and treated with 1.7 ml (0.01 mole) of benzoyl chloride. The solution was acidified, leading to DL-α-phenyl-α-benzamidoacetic acid: 1.60 g (0.0063 mole), 63% yield; mp 177.5° (lit.[15] 175.5°).

D-(−)-α-Phenyl-α-benzamidoacetic acid was prepared in the same way from D-(−)-α-phenylglycine: mp 186–187° (lit.[25] 187–188°); α_{obsd} −1.18°, 1.40% in 1 *N* NaOH; $[\alpha]^{33}D$ −84.3° (lit.[25] $[\alpha]^{15}D$ −84.1°).

Anal. Calcd for $C_{15}H_{13}O_3N$: C, 70.58; H, 5.13; N, 5.49. Found: C, 70.81; H, 5.25; N, 5.39.

Ethyl DL-α-phenyl-α-benzamidoacetate was prepared from 2.50 g (0.098 mole) of the benzamido acid as described for the acetamido ester, in 90% yield, mp 89° (lit.[15] 84°).

Ethyl D-(−)-α-phenyl-α-benzamidoacetate was prepared similarly from the D-(−) benzamido acid: mp 108–108.5°; α_{obsd} −2.05°, 2.23% in ethanol; $[\alpha]^{32}D$ −92.0°.

Anal. Calcd for $C_{17}H_{17}O_3N$: C, 72.07, H, 6.05; N, 4.94. Found: C, 71.90; H, 6.18; N, 5.26.

Treatment of Ethyl DL-α-Phenyl-α-benzamidoacetate with α-Chymotrypsin. A solution of 0.102 g of α-chymotrypsin in 5 ml of water was added to a stirred suspension of 0.204 g (0.72 mmole) of ethyl DL-α-phenyl-α-benzamidoacetate in 15 ml of 0.1 *N* NaCl and 5 ml of dioxane. The hydrolysis was followed at pH 7.8 in a pH-Stat, and the reaction was interrupted after 140 min, when 0.288 ml of 1 *N* NaOH was consumed, corresponding to hydrolysis of 80% of one enantiomorph. The products were isolated as described above for the acetamido compounds: unreacted ethyl α-phenyl-α-benzamidoacetate: 0.071 g (0.25 mmole), 60% yield; mp 102–103°; α_{obsd} −0.63°, 0.85% in ethanol; $[\alpha]^{27}D$ −74°; and L-(+)-α-phenyl-α-benzamidoacetic acid: 0.043 g (0.17 mmole), 58% yield; mp 192°; α_{obsd} +0.37°, 0.517% in 1 *N* NaOH; $[\alpha]^{23}D$ +72°.

A suspension of 0.0212 g (0.075 mmole) of ethyl DL-α-phenyl-α-benzamidoacetate in 15 ml of 0.1 *N* NaCl was allowed to react in a pH-Stat at 25.5° at pH 7.8, the nonenzymic hydrolysis proceeding at a rate of 2×10^{-5} mmole min^{-1}. A solution of 0.0206 g of α-chymotrypsin in 0.1 *N* NaCl was added, 0.1024 ml of 0.1 *N* NaOH being consumed in 60 min, corresponding to 24% hydrolysis of one enantiomorph by the enzyme, 1.5×10^{-4} mmole min^{-1}.

A similar study was carried out at pH 7.8 on 0.0507 g (0.18 mmole) of the ester in 11 ml of 0.1 *N* NaCl and 4 ml of dioxane. The nonenzymic rate was determined, 1×10^{-4} mmole min^{-1}. A solution of 0.0204 g of α-chymotrypsin in 5 ml of 0.1 *N* NaCl was added, 0.544 ml of 0.1 *N* NaOH being consumed in 60 min corresponding to 52% hydrolysis of one enantiomorph by the enzyme, 8×10^{-4} mmole min^{-1}.

Ethyl L-(+)-Mandelate. L-(+)-Mandelic acid (2.03 g, 0.013 mole) was boiled for 4.5 hr in 100 ml of ethanol containing 5 drops of sulfuric acid. The solution was diluted, treated with 5% sodium bicarbonate, and extracted with methylene chloride, leading to ethyl L-(+)-mandelate: 2.2 g (0.012 mole), 92% yield; bp 109° (2 mm); α_{obsd} +3.63°, 2.68% in chloroform; $[\alpha]^{28}D$ +136° (lit.[26] bp 106–107° (4 mm); $[\alpha]^{25}D$ +136.6° in chloroform). **Ethyl D-(−)-mandelate** was similarly prepared: bp 109° (2.4 mm); α_{obsd} −3.53°, 2.61% in chloroform; $[\alpha]^{26}D$ −136° (lit.[26] bp 103–105° (2 mm); $[\alpha]^{25}D$ −136.6° in chloroform).

Ethyl DL-mandelate was similarly prepared: bp 104° (1.5 mm) (lit.[27] 155–156° (35 mm)).

Treatment of Ethyl Mandelate with α-Chymotrypsin. A suspension of 1.00 g (5.55 mmoles) of ethyl DL-mandelate in 15 ml of 0.1 *M* NaCl was treated with a solution of 0.080 g of α-chymotrypsin in 5 ml of 0.1 *M* NaCl under nitrogen at 25° in a pH-Stat at pH 7.8. The reaction was stopped after 3.75 hr, 1.99 ml of 1 *N* NaOH having been consumed, 36% reaction. Unreacted ester was extracted with ether, 0.63 g, 98% recovery. Its infrared spectrum was identical with that of the starting material. It showed no optical activity beyond experimental error, α_{obsd} −0.03°, 2.82% in chloroform. The aqueous reaction solution was acidified and lyophilized, and the residue was extracted with acetone, leading to DL-mandelic acid: 0.101 g (0.67 mmole), 33% yield; mp 115–116.5°; mmp 117.5–118.5°; α_{obsd} +0.01°, 1.25% in water, within experimental error.

