine, 3′-*O*-carboxymethylthymidine, and 2′,3′-*O*-isopropylideneadenosine. Strong acid hydrolysis (98% formic acid at 175°) gave thymine and adenine in a molar ratio of 2 : 1. The trityl and isopropylidene groups were removed under acidic conditions to give compound (III) ($R^1 = R^2 = R^3 = H$, X = adenine), thymidinylacetyl-(3′ → 5′)-thymidinylacetyl-(3′ → 5′)-adenosine.



(III)

The corresponding uracil derivative (III; $R^1 = R^2 = R^3 = H$, X = uracil) was obtained from 2′,3′-*O*-isopropylideneuridine, and the compound was characterised in a similar manner.

Attempts to obtain the guanine- and hypoxanthine-containing compounds were not successful, however, because removal of the terminal isopropylidene group resulted in partial hydrolysis of the purine glycosidic linkages. In these cases, therefore, the anisylidene nucleosides were used because of the greater lability of the protecting group in acid. 2′,3′-*O*-Anisylideneguanosine has been prepared before[4] but not, as far as we could ascertain, the corresponding inosine compound. We obtained the latter without difficulty, however, by a standard procedure. Condensation of compound (II) (R^1 = trityl, $R^2 = H$) with the anisylidene nucleoside in the usual way and removal of the protecting groups with acid gave thymidinylacetyl-(3′ → 5′)-thymidinylacetyl-(3′ → 5′)-guanosine (III; $R^1 = R^2 = R^3 = H$, X = guanine) and the corresponding inosine derivative.

In order to obtain the cytidine derivative it was necessary to protect the cytosine residue because of its susceptibility to acylation. Initially 4-*N*-acetyl-2′,3′-*O*-isopropylidenecytidine was used. This condensed readily with the dimer (II; R^1 = trityl, $R^2 = H$) but it was not possible to remove the *N*-acetyl group without causing considerable hydrolysis of the internucleoside linkages. The protecting group used, therefore, was the dimethylaminomethylene group, which can be introduced and removed under very mild conditions.[5] 2′,3′-*O*-Isopropylidene-4-*N*-dimethylaminomethylenecytidine was condensed with the dimer (II; R^1 = trityl, $R^2 = H$) in the usual way. The isopropylidene and trityl groups were removed with dilute acid and the dimethylaminomethylene group was removed with boiling ethanol. The latter process caused some scission of the internucleoside linkage and it was necessary to purify the final product carefully. Thymidinylacetyl-(3′ → 5′)-thymidinylacetyl-(3′ → 5′)-cytidine (III; $R^1 = R^2 = R^3 = H$, X = cytosine) was finally obtained and characterised in the usual way.

One of the characteristics of the natural oligonucleotides is that their extinction coefficients are lower than would be expected from the summation of the extinction coefficients of the nucleotide units. This hypochromic effect is present even in dinucleotides and has been attributed to base stacking.[6] The effect is not confined to oligonucleotides; it has also been shown to occur in compounds in which purines and pyrimidines are joined by a polymethylene chain (especially $-[CH_2]_3-$).[7] The hypochromic effects at 260 nm. in 1% dimethylformamide–water shown by the analogues of trinucleoside diphosphates are given in Table 1. They are in general of about the same magnitude as shown by a series of uracil-containing triribonucleotides.[8] A direct comparison, cannot be made, however, because the analogues do not have the same sequence or composition as the natural compounds and the conditions for measurement were different. The presence of the effect is evidence, nevertheless, of base stacking in the analogues.

N.m.r. spectroscopy has been used to study the interaction between the bases in oligonucleotides. An upfield shift for protons on stacked aromatic or heteroaromatic rings is known to occur and has been observed in di- and tri-nucleotides.[9,10] The n.m.r. spectra of the analogues synthesised in the present work have been measured and the relevant results are shown in Table 2. A direct correlation between the shielding effects in the analogues and in the natural oligomers does not appear to be possible because Scheit and his co-workers[9] used a different solvent, and T'so and his collaborators[10] an external reference, and found that the phosphate

