CHEMICAL SYNTHESIS OF GUANOSINE OLIGORIBONUCLEOTIDES

CHEMICAL SYNTHESIS OF GUANOSINE OLIGORIBONUCLEOTIDES: THE SYNTHESIS OF N^2-BENZOYL-2'-O-TETRAHYDROPYRANYLGUANOSINE AND N^2-BENZOYL-2'-O-TETRAHYDROPYRANYL-5'-O-TRIPHENYLMETHOXYACETYLGUANOSINE

By

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SCOPE AND CONTENTS:

Synthesis of oligoribonucleotides of defined sequence and length corresponding to regions within biologically active RNA species is an attractive proposition. The specific objective is the synthesis of oligoribonucleotides corresponding to the amino acyl synthetase acceptor stem region of yeast $tRNA^{ala}$. This sequence contains many guanosine residues but guanosine derivatives necessary for incorporation into a general synthesis were not at hand. The synthesis of N^2-benzoyl-2'-O-

tetrahydropyranylguanosine (VI) and N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanosine (VIII) are carried out. Their utilization in the synthesis of guanylyl(3'-5')guanosine (GpG) is described. These precursors, VI and VIII, can now participate in a fragment coupling synthesis of the duplex corresponding to the acceptor stem region of yeast $tRNA^{ala}$.

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ABBREVIATIONS

A = adenosine

ala = alanine

bz = benzoyl

C = cytidine

Cl_3Et = 2,2,2-trichloroethyl

D = dihydrouridine

DCC = dicyclohexylcarbodiimide

DMF = N,N-dimethylformamide

DMSO = dimethylsulfoxide

fmet = N-formylmethionine

G = guanosine

Gp = 3' guanylic acid

pG = 5' guanylic acid

$m^{2'}G$ = 2'-O-methylguanosine

m^7G = N^7-methylguanosine

m_2^2G = N^2-dimethylguanosine

I = inosine

meOac = methoxyacetyl

MMTr = monomethoxytriphenylmethyl

N(O, O-ME) = 2', 3'-O-methoxyethylidine derivative of nucleoside

p = phosphate

p.c. = paper chromatography

p.m.r. = proton magnetic resonance

mRNA = messenger ribonucleic acid

tRNA = transfer ribonucleic acid

$tRNA^{ala}$ = tRNA specific for amino acid alanine

ala-$tRNA^{ala}$ = $tRNA^{ala}$ esterified ('charged') with alanine

aa-tRNA = amino acylated tRNA

RNase = ribonuclease

Ψ = pseudouridine

Syn^{ala}(yeast) = alanyl tRNA synthetase from yeast

T-1 = ribonuclease T-1 specific for guanosine residues.

thp = tetrahydropyranyl

tlc. = thin layer chromatography

TMS = tetramethylsilane

TPS = tri-isopropylbenzenesulfonyl chloride

trityl = triphenylmethyl

trOac = triphenylmethoxyacetyl

U = uridine

To condense the huge structural formulae necessary to describe protected oligoribonucleotides and nucleosides, a shorthand method suggested by the IUPAC-IUB Commission on Biochemical Nomenclature is adopted (Biochem, 9, 4022 (1970)). For example, N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanylyl(3'-2,2,2-trichloroethyl-5')N^2-benzoyl-2'-O-tetrahydropyranylguanosine can be written as trOac-G^{bz}(thp)-p(Cl_3Et)G^{bz}(thp)-OH.

HISTORICAL INTRODUCTION

Attention is first directed to the structure and function of the tRNA molecule. The second section will serve to characterize the procedures of oligoribonucleotide synthesis that have been developed.

1.1. THE tRNA MOLECULE--STRUCTURE

In 1958, Hoagland (1) demonstrated that protein biosynthesis involved an RNA containing fraction of estimated molecular weight 20,000. This fraction was described to bind amino acids in their activated state which could then be precipitated, stored, and would later directly donate their amino acids to protein when added to an in vitro system containing microsomes. This activated amino acid intermediate could be deactivated by mild KOH treatment or RNase treatment, indicating that the amino acid was attached by a base labile bond to some functional group on RNA.

Work on this transfer RNA (tRNA) fraction revealed that it was a heterogenous mixture of species. Purification into species of tRNA that would accept only one amino acid could be accomplished, and in 1965, Holley (2) elaborated the primary sequence of the nucleoside bases in a yeast tRNA which would carry the activated amino acid alanine (Fig. 1).

Several such species of tRNA from different organisms have now been sequenced; all have a sequence length of between 70 to 90 nucleotides (3). These can all be arranged having a 'cloverleaf' secondary structure of hydrogen bonding between the bases C and G, and

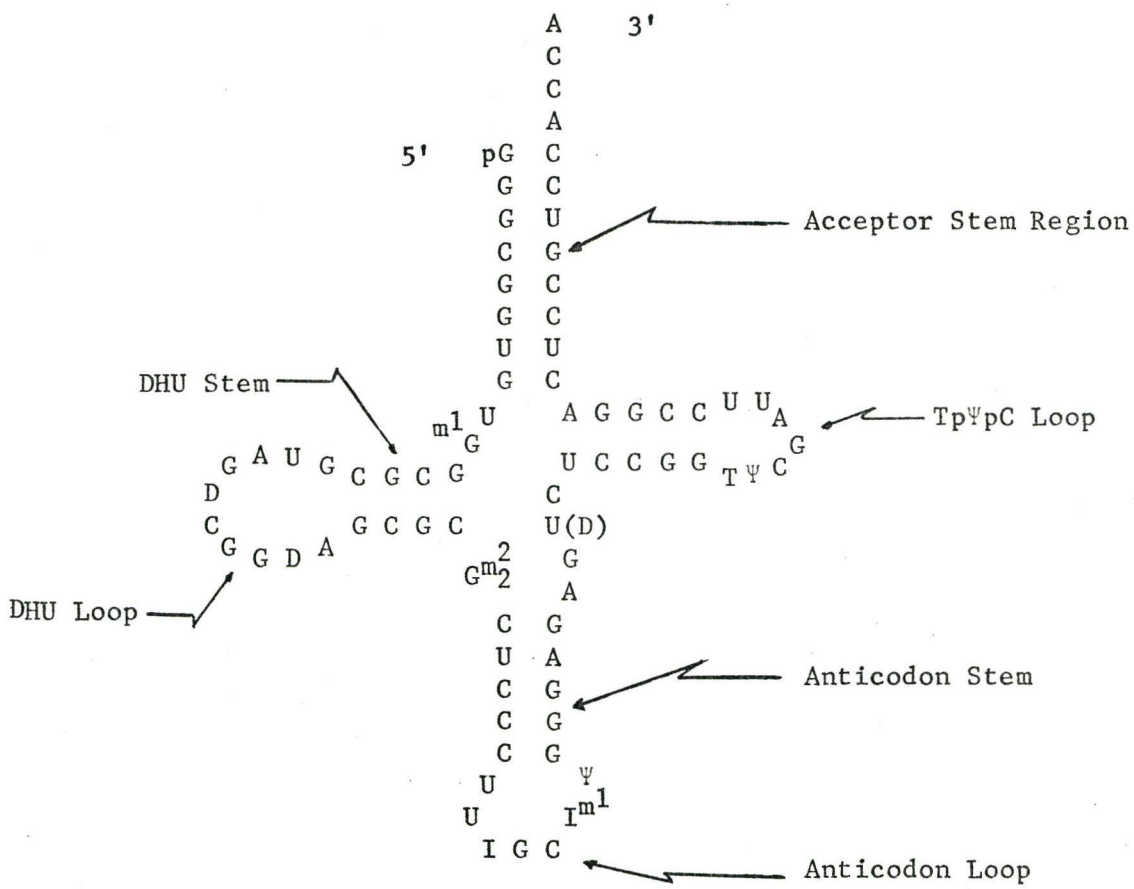


Figure 1: Primary structure of yeast $tRNA^{ala}_{Iab}$ (2)

A and U. The 3' terminal adenosine is the one esterified to the amino acid; the anticodon triplet which can hydrogen bond with a complementary codon on a messenger RNA, is contained in the center loop region.

Evidence has been gleaned from X-ray work that the arms of this cloverleaf are folded over one another to give a compact molecule 80 Å by 35 Å by 25 Å (4). The exact nature of the folding has not been elucidated as yet, but evidence from chemical modification, photochemical studies, and partial enzyme digests indicate that the anticodon region is exposed at one end of the molecule, and that the 3' terminal CpCpA is exposed at the other end; the TpψpC sequence is imbedded inside the molecule and the DHU loop is folded to bring the 4-thiouridine residue at position 8 near a cytidine residue at position 13 in several species of tRNA (4).