(25) S. Minovici, *Bull. Soc. Chim. Romania*, **2**, 8 (1920); *Chem. Abstr.*, **14**, 3228 (1920).

(26) W. Bonner and C. Hurd, *J. Am. Chem. Soc.*, **73**, 4293 (1951).

(27) J. Berson and M. Greenbaum, *ibid.*, **81**, 6458 (1959).

Stepwise Synthesis of Oligodeoxyribonucleotides on an Insoluble Polymer Support[1,2]

Robert L. Letsinger and V. Mahadevan

Contribution from the Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received August 5, 1966*

Abstract: Procedures are described for synthesizing oligonucleotide derivatives (*e.g.*, TrdCpTpTpT) on an insoluble support polymer.

The comprehensive studies by Khorana and his co-workers have provided elegant and effective methods for the chemical synthesis of a wide variety of oligonucleotides.[3] Nevertheless, the stepwise synthesis of relatively long chain polynucleotides remains a formidable task. Some time ago it occurred to us that the labor involved in repetitive step syntheses of this type might be materially reduced if the syntheses were carried out on an insoluble polymer support. In the initial step a nucleoside would be joined covalently to the support. Nucleotide units would subsequently be added stepwise to this nucleoside, and in the final reaction the covalent bond joining the oligonucleotide chain to the support would be broken and the oligonucleotide eluted. This technique would enable one to separate the products in the building stages from the solvents, excess reagents, and soluble by-products simply by filtration, thus avoiding numerous time-consuming isolation steps. In testing this idea we first developed an insoluble, functionalized polymer and

(1) Part III, Nucleotide Chemistry. A preliminary account of some of this work was published in *J. Am. Chem. Soc.*, **87**, 3526 (1965).

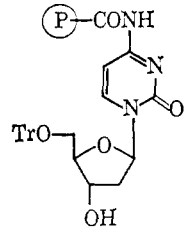
(2) This research was supported by the Division of General Medical Sciences, National Institutes of Health, Grant 10265.

(3) For leading references see: (a) H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **87**, 2954 (1965); (b) T. M. Jacob and H. G. Khorana, *ibid.*, **86**, 1630 (1964); (c) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963); (d) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958). Since completion of the present paper a procedure for synthesizing oligonucleotides on a soluble polymer support has been described by H. Hayatsu and H. G. Khorana, *ibid.*, **88**, 3182 (1966).

demonstrated that chemical transformations could be carried out satisfactorily on it when suspended in an organic solvent.[4] The present paper reports the utilization of a support polymer in the synthesis of oligonucleotide derivatives.

The support chosen for the synthetic work was a popcorn copolymer prepared from styrene (88%), *p*-vinylbenzoic acid (12%), and *p*-divinylbenzene (0.2%). It was insoluble in water, alkaline solutions, and all organic solvents examined. Promising results have also been obtained recently with a polymer that contained 0.02% *p*-divinylbenzene as the cross-linking agent. It swelled considerably more in benzene and in pyridine than did the higher cross-linked polymer yet it remained insoluble and could be separated satisfactorily from the solvents by centrifugation or by filtration. Popcorn-type polymers were obtained from styrene and *p*-vinylbenzoic acid mixtures that contained as little as 0.01% *p*-divinylbenzene; however, these products were partially soluble in pyridine. Other materials considered as supports were a bead copolymer of acrylonitrile and acrylic acid and a popcorn copolymer of 2,3-dimethylbutadiene and *p*-vinylbenzoic acid.[5] While usable, these polymers were somewhat less stable than the styrene-based supports and offered no apparent advantage over them.

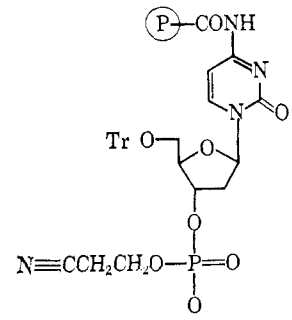
5′-O-Trityldeoxycytidine was utilized as the anchor group in these syntheses. It was joined to the support by reaction with the acid chloride form of the polymer (Ⓟ–COCl).[6] That the juncture was through the amino group of the cytosine moiety was shown by enzymatic degradation of the oligonucleotide derivatives synthesized on the support.[7] Approximately 40% of the acid chloride functional groups reacted when Ⓟ–COCl was stirred with excess 5′-O-trityldeoxycytidine in pyridine for 48 hr. Since the remaining acid chloride groups would have been undesirable in later synthetic steps, they were esterified by treatment with methanol in pyridine. The resulting polymer is indicated by I.



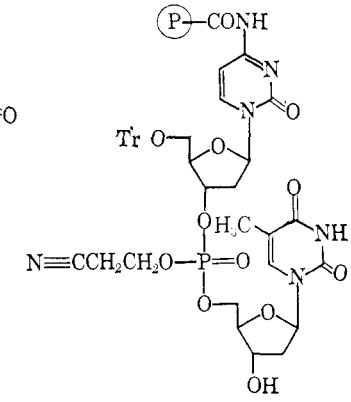
I

A number of experiments were carried out to establish conditions for removing the trityldeoxycytidine from the support. The reagent selected was 0.4 *M* sodium hydroxide in dioxane–ethanol–water. At room temperature it removed all the trityldeoxycytidine within 36 hr. Solutions of ammonium hydroxide in pyridine were less satisfactory. When the polymer was heated in the ammonia mixture at atmospheric pressure, the cleavage was very slow; when the reaction was carried out in an autoclave at 100°, extensive degradation of oligonucleotides joined to the support took place.