[4] S. Chladek and J. Smrt, *Coll. Czech. Chem. Comm.*, 1963, **28**, 1301.
[5] J. Zemlicka and A. Holy, *Coll. Czech. Chem. Comm.*, 1967, **32**, 3159.
[6] A. M. Michelson, *Biochim. Biophys. Acta*, 1962, **55**, 841.
[7] D. T. Browne, J. Eisinger, and N. J. Leonard, *J. Amer. Chem. Soc.*, 1968, **90**, 7302.
[8] A. M. Michelson, *J. Chem. Soc.*, 1959, 3655.
[9] K. H. Scheit, F. Cramer, and A. Franke, *Biochim. Biophys. Acta*, 1967, **145**, 21.
[10] P. O. P. T'so, N. S. Kondo, M. P. Schweizer, and D. P. Hollis, *Biochemistry*, 1969, **8**, 997.

group has a shielding effect on 8-H of a purine nucleoside-5′-phosphate and 6-H of a pyrimidine nucleoside-5′-phosphate. However, certain features obtained with the analogues are similar to those obtained with oligonucleotides containing thymine residues. For instance, two adjacent thymine residues show separate signals for 6-H. There is also a difference in the 6-H signal of the uridine residues between uridine itself (δ 7·88) and the uridine-containing trinucleoside diphosphate analogue (δ 7·60 p.p.m.). A shift in the 8-H signal of the purine nucleoside derivatives was also observed. That this was smaller than expected could have been due to the use of dimethyl sulphoxide as solvent; T'so and his co-workers [10] found that in this solvent the chemical shift of the C-8-protons in adenylyl-(3′ → 5′)-adenosine was almost identical to that of 8-H in adenosine, whereas in deuterium oxide these protons are shielded and the signals were upfield relative to that of the parent nucleoside. These shifts in the 6-H signal of the pyrimidines and the 8-H signal of the purines in the synthetic analogues are in accordance, therefore, with the occurrence of base stacking.

EXPERIMENTAL

The following chromatographic solvents were used: (1) butan-1-ol–ethanol–water (4 : 1 : 5) (organic phase); (2) butan-2-ol saturated with water; (3) propan-2-ol–ammonia (*d* 0·88)–water (35 : 3 : 10). Propan-2-ol–10N-hydrochloric acid–water (136 : 33 : 31) was used to separate purines and pyrimidines formed after hydrolysis with 98% formic acid at 175° for 1 hr.

3′-O-Carboxymethyl-5′-O-tritylthymidine.—5′-*O*-Tritylthymidine (monobenzene adduct; 2·27 g., 4 mmoles) was dried *in vacuo* (P_2O_5) and dissolved in dry dimethyl sulphoxide (20 ml.). Sodium hydride (240 mg., 10 mmoles) was added to the solution, which was stirred under anhydrous conditions until evolution of hydrogen was complete (1 hr.). Sodium chloroacetate (519 mg., 4·4 mmoles) was added and the mixture was shaken at room temperature to give a yellow solution, which was stirred at room temperature for 3 days. A white precipitate which formed was dissolved by addition of water (40 ml.) and ethanol (40 ml.). Paper chromatography [solvent (1)] showed that all of the starting material had been consumed and that the major product was the required carboxylic acid. The solution was adjusted to pH 8 with N-hydrochloric acid and evaporated under reduced pressure. Dimethyl sulphoxide was removed under high vacuum at 40°. The residue was dried *in vacuo* (P_2O_5) and extracted with anhydrous chloroform–ethanol (1 : 3 v/v; 6 × 200 ml.). The solid was pulverised before extraction and treated with the boiling solvent for 30 min. The hot extracts were filtered, allowed to cool to room temperature, and then stored at 4° overnight. The resulting gelatinous mass was filtered to give a chromatographically homogeneous product (0·92 g.). The filtrate was concentrated to dryness and the residue was extracted as before. This procedure was repeated. 3′-*O*-Carboxymethyl-5′-*O*-tritylthymidine was thus obtained (85—95%) as the sodium salt, identical with that previously obtained.