Regions that are looped in the 'cloverleaf' secondary structure are found to contain many modified nucleosides. The DHU loop has dihydrouridine residues in most tRNA's sequenced; the pseudouridine in the TpψpC sequence is ubiquitous. The purine on 3' edge of the anticodon is modified in many tRNA species; frequently, methylated minor bases also occur in the single strands of the molecule (5).

As a gene product, tRNA have longer sequences than the mature species; the sequence of these additional nucleotides on pre-tRNA of E. Coli. for tyrosine has been elucidated (6). Also, the primary sequence of pre-tRNA consists of only the regular four ribonucleosides; viz, guanosine, adenosine, cytidine, and uridine. The first interactions of this pre-tRNA must then be with maturation enzymes;

these interactions may occur in a species specific or a species non-specific manner.

1.2. THE tRNA MOLECULE--FUNCTION

1.2.1. Some Species Non-specific Interactions of tRNA

A region in the 3'-quarter of all tRNA's so far sequenced has the TpΨpC sequence. The specific modification on the tRNA molecules in this region would require that the maturation enzymes involved recognize the molecule as a tRNA plus some structural feature that is general for tRNA (3).

In order for a tRNA to be esterified with an amino acid, the 3' terminal CpCpA sequence must be intact. A CpCpA nucleotidyl transferase that reversibly cleaves and adds this sequence to the 3' terminus of tRNA is found to interact with several species of tRNA. The yeast enzyme was found to catalyze the incorporation of ATP and CTP into the terminal sequence of 3' quarter molecules of yeast $tRNA^{ser}$ and yeast $tRNA^{phe}$ when the 5' quarter was present (7, 8). Only a small amount of such incorporation was found for the 3' quarter alone. This enzyme must recognize some common structural feature near the acceptor stem region of tRNA molecules.

All tRNA's interact in some fashion with ribosomes in the process of protein biosynthesis. This interaction occurs in what has been defined as two sites; the A or acceptor site, and the P or donor (peptidyl) site (9). For the binding to the A site to occur all tRNA's must interact

with a translation factor complex, Tu-GTP:

$$\text{aa-tRNA} + \text{Tu-GTP} \longrightarrow \text{aa-tRNA-Tu-GTP}. \qquad (1)$$

This complex then binds to the A site on the ribosomes. Hydrolysis of GTP to GDP occurs and the aa-tRNA is in position for its amino acid to be incorporated into the growing peptide (10).

1.2.2. Some Species Specific Interactions of tRNA

Each amino acid accepting tRNA species is recognized specifically by its amino acyl tRNA synthetase. There is at least one synthetase for each amino acid, and the recognition of this enzyme for the correct tRNA ensures errorless, high fidelity translation of a messenger RNA. The interactions of the amino acyl synthetases indicate a greater specificity of recognition for the correct tRNA species than for the amino acid (9, 11).

The formyl transferase in E. Coli. is specific for met-tRNA$^{met}_{f}$ of E. Coli. The formylation of this species is necessary for initiation of protein synthesis to occur in prokaryotes (9).

An amino acyl-tRNA recognizes a codon or codons on the messenger RNA which signals the insertion of the amino acid into that position of a growing peptide chain. This recognition has been shown to occur by base pairing (Watson-Crick hydrogen bonding) between the codon on mRNA and the anticodon on the tRNA. The fidelity of this hydrogen bonding is less than the base pairing occurring in double stranded helical RNA and DNA. The possibility of such 'wobble'

pairing (12) in this recognition allows for the presence of isoaccepting tRNA's for the same amino acid but which have differing anticodons. This allows for the incorporation of amino acids in response to more than one codon of the 64 triplet codons in the genetic code (13).

Species specific modifications of some of the bases in tRNA also occur. Such a modification is the presence of a hypermodified base at the 3' edge of the anticodon of yeast $tRNA^{phe}$ (5, 13).

The combination of features of a tRNA molecule that allow it to be general in some interactions, and yet enable discrimination from all the other species in other interactions, must dictate an overall tertiary and secondary structure that is isostructural in some regions of surface groups, but yet other regions of surface accessible groups must vary significantly from species to species for accurate, high fidelity recognition (4, 11, 14).

In order to study a structural region which is involved in a species specific interaction, one must first locate that region as part of the nucleotide sequence of the whole molecule. Specific replacement or chemical modification of some of the bases in this important RNA sequence could then be carried out in attempts to relate structure to function. Chemical synthesis of oligoribonucleotide sequences is currently the best approach to the study of these elements of interaction.

1.3. THE RECOGNITION OF AMINO ACYL tRNA SYNTHETASES FOR THEIR tRNA

A variety of techniques capable of yielding information on the recognition site of amino acyl synthetases for various tRNA's, whose primary sequence is known, have been used. These techniques include chemical modification, heterologous combinations of half molecules, combinations of acceptor stem region quarter molecules with synthetic oligoribonucleotides, and the study of heterologous charging reactions. Table I summarizes some of the data obtained in these ways for differing tRNA species.

1.4. THE RECOGNITION OF YEAST ALANINE tRNA SYNTHETASE FOR $tRNA^{ala}$(YEAST)

Evidence indicates that the acceptor stem region in yeast $tRNA^{ala}$ is the recognition or R site for yeast alanine tRNA synthetase, deduced from the results of two quite different studies done by Chambers and his coworkers (11).

1.4.1. Photolysis of $tRNA^{ala}_{Iab}$ (Yeast)

Under conditions of photolysis on RNA at 254 nm, possible reactions that can occur are: (a) photohydration of pyrimidines, (b) the dimerization of pyrimidines, (c) cleavage at pseudouridine. After photolysis of yeast $tRNA^{ala}_{Iab}$ (24), those molecules that were still active in the aminoacylation reaction were separated from the inactive ones; by studying T-1 ribonuclease digest fragments of these two groups of molecules, one could conclude that reaction at residues 5, 6 and/or 7

TABLE I

Postulated R sites of some tRNA species for amino acyl synthetases are summarised.

tRNA species	Method of Study	Postulated R Site (If any)	Ref.
yeast $tRNA^{phe}$ wheat $tRNA^{phe}$	Hom. & Het. comb- of half molecules	Syn^{phe} (yeast) active if acceptor stem intact	16
yeast $tRNA^{val}$	reconstituted fragments	anticodon stem and acceptor stem in R site	15
yeast $tRNA^{phe}$	5' quarter with 3'quarter	intact acceptor stem inhibits charging	17
E. Coli. $tRNA^{tyr}_{II}$	3' quarter with oligo G(n = 12)	intact acceptor stem inhibits charging	18
yeast $tRNA^{phe}$	excise anticodon stem	reconstituted halves remain chargeable	19
yeast $tRNA^{phe}$ wheat $tRNA^{phe}$ E. Coli. $tRNA^{val}$ E. Coli. $tRNA^{phe}$	heterologous charging by Syn^{phe} (yeast)	stem of DHU loop is R site	20
yeast $tRNA^{val}$	complex with Syn^{val} (yeast)	removal of 7 bases from 3' end does not affect complex formation	21
E. Coli. $tRNA^{phe}$	$NaBH_4$ reduction of D residues	R site in DHU loop	22
E. Coli. $tRNA^{ile}$	binding of GpGpU to acceptor stem CpCpA	Syn^{ile} (E. Coli.) blocks binding of GpGpU	23

caused inactivation (Fig. 2). Inactivation was first order when Mg^{++} was present indicating the formation of a single photoproduct was the inactivating event. No kinetic isotope effect was observed for this inactivation when D_2O was used; this single inactivation event was therefore not due to photohydration. Though a significant difference in the extent of damage to the amino-acyl acceptor stem region of inactive molecules was observed compared to that region in still active molecules, this does not mean that this is the only such target in the molecule (11).

1.4.2. Reconstitution of Fragments of Yeast $tRNA^{ala}_{II}$

Enzymatic digestion by RNase T-1 of yeast $tRNA^{ala}_{II}$ yielded half and quarter pieces that could be purified by chromatography. Those quarter molecules that derived from the 5' end and the 3' end of the tRNA could be reconstituted to accept only alanine of the 19 amino acids tested (Fig. 3) (25). The aminoacylation of these structures (I and II, fig. 3) with C^{14}-alanine in the presence of the 19 other C^{12}-amino acids did not lower the amount incorporated compared with a control amino-acylation on the whole molecule (11). The esterification reaction is still specific for these structures implying that some recognition information is in the stem region of this tRNA.

The structure recognized by yeast alanine tRNA synthetase would appear to be secondary in nature, for it would be difficult to reconstruct any of the original tertiary structure in the above fragments, especially in II (Fig. 3) (11). Tertiary structure would stabilize further the seven base pairs which hold duplex II together.

