Synthesis of Some Monofunctional Guanosine Nucleotides[1]

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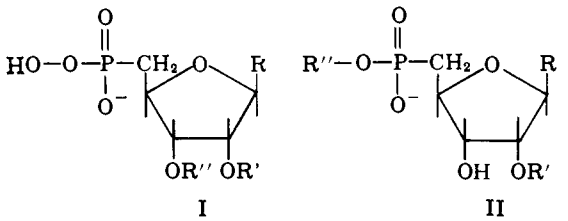
Ribonucleotides with only one reactive group are useful as chain-terminating or -initiating monomers in the chemical synthesis of polyribonucleotides and related substances. Several guanosine derivatives containing a 5'-phosphomonoester as the only functional group were synthesized including: (1) N,2',3'-O-tribenzoylguanosine-5'-phosphate by reaction of guanosine-5'-phosphate with benzoyl chloride; (2) N-benzoyl-2',3'-di-O-tetrahydropyranylguanosine-5'-phosphate by acid-catalyzed reaction of N-benzoylguanosine-5'-phosphate with dihydropyran; (3) 2',3'-O-isopropylideneguanosine-5'-phosphate by phosphorylation of 2',3'-O-isopropylideneguanosine with 2-cyanoethyl phosphate and dicyclohexylcarbodiimide; and (4) N-benzoyl-2',3'-O-isopropylideneguanosine-5'-phosphate by reaction of (3) with benzoyl chloride. The synthesis of N-benzoyl-2'-O-tetrahydropyranyl-3'-O-acetylguanosine-5'-phosphate, a nucleotide with differentially removable blocking groups, was also investigated. N-Benzoyl-2'-O-tetrahydropyranyl-5'-O-(2''-cyanoethyl)phosphorylguanosine, which contains a 3'-hydroxyl as the only reactive group, was synthesized by treating N-benzoyl-2'-O-tetrahydropyranylguanosine-5'-phosphate with dicyclohexylcarbodiimide and a large excess of 3-hydroxypropionitrile as solvent.

In addition to bifunctional nucleotides such as N-benzoyl-2'-O-tetrahydropyranylguanosine-5'-phosphate, described in the accompanying paper,[2] monofunctional nucleotides containing either a phosphomonoester group or a hydroxyl group as their only reactive group have been found useful in carbodiimide-activated polymerization reactions.[3] These substances, as chain initiators, minimize the formation of macrocyclic oligonucleotides, which otherwise form by esterification of the terminal phosphomonoester with the hydroxyl group at the other terminus. A further use of the monofunctional nucleotides lies in the synthesis of specific oligonucleotides by condensation of a monofunctional nucleotide and a monofunctional alcohol, either nucleoside or blocked nucleotide.[4]

Two types of monofunctional nucleotide are possible for use as end groups in the chemical synthesis of polyribonucleotides from 5'-nucleotide monomers: a 5'-nucleotide with both 2'- and 3'-hydroxyls blocked (I), and a 5'-nucleotide with both the 2'-hydroxyl and the phosphomonoester group blocked (II). In this report, the synthesis of a number of derivatives of Guo-5'-P[5] of the first type are described as well as a derivative of the second type.



I II

The synthesis of guanosine derivatives of the first type is simple and straightforward, utilizing ester, tetrahydropyranyl ether, or ketal protecting groups. Thus, N,2',3'-O-tribenzoyl-Guo-5'-P (I, R = N-benzoylguanine, R' = R'' = benzoyl) is the initial product in the preparation of N-benzoyl-Guo-5'-phosphate according to the method of Smith, *et al.*,[6] and can be isolated from the reaction mixture if the alkaline hydrolysis step of these workers is omitted. Similarly, N-benzoyl-2',3'-di-O-THP-Guo-5'-P (I, R = N-benzoylguanine, R' = R'' = tetrahydropyranyl) is easily prepared by the acid-catalyzed reaction of dihydropyran with N-benzoyl-Guo-5'-P, analogous to the synthesis of N-benzoyl-2'-O-tetrahydropyranyl-Guo-3',5'-cyclic phosphate.[2] Synthesis of 2',3'-O-isopropylidene-Guo-5'-P (I, R = guanine, R' + R'' = isopropylidene) has also been accomplished by reaction of the readily available 2',3'-O-isopropylideneguanosine with 2-cyanoethyl phosphate,[7] followed by removal of the cyanoethyl group using alkaline hydrolysis under conditions where the isopropylidene group is stable.