3′-O-(2-Cyanoethoxycarbonylmethyl)-5′-O-tritylthymidine.—3′-*O*-Carboxymethyl-5′-*O*-tritylthymidine (sodium salt) (0·8 g.) was converted into the pyridinium salt by means of the pyridinium form of Zeo-Karb 225 resin. The pyridinium salt was dried and dissolved in dry pyridine (10 ml.), 3-hydroxypropiononitrile (0·8 ml.) and dicyclohexylcarbodiimide (1·2 g.) were added and the solution was kept at room temperature overnight. Water (5 ml.) was then added and the solution was set aside for 2 hr. at room temperature and filtered. The filtrate was evaporated to a brown oil, which was extracted with ether (2 × 100 ml.) to remove 3-hydroxypropiononitrile. The residue was then partly dissolved in benzene. The insoluble material (dicyclohexylurea) was filtered off and the filtrate was added dropwise to light petroleum (b.p. 60—80°). The precipitate so formed was filtered off and purified by repeatedly dissolving in benzene and reprecipitating with light petroleum. 3′-O-(2-*Cyanoethoxycarbonylmethyl*)-5′-O-*tritylthymidine* (410 mg., 55%) was obtained chromatographically homogeneous (Found: C, 68·3; H, 6·2; N, 7·3. $C_{34}H_{33}N_3O_7$ requires C, 68·6; H, 5·6; N, 7·1%), $\lambda_{max.}$ (EtOH) 265 nm. (ε 9600), $\nu_{max.}$ 2242 (C≡N), and 1665 cm.$^{-1}$ (C=O).

3′-O-(2-Cyanoethoxycarbonylmethyl)thymidine.—3′-*O*-(2-Cyanoethoxycarbonylmethyl)-5′-*O*-tritylthymidine (280 mg.) was dissolved in acetic acid–water (4 : 1) and the solution was heated at 100° for 15 min. Paper chromatography in solvent (1) showed that the starting material had been converted into a product (R_F 0·63) which did not have a trityl group (absence of a yellow colour when the chromatogram was sprayed with perchloric acid and heated). A small amount of 3′-*O*-carboxymethylthymidine (R_F 0·09) was also present. The acetic acid solution was evaporated *in vacuo* to dryness and all of the acetic acid was removed by repeated co-evaporation with water. The residue was extracted with water and the solid residue of triphenylmethanol was filtered off. The filtrate was again evaporated to dryness and the residue was dissolved in acetone–chloroform (1 : 1) and fractionated on a column of silica gel (Kieselgel, 0·05—0·2 mm., 70—325 mesh ASTM, type 7734, Merck) (20 g.) by use of the same solvent. The fractions containing the pure product gave an oil, which yielded *crystals* (100 mg., 60%) from acetone–light petroleum, m.p. 125—127° (Found: C, 51·2; H, 5·3; N, 11·8. $C_{15}H_{19}N_3O_7$ requires C, 51·0; H, 5·4; N, 11·9%), $\lambda_{max.}$ (H_2O) 266 nm. (ε 10,400), $\nu_{max.}$ 2242 (C≡N), 1750, and 1665 cm.$^{-1}$ (C=O).

For subsequent large-scale preparations, the compound was obtained without the intermediate isolation of the 3′-*O*-(2-cyanoethoxycarbonylmethyl)-5′-*O*-tritylthymidine. The material was used immediately after the excess of 3-hydroxypropionitrile had been removed with ether. The overall yield from 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine was 62%.

2-Cyanoethyl 5′-O-Tritylthymidinylacetyl-(3′ → 5′)-thymidin-3′-ylacetate (II; R^1 = trityl, R^2 = $CH_2{\cdot}CH_2{\cdot}CN$).—To 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine [pyridinium salt, obtained from the sodium salt (1·05 g., 1·8 mmole)] in anhydrous pyridine (40 ml.) were added 3′-*O*-(2-cyanoethoxycarbonylmethyl)thymidine (635 mg., 1·8 mmole) and dicyclohexylcarbodi-imide (3 g.). The solution was kept at room temperature overnight, then water (10 ml.) was added and the solution was left at room temperature for a further 2 hr. The solid produced (dicyclohexylurea) was filtered off and the filtrate was evaporated to dryness. The residue was extracted with acetone and insoluble material was filtered off. The acetone solution was again evaporated to dryness and the extraction procedure was repeated until all of the residue was soluble in acetone. Silica gel t.l.c. [acetone–ethyl acetate (1 : 19)] of this solution showed that

a major product (R_F 0·69), two minor products (R_F 0·86 and 0·96), and a small amount of 3′-*O*-(2-cyanoethoxycarbonylmethyl)thymidine (R_F 0·35) were present.