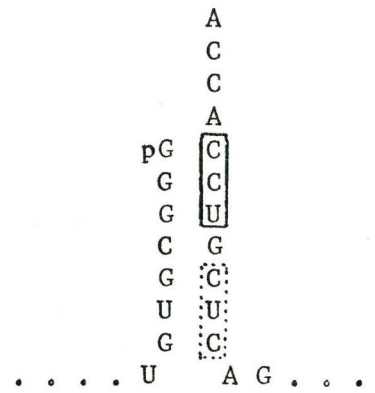
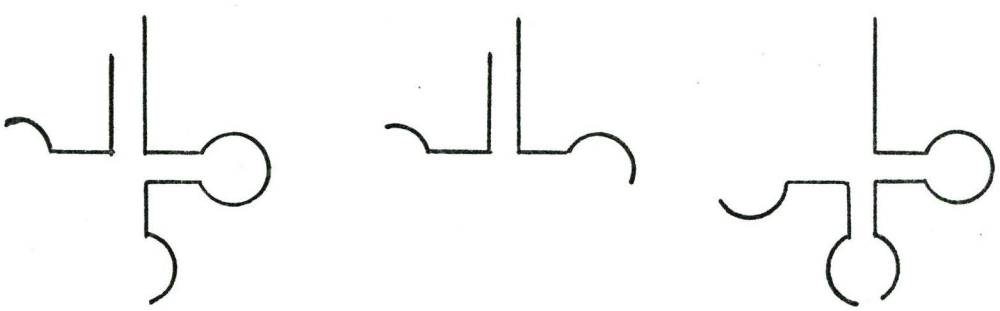


Figure 2: Charging inactivation target of yeast $tRNA^{ala}_{Iab}$ (11)
(▭ : major target; ⋯ : possible target)



T(°C)	Activity (%)		
25	7	3	0
0	25	6	0

Figure 3: Acceptor activity of reconstituted fragments of yeast $tRNA^{ala}_{II}$ (11)

Data on the incorporation of CpCpA, catalyzed by tRNA nucleotidyl transferase, were not given for the above study. The ability of the transferase to recognize the fragments in this case is not known, though in other cases such fragments are recognized (7). The lack of an intact CpCpA 3' terminus on these recombined fragments would show up as reduced acceptor activity and yet not represent lack of recognition (26).

1.5. CHEMICAL SYNTHESIS OF STRUCTURES CONTAINING A RECOGNITION SITE

The primary structure on the amino acid acceptor stem region of yeast $tRNA^{ala}$ is amenable to chemical synthesis (27). The insertion of analogues of the nucleoside bases then becomes feasible; the activity of these synthetic fragments should then provide the elements of interaction between tRNA and enzyme.

Procedures for chemical synthesis of oligoribonucleotides have been developed in this laboratory that are capable of readily providing fragments of defined sequence and length. Application of these methods in the synthesis of oligoribonucleotides corresponding to the amino acid acceptor stem region of yeast $tRNA^{ala}$ should provide some insight into the elucidation of that particular structure-function relationship.

2.1. APPROACHES TO SYNTHESIS OF OLIGORIBONUCLEOTIDES

The 2', 3' diol system of the ribose moiety in a nucleoside presents a challenging problem in the synthesis of a specific, natural 3'-5- internucleotide phosphodiester linkage. However, a distinction between these two secondary hydroxyls, so alike in chemical reactivity, can be made both by enzymes, and by certain physico-organic systems.

2.1.1. Enzymatic Distinction of 2' and 3' Hydroxyl

In 1955, Grunberg-Manago and coworkers discovered a polynucleotide phosphorylase that would polymerize the nucleoside 5' diphosphates into large polymers, releasing inorganic phosphate. Random statistical polymers could be made with all the nucleotides; homopolymers of adenosine, uridine, and cytidine could be made efficiently, all having the natural 3'-5' internucleotidic linkage (28).

RNA hydrolyzing enzymes that go through the intermediate 2', 3'-cyclic phosphate, can be used to synthesize the 3'-5' phosphodiester linkage by reversal of the reaction. Thus, it was found that RNase T-1 would synthesize guanylyl(3'-5')guanosine (GpG) under favorable conditions (eq. 2) (29).

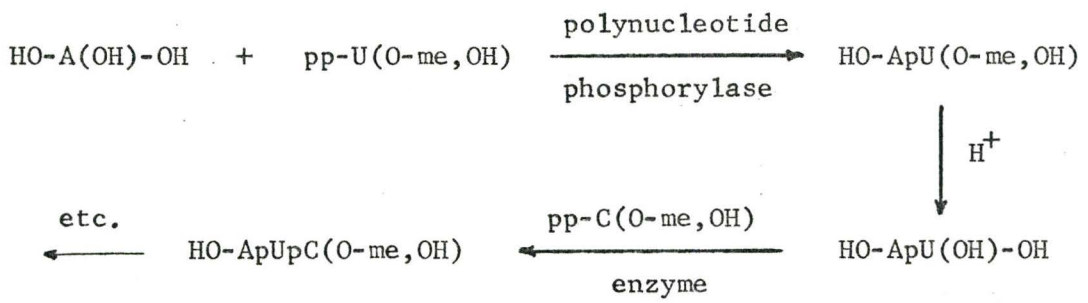
$$\text{HO-G(O,O-cp)} + \text{HO-G(OH)-OH} \xrightarrow{\text{T-1}} \text{GpG} \qquad (2)$$

Synthetic reactions with this enzyme were extended to include block coupling (eq. 3) (30). These reactions were done on the micromolar scale but may possibly be scaled up.

$$\text{HO-ApUpG(O,O-cp)} + \text{HO-ApC(OH)-OH} \xrightarrow{\text{RNase T-1}} \text{ApUpGpApC} \qquad (3)$$

The products of RNase hydrolysis are specifically the 3' nucleoside phosphates. These products could then be used in a specific chemical synthesis of oligoribonucleotides containing the 3'-5' internucleotide linkage if the 2' hydroxyl was blocked (31, 32).

Recently, Gilham and coworkers found that the polynucleotide phosphorylase can accommodate a small 2' (3') blocking group, (methoxyethyl), on the nucleoside 5' diphosphates. The reaction could then be carried out specifically, adding one base at a time in a stepwise manner to the growing oligoribonucleotide chain (scheme I) (33).



SCHEME I

2.1.2. Chemical Distinction of 2' and 3' Hydroxyl

In 1955, Todd and coworkers found that only the 3', 5'-O-diacetyladenosine could be isolated from a transacetylation melt of 2', 3', 5'-O-triacetyladenosine and 5'-O-acetyladenosine. (This 3', 5'-O-diacetyladenosine was then used in the specific chemical

synthesis of adenylyl(2'-5')uridine (34)). This was the first indication of a general rule of thumb, that 3' acetates are preferentially deposited on crystallization from 2'(3') mixtures of acetates of nucleosides.

Reese and coworkers used a convienent ortho ester exchange method to synthesize the 3'(2')-O-acetates. Trimethylorthoacetate reacts readily with the 2', 3' diol system to form the methoxyethylidine derivatives. Mild acid hydrolysis then generates an acetate group at the 2'(3') position (35).

Reese and coworkers also established that a base catalyzed equilibrium exists between the 2' and 3'-O-acetates (36). By taking advantage of this equilibrium, 2'-O-acetates can be converted to the 3'-O-acetates if this species is removed from solution by crystallisation.

Khorana and coworkers found that barium hydroxide hydrolysis of nucleoside 3', 5'-cyclic phosphates yielded 3' phosphates preferentially. Reaction of this mixture (ca. 5 : 1 = Np : pN) with trityl chloride in pyridine yielded the 5' trityl ether derivative of the nucleoside 3' phosphate. This product could be readily separated from the 5' phosphate impurity which cannot be tritylated at its secondary hydroxyls (37).

2.1.3. Acid Labile 2' Hydroxyl Protection

2'-O-tetrahydropyranyl derivatives of the nucleotides were used in the first reported specific chemical synthesis of oligoribonucleotides

containing 3'-5' interribonucleotidic linkages (37). Deblocking conditions with 50-100% acetic acid-water at elevated temperatures was later found to result in appreciable phosphate migration to the 2' hydroxyl (38).

An attempt to find the optimum conditions for hydrolysis of 2'-O-tetrahydropyranyl derivatives by Reese and coworkers, determined that 0.003 N HCl gave complete hydrolysis of the tetrahydropyranyl group of a UpA derivative in 24 hours at room temperature. Treatment of 2'-O-tetrahydropyranyluridylyl(3'-5')2', 3'-O-methoxymethylidine-uridine with 0.01 N HCl for 216 hours at 25 ^{o}C resulted in only ca. 1% phosphoryl migration (39), the tetrahydropyranyl group being removed completely in 6 hours. The tetrahydropyranyl protecting group therefore had favorable characteristics for use in oligoribonucleotide synthesis.