Nucleotide units were built onto the terminal hydroxyl groups in polymer I in two steps: (1) phosphorylation and (2) condensation of the phosphoryl derivative with a nucleoside. Phosphorylation could be effected by a variety of reagents, including phosphorus oxychloride, *p*-nitrophenyl phosphorodichloridate,[8] β-cyanoethyl phosphorodichloridate, *p*-nitrophenyl phosphate plus dicyclohexylcarbodiimide, and β-cyanoethyl phosphate plus dicyclohexylcarbodiimide.[9] For construction of oligonucleotide chains the β-cyanoethyl phosphate–dicyclohexylcarbodiimide combination proved to be the most satisfactory. Step 2 was accomplished by activating the phosphodiester resulting from the β-cyanoethyl phosphate reaction (II) with mesitylenesulfonyl chloride and then adding the appropriate nucleoside derivative. The β-cyanoethyl group in the resulting phosphotriester was subsequently removed by the alkali used to cleave the oligonucleotides from the support polymer. Initially, thymidine blocked at the 3′ position by the dinitrobenzenesulfenyl group[10] was used in order to assure attack at the hydroxyl at the 5′ position; however, later experiments demonstrated that polymer II, activated by mesitylenesulfonyl chloride, attacked thymidine itself at the 5′ position. Consequently thymidine was used directly in the preparative scale reactions.



II



III

The over-all sequence of reactions may be illustrated by the synthesis of 5′-O-trityldeoxycytidylyl-(3′–5′)-thymidine (TrdCpT). Starting with Ⓟ–COCl, the insoluble polymer was subjected successively to the following reagents: 5′-O-trityldeoxycytidine, methanol (yielding I), β-cyanoethyl phosphate and dicyclohexylcarbodiimide, water (yielding II), mesitylenesulfonyl chloride, thymidine (yielding III), and sodium hydroxide (yielding TrdCpT). Pyridine was used as the liquid medium throughout except in the alkaline cleavage reaction. At the end of each step the polymer

(4) R. L. Letsinger and M. J. Kornet, *J. Am. Chem. Soc.*, **85**, 3045 (1963); R. L. Letsinger, M. J. Kornet, V. Mahadevan, and D. M. Jerina, *ibid.*, **86**, 5163 (1964).

(5) Work of Jerina and Becker.

(6) In this notation the relevant functional group is indicated explicitly and the remainder of the support is denoted by Ⓟ–. Other abbreviations used in this paper are: Tr, triphenylmethyl; dC, deoxycytidine; T, thymidine; dA, deoxyadenosine; p, the phosphate in a nucleotide or oligonucleotide derivative. The symbolism is typified by TrdCpTpdA, which refers to 5′-O-trityldeoxycytidylyl-(3′–5′)-thymidylyl-(3′–5′)-deoxyadenosine.

(7) The oligonucleotide derivatives obtained were degradable with snake venom phosphodiesterase. If the amino group had not been blocked, nondegradable products should have been obtained.

(8) A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4651 (1959).

(9) P. T. Gilham and G. M. Tener, *Chem. Ind.* (London), 542 (1959); G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).

(10) R. L. Letsinger, J. Fontaine, V. Mahadevan, D. A. Schexnayder, and R. E. Leone, *J. Org. Chem.*, **29**, 2615 (1964).

was separated from the liquid medium, washed, and resuspended in a fresh solution containing the next reagent. The final alkaline solution, which contained the nucleotidic material, was neutralized, concentrated, and chromatographed on DEAE-cellulose. From 2.2 g of polymer III was obtained 0.15 g of TrdCpT, a 53% yield based on the amount of tritydeoxycytidine bound to the support.

Since polymer III, like polymer II, possessed free 3′-hydroxyl groups, additional nucleotide units could be added to the chains by repetition of the phosphorylation, activation, and condensation steps utilized in building III. Thus, 5′-O-tritydeoxycytidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine (TrdCpTpT) and 5′-O-tritydeoxycytidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine (TrdCpTpTpT) were obtained in yields of 16 and 14%, respectively, based on the weights of the products isolated and the amount of tritydeoxycytidine bound to the support. 5′-O-Tritydeoxycytidylyl-(3′–5′)-thymidylyl-(3′–5′)-deoxyadenosine (TrdCpTpdA) was similarly prepared by substituting N-benzoyldeoxyadenosine for thymidine in the final condensation step.

These oligonucleotide derivatives behaved as pure compounds on electrophoresis and chromatography on paper, and the ultraviolet spectra in neutral, acidic, and basic solutions were in accord with the proposed structures. Pertinent data are summarized in Table I.

Table I. Properties of Synthetic Trityloligonucleotides[a]

	TrdCpT[b]	TrdCpTpT	TrdCpTpTpT
R, solvent A	0.72	0.50	0.29
R, solvent B	0.74	0.57	0.48
Electrophoretic mobility rel. to pdC	0.37	0.59	0.80
Ultraviolet in 0.01 *M* HCl[c]	275 (17900)	271 (24800)	269 (32000)
	243 (5500)	241 (9200)	237 (12800)
Ultraviolet in water[c]	270 (15600)	270 (23000)	269 (30600)
	247 (9600)	246 (13000)	242 (16400)
Ultraviolet in 0.01 *M* NaOH[c]	269 (14300)	269 (20200)	268 (26300)
	249 (10200)	249 (14400)	249 (19800)

[a] The values for ϵ depend upon the molecular weights assumed for the nucleotide derivatives. Since these products were hygroscopic, varying amounts of water could have been taken up; consequently the absolute values are not very precise. The molecular weights used in the calculations were 828 for TrdCpT, 1204 for TrdCpTpT, and 1436 for TrdCpTpTpT, corresponding to the formulas calculated from the combustion analyses (see Experimental Section). [b] The solvent for the ultraviolet determinations on TrdCpT contained a small amount of ethanol (5% v/v). [c] λ_{max} (ϵ) and λ_{min} (ϵ) are given.

That the nucleotides were joined in 3′–5′ phosphodiester linkages was shown by degradation by snake venom phosphodiesterase, an enzyme which acts on oligonucleotides bearing a free 3′-OH group. In each case the trityloligonucleotide was hydrolyzed to 5′-O-tritydeoxycytidine and thymidine 5′-phosphate in the appropriate mole ratio.