The acetone solution was evaporated to dryness and the residue was dissolved in the minimum volume of ethyl acetate and applied to a column of silica gel (200 g.). The column was eluted with acetone–ethyl acetate (1 : 19). The fractions containing the major product were pooled and evaporated to dryness to give a colourless glass. Water was added and the *product* so obtained was filtered off and dried (960 mg., 60%) (Found: C, 62·4; H, 5·3; N, 7·9. $C_{46}H_{47}N_5O_{13}$ requires C, 63·0; H, 5·4; N, 8·0%). It contained a trityl group and a cyanide group (i.r. spectrum); $\lambda_{max.}$ (ethanol, acid, and alkali) 266 nm., ε_{266} (ethanol) 18,000, (0·01N-NaOH) 15,500; δ 1·45 (3H, Me), 1·76 (3H, Me), 6·20 (2H, t, 1′-H), 7·43 (17H, m, 6-H and trityl protons), and 11·15 (2H, s, NH).

Hydrolysis of this compound with 0·1N-sodium hydroxide in aqueous acetone at room temperature for a few minutes gave equimolar amounts of 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine and 3′-*O*-carboxymethylthymidine.

5′-O-*Tritylthymidinylacetyl*-(3′ → 5′)-*thymidin*-3′-*ylacetic Acid* (II); R^1 = trityl, R^2 = H).—The 2-cyanoethyl ester (9·4 mg.) was dissolved in anhydrous dimethylformamide (1 ml.) and to this was added potassium t-butoxide (2·4 mg., 2 mol.). The solution was heated at 100° for 2 hr., cooled, and poured into a thick slurry of Zeo-Karb 225 (pyridinium form) in pyridine. The mixture was left at room temperature for 3 hr. The resin was filtered off and washed with pyridine, and the filtrate and washings were evaporated *in vacuo* to a brown gum. This was then fractionated by paper chromatography in solvent (2). Three components were obtained: 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine (R_F 0·66), 3′-*O*-carboxymethylthymidine (R_F 0·13), and a component (A) of R_F 0·45. The last comprised 88% of the u.v. absorption of the products and the other two components were present in almost equal amounts and comprised the remaining 12%. Substance (A) was chromatographically homogeneous [solvent (1), R_F 0·36]. It was eluted from paper chromatograms and the amount of trityl residue present was determined by measuring the optical density at 436 nm. of a solution in glacial acetic acid–conc. sulphuric acid (1 : 1). Trityl chloride was used as standard. Full details of this procedure will be published elsewhere. The amount of thymidine present was determined by measuring the u.v. absorption. The ratio of trityl residues to thymidine residues was found to be 0·96 : 2.

Substance (A) was hydrolysed with 0·1N-sodium hydroxide at room temperature for 18 hr.; the solution was neutralised with acidic ion-exchange resin and chromatographed in solvent (1). Equimolar amounts of 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine and 3′-*O*-carboxymethylthymidine were obtained.

A solution of substance (A) in acetic acid–water (4 : 1) was heated at 100° for 15 min. The acetic acid was removed *in vacuo* and the residue was subjected to paper electrophoresis at pH 6·8. The product moved to the anode with a mobility of 3·6 cm./kV hr. 3′-*O*-Carboxymethylthymidine moves with a mobility of 6·3 cm./kV hr.

5′-O-*Tritylthymidinylacetyl*-(3′ → 5′)-*thymidinylacetyl*-(3′ → 5′)-(2′,3′-O-*isopropylidene*)*adenosine* (III; R^1 = trityl, R^2R^3 = isopropylidene, X = adenine).—Compound (II; R^1 = trityl, R^2 = CH_2·CH_2·CN) (351 mg., 0·4 mmole) was dissolved in anhydrous dimethylformamide (20 ml.), potassium t-butoxide (90 mg., 0·8 mmole) was added, and the solution was heated at 100° for 2 hr., cooled to room temperature, then poured on to Zeo-Karb 225 resin (pyridinium form; 100 ml.) and left at room temperature for 2 hr. The resin was filtered off and washed with pyridine–water (1 : 1; 4 × 50 ml.). The combined filtrate and washings were evaporated to dryness below 40°, pyridine being added during the evaporation to dissolve any precipitate formed. The final residue (II; R^1 = trityl, R^2 = H) was dried *in vacuo* at room temperature (conc. H_2SO_4) and then dissolved in anhydrous pyridine (5 ml.). To this solution, 2′,3′-*O*-isopropylideneadenosine (123 mg., 0·4 mmoles) and dicyclohexylcarbodi-imide (1·2 g.) were added, and mixture was left at room temperature overnight. Water (3 ml.) was then added, and after 3 hr. the dicyclohexylurea formed was filtered off. The filtrate was evaporated to dryness, the residue was extracted with acetone, and acetone-insoluble material was filtered off. The acetone was evaporated from the filtrate and the residue was dissolved in chloroform and fractionated on a column of silica gel (70 g.). Unchanged dicyclohexylcarbodi-imide and dicyclohexylurea were eluted with chloroform, and the product was then eluted with ethanol–chloroform (1 : 9). The fractions containing the pure product were combined, evaporated to dryness, and dissolved in chloroform, and the product (III; R^1 = trityl, R^2R^3 = isopropylidene, X = adenine) was precipitated with light petroleum (b.p. 60—80°) and filtered off (230 mg., 55%). Hydrolysis of a sample (0·1N-NaOH) at room temperature gave 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine, 3′-*O*-carboxymethylthymidine, and 2′,3′-*O*-isopropylideneadenosine in equimolar proportions. Hydrolysis with 98% formic acid at 175° for 1 hr. gave thymine and adenine in the molar ratio of 2·1 : 1.