Recently, Reese et. al. have introduced the 4-methoxytetrahydropyran-4-yl blocking group. This group does not create a chiral center at the point of attachment to the hydroxyl to be protected; the tetrahydropyranyl group forms such a chiral center, giving rise to a diastereoisomeric pair with optically active alcohols, and it is thus less convenient (e.g. see Fig. 8). The hydrolysis characteristics of the new group are very similar to those of the tetrahydropyranyl group (40).

2.1.4. Base Labile 2' Hydroxyl Protection

The 2'-O-acetates and benzoates have been used in this role. Khorana and coworkers found that acylation could be effected on the

nucleoside 3' phosphates by reaction with the acid anhydride in the presence of a quaternary ammonium salt of the corresponding carboxylate. Under these conditions no phosphoryl migration occurred. Cleavage of the 2' ester occurs under mild alkaline conditions which do not affect phosphodiester linkages (31).

Reese and coworkers studied the 2'-O-acyl derivatives of guanosine and cytidine in pyridine solution, the medium normally used for phosphorylations. The benzoyl group displayed 5% migration to the 3' hydroxyl after 5 days in pyridine at room temperature; however, specific phosphorylation could be effected in 8 hours. The acetate group displayed significant migration in 8 hours. Phosphorylation of free 3' hydroxyls in the presence of an immobile 2'-O-acyl group could be a feasible approach to oligoribonucleotide synthesis; however, a simple method for the preparation of the 2'-O- esters of nucleosides in good yields is lacking (41).

2.1.5. Protection of 5' Hydroxyl Function

If selective removal of 5' hydroxyl protection is to be carried out, a blocking group susceptible to very mild alkaline or acidic conditions must be used. If acid-labile 2' hydroxyl protection is used, alkaline labile 5' hydroxyl protection is required (and vice versa) for maximum hydrolytic selectivity.

Acid labile dimethoxytrityl ether derivatives were inserted by Khorana and coworkers since they used the base labile 2' ester protection. Treatment of nucleoside 3' phosphates with dimethoxytrityl

chloride gave specifically 5'-O-dimethoxytrityl ethers. The remaining free functional groups--2' hydroxyls, base amino functions--can then be acylated, thus providing a direct route to precursor 5' blocked mononucleotides necessary for a chemical synthesis (31).

The triphenylmethoxyacetyl group has recently been introduced by Neilson and coworker as alkaline labile 5' protection. Mild alkaline hydrolysis was shown to deblock selectively the 5' hydroxyl on a blocked oligoribonucleotide. This lipophilic group aided solubility of ionic blocked nucleoside phosphates in methylene chloride; detection of the blocked nucleotide on tlc. plates was aided by the yellow color of this group when sprayed with ceric sulfate-sulfuric acid and heated (42).

2.1.6. Protection of the Phosphate Residue

Methods of oligoribonucleotide synthesis have been developed by Khorana and coworkers which do not use a blocking group to remove the ionic charge, or reduce the reactivity of this oxyanion on a phosphodiester linkage (31). Recent studies indicate, however, that a major side reaction occurs when these oxyanions react with the activation agent, arylsulfonyl chloride, during the course of a coupling reaction. As a result, cleavage of preformed oligoribonucleotide chains and oligodeoxyribonucleotide chains have been observed to occur (27, 43). Thus, it is advantageous to inactivate the phosphodiester oxyanion.

Todd and coworkers used the benzyl group to form phosphotriesters in their synthesis of adenylyl(2'-5')uridine. The removal of this group by hydrogenolysis or anionic treatment occurred readily but under drastic hydrolytic conditions (34). Use of this group has not been extended.

The synthesis of phosphites in the ribonucleotide series has also been reported (44). However, the phosphite diesters are both acid and base labile making their use less convenient for selective deblocking procedures of other functional groups.

The cyanoethyl (45) and the phenyl (46) are two alkaline labile groups that have been used to block the phosphate. Their disadvantage is that selective removal of other blocking groups by alkaline conditions cannot be used.

The 2,2,2-trichloroethyl group was shown by Eckstein to be suitable for the formation of phosphotriesters in the chemical synthesis of deoxyribonucleotides. This group could be selectively removed by copper-zinc couple in neutral polar solvent such as N,N-dimethylformamide (DMF). This group gave stability to the phosphotriester system during mild alkaline or acid treatment (47, 48).

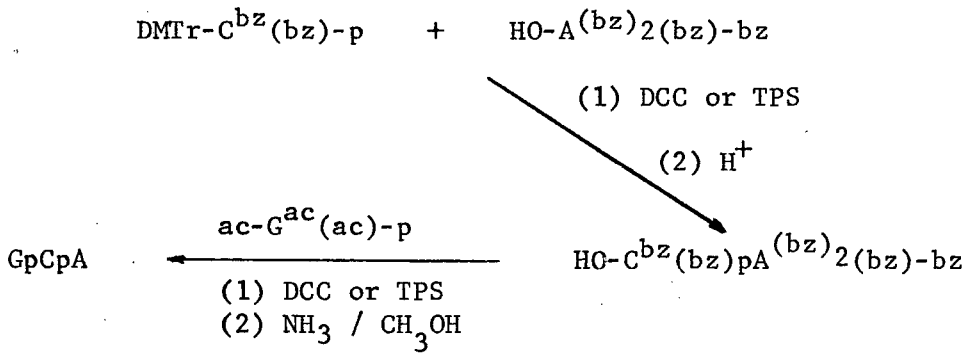
2.1.7. Protection of Base Amino Functions

The nucleophilic free amino groups on cytidine, guanosine, and adenosine are reactive in the phosphorylation reactions. These can be conveniently protected by base labile acyl groups (31). The benzoyl

group is most favored for its stability and ease of removal. It also adds lipophilicity to the protected compounds.

2.2 SUMMARY OF METHODS OF OLIGORIBONUCLEOTIDE SYNTHESIS

Khorana reported the small scale preparation of the 64 triribonucleotides. The general scheme of synthesis featured a 3' to 5' stepwise growth of the chain by the addition of the nucleoside 3' phosphate monomers (scheme II) (31).

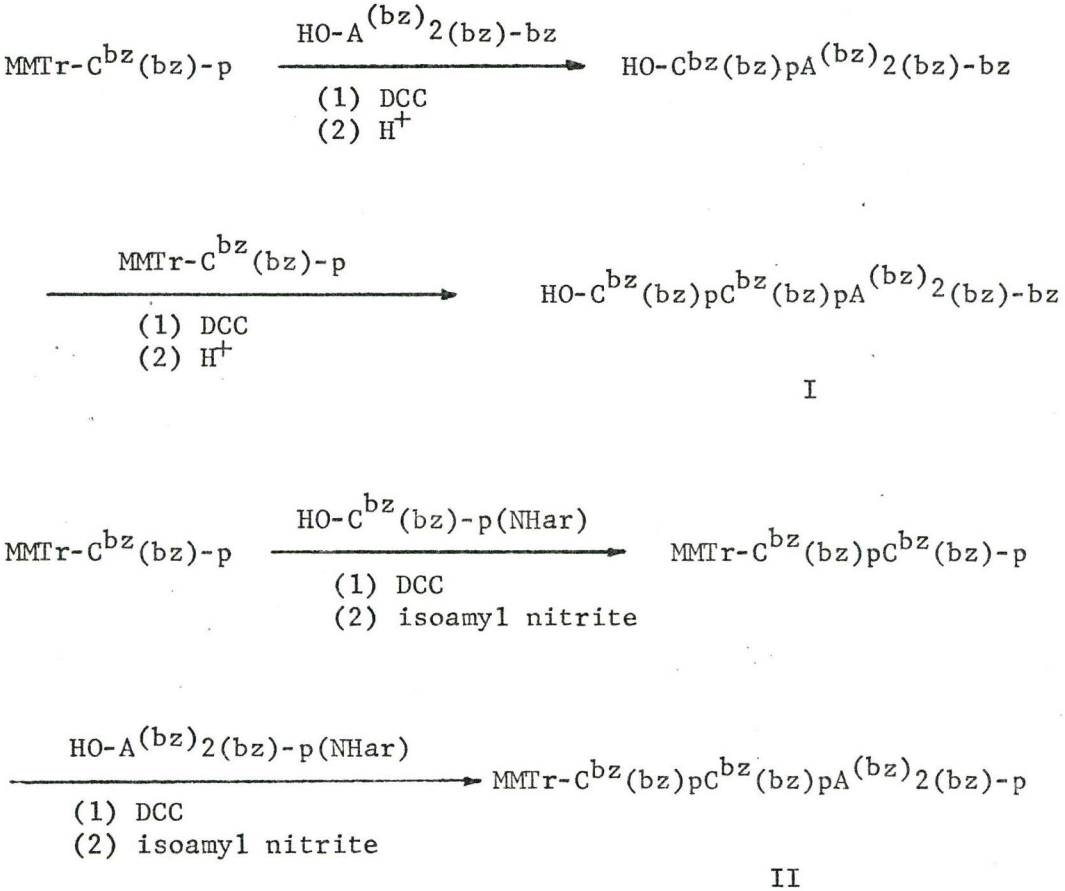


SCHEME II

A similar series of reactions was used by Ohtsuka to synthesize the hexanucleotide CpCpApCpCpA (scheme III) (27).