Synthesis of monofunctional 5'-nucleotides like I, where R' and R'' differ, would enable selective removal of one or the other of such groups from the terminal nucleotide after polymerization or after specific coupling reactions have been carried out. The unblocked hydroxyl could then be reacted further as, for example, in chain-lengthening with specific nucleotides.[8] A preliminary experiment, on the 0.4-μmole level, has been carried out to test the feasibility of such syntheses. Triethylammonium N-benzoyl-2'-O-THP-Guo-5'-P[2] was treated with acetic anhydride in anhydrous dioxane. Paper chromatography of the product mixture showed 80% removal of starting material. However, two new nucleotide derivatives were observed which have

(1) This work was supported by grants from the National Institutes of Health to J. R. Fresco.

(2) D. B. Straus an J. R. Fresco, *J. Am. Chem. Soc.*, 87, 1364 (1965).

(3) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, pp. 115–117.

(4) Some examples in regard to oligoribonucleotides include: (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, 84, 430 (1962); (b) D. H. Rammler and H. G. Khorana *ibid.*, 84, 3112 (1962); (c) R. Lohrmann and H. G. Khorana, *ibid.*, 86, 4188 (1964); (d) F. Cramer and K. H. Scheit, *Angew. Chem.*, 74, 717 (1962); (e) F. Cramer, R. Witmann, K. Daneck, and G. Weimann, *ibid.*, 75, 92 (1963); (f) J. Smrt and F. Sorm, *Collection Czech. Chem. Commun.*, 27, 73 (1962).

(5) Abbreviations for polynucleotides are those given by Khorana (ref. 3, pp. 93–95). Also, Guo is the abbreviation used for guanosine (see IUPAC, Tentative Rules, Abbreviations and Symbols for Chemical Names of Special Interest in Biological Chemistry, *J. Biol. Chem.*, 237, 1381 (1962), paragraph 5.4), THP for the tetrahydropyranyl group, P for the phosphomonoester group, DCC for N,N'-dicyclohexylcarbodiimide, and DEAE for diethylaminoethyl.

(6) M. Smith, G. I. Drummond, and H. G. Khorana, *J. Am. Chem. Soc.*, 83, 691 (1961).

(7) G. M. Tener, *ibid.*, 83, 159 (1961).

(8) For example, G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, 85, 3835 (1963), and references therein.

not been characterized. The degree of reaction and the limited number of products suggest that this approach holds promise for preparation of monofunctional derivatives of Guo-5′-P with selectively removable blocking groups.

Synthesis of nucleotides of type II, containing a 3′-hydroxyl as the only functional group, is inherently more complicated than preparation of derivatives of type I since different blocking groups are required for the 2′-hydroxyl and the phosphomonoester groups. The most difficult problem, that of selectively blocking the 2′-hydroxyl, has been solved in the case of the guanosine nucleotides with the synthesis of N-benzoyl-2′-O-THP-Guo-5′-P,[2] but it remained to block the phosphomonoester group of this derivative. Two different ester blocking groups have been reported for protecting phosphomonoesters. The benzhydryl esters of some 3′-nucleotides have been synthesized by Cramer, *et al.*,[9] and such a protected uridylic acid was used in the synthesis of UpUp.[4d] Alternatively, the phosphomonoester can be further esterified with 3-hydroxypropionitrile to form the 2-cyanoethyl phosphodiester as reported by Weimann, *et al.*[8] Of the two direct methods of phosphate esterification, one involving the reaction of nucleotide with DCC and a stoichiometric quantity of alcohol[7,8,10] and the other using DCC and a large excess of alcohol,[7,10,11] the latter appeared more attractive in the present work because it gives very high yields without the severe cation and solvent restrictions of the DCC-stoichiometric alcohol method.[10]