Thymidinylacetyl-(3′ → 5′)-*thymidinylacetyl*-(3′ → 5′)-*adenosine*.—The compound just described (200 mg.) was dissolved in formic acid (10 ml.) and water (5 ml.) was added. The resulting suspension was left at room temperature for 24 hr. and then filtered. The filtrate was evaporated to dryness under reduced pressure, the last traces of formic acid being removed by repeated evaporation to dryness with water. The residue was dissolved in dimethylformamide (5 ml.), the solution was filtered, and the filtrate was diluted with water (200 ml.). This solution was extracted with ether (3 × 100 ml.) and the aqueous phase was evaporated under reduced pressure to remove any ether. The solution was then left overnight at 4° and the product (III; $R^1 = R^2 = R^3 = H$, X = adenine) (80, mg., 56%) was obtained as white *crystals*, m.p. 162—164° (Found: C, 48·9; H, 5·3; N, 15·0. $C_{34}H_{41}N_9O_{16}$ requires C, 49·1; H, 4·9; N, 15·2%), chromatographically homogeneous in solvent (2) (R_F 0·26). Upon hydrolysis with 98% formic acid at 175° for 1 hr. the product gave thymine and adenine in the molar ratio of 2·1 : 1. Hydrolysis with 0·1N-sodium hydroxide gave 3′-*O*-carboxymethylthymidine and adenosine.

Thymidinylacetyl-(3′ → 5′)-*thymidinylacetyl*-(3′ → 5′)-*uridine*.—Condensation between compound (II; R^1 = trityl, R^2 = H) (0·4 mmole) and 2′,3′-*O*-isopropylideneuridine (118 mg., 0·4 mmole) in the presence of dicyclohexylcarbodiimide was carried out similarly. The products were fractionated on silica gel (70 g.), the required material being eluted with ethanol–chloroform (1 : 9). The solvents were evaporated off, the residue was dissolved in ethanol, and the product (280 mg.) was precipitated with ether. [Hydrolysis of this compound (0·1N-NaOH) gave 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine, 3′-*O*-carboxymethylthymidine, and

2′,3′-*O*-isopropylideneuridine.] It was dissolved in 98% formic acid (10 ml.), water (5 ml.) was added, and the solution was left at room temperature for 4 hr. The precipitate of triphenylmethanol was filtered off, and the filtrate was evaporated to dryness, the last traces of formic acid being removed in the usual way. The residue was dissolved in dimethylformamide (5 ml.), and ethyl acetate (25 ml.) and then light-petroleum (b.p. 100—120°; 200 ml.) were added. The white *precipitate* was filtered off and dried (180 mg., 85%) (Found: C, 48·4; H, 5·0; N, 10·5. $C_{33}H_{40}N_6O_{18}$ requires C, 49·0; H, 5·0; N, 10·4%). This compound gave 3′-*O*-carboxymethylthymidine and uridine when hydrolysed with 0·1N-sodium hydroxide. Hydrolysis with 98% formic acid at 175° gave thymine and uracil in the molar ratio of 2·1 : 1.