The stepwise synthesis of II in a 5' to 3' fashion was made possible by the discovery that nucleoside 3' phosphoramidates (-p(NHar)) do not enter into coupling reactions of normal phosphomonoesters when dicyclohexylcarbodiimide (DCC) is the activating agent (49). The aryl

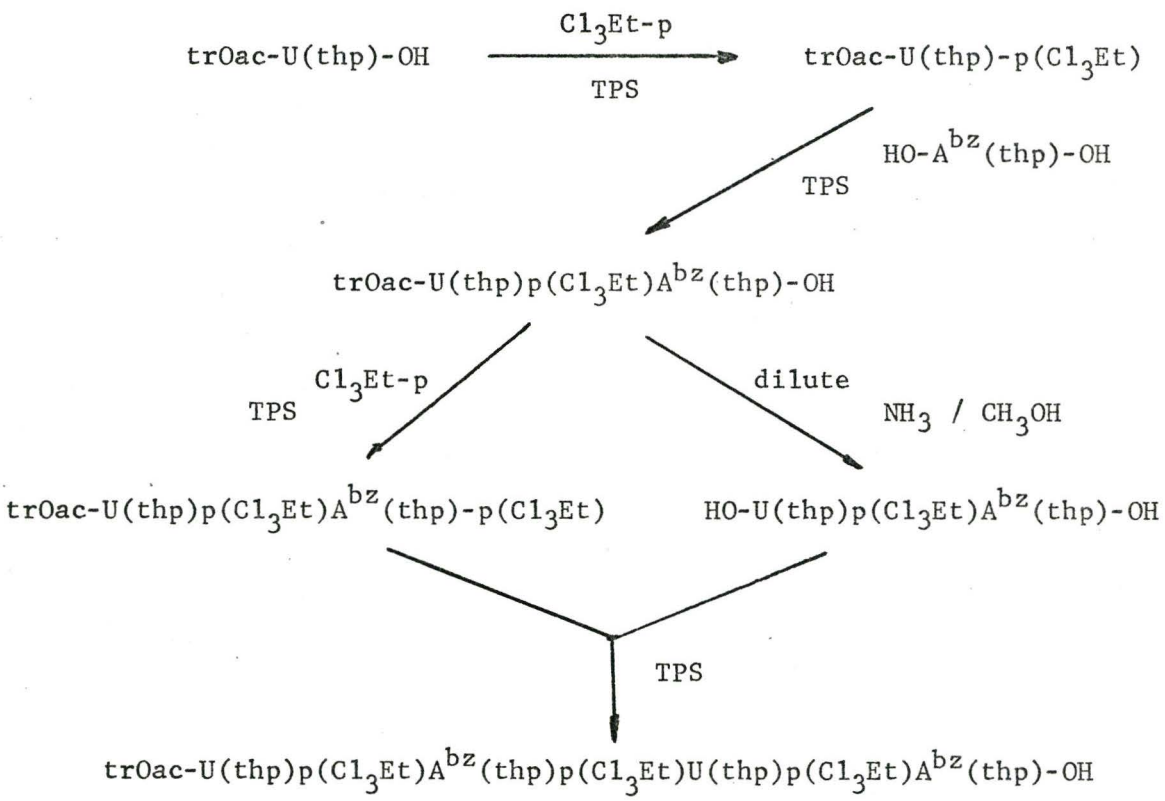
amidate function can be selectively removed by careful isoamyl nitrite treatment and the consequent 3' phosphate monoester coupled to incoming 5' hydroxyl.



SCHEME III

The TPS-activated coupling of I with II, then deblocking, gave the hexanucleotide, CpCpApCpCpA (27). A side product from this reaction was CpA, which could only have arisen through chain cleavage at one of the phosphodiester bonds of I or II.

Neilson has demonstrated a method using phosphotriester intermediates as well as block coupling for the large scale synthesis of oligoribonucleotides (scheme IV) (42). This scheme features a 5' to 3' direction of chain growth by the addition of nucleosides; chain growth in the 3' to 5' direction could also be accomplished by the addition of the suitably blocked nucleoside 3' phosphates.



SCHEME IV

The convenience and versatility of this method promises to make it generally applicable for the large scale synthesis of oligoribonucleotides. Blocked nucleoside derivatives for adenosine, cytidine, and uridine, necessary for incorporation into oligoribonucleotides by scheme IV, have been prepared in this laboratory (50). The first objective of this present undertaking was to develop protected derivatives of guanosine, so that the above method could be extended to a general synthesis of oligoribonucleotides.

RESULTS AND DISCUSSION

The general synthesis for oligoribonucleotides of defined sequence outlined by scheme IV required the availability of guanosine derivative precursors, N^2-benzoyl-2'-O-tetrahydropyranylguanosine, and N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanosine. Since the oligoribonucleotides corresponding to the stem region sequence of yeast $tRNA^{ala}$, a possible alanyl synthetase recognition site, contains several guanosine moities, the development of an efficient synthesis of these intermediates was given first priority.

Direct reaction of guanosine with trimethylorthoacetate to form its 2', 3'-O-methoxyethylidene derivative was attempted, followed by benzoylation with benzoyl chloride in pyridine of the remaining, free 5' hydroxyl and N^2 amino functions. Mild acid hydrolysis would then give the 2'(3')-O-acetate derivative (III) (eq. 4). However,

$$\text{HO-G(OH)-OH} \xrightarrow[H^+]{CH_3C(OCH_3)_3} \text{HO-G(O,O-ME)} \xrightarrow[(2)\ H_3O^+]{(1)\text{bzCl/pyr.}} \underset{\text{III}}{\text{bz-G}^{bz}\text{(OH)-ac}} \quad (4)$$

guanosine did not react efficiently with trimethylorthoacetate even when DMF was added to aid solution, and when several different equivalents of acid were added as the catalyst. This route was abandoned.

Perhaps the poor solubility of guanosine in trimethylortho-acetate-DMF mixtures accounted for partial reactions. Guanosine could be made more lipophilic if the N^2 amino function was first benzoylated.

A literature method existed for preparing N^2-benzoylguanosine (51). However, this was a small scale method and required scaling up for 30 g. quantities of N^2-benzoylguanosine(I).

Benzoylation of guanosine in pyridine at 0 °C with benzoyl chloride readily gave the tetrabenzoylguanosine in high yields (eq. 5). The purity of this intermediate was not critical so only a simple tituration with methylene chloride was carried out to remove most of the excess benzoic acid formed.

$$\text{HO-G(OH)-OH} \xrightarrow[\text{pyridine}]{\text{bzCl}} \text{bz-G}^{\text{bz}}\text{(bz)-bz} \xrightarrow{\text{NaOCH}_3} \underset{\text{I}}{\text{HO-G}^{\text{bz}}\text{(OH)-OH}} \qquad (5)$$

Preliminary de-O-benzoylation experiments indicated that the literature method (51) was unsatisfactory for large quantities, giving low yields with difficult isolation. Poor solution of tetrabenzoyl-guanosine and N^2-benzoylguanosine (I) in dioxane-methanol was responsible.

Addition of chloroform or methylene chloride to the dioxane-methanol system gave a solvent that would dissolve large quantities of tetrabenzoylguanosine. Reaction of this solution by slow addition of sodium methoxide-methanol at 0 °C occurred smoothly, with sodium benzoate and N^2-benzoylguanosine precipitating out of solution.

Neutralization of the reaction with addition of aqueous acidic solution (pH = 2) and Dowex 50W-X8 (pyridinium form) redissolved the precipitate while forming a two phase system, the methylene chloride solution as the bottom layer. The resin could readily be filtered off from the now neutral solution. Simple partitioning of the already two phase system with more methylene chloride removed the benzoic acid and other lipophilic impurities. This extraction also lowered the methanol concentration in the aqueous layer, with the consequent precipitation of N^2-benzoylguanosine (I) as a clean white wool. No N^2-benzoylguanosine was extracted into the organic layers. This method constituted a simple, large scale procedure for synthesizing N^2-benzoylguanosine (I) in high yields.