These synthetic experiments demonstrate the ultility of a polymer support in building oligonucleotide chains. The principal limitations stem from the yields, which are lower than desirable, and the time required in the reaction with β-cyanoethyl phosphate. Recent experiments have shown that both aspects may be somewhat improved. On the assumption that poor diffusion through the cross-linked support polymer was responsible for the inefficient conversions, we have repeated the synthesis of TrdCpT on a polymer support prepared from a monomer mixture that contained only 0.02% divinylbenzene. Manipulations with this support were quite satisfactory, and the yield of TrdCpT was 61% of the theroretical based on the millimoles of tritydeoxycytidine bound to the support. It has also been found that the condensation of β-cyanoethyl phosphate with a 3′-hydroxyl group on a nucleoside joined to the support may be effected with mesitylenesulfonyl chloride in place of dicyclohexylcarbodiimide. In this case the time required to join a full nucleotide unit to the chain on the support may be reduced to 2 days or less. By use of mesitylenesulfonyl chloride throughout with the low cross-linked support, TrdCpT and TrdCpTpT were prepared from polymer–TrdC in yields of 44 and 21%, respectively.

Experimental Section

Ultraviolet spectra were obtained with a Cary 14 or a Beckman DU spectrophotometer. Infrared spectra were obtained with a Baird Model AB2 spectrometer with the sample in a potassium bromide disk. Melting points were determined with a Fisher-Johns apparatus. Descending paper chromatography was carried out on Whatman 3MM paper with solvent A (isopropyl alcohol, concentrated ammonium hydroxide, and water in the proportions 7:1:2 by volume) and in solvent B (1-butanol, acetic acid, and water in the proportions 5:2:3 by volume). Nucleosides and nucleotides were observed by their fluorescence in ultraviolet light (~2537 A), and trityl-containing compounds were observed by the yellow color which developed when the paper was sprayed with 10% aqueous perchloric acid and subsequently warmed in a drying oven at 60° for 30 min.

Electrophoretic separations were made at pH 8.1 on Whatman 3MM paper strips with a Savant flat plate electrophoresis apparatus operated at 2000 v. The buffer solution contained 9.03 g of disodium hydrogen phosphate and 0.453 g of potassium hydrogen phosphate per liter.

Diethylaminoethyl- (DEAE) cellulose and Sephadex G-25 and G-15 were used in separating the reaction products. For the former, 80 g of DEAE-cellulose (Calbiochem, 0.93 mequiv/g) was washed[11] and added with stirring to a glass column (4 × 80 cm) as a slurry in 1.5 l. of 0.2 *M* ammonium bicarbonate buffer. The height, after a washing with 6 l. of 0.02 *M* ammonium bicarbonate, was approximately 35 cm. The Sephadex column was 1.5 × 120 cm and was packed with 0.02 *M* ammonium bicarbonate as the liquid phase. Effluent from the columns was monitored with a Gilson UV-2651F ultraviolet absorption meter and a Texas instrument rectiriter recorder; fractions (~13 ml) were collected automatically with a Gilson Model VL fractionator.

Chemicals. Nucleosides were purchased from Nutritional Biochemicals Corp. 5′-O-tritydeoxycytidine,[12] mp 249°, and N-benzoyldeoxyadenosine,[3c] mp 113–114°, were prepared by procedures described in the literature. Barium β-cyanoethyl phosphate was converted to the pyridinium salt[9] in aqueous solution by exchange with the pyridinium form of Dowex 50 resin. After evaporation of the resulting solution to dryness the salt was rendered anhydrous by repeated additions of dry pyridine and concentration of the solution; then a 0.8 *M* solution of the salt was prepared by addition of the appropriate amount of pyridine. Mesitylenesulfonyl chloride, mp 57°, was prepared by the method of Wang and Cohen.[13] Pyridine was dried over calcium hydride and fractionated through a 35-cm helices-packed column immediately before use.

General Procedure. The reactions were carried out at room temperature, unless otherwise specified, in glass-stoppered flasks equipped with a magnetic stirrer. Pyridine was employed as a solvent for the reagents except in the cleavage step. After the appropriate period of stirring, the polymer was separated from the

(11) G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Am. Chem. Soc.*, **80**, 6223 (1958).
(12) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, **34** (1954).
(13) C. Wang and S. G. Cohen, *J. Am. Chem. Soc.*, **79**, 1924 (1957).

solution of reagents and by-products by filtration, centrifugation, or settling under gravity. Filtration was employed when exposure to air and moisture was not detrimental; otherwise, the centrifugation or settling technique was used. Since the support with adjoining nucleotides was insoluble in pyridine, the reaction products could in principle be recovered quantitatively, though in practice small mechanical losses were experienced.

Polymer I. A support made from styrene (88%), *p*-vinylbenzoic acid (12%), and *p*-divinylbenzene (0.2%) was converted to the acid chloride form by treatment with excess thionyl chloride in benzene[4] and stored in a vacuum desiccator over phosphorus pentoxide. The infrared spectrum had two strong bands in the carbonyl region at 5.65 and 5.75 μ.

A portion (12 g) of the polymer acid chloride was stirred with 7.0 g (14.9 mmoles) of 5'-O-trityldeoxycytidine in 100 ml of dry pyridine for 2 days. To esterify any acid chloride groups that remained, 50 ml of methanol was added and stirring continued for 12 hr. The polymer (I) was then collected by filtration, extracted with methanol–pyridine (9:1 v/v) in a Soxhlet extractor for 4 hr to remove residual trityldeoxycytidine, and dried under vacuum. At this stage the polymer weighed 13.3 g and exhibited a band at 5.9 μ in the infrared.

To recover excess trityldeoxycytidine, the filtrate from the polymer reaction was concentrated and mixed with 1 l. of water and ice containing 5 ml of ammonium hydroxide. Filtration afforded 5.6 g of 5'-O-trityldeoxycytidine, identified by its melting point and infrared spectrum.