Thymidinylacetyl-(3′ → 5′)-thymidinylacetyl-(3′ → 5′) cytidine.—Condensation between compound (II) (R^1 = trityl, R^2 = H) (0·8 mmole) and 4-*N*-dimethylaminomethylene-2′,3′-*O*-isopropylidenecytidine [5] (270 mg., 0·8 mmole) in the presence of dicyclohexylcarbodi-imide was carried out similarly. The major product isolated had $\lambda_{max.}$ 267 and 315 nm. The latter absorption is characteristic of a dimethylaminomethylenecytidine derivative. Hydrolysis of a sample with alkali and paper chromatography of the hydrolysate in solvent (2) showed the presence of 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine, 3′-*O*-carboxymethylthymidine, and 2′,3′-*O*-isopropylidenecytidine. The product obtained from the condensation was dissolved in ethanol (100 ml.) and the solution was boiled under reflux until the absorption at 315 nm. had disappeared. Silica gel t.l.c. at this stage showed that, in addition to the removal of the dimethylaminomethylene group, some hydrolysis of internucleoside linkages had taken place. The ethanolic solution was evaporated to dryness and the residue was dissolved in chloroform and fractionated on silica gel (70 g.); elution was carried out with ethanol–chloroform (3 : 22). Fractions containing the major product were evaporated to dryness, the residue was dissolved in 98% formic acid (20 ml.), and water (10 ml.) was added. After 8 hr. at room temperature the solution was extracted with chloroform (3 × 50 ml.). The aqueous phase was evaporated to dryness, the residue was dissolved in dimethylformamide, and the *product* (III; $R^1 = R^2 = R^3$ = H, X = cytidine) was precipitated with an excess of ethyl acetate (yield 70 mg., 10%) (Found: C, 46·3; H, 4·6; N, 11·1. $C_{33}H_{41}N_7O_{17},HCO_2H,1{\cdot}5H_2O$ requires C, 46·4; H_5 5·2; N, 11·1%). Hydrolysis with 98% formic acid at 175° gave thymine and cytosine in a molar ratio of 2·14 : 1.

2′,3′-O-*Anisylideneinosine.*—A suspension of inosine (1 g.) in anisaldehyde (10 ml.) was shaken overnight at room temperature with zinc chloride (2 g.). The product was triturated with ether (200 ml.) and the resulting solid was filtered off, washed with water, and crystallised from boiling water containing a little ethanol to give 2′,3′-O-*anisylideneinosine* (0·6 g., 40%), m.p. 228—229° (Found: C, 55·9; H, 5·0; N, 14·3. $C_{18}H_{18}N_4O_6$ requires C, 56·0; H, 4·7; N, 14·5%). The product was chromatographically homogeneous; R_F 0·62 in solvent (1) and 0·74 in solvent (3). It showed no mobility in borate electrophoresis. On treatment with formic acid–water (2 : 1) at room temperature it was converted into inosine in 5 min.

Thymidinylacetyl-(3′ → 5′)-thymidinylacetyl-(3′ → 5′)inosine.—Condensation between compound (II; R^1 = trityl, R^2 = H) (0·4 mmole) and 2′,3′-*O*-anisylideneinosine (154 mg., 0·4 mmole) was carried out as in the previous cases. The major product was isolated by chromatography on silica gel; the required compound eluted with ethanol–chloroform (1 : 19), was finally precipitated from chloroform solution with light petroleum (b.p. 40—60°) (yield 205 mg., 55%). [Hydrolysis of a sample of this substance (0·1N-NaOH) gave 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine, 3′-*O*-carboxymethylthymidine, and 2′,3′-*O*-anisylideneinosine.] This solid (200 mg.) was dissolved in chloroform (10 ml.) and 98% formic acid (20 ml.) and water (10 ml.)

TABLE 1

Compound *	$\varepsilon_{260} \times 10^{-3}$ (1% $Me_2N{\cdot}CHO$–H_2O) Found †	Calculated ‡	Hypochromicity (%)
dThd-a-dThd-a-Ado	30·2	33·3	9·3
dThd-a-dThd-a-Urd	26·2	27·8	5·7
dThd-a-dThd-a-Cyd	24·2	25·3	4·4
dThd-a-dThd-a-Ino	23·3	25·1	7·2
dThd-a-dThd-a-Guo	28·0	29·6	5·4

* Abbreviations for thymidinylacetyl-(3′→ 5′)-thymidinylacetyl-(3′→ 5′)-adenosine and the corresponding uridine, cytidine, inosine, and guanosine compounds. † Values corrected as described in text. ‡ Summation of extinction coefficient of the nucleosides measured under identical conditions.