1. SYNTHESIS AND CHARACTERIZATION OF 3'-O-ACETYL-N^2, 5'-O-DIBENZOYL-GUANOSINE (III)

The anhydrous acid catalyzed reaction of N^2-benzoylguanosine with trimethylorthoacetate was next attempted. The N^2-benzoylguanosine (I) did not dissolve in DMF/trimethylorthoacetate solvent but was reacted slowly as a suspension. Literature (52) claimed success; however, the stated conditions resulted in partial reaction, as followed by tlc. in 10% methanol in methylene chloride. Only 0.1 mole-equivalent of acid catalyst, p-toluenesulfonic acid, was used and this appeared to be inadequate.

Addition of 1.0 mole-equivalent of acid was attempted in analogy to the observations in the adenosine series (35), resulting in

the complete disappearance of starting material (R_f = 0.1 in 10% methanol-methylene chloride). However, only low yields (30%) of the final product (III) were obtained after benzoylation and ring opening (eq. 6).

$$\underset{\text{I}}{\text{HO-G}^{bz}\text{(OH)-OH}} \xrightarrow[H^+]{CH_3C(OCH_3)_3} \underset{\text{II}}{\text{HO-G}^{bz}\text{(O,O-ME)}} \xrightarrow[(2)\ H_3O^+]{(1)\text{bzCl/py}} \underset{\text{III}}{\text{bz-G}^{bz}\text{(OH)-ac}} \quad (6)$$

A side product of the reaction was depurination; a compound was isolated in good yield showing a p.m.r. spectrum for a benzoylated ribose but no anomeric proton or guanosine H(8) proton resonance could be seen. At this time, the structure of the Y base from yeast $tRNA^{phe}$ was published (53); this is an N^2- substituted guanine which has a very acid labile glycosidic bond. Cleavage of Y base from yeast $tRNA^{phe}$ occurs readily in mild acid conditions (37 °C, 2-4 h, pH = 2.9(54)). N^2-benzoylguanosine might be expected to react similarily, thus accounting for the appearance of depurinated products in the above reaction.

Thus, the concentration of acid catalyst used to effect reaction of N^2-benzoylguanosine (I) with trimethylorthoacetate is critical. Too little gave only partial reaction; too much gave depurination as a side reaction.

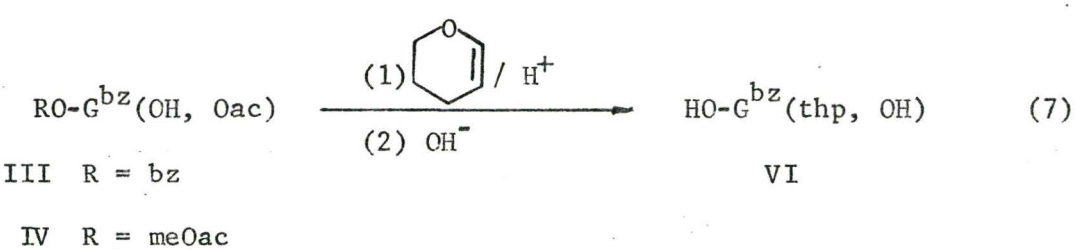
In one experiment, the reaction proceeded very fast after the initial addition of p-toluenesulfonic acid (0.3 equivalent) (the suspension would clear up noticeably), as monitored by tlc. (R_f: 0.1 → 0.5

in 10% methanol-methylene chloride). The reaction then appeared to stop with virtually no further reaction over the next 5 hours. More acid was then added (0.3 equivalent), and again a noticeable clearing of the suspension occurred immediately. However, the yield of product was again low with depurination as the major side reaction. However the observation that reaction occurred immediately upon addition of acid suggested that the titration of acid into the reaction mixture might drive the reaction to completion quickly without any side reactions.

Thus, titration of a DMF solution of p-toluenesulfonic acid into a suspension of N^2-benzoylguanosine (I) in trimethylorthoacetate/DMF effected complete reaction; 0.8 equivalent of acid had been added over 1.5 hours. Tlc. on silica gel in 10% methanol-methylene chloride revealed quantitative appearance of a new product (II) at R_f = 0.5.

Benzoylation of IV (eq. 6) occurred smoothly in pyridine at 0 °C. (R_f: 0.5 → 0.9 in 10% methanol-methylene chloride). Evaporation of solvent and treatment of the resulting syrup with 10% acetic acid-water at room temperature for 16 hours gave hydrolytic ring opening of the 2', 3'-O-methoxyethylidine ring to yield III (R_f = 0.55 in 10% methanol-methylene chloride). Purification on a silica gel column gave a crude yield of 80% of a mixture of 2' and 3'-O-acetate isomers of III, on elution with 3% methanol in methylene chloride. One of the isomers, readily crystallized from absolute ethanol. The crystals had a sharp melting point (214-216 °C) and the correct elemental analysis for III.

Direct chemical proof for the position of the acetyl was attempted. Dihydropyran treatment followed by de-O-acylation would provide either the 2'- or the 3'-O-tetrahydropyranyl-N^2-benzoyl-guanosine (eq. 7). Literature (55) provided N^2-benzoyl-3'-O-acetyl-5'-O-methoxyacetylguanosine (IV) which on similar treatment would provide the 2'-O-tetrahydropyranyl derivative (VI). Unfortunately, isolation of this 3'-O-acetate derivative (IV) by fractional crystallization from its 3'-(2')-O-acetate mixture could not be accomplished in our hands, even after careful silica gel chromatography of the isomeric mixture.



The position of the acetyl group in III, however, could be unequivocally determined from analysis of its p.m.r. spectrum.

Reese and coworkers studied the p.m.r. spectra of 2'- and 3'-derivatives of uridine, cytidine, and adenosine; only the spectral data for a mixture of 2'(3')-O-acetate isomers of guanosine was given (56). The results showed that the H(1') resonance signal appeared at lower field for the 2'- than for the 3'- isomer. The coupling constant ($J_{2'-1'}$) between H(2') and H(1') was larger for the 3'- than for the 2'- isomer. Similar data should be expected for the guanosine derivatives.