The amount of trityldeoxycytidine which had been covalently bound to the support was determined from the optical density of a solution prepared from the alkaline cleavage products. For this purpose 100 mg of polymer (I) was stirred with approximately 30 ml of 0.4 *M* sodium hydroxide in dioxane–ethanol–water (10:10:1, v/v/v) for 36 hr and then separated from the solution by filtration. That all trityldeoxycytidine had been cleaved from the polymer under these conditions was indicated by the absence of absorption characteristic of this nucleoside in the infrared spectrum of the support. The alkaline solution was neutralized with Dowex 50 (pyridinium form) and evaporated to dryness in a rotary evaporator at about 35°. Ethanol (50 ml) and ammonia (2 ml) were added and the solution again taken to dryness *in vacuo* in order to remove residual pyridine. The process was repeated three more times; then the residue was dissolved in 100 ml of ethanol which was 0.01 *M* in hydrochloric acid. The optical density at 280 mμ of 2 ml of this solution, after dilution to 10 ml, was 0.44. On the basis that the extinction coefficient at 280 mμ is 1.33×10^4, this corresponds to 78 mg (0.166 mmole) of trityldeoxycytidine bound per gram of polymer I.

Polymer II. A mixture containing 12 g of polymer–trityldeoxycytidine (I) (2.0 mmoles of bound trityldeoxycytidine), 12.5 mmoles of pyridinium β-cyanoethyl phosphate, 5.16 g (25 mmoles) of dicyclohexylcarbodiimide, and 60 ml of dry pyridine was stirred for 8 days. Water (100 ml) was added and after an additional 24-hr period of stirring the solids (polymer plus dicyclohexylurea) were separated by filtration, washed with 20% aqueous pyridine, and stirred for 3 hr with 100 ml of 1:1 cyclohexane–ethanol. Filtration and two additional washes with cyclohexane–ethanol removed the dicyclohexylurea. The polymer was then washed with methanol and ether and dried in a desiccator over phosphorus pentoxide.

Polymer III. Activation of the phosphodiester was achieved by stirring 5.5 g of the polymer (II) with 3.27 g (15 mmoles) of mesitylenesulfonyl chloride in 50 ml of pyridine for 24 hr. An additional 50-ml portion of pyridine was then added, the polymer was allowed to settle out, and the supernatant liquid was withdrawn. This process was repeated two times to remove the major portion of the excess mesitylenesulfonyl chloride. Condensation with thymidine was effected by stirring the residual slurry with 0.600 g (2.48 mmoles) of thymidine and 25 ml of pyridine for 48 hr. Methanol (50 ml) was then added and after 3 hr of stirring the mixture was filtered and the polymer washed successively with 30% aqueous pyridine, methanol, and ether.

5'-O-Trityldeoxycytidylyl-(3'–5')-thymidine. Nucleotidic material was cleaved from a 2.2-g portion of III by stirring the polymer successively with six 50-ml portions of 0.4 *M* sodium hydroxide in dioxane–ethanol–water (10:10:2.2, v/v/v). After approximately 6 hr of stirring each portion was filtered and the filtrate was neutralized with Dowex 50 resin in the pyridinium form (approximately 12 g of resin required for each portion). The resin was removed by filtration and washed with 50% aqueous pyridine and ethanol. The combined filtrates and washings were concentrated to 10–20 ml in a rotary evaporator. After addition of 50 ml of water and reconcentration, the pH was brought to 10 with ammonium hydroxide, and the mixture was centrifuged to remove the insoluble trityldeoxycytidine (56 mg, 0.12 mmole, identified by mp 232–235°, infrared spectrum, and R_f 0.86 in solvent A). The solution (~25 ml) was applied to the top of a DEAE-cellulose column and elution effected by a linear gradient of ammonium bicarbonate buffer (2.2 l. of 0.02 *M* ammonium bicarbonate buffer in the mixing vessel and 2.2 l. of 0.25 *M* ammonium bicarbonate in the reservoir). Fractions were collected approximately every 7 min. After an initial peak, due primarily to pyridine, two peaks were obtained. The tubes within each were pooled and the absorbance was determined (see Table II).

Table II. Products from Preparation of TrdCpT

Peak	Tube no.	Total vol., ml	OD_{270}[a]	OD_{270} × vol.[b]
i	62–125	400	0.42	168
ii	135–310	2400	1.20	2900

[a] OD_{270} refers to the optical density measured at 270 mμ. [b] This product gives the number of optical density units present.

Paper chromatography of a sample of i in solvent A showed one spot, R_f 0.49, positive for the trityl group. The substance is probably TrdCp. Peak ii similarly gave a single spot on chromatography in solvent A, R_f 0.78, positive for trityl. Concentration and lyophilization of ii afforded 150 mg (0.181 mmole) of trityldeoxycytidylylthymidine as a fluffy white powder. It was homogeneous in solvents A and B and on electrophoresis at pH 8.1. *Anal.*[14] Calcd for $C_{38}H_{40}N_6O_{11}P \cdot 3H_2O$: C, 55.13; H, 5.60; N, 8.46; P, 3.75. Found: C, 54.85; H, 5.61; N, 8.11; P, 4.18.

The yield may be estimated from (a) the weight of TrdCpT recovered per gram of polymer hydrolyzed or (b) the OD_{270} units in fraction peak ii relative to the number of OD_{270} units eluted from the polymer by the alkaline treatment. On the weight basis, 150 mg of TrdCpT corresponds to ~53% of the amount calculated from the number of TrdC units bound to the polymer. In terms of OD_{270} units, the units obtained in peak ii amount to 60% of the total OD_{270} units eluted from the support, including the trityldeoxycytidine collected as a solid prior to fractionation. If the reactions had been quantitative, of course, all of the OD_{270} units would have been in fraction ii.