were added. The solution was left at room temperature for 15 min. and then extracted with chloroform (3 × 60 ml.). The aqueous phase was evaporated to dryness, the residue was dissolved in dimethylformamide, ethyl acetate was added, and the precipitated material was centrifuged off, washed with ethyl acetate, and dried to give the *product* (III; R^1 = trityl, $R^2 = R^3$ = H, X = hypoxanthine) (110 mg., 72%) (Found: C, 47·1; H, 4·5; N, 12·8. $C_{34}H_{40}N_8O_{17},2H_2O$ requires C, 47·0; H, 5·0; N, 12·9%). Hydr-

TABLE 2

	δ (p.p.m.)					
Compound *	Thymine Me	H-5	H-6	H-8	H-2	H-1′
Thymidine	1·79		7·69			6·18
Uridine		5·65	7·88			5·79
Adenosine				8·36	8·16	5·90
Guanosine				7·97		5·72
Inosine				8·32	8·08	5·90
dThd-a-dThd-a-Ado	1·79		7·68	8·30	8·16	6·14
			7·44			
dThd-a-dThd-a-Urd	1·79		7·66			6·15
			7·43			
		5·66	7·60			
dThd-a-dThd-a-Guo	1·79		7·72	7·92		6·18
			7·47			
dThd-a-dThd-a-Ino	1·79		7·67	8·27	8·06	6·14
			7·44			5·90

* Abbreviations as for Table 1.

olysis (0·1N-NaOH) and separation of the products by paper chromatography in solvent (2) showed that 3′-*O*-carboxymethylthymidine and inosine had been produced. Hydrolysis with 98% formic acid gave thymine and hypoxanthine in a molar ratio of 1·9 : 1.

Thymidinylacetyl-(3′ → 5′)-thymidinylacetyl-(3′ → 5′)-guanosine.—2′,3′-*O*-Anisylideneguanosine [4] (320 mg., 0·8 mmole) in anhydrous dimethylformamide (5 ml.) was added to a solution of compound (II; R^1 = trityl, R^2 = H) (0·8 mmole) and dicyclohexylcarbodi-imide (4 g.) in pyridine (10 ml.) and the solution was left at room temperature overnight. The solvents were removed by evaporation *in vacuo* and the residue was chromatographed on silica gel

(70 g.). The product was eluted with ethanol–chloroform (1 : 9), dissolved in chloroform, and precipitated with light petroleum (b.p. 40—60°) (yield 260 mg., 35%). Hydrolysis of a sample (0·1N-NaOH) gave 3′-*O*-carboxymethyl-5′-*O*-tritylthymidine, 3′-*O*-carboxymethylthymidine, and 2′,3′-*O*-anisylideneguanosine. The compound was dissolved in chloroform (20 ml.), 98% formic acid (25 ml.) was added, and the solution was kept at room temperature for 5 min. Water (15 ml.) was then added and the mixture was kept at room temperature for a further 15 min. The chloroform layer was then removed and the aqueous layer was washed with chloroform (3 × 60 ml.) and evaporated to dryness. The residue was dissolved in dimethylformamide and ethyl acetate was added. The resulting white precipitate was filtered off, washed, and dried to give the *product* (III; $R^1 = R^2 = R^3 = H$, X = guanine) (140 mg., 70%) (Found: C, 48·5; H, 4·6; N, 14·6. $C_{34}H_{41}N_9O_{17}$ requires C, 48·2; H, 4·8; N, 14·9%).

Measurement of Hypochromic Effects.—The analogues and standards were dissolved in dimethylformamide and the solutions were diluted (100 ×) with water. This procedure was used because the analogues dissolved very slowly in water. The extinction coefficients at 260 nm. were then measured. The solutions were then made 0·01N in sodium hydroxide and set aside until all the internucleoside linkages had been hydrolysed; the extinction coefficients were then measured again. This last operation was a check on the accuracy of the instruments and the weighing procedure. It also eliminated any errors due to the presence of non-u.v.-absorbing impurities (*e.g.* water, solvent). With one exception the value thus obtained was lower than the theoretical one, so a small correction (×1·02—1·04) was applied to the extinction coefficient at neutrality. The results are shown in Table 1.

N.m.r. Spectra.—The spectra were recorded for solutions in [$^2H_6$]dimethyl sulphoxide, with tetramethylsilane as internal reference. The spectrum of the cytosine-containing oligomer had a complex series of peaks which did not enable the signals for the thymidine 6-H and cytosine 6-H to be assigned accurately. The results are shown in Table 2.

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