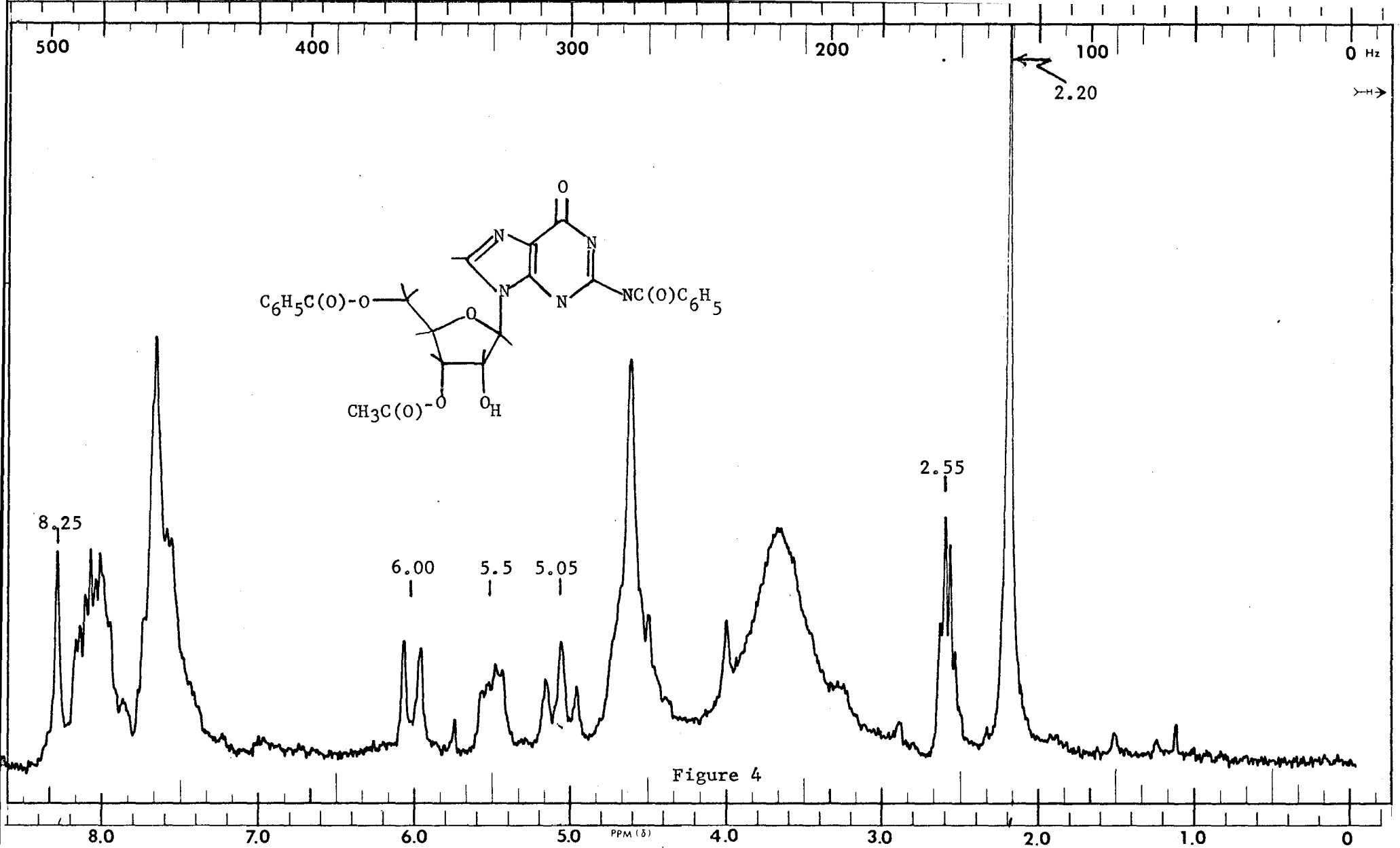


Figure 4

The p.m.r. spectrum for the compound (III) that crystallized from solution is given in Figure 4. The signals in the region 5.0 to 6.5 δ can be assigned unequivocally for this derivative. The doublet at δ = 6.00 is the expected position of H(1') (56). The triplet at δ = 5.05 can be assigned to H(2') by the following considerations (Fig. 5).

The H(2') is coupled vicinally to both H(1') and H(3'). These two couplings would give a clean triplet signal for H(2') only if $J_{2'-1'} = J_{3'-2'}$ (Fig. 6a). This would be the case if the dihedral angle, $\phi_{2'-3'}$, between the vicinal protons H(3')-H(2') supplements the dihedral angle, $\phi_{2'-1'}$, between the vicinal protons H(2')-H(1') (57).

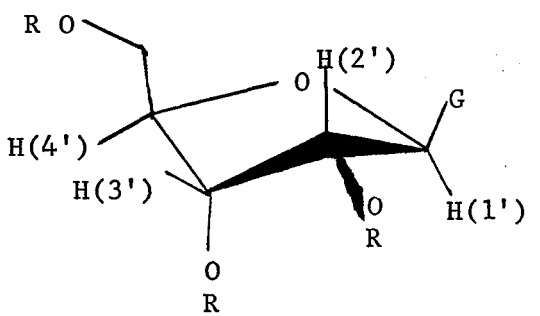


Figure 5

The required dihedral angles can be estimated from the observed coupling constant, $J_{2'-1'}$ = 6.0 hz, and the above condition, that $J_{2'-1'} = J_{2'-3'}$, by the use of the emperical graph of J vs. ϕ (57). For J = 6.0 hz it is required that $\phi_{1'-2'} = 150^{o}$ and $\phi_{2'-3'} = 30^{o}$ (Fig. 6b).

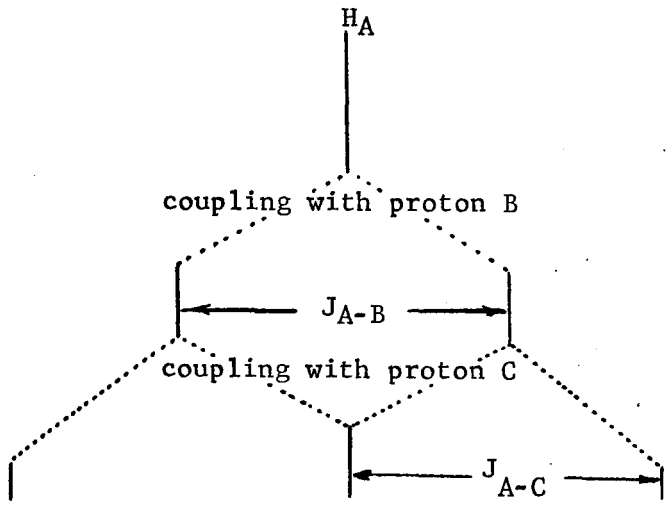


Figure 6a: Proton coupling of H_A with H_B and H_C

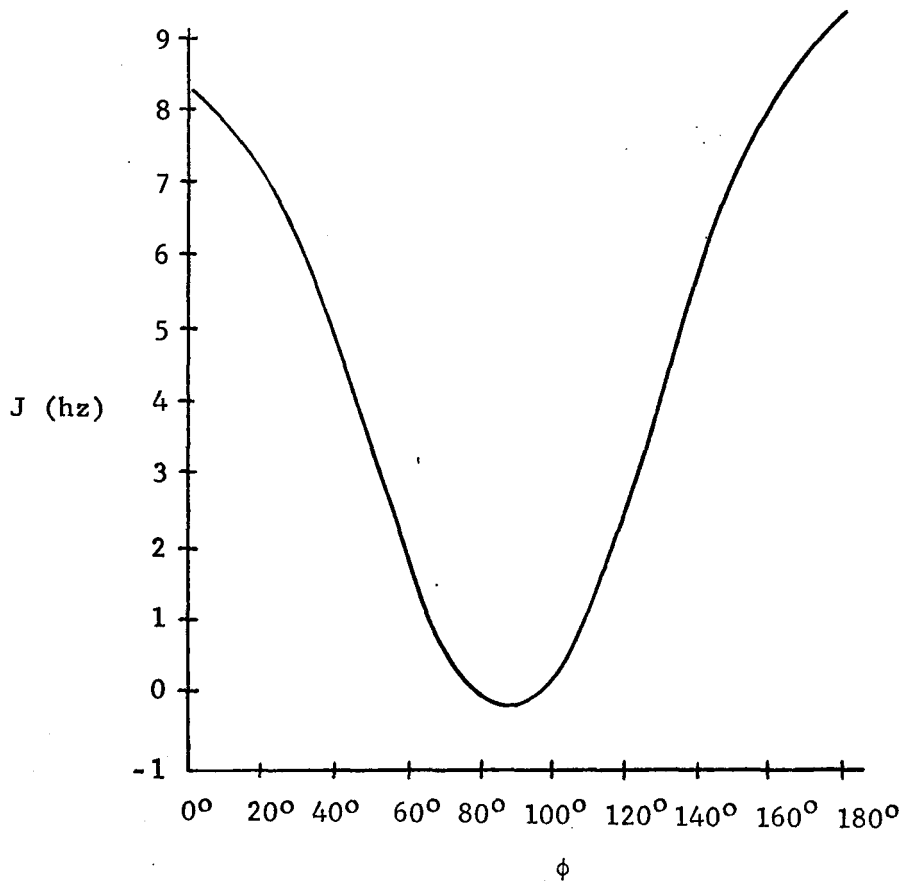


Figure 6b: J vs. ϕ for substituted ethanes (57)

Molecular models show this situation to be the energetically favorable C(2') endo conformation (58)*. This triplet at δ = 5.05 is then assigned to the resonance of H(2').

The remaining signal, a multiplet at δ = 5.5, is assigned, by elimination, to the resonance of H(3'). The H(3') is coupled to H(2') ($J_{2'-3'}$ = 6.0 hz from the above analysis) and to H(4') (Fig. 5). However, the dihedral angle between H(2')-H(3') ($\phi_{2'-3'}$) does not supplement the dihedral angle between H(3')-H(4') ($\phi_{3'-4'}$) in the energetically favorable model constructed above; consequently, $J_{2'-3'} \neq J_{3'-4'}$ and a multiplet signal results for H(3') (Fig. 6) (57).

The alternative assignment, where the multiplet signal results from vicinal coupling of H(2') with H(1') (J = 6.0 hz--observed) and H(3') (Fig. 5) must assume that $J_{2'-3'} \neq J_{2'-1'}$. Yet, assigning, by elimination, the remaining triplet to H(3'), requires that $J_{3'-4'} = J_{3'-2'}$ = 6.0 hz (the observed coupling value). But then $J_{2'-1'} = J_{2'-3'}$, and no multiplet signal should be observed. Thus, this assignment cannot hold, and the above assignment is correct.