5'-O-Trityldeoxycytidylyl-(3'–5')-thymidylyl-(3'–5')-thymidine. A sample of polymer III (3 g) was stirred with 4 mmoles of pyridinium β-cyanoethyl phosphate and 1.65 g (8 mmoles) of dicyclohexylcarbodiimide in pyridine for 7 days. By the procedure followed in preparing III, the resulting polymer was activated with 1.32 g (6 mmoles) of mesitylenesulfonyl chloride and condensed with 0.300 g (1.24 mmoles) of dry thymidine. Alkaline cleavage of 2.85 g of the polymer and work-up as in the previous case afforded 70 mg of trityldeoxycytidine and the products indicated in Table III.

Table III. Products from Preparation of TrdCpTpT

Peak	Tube no.	Total vol., ml	OD_{270}	OD_{270} × vol.
i	50–101	420	0.59	248
ii	102–140	600	1.30	858
iii	145–160	290	0.96	278
iv	163–222	850	1.30	1110
v	225–325	1360	1.15	1565

A forerun from the fractionation, containing as ultraviolet absorbing material mainly pyridine with trace amounts of thymidine and a substance which appeared to be deoxycytidine, was also obtained.

(14) The compounds were prepared for analysis by drying under vacuum over phosphorus pentoxide at room temperature. After drying they were hygroscopic, taking up moisture slowly when exposed to air, and they gave acidic solutions when redissolved in water. The theoretical values for the analyses were calculated for hydrates of the nucleotides in the acid form. The C, H, and N analyses were made by the Micro-Tech Laboratories, Skokie, Ill. Phosphorus was determined by the method of P. S. Chen, T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

Anal.[14] Calcd for $C_{48}H_{53}N_7O_{18}P_2 \cdot 7H_2O$: C, 47.88; H, 5.61; N, 8.14; P, 5.23. Found: C, 47.74; H, 5.58; N, 7.51; P, 5.47.

Paper chromatography showed that i contained thymidine as the single fluorescent material (R_f 0.70 in solvent A), ii had one component (R_f 0.48, trityl positive, probably TrdCp), iii had two components (R_f 0.1 and 0.86, both trityl free), iv had one component (R_f 0.77, trityl positive, probably TrdCpT), and v had one component (R_f 0.61, trityl positive). Concentration and lyophilization of v afforded 80 mg of TrdCpTpT as a white powder (~16% of the weight calculated on the basis of the weight of polymer hydrolyzed). The OD_{270} units in v correspond to 29% of the OD_{270} units removed from the support by the alkaline treatment.

5′-O-Trityldeoxycytidylyl-(3′-5′)-thymidylyl-(3′-5′)-thymidylyl-(3′-5′)-thymidine. This substance was prepared from a fresh batch of polymer-tritydeoxycytidine. Polymer I (8.25 g.) was phosphorylated with 10 mmoles of β-cyanoethyl phosphate and 4.54 g (22 mmoles) of dicyclohexylcarbodiimide over a period of 7 days as in the previous synthesis. Subsequent activation with 4.38 g (20 mmoles) of mesitylenesulfonyl chloride and treatment with 0.85 g (3.5 mmoles) of thymidine afforded the dinucleotide bound to the polymer (polymer type III). This sequence was repeated twice to build up the polymer-tetranucleotide derivative (7.7 g), which was then cleaved with 0.4 *M* sodium hydroxide in dioxane–ethanol–water and worked up as before. The trityldeoxycytidine recovered amounted to 82 mg. From the fractionation on DEAE-cellulose was obtained an initial fraction, which contained pyridine and the products indicated in Table IV. Paper chromatography

Table IV. Products from Polymer Support Synthesis of TrdCpTpTpT

Peak	Tube no.	Total vol., ml	OD_{270}	OD_{270} × vol.
i	50–85	315	6.25	1970
ii	86–100	200	2.3	460
iii	101–140	560	8.5	4760
iv	141–245	1420	4.2	5960
v–viii	250–420	2340		5506
ix	425–550	1900	1.65	3140

with solvent A revealed a single ultraviolet-absorbing product in i (R_f 0.70, thymidine), ii (R_f 0.59, probably deoxycytidine), iv (R_f 0.27, trityl positive, possibly TrdCpTp), and ix (R_f 0.35, trityl positive, identified as TrdCpTpTpT). Fraction iii contained a substance with R_f 0.38 and a trace amount of a material with R_f 0.78. Portions v–viii contained a total of three components with R_f values of 0.61, 0.15, and 0.04, the former two being positive for trityl and the last negative for trityl.

The yield of TrdCpTpTpT calculated from the weight of isolated material (220 mg) corresponds to 14% of the amount calculated on the basis of the amount of trityldeoxycytidine bound per gram of polymer. For comparison, the OD_{270} units in fraction ix correspond to 15% of the total OD_{270} units removed from the polymer support.

Anal.[14] Calcd for $C_{58}H_{66}N_9O_{25}P_3 \cdot 3H_2O$: C, 48.50; H, 5.05; N, 8.78; P, 6.46. Found: C, 48.53; H, 5.29; N, 8.50; P, 6.10.

5′-O-Trityldeoxycytidylyl-(3′-5′)-thymidylyl-(3′-5′)-deoxyadenosine. Essentially the same procedure was used as in the preparation of TrdCpTpT except for the cleavage step. In this case 2 g of polymer at the second phosphorylation stage was activated by 0.750 g (3.63 mmoles) of mesitylenesulfonyl chloride (24 hr) and condensed with 0.711 g (2 mmoles) of N-benzoyldeoxyadenosine (2 days). Cleavage was effected by three successive 5-hr treatments of the polymer with 100 ml of 1:1 pyridine–concentrated ammonium hydroxide on a steam bath. During the heating periods, ammonia was replenished periodically (0.5-hr intervals) by addition of 10–20 ml of ammonium hydroxide. This work was done before the cleavage method utilizing sodium hydroxide was developed. The method is inefficient in that not all the nucleotidic material is removed from the polymer support. Fractionation of the products afforded 525 OD_{270} units of TrdCpTpA, ~26% of the total of ~2000 OD_{270} units eluted from the polymer support. This material was homogeneous by all criteria applied: R_f (solvent A) 0.60; R_f (solvent B) 0.64; electrophoretic mobility relative to pdC at pH 10.8, 0.67. In the ultraviolet in 0.01 *M* HCl, water, and 0.01 *M* NaOH λ_{max} ($\epsilon \times 10^{-4}$) was 272 (2.5), 269 (2.2), and 267 (2.0), respectively, and λ_{min} ($\epsilon \times 10^{-4}$) was 239 (1.1), 246 (1.5), and 267 (1.6), respectively.