Reaction of triethylammonium N-benzoyl-2′-O-THP-Guo-5′-P with a 5800-fold molar excess of 3-hydroxypropionitrile and DCC with no other solvent gave a product identified as the desired N-benzoyl-2′-O-THP-5′-O-(2′′-cyanoethyl)phosphorylguanosine (II, R = N-benzoylguanine, R′ = tetrahydropyranyl, R′′ = 2-cyanoethyl). This product was contaminated by 5–10% of an unidentified nucleotide derivative that did not separate from the main product by electrophoresis at pH 8.5 or by chromatography on DEAE cellulose (HCO_3^-), indicating ionic properties quite similar to the main product. However, on paper chromatography, in two solvents, the side product migrated near the solvent front, well separated from the main product. Further characterization of the side product was not attempted.

Alkaline hydrolysis of the product mixture gave 2′-O-THP-Guo-5′-P as expected,[12] but the main product of hydrolysis with 0.01 *N* acetic acid was N-benzoyl-Guo-5′-P, not N-benzoyl-5′-O-(2′′-cyanoethyl)phosphorylguanosine as anticipated, though the latter was identified as a minor product of the acid hydrolysis. Thus, the cyanoethyl phosphodiester is readily hydrolyzed by both very weak acid and alkali.[7,8,13] Acid hydrolysis should be advantageous in unblocking terminal phosphomonoester groups of alkaline labile polyribonucleotides containing this protecting group, and use of acid hydrolysis might well be examined as a means of overcoming the problem of overlapping rates of hydrolysis of acyl- and cyanoethyl-protecting groups in selective removal of such groups in the synthesis of specific polydeoxynucleotides.[13]

The stability of protecting groups must be considered in choosing monofunctional nucleotides for polynucleotide synthesis. The O-benzoyl substituent is difficult to maintain, even at low temperatures, when the pH of aqueous solutions is greater than 9. This complicates use of such protected nucleotides since higher pH is encountered during work-up of fractions from ion-exchange chromatography using triethylammonium bicarbonate for elution and also in several paper chromatographic solvent systems. The stability of the di-O-tetrahydropyranyl nucleotide to acid is similar to the mono-O-tetrahydropyranyl-Guo-5′-P derivatives which have been studied.[2] Thus, both tetrahydropyranyl groups are quantitatively hydrolyzed in 2 hr. at 100° at pH 4. The precautions described in the accompanying paper[2] for maintaining N-benzoyl-2′-O-THP-guanosine nucleotides apply equally to maintaining 2′,3′-di-O-THP-guanosine nucleotides. The 2′,3′-O-isopropylidene group is quite stable under the conditions of work-up of 2′,3′-O-isopropylidene-Guo-5′-P. However, the conditions for complete removal of the isopropylidene group, hydrolysis for about 60 min. at 100° and pH 3.25,[14] are close to those reported to bring about migration of phosphodiesters from $C_{3'} \rightarrow C_{2'}$ (*cf.* ref. 4a) which would preclude use of this protecting group in polyribonucleotide syntheses unless conditions for isopropylidene hydrolysis could be devised where no phosphodiester migration occurs.

Experimental

Materials. Guanosine-5′-P and 2′,3′-O-isopropylideneguanosine were obtained from the California Corporation for Biochemical Research. N-Benzoyl-Guo-5′-P was synthesized using the method of Smith, *et al.*[6] The 2-cyanoethyl phosphate was prepared by the method of Tener.[7] Pyridine, dimethyl sulfoxide, dihydropyran, dioxane, DEAE cellulose, and triethylammonium bicarbonate were prepared as described previously.[2] All other reagents were of the highest commercial grade obtainable and used without further purification.

General Methods. As in previous work, new compounds were identified by their chromatographic, electrophoretic, and spectral properties coupled with analysis for total phosphorus, and derivatization to known compounds.

Ultraviolet spectra, routine determinations of absorbance and other colorimetric analyses, and total phosphorus analyses were performed as described previously.[2]

Molar extinction coefficients were determined on the basis of absorbance and the concentration of phosphorus in the spectral sample. Above 250 mμ, extinction coefficients of the compounds studied were close to those of the purine chromophore, *i.e.*, at 260 mμ, 11.8×10^3 for guanosine[15] and 17.1×10^3 for the N-benzoylguanosine chromophore.[2]

(9) F. Cramer, H. Neunhoeffer, K. H. Scheit, G. Schneider, and J. Tennigkeit, *Angew. Chem.*, 74, 387 (1962).
(10) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, 80, 6204 (1958).
(11) D. B. Straus and E. Goldwasser, *Biochim. Biophys. Acta*, 47, 186 (1961).
(12) The side product was also completely converted to new substances which were not identified.
(13) H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, 85, 3841 (1963).
(14) A. Hampton and M. H. Maguire, *ibid.*, 83, 150 (1961).
(15) W. Cohn, *Methods Enzymol.*, 3, 740 (1957).

Samples of the synthesized guanosine nucleotide derivatives were hydrolyzed with 2 *N* NH_4OH and 0.01 *N* acetic acid and then with both base and acid as previously described,[2] and the hydrolysates chromatographed on paper along with appropriate standards. Table I gives R_f values and relative electrophoretic mobilities of various compounds studied in this work.

Table I. Paper Chromatography and Electrophoresis of Various Compounds

Substance[c,d]	R_f Solvent[b] A[e]	R_f Solvent[b] B	R_M[a]
Guo-5'-P	0.03	0.07	1.00
Benzoic acid	0.84	0.67	
N,2',3'-O-Tri-Bz-Guo-5'-P	0.60	0.83	
N-Bz-2',3'-Di-O-THP-Guo-5'-P	0.50	0.62	
N-Bz-2'-O-THP-Guo-5'-P	0.34	0.54	0.92
2',3'-O-Isopropylidene-Guo	0.67		
2',3'-O-Isopropylidene-Guo-5'-P	0.19		
N-Bz-2'-3'-O-Isopropylidene-Guo-5'-P	0.40		
N-Bz-2'-O-THP-3'-O-Acetyl-Guo-5'-P[f]	0.45		0.73
2'-O-THP-Guo-5'-P	0.16	0.28	0.88
2'-O-THP-5'-O-(2''-CNEth)Phos-Guo[f]	0.38	0.45	
N-Bz-5'-O-(2''-CNEth)Phos-Guo[f]	0.48	0.35	0.66
N-Bz-2'-O-THP-5'-O-(2''-CNEth) Phos-Guo	0.54	0.63	0.73

[a] Electrophoresis was carried out as described.[2] The buffer was 0.05 *M* ammonium acetate, pH 8.5, and electric fields of about 40 v./cm. were applied for 2 hr. The reference substance was Guo-5'-P. [b] Compositions were as follows: solvent A, 7:1:2 2-propanol–concentrated NH_4OH–H_2O; solvent B, 5:2 2-propanol–0.5 *M* ammonium acetate, pH 6.0. Chromatograms were run descending. [c] The abbreviations used in the table are as follows: Guo- and -Guo for guanosine, -Bz- for benzoyl, -THP- for tetrahydropyranyl, 2''-CNEth for 2''-cyanoethyl, Phos for phosphoryl, and P for phosphate as the appropriate phosphomonoester. [d] Materials were visualized with ultraviolet light (2537 Å.). Guanosine derivatives appeared as dark blue spots from light absorption and N-benzoylguanosine derivatives showed a pink fluorescence. [e] Benzoyl esters and amides are partially hydrolyzed during chromatography in solvent A. [f] Tentative.

Benzoyl substituents were spectrophotometrically determined by first taking to dryness in a centrifuge tube (with the aid of a gentle stream of dry air) a volume of sample containing 0.5–2 μmoles of benzoyl substituent. The dry residue was mixed with 1.00 ml. of 0.30 *N* NaOH and the tube was sealed and incubated at 100° for 60 min.; after cooling, the hydrolysate was carefully mixed with 0.40 ml. of 1 *N* HCl bringing the pH of the solution to about 1; slight precipitation of the sparingly soluble benzoic acid occurred. The acidified hydrolysate was extracted six times with 2–3 ml. volumes of ether, the ether layers being quantitatively transferred to another container. After removal of the ether by evaporation, the residue was dissolved in 5.00–25.00 ml. of 0.01 *N* NaOH and the absorbance at 225 mμ was determined (λ_{max} for benzoate, ϵ 8.4 × 10^3). The nucleotide which remains in the aqueous layer after ether extraction was also determined spectrophotometrically.