Preparation of TrdCpT on a Low-Cross-Linked Support. A 5.5-g sample of support polymer acid chloride (the support was a popcorn polymer made from styrene (90%), *p*-vinylbenzoic acid (10%), and *p*-divinylbenzene (0.02%)) was stirred with 3 g (6.5 mmoles) of 5′-O-trityldeoxycytidine in 50 ml of pyridine for 2 days. Methanol (50 ml) was added and the polymer was filtered and washed with 9:1 methanol–pyridine in a Soxhlet extractor. From the filtrate and washings was obtained 1.95 g of trityldeoxycytidine. The polymer, after drying in a desiccator over phosphorus pentoxide, weighed 6.48 g and contained 0.245 mmole of TrdC per gram of polymer.

One portion of this polymer (2.18 g) was used to build TrdCpT by the procedure previously described. In this case phosphorylation with 4 mmoles of pyridinium β-cyanoethyl phosphate and 12 mmoles of dicyclohexylcarbodiimide (7 days) afforded 2.3 g of a polymer–phosphorylated derivative. Activation (1 day) of 2.1 g of this product with 1.05 g (48 mmoles) of mesitylenesulfonyl chloride, condensation (2 days) with 0.480 g (2 mmoles) of thymidine, and cleavage of 2.12 g of the resulting derivative with 0.4 *M* sodium hydroxide in dioxane–ethanol–water (40 *m*) yielded 560 OD_{270} units (~26 mg) of trityldeoxycytidine and the fractionation products shown in Table V. Evaporation to dryness and analysis

Table V. Products from Preparation of TrdCpt on Low-Cross-Linked Support

Peak	Tube no.	Total vol., ml	OD_{270}	OD_{270} × vol.
i	8–41	450	2.5	1120
ii	60–80	320	0.6	192
iii	81–105	350	0.82	287
iv	125–410	4090	1.02	4170

of the residue from i indicated that i contained pyridine (370 OD units) and three substances with R_f 0.69 (thymidine), 0.56 (deoxycytidine), and 0.84 (probably mesitylenesulfonic acid). On paper chromatography in solvent A, ii showed three components (R_f 0.50, 0.60, 0.84), iii showed two components (R_f 0.40 and 0.84), and iv showed one component (R_f 0.73, TrdCpT). The yield of TrdCpT (240 mg of solid isolated) corresponded to 61% conversion of the polymer–TrdC to TrdCpT. Furthermore, the mixtures obtained by base treatment of the polymer was relatively simple, 77% of the OD_{270} units being in the TrdCpT fraction.

Another portion (1.0 g) of the low-crossed-linked support polymer with adjoined trityldeoxycytidine was phosphorylated by stirring with 3 mmoles of pyridinium β-cyanoethyl phosphate and 1.96 g (9 mmoles) of mesitylenesulfonyl chloride in 15 ml of pyridine for 24 hr. After centrifugation and removal of the supernatant liquid, the jelly-like polymer was hydrolyzed by stirring with 100 ml of 30% pyridine for 4 hr and then with 100 ml of 10% aqueous pyridine for 30 min on a steam bath. The polymer was then separated by filtration, washed with methanol and ether, and dried in a vacuum desiccator over phosphorus pentoxide; polymer weight, 1.01 g. Part of the resulting polymer (0.86 g) was then activated by 0.306 g (1.4 mmoles) of mesitylenesulfonyl chloride and condensed with thymidine as in the previous synthesis of TrdCpT; polymer weight recovered, 0.95 g. Cleavage of 0.90 g of this polymer and work-up as usual afforded 400 OD_{270} units of trityldeoxycytidine, 1390 OD_{270} units of TrdCpT (53% of total), and 817 OD_{270} units of other material absorbing in the ultraviolet region.

Preparation of TrdCpTpT on Low-Cross-Linked Support. The technique and procedures were the same as employed in the synthesis of TrdCpT. In the first stage 1.5 g of support polymer bearing trityldeoxycytidine (0.02% divinylbenzene in polymer; 0.245 mmole of trityldeoxycytidine per gram of polymer) was phosphorylated with β-cyanoethyl phosphate and mesitylenesulfonyl chloride in pyridine and then condensed with thymidine by means of mesitylenesulfonyl chloride. The resulting polymer slurry was mixed with 150 ml of methanol and stirred for 1 hr. The solid was then filtered off, stirred with 20% aqueous pyridine for 3 hr at room temperature and 1 hr at 90°, separated by filtration, and dried under vacuum. The second nucleotide was added to the chain by repetition of the steps used in the first stage, and the nucleotide products were removed from the final polymer (weight, 1.68 g)

by conventional treatment with 0.4 *M* sodium hydroxide. Work-up of the products afforded 21 mg of trityldeoxycytidine and the fractions listed in Table VI.

Table VI. Products from Preparation of TrdCpTpT on LowCross-Linked Support

Peak	Tube no.	Total vol., ml	OD_{270}	OD_{270} × vol.
i	6–38	430	1.62[a]	700
ii	42–105	850	1.375	1170
iii	121–200	1040	1.175	1220
iv	212–240	375	0.67	251
v	242–345	1500	1.15	1720

[a] OD after evaporation to remove pyridine and then redilution.