N,2',3'-O-Tribenzoylguanosine-5'-phosphate. An aqueous solution of pyridinium Guo-5'-P containing 200 μmoles was mixed with pyridine and taken to dryness under high vacuum. The dry residue was dissolved in 3 ml. of anhydrous pyridine, 0.5 ml. of benzoyl chloride was added, and the reaction mixture was placed in the dark. After 2 hr., the pale yellow solution containing a small amount of white precipitate was mixed with 10 ml. of H_2O, whereupon it turned milky, an oil separating in a few minutes. This mixture was extracted three times with 40-ml. volumes of chloroform. The solvent was evaporated leaving a pink oil which changed to a white water-insoluble precipitate which dissolved readily in ether or methanol. Chromatography of a methanolic solution of the product in solvents A and B showed one nucleotide spot but also a benzoic acid spot. Attempts to remove the benzoic acid contaminant by extraction of the residue with water were not completely successful. Some benzoate also entered the water phase, and nucleotide solubility was low. Hence, the product after this step was still contaminated with benzoic acid, showing a ratio of benzoate:total P of 4.5 instead of 3.0. The yields of N-benzoylguanosine nucleotide, based on $A_{290m\mu}$, where neither benzoic acid nor benzoate absorbs, were quantitative after ether extraction and 68% after the purification with water.

The water-solubilized nucleotide product was next acidified to pH 4 in about 200 ml. and the resulting suspension was extracted five times with 200-ml. volumes of ether. The ether extracts were found to contain benzoic acid but no nucleotide, while the slightly turbid water layer contained all the nucleotide and no benzoic acid. Paper chromatography indicated two new N-benzoylguanosine nucleotides of lower R_f in addition to the original product. The evident hydrolysis of benzoyl groups during this purification step, where pH was kept between 4 and 7 except for a few minutes between 2.5 and 4, was surprising. These problems precluded further work with this compound.

N-Benzoyl-2',3'-di-O-tetrahydropyranylguanosine-5'-phosphate. Anhydrous ammonium N-benzoyl-Guo-5'-P (170 μmoles) was rapidly mixed with 5 ml. each of freshly distilled dimethyl sulfoxide and dihydropyran so as to minimize contact with atmospheric moisture, and the reaction was started by the addition of 700 μmoles of anhydrous HCl dissolved in dry dioxane. The reaction mixture was tightly sealed and allowed to stand overnight at 40°. The brown reaction mixture was cooled to 0°, and 800 μmoles of NH_4OH was added.[16] Under high vacuum, the unreacted dihydropyran was rapidly removed and the dimethyl sulfoxide was largely removed in 5 hr. The resulting brown gum was dissolved in 10 ml. of 70% aqueous methanol and 5 ml. of an aqueous suspension of lithium Dowex-50W added (*ca.* 3 mequiv.). The mixture was filtered and the resin washed thoroughly with H_2O. The filtrate and washes, containing both the lithium nucleotide product, LiCl, and some dimethyl sulfoxide, were taken to dryness and then dissolved in 17 ml. of methanol. Paper chromatography of the crude product in solvent A showed about 95% of the nucleotide material in a new spot, and the remainder was divided between two spots, one of which migrated like N-benzoyl-2'-O-THP-Guo-5'-P, but probably consisted of a mixture of the 2'- and 3'-THP derivatives; the other spot was not

(16) If the base is added at room temperature, hydrolysis of the benzoyl group occurs.[2]

identified. Hydrolysis with 0.01 *N* acetic acid yielded N-benzoyl-Guo-5′-P exclusively, and Guo-5′-P was the only product when both NH_4OH and acetic acid hydrolyses were used. The yield of N-benzoylguanosine derivative, based on A_{290}, was 153 μmoles.

The methanol solution of the crude product was diluted with H_2O to 21% methanol and the slightly turbid solution was passed through a 2 × 5 cm. column of DEAE cellulose (HCO_3^-). The column was washed with 300 ml. of H_2O, and then eluted with 0.027 *M* triethylammonium bicarbonate buffer, pH 7.5, until the eluate was free of chloride. The column was then eluted with 0.2 *M* triethylammonium bicarbonate, pH 7.5, and the fractions containing the nucleotide material were taken to dryness and repeatedly coevaporated with water at 20° and pressure of 0.1 torr until no more CO_2 boiled off from concentrated solutions.[17]

The purified product was chromatographed at the 0.2-μmole level in both solvents A and B. In the former a single pink fluorescing spot was seen but in solvent B a trace of N-benzoyl-2′(3′)-mono-O-THP-Guo-5′-P was also revealed; this difference between the two solvents might be due to a slight hydrolysis of the product in solvent B. None of the high R_f material seen in chromatograms of the crude product was found. Hydrolysis of the product with 0.01 *N* acetic acid under the standard conditions gave N-benzoyl-Guo-5′-P as the only product and Guo-5′-P resulted from combined acid and alkaline hydrolyses. The spectrum of the product above 250 mμ is identical with that of other N-benzoylguanosine nucleotides studied.[18] The recovered yield of product, based on A_{260} or total phosphorus, was 126 μmoles (74%).

2′,3′-O-Isopropylideneguanosine-5′-phosphate. 2′,3′-O-Isopropylideneguanosine (161 mg., 0.5 mmoles) was mixed with 5 ml. of 50% aqueous pyridine and 5 ml. of 0.4 *M* pyridinium 2-cyanoethyl phosphate in aqueous pyridine[7] and taken to dryness. The solid was mixed with 5 ml. of anhydrous pyridine, taken to dryness, and the coevaporation repeated three times. Finally, the residue was dissolved in 5 ml. of anhydrous pyridine and 1 g. (4.8 mmoles) of DCC was added. After 20 hr. at room temperature, 0.5 ml. of H_2O was added and the reaction mixture was allowed to stand 30 min. The mixture, containing a small amount of crystalline dicyclohexylurea, was taken to dryness and mixed with 10 ml. of H_2O; this aqueous mixture was filtered, and the residue was washed thoroughly with H_2O. The pooled filtrate and washes (20 ml.) were adjusted to pH 7.5 with dilute $Ba(OH)_2$ and mixed with 80 ml. of absolute ethanol to precipitate barium phosphates; the mixture was filtered, and the residue was washed three times with 10-ml. volumes of 80% ethanol. Chromatography of an aliquot of the combined filtrate and washes showed a single ultraviolet-absorbing spot (R_f 0.55) in solvent A, which is presumably 2′,3′-O-isopropylidene-5′-O-(2′′-cyanoethyl)-phosphorylguanosine.

This ethanolic solution was taken to dryness, the residue was dissolved in 20 ml. of H_2O and taken to dryness again, and the final residue was dissolved in 70 ml. of 0.4 *N* LiOH and the clear yellow solution kept under reflux for 1 hr. After cooling to room temperature, the mixture was filtered free of acrylic polymers[19] and the filtrate neutralized by careful addition of Dowex-50W H^+ to pH 6.8. This solution of lithium 2′,3′-O-isopropylidene-Guo-5′-P chromatographed as a single spot in solvent A and yielded Guo-5′-P exclusively after hydrolysis for 90 min. at pH 3.25, 100°.[14] The absorption spectrum of the protected product was identical with that of Guo-5′-P and the yield based on A_{260} was 448 μmoles (90%).