Paper chromatography in solvent A indicated that i was mainly thymidine with a trace of trityldeoxycytidine; ii contained two components, R_f 0.49 (probably TrdCp) and 0.71; iii was largely TrdCpT (R_f 0.73) with a trace of material with R_f 0.15; iv had one compound with R_f 0.29; and v showed one component with R_f 0.62, which was the desired TrdCpTpT. Fraction v contained 34% of the ultraviolet-absorbing material removed from the the support polymer. The OD units of TrdCpTpT correspond to 21% of the amount calculated assuming quantitative conversion of the TrdC on the polymer to TrdCpTpT.

Spectral Determinations and Enzyme Assays. For spectral determinations and enzyme assay, the oligonucleotide derivatives were dissolved in water and rechromatographed on DEAE-cellulose. Better than 90% of the solids were recovered in all cases (Table I). This purification procedure had little effect on the ϵ value for TrdCpT, but increased the ϵ values for TrdCpTpT and TrdCpTpTpT by about 10%.

Table VII. Products of Enzymatic Degradation

Compound	TrdC OD_{278} units	pT OD_{267} units	TrdC/pT Found, %	TrdC/pT Calcd, %
TrdCpt	2.45	1.9	1.1	1
TrdCpTpT	0.75	1.15	2.1	2
TrdCpTpTpT	0.60	1.35	3.1	2

Hydrolyses were carried out by incubating 0.4–1.0-mg samples of the nucleotide derivatives with 0.1 ml of an aqueous solution of snake venom phosphodiesterase for 7 hr at 37°. The enzyme solution was made up by dissolving 500 units of phosphodiesterase (Russel's viper venom, lyophilized)[15] in 2.5 ml of 0.33 *M* Tris buffer at pH 9.1. Turbidity which developed due to formation of trityldeoxycytidine was cleared by addition of two drops of pyridine and the clear solution was chromatographed on 3MM paper in solvent A. Product spots were cut out and eluted by soaking for 24 hr in 30% ethanol–water 0.01 *M* in HCl (for the phosphates) or 90% ethanol–water 0.01 *M* in HCl (for trityldeoxycytidine). Appropriate blanks were cut from other areas of the chromatograms and soaked in the same solvents. Concentrations of the nucleotides and trityldeoxycytidine were determined from differences between the absorbances of solutions resulting from elution of the spots and the blanks. Values used for extinction coefficients in these calculations were 13,200 for trityldeoxycytidine and 9600 for thymidine 5′-phosphate. A similar hydrolysis of TrdCpTpA proceeded well and afforded approximately equal amounts of trityldeoxycytidine, thymidine 5′-phosphate, and adenosine 5′-phosphate, as judged visually from the spots produced on paper chromatography.

(15) Calbiochem.

Communications to the Editor

A New Series of Four-, Five-, and Six-Coordinated Iridium Complexes

Sir:

In the course of our continuing inquiries into the determinants of activation of molecular hydrogen,[1] oxygen,[2] nitrogen,[3] and related molecules[4] by transition metal complexes, we have discovered a *cationic* Ir(I) complex, $[Ir(P\text{–}P)_2]X$ (P–P = bis(1,2-diphenylphosphino)ethane,[5] X = univalent anion), which displays a reactivity and versatility reminiscent of the notable compound, *trans*-$[IrX(CO)(Ph_3P)_2]$,[1–4,6] from which it has been synthesized (eq 1 and 2).

$$[IrCl(CO)(Ph_3P)_2] + 2P\text{–}P \longrightarrow [Ir(CO)(P\text{–}P)_2]Cl + 2Ph_3P \quad (1)$$

$$[Ir(CO)(P\text{–}P)_2]Cl \underset{}{\overset{\Delta}{\rightleftharpoons}} [Ir(P\text{–}P)_2]Cl + CO \quad (2)$$

The four-coordinated bisphosphine chelate is best prepared by a two-step synthesis involving a five-coordinated carbon monoxide adduct as intermediate (eq 1 and 2). White crystals of the latter are formed[7,8] upon mixing benzene solutions of the reactants at 25° (eq 1). Reaction 2 is carried out by heating the CO complex in a 10:1 mixture of toluene–1-propanol[9] to 100° which results in a bright orange precipitate of $[Ir(P\text{–}P)_2]Cl$.[7,8]

The crystals of the novel d^8 complex (**1**, Table I)[10] are moderately air stable, but they do react with atmospheric oxygen slowly on standing. In solution (*e.g.*, $CHCl_3$, alcohols), a conversion to a cream-colored oxygen adduct takes place within minutes (eq 3). The reaction is

$$[Ir(P\text{–}P)_2]Cl + O_2 \longrightarrow [O_2Ir(P\text{–}P)_2]Cl \quad (3)$$

accompanied by appearance of an infrared band at 845 cm^{-1} which, by analogy with $[O_2IrCl(CO)(Ph_3P)_2]$,[2] is tentatively assigned to the Ir–O_2 stretching vibration.

(1) L. Vaska, *Inorg. Nucl. Chem. Letters*, **1**, 89 (1965), and references quoted.
(2) L. Vaska, *Science*, **140**, 809 (1963).
(3) Unpublished results.
(4) L. Vaska, *Science*, **152**, 769 (1966), and references cited therein.
(5) P–P = $Ph_2PCH_2CH_2PPh_2$ throughout this paper.
(6) L. Vaska and J. W. DiLuzio, *J. Am. Chem. Soc.*, **83**, 2784 (1961).
(7) Yields, based on $[IrCl(CO)(Ph_3P)_2]$, range from 85 to 95%.
(8) The chlorides tend to be hygroscopic and acquire one to four molecules of water (on standing in air); these can be removed on heating the crystals near 90°.
(9) When CH_3OH or C_2H_5OH is used in step 2, the chelate precipitates with alcohol of crystallization.
(10) The numerals cited in text refer to the complexes given in Table I.