N-Benzoyl-2′,3′-O-isopropylideneguanosine-5′-phosphate. Pyridinium 2′,3′-O-isopropylidene-Guo-5′-P (100 μmoles) was rendered anhydrous by coevaporation with anhydrous pyridine and suspended in 1.5 ml. of this solvent. Benzoyl chloride (0.25 ml., 2.16 mmoles) was added with mixing, but a homogeneous reaction mixture did not result. The mixture was stored in the dark for 2 hr. with periodic mixing, during which time brown and yellow solids were deposited. Additional benzoyl chloride (0.25 ml.) was added and the mixture was kept another 1.5 hr. before the reaction was stopped by addition of 5 ml. of H_2O. This gave a dark yellow solution from which an oil separated. This mixture was extracted three times with 20-ml. volumes of chloroform. The combined chloroform extracts were washed twice with 10-ml. volumes of water and taken to dryness. The residue was suspended in water and taken to dryness again to remove traces of chloroform, and then resuspended in 10 ml. of H_2O and extracted twice with 10-ml. volumes of ether to remove benzoic acid. Chromatography showed that these aqueous and ether extracts contained no nucleotide material. The aqueous suspension of the chloroform-soluble material was then taken to dryness to eliminate traces of ether, and the residue containing the pure N-benzoyl-2′,3′-O-isopropylidene-Guo-5′-P was dissolved in 10 ml. of methanol. A single pink fluorescing spot (R_f 0.40) was found in solvent A, and the expected products were formed exclusively on hydrolysis with NH_4OH (2′,3′-O-isopropylidene-Guo-5′-P), acetic acid, pH 3.2 (N-benzoyl-Guo-5′-P), and both base and acid (Guo-5′-P). Yield was essentially quantitative.

Attempted Synthesis of N-Benzoyl-2′-O-tetrahydropyranyl-3′-O-acetylguanosine-5′-phosphate. Triethylammonium N-benzoyl-2′-O-THP-Guo-5′-P,[2] (0.4 μmole) was dried under high vacuum and mixed with 0.4 ml. of anhydrous dioxane; after 30 min. at 50°, most of the solid had dissolved, and 2 μl. (about 20 μmoles) of acetic anhydride was added. By 4 hr. at 50° all solid had disappeared, and after 8 hr. a small amount of white needles were deposited; no other changes were observed up to 26 hr., when the reaction was terminated by the addition of 1 ml. of methanol. After another hour, the solvents and methyl acetate were evaporated under high vacuum. This treatment

(17) Because of the very slow removal of dimethyl sulfoxide at low temperatures,[2] for larger reactions requiring more of this solvent the anion-exchange purification step should be used directly after dihydropyran removal (5 min. *in vacuo*). Conversion of the crude product to the lithium salt, soluble in methanol, is convenient for storage, but this step can be eliminated if the product is to be purified and used immediately after synthesis.

(18) The weak absorption band around 240 mμ, which appears as a shoulder in the spectrum of N-benzoyl-Guo-3′,5′-cyclic phosphate (ref. 2, Figure 1) with A_{238}/A_{260} = 0.83, shows increased intensity for N-benzoyl-2′-O-THP-Guo-5′-P, A_{238}/A_{260} = 0.85, and is still more pronounced in N-benzoyl-2′,3′-di-O-THP-Guo-5′-P, A_{238}/A_{260} = 0.90.

(19) Any 2-cyanoethyl phosphate present would be hydrolyzed and the resulting insoluble lithium phosphate would be removed here. Also, if the product nucleotide was hydrolyzed, the orthophosphate should be removed similarly, but chromatography showed that no guanosine or 2′,3′-O-isopropylideneguanosine was formed in the hydrolysis.

with methanol was repeated, and the residue was then suspended in 1 ml. of methanol and 0.05 ml. of triethylamine, which was removed under high vacuum. This residue was dissolved in H_2O and examined by paper chromatography (solvent A), paper electrophoresis, and by the standard hydrolytic derivatization reactions.

Approximately 80% of the starting material reacted to give two products, separated in solvent A (R_f 0.10 and 0.45, compared with 0.34 for starting material) in the ratio of about 2:1. On paper electrophoresis all nucleotidic material moved in a long spot slightly slower than the starting material (Table I) which corresponded, nevertheless, to a dianion. Hydrolysis with NH_4OH gave Guo-5′-P and 2′-O-THP-Guo-5′-P, again in about 2:1 ratio; with HOAc yielded unchanged R_f 0.10 material as well as N-benzoyl-Guo-5′-P; and with both acid and base gave Guo-5′-P. These results suggested that either the THP group was partially removed during the reaction or work-up or that hydrolysis of this group was very much slower in the R_f 0.10 material than in the starting nucleotide. The changes in the R_f 0.45 material are consistent with its being the desired N-benzoyl-2′-O-THP-3′-O-acetyl-Guo-5′-P, but this identification can only be considered tentative.

N-Benzoyl-2′-O-tetrahydropyranyl-5′-O-(2′′-cyanoethyl)phosphorylguanosine. An aqueous solution containing 36 μmoles of triethylammonium N-benzoyl-2′-O-THP-Guo-5′-P was rendered anhydrous and the dry solid mixed with 14 ml. (208 mmoles) of 3-hydroxypropionitrile giving a clear solution. A mixture of this solution with 72 mg. (350 μmoles) of DCC was agitated for 5 min. to dissolve the reagent and then allowed to stand 96 hr. at room temperature. The reaction was stopped by the addition of 16 ml. of H_2O. Because dicyclohexylurea is appreciably soluble in aqueous 3-hydroxypropionitrile, the reaction mixture was then diluted to about 200 ml. with H_2O, filtered, and the residue thoroughly washed with water. The filtrate and washes were mixed with an aqueous suspension of several grams of DEAE cellulose (HCO_3^-) (to bind the nucleotides) which was then filtered. The DEAE cellulose was washed with water until the filtrate no longer gave a precipitate when mixed with excess NaOH, indicating the absence of 3-hydroxypropionitrile, and then eluted with 0.1 *M* triethylammonium bicarbonate until the absorbance at 260 mμ fell to a low value.[20] The buffer was removed in the usual way, and the product was dissolved in H_2O and characterized.

Two spots of N-benzoylguanosine derivatives were observed on paper using solvent A, the major one (80% of total nucleotide present) at R_f 0.53 and another at R_f 0.79. A small amount of a substance, tentatively identified as 2′-O-THP-5′-O-(2′′-cyanoethyl)phosphorylguanosine, was also observed at R_f 0.38; this material probably arose by hydrolysis of the benzoyl group during removal of triethylammonium bicarbonate. The principal product resulting from NH_4OH hydrolysis of the aqueous product mixture was 2′-O-THP-Guo-5′-P, though traces of other nucleotides were also observed (also see ref. 13). With acetic acid hydrolysis, the principal product was N-benzoyl-Guo-5′-P; a new substance, in small amounts, tentatively identified as N-benzoyl-5′-O-(2′′-cyanoethyl)-phosphorylguanosine was also detected. As expected Guo-5′-P was the product of sequential acid and alkaline hydrolyses. Paper electrophoresis at pH 8.5 also showed that all nucleotide material in the product mixture was diesterified. The spectrum of the aqueous product mixture was close to that of the other N-benzoylguanosine derivatives studied, though the A_{290}/A_{260} ratio was reduced slightly indicating about 10% hydrolysis of the N-benzoyl group, in accord with the chromatographic results. The yield of total nucleotide, of which about 80% was the desired compound, based on total phosphorus was 18.9 μmoles (53%).[21] Since no starting material was detected in these characterizations, all losses must have occurred in purification steps.

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(20) The elution of the nucleotide product in this experiment was inefficient due to the relatively low anion concentration of the eluent, and to the uniform distribution of nucleotide on the exchanger occuring in the batchwise sorption procedure. In later syntheses this step was carried out on columns with resultant increased efficiency and higher recovered yields.

(21) In another synthesis, identical with this one except for column rather than batchwise purification on DEAE cellulose,[20] an 80% yield of diesterified nucleotide was recovered.

Communications to the Editor

Fluorenyllithium–Lewis Base Complexes

Sir:

It has been known for some time from anionic polymerization[1-4] and other studies[5,6] that organolithium compounds complex with Lewis bases and the reactivity of the resulting complexes is significantly different from that of the respective uncomplexed RLi. However, little

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