In the free 2', 3' diol case, it is expected that the H(2') resonance appear at lower field than the H(3') on the basis that C(2') is one covalent bond away from a carbon atom, C(1'), that is

---

*The endo position is defined as the out of plane atom (carbon) located on the same side as the C(5'); exo position is defined as the out of plane atom located on the side opposite to C(5'). The plane is defined by O(1')-C(1')-C(2') (58).

attached to two electronegative groups, viz. $-NR_2$ and -OR. Such inductive deshielding by electronegative groups has been observed up to four covalent bonds distant; the magnitude ($\Delta\delta$) of this effect decreases drastically with number of covalent bonds (57). For the crystalline compound (III), the H(3') resonance is at lower field, $\delta = 5.5$, than the H(2') resonance, $\delta = 5.05$. The presence of an electronegative carbonyl of the 3'-O-acetate derivative (III) would inductively deshield the H(3') more than the H(2'), and thus lead to the observed chemical shifts. The effect should extend to a small extent to the H(1'); and indeed, its chemical shift is to lower field ($\delta = 6.00$) compared with the 2', 3' diol case ($\delta = 5.95$ for H(1') of N^2-benzoylguanosine (I)).

The p.m.r. spectrum of the mixture of isomers (Fig. 7) shows a new signal for the H(1') ($\delta = 6.20$, $J_{2'-1'} = 4.0$ hz) downfield from H(1') of the 3'-O-acetate derivative analyzed above. Also, a new triplet appears at $\delta = 5.75$ ($J_{2'-1'} = 4.0$ hz); this can be assigned to the resonance of H(2') analogously to the above. This downfield shift ($\Delta\delta = 0.7$) of the H(2') resonance can only be attributed to inductive deshielding of the H(2') by electronegative carbonyl of a 2'-O-acetate ester (57). This inductive deshielding extends more powerfully from the 2'-O-acetate to the H(1') which appears downfield ($\Delta\delta = 0.20$) of that signal for the 3'-O-acetate as would be expected for a more near electronegative group*.

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*Similar spectra are published for pure adenosine derivatives (59).

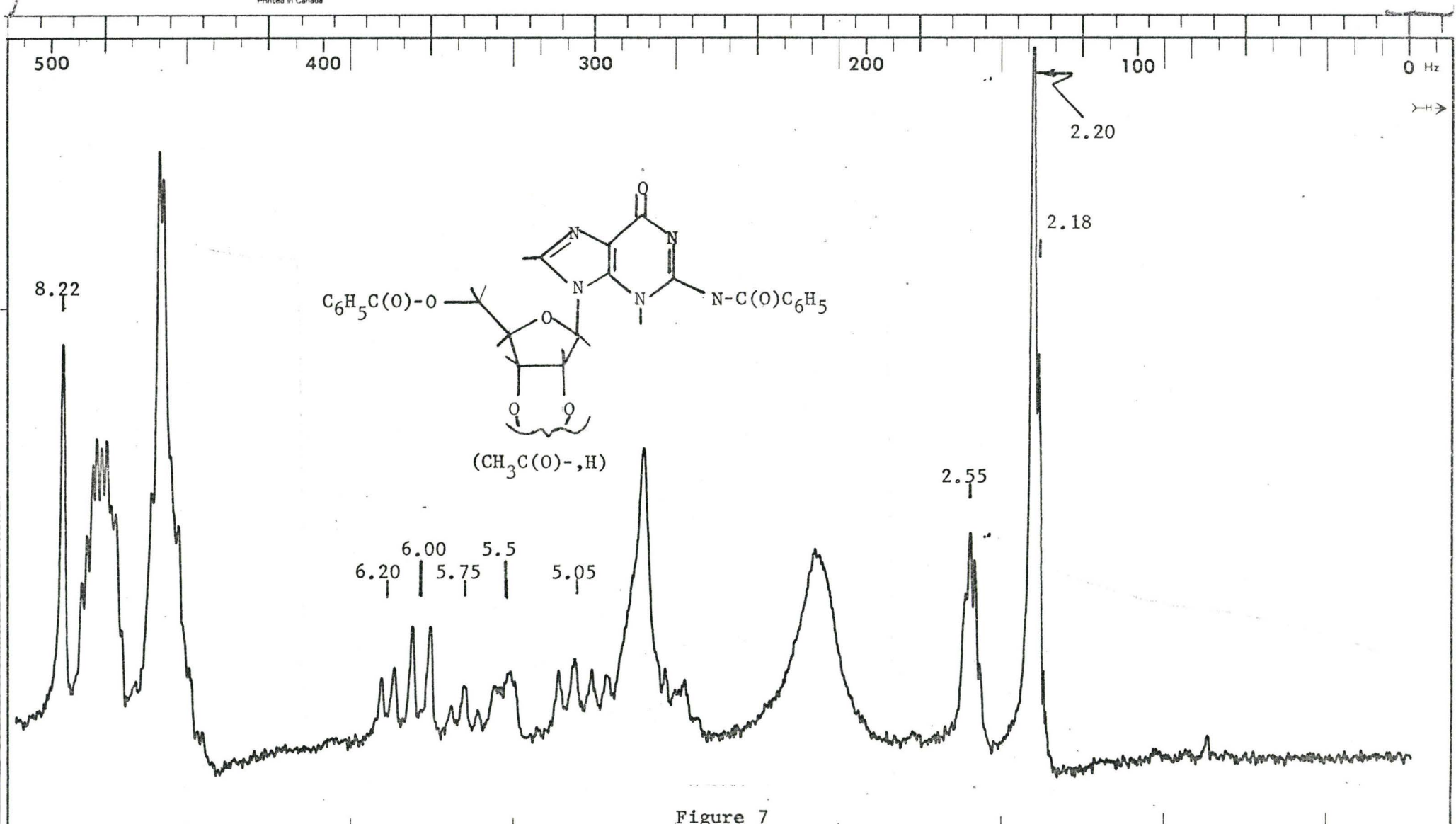
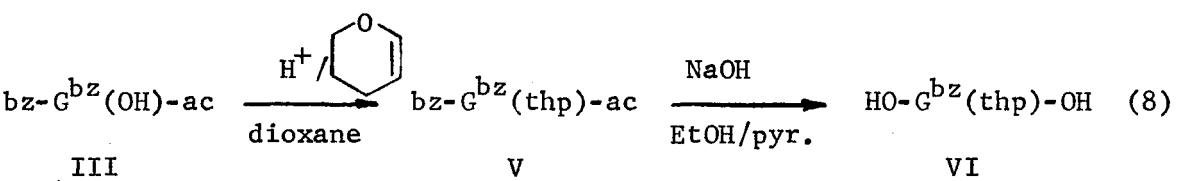


Figure 7

This analysis establishes that the 3'-O-acetyl-N^2, 5'-O-dibenzoylguanosine (III) crystallized from an absolute ethanol solution of the mixture of isomers.

2. SYNTHESIS AND CHARACTERIZATION OF N^2-BENZOYL-2'-O-TETRAHYDROPYRANYLGUANOSINE (VI)

The acid catalyzed reaction of dihydropyran with free hydroxyl groups occurs readily in anhydrous non-polar solvents. Though III is insoluble in dioxane, the suspension was found to react readily to form the soluble derivative V (eq. 8).



(8)

p-Toluenesulfonic acid was found most convenient as catalyst since it is soluble in dioxane; simple neutralization with a few drops of conc. ammonium hydroxide formed the insoluble salt, ammonium p-toluenesulfonate, which could be filtered off.

The de-O-acylation of V was next studied. The mild condition of ammonolysis by one-half saturated (at 0 °C) methanolic ammonia might be expected to cleave the esters without affecting the N^2 benzoyl amide linkage (55). However, in this instance, tlc. of the ammonolysis showed that cleavage of benzoyl and acetyl ester linkages could not be completed without partial cleavage of the N^2-benzoyl amide linkage.

Hydrolysis of the ester groups of V was then studied. Conditions are available which preferentially cleave esters in the presence of a benzamido group in the cytidine series (50). These conditions were extended to this case.

Reaction of V with ethanolic sodium hydroxide in ethanol/pyridine was found to give good yield of de-O-acylated product VI very quickly (10 min.); cleavage of the N^2 benzamido could also be observed if reaction continued after 20 min. Only a trace amount of V was left unreacted in the optimal conditions worked out for this reaction (eq. 8).

A new chiral center is introduced into the nucleoside derivatives by the attachment of the tetrahydropyranyl group (Fig. 8). Thus two diasterioisomers of VI were isolated: a high R_f (low melting) and a low R_f (high melting) isomer. These diasterioisomers have sufficiently different physical properties that they can be separated on silica gel column chromatography. The fractions of each isomer readily gave crystalline material from ethyl acetate-methanol. Each diasterioisomer crystallized with one mole of water.

Though the ratio of formation of those isomers would be expected to be 1 : 1, the low R_f isomer occurred in a greater amount (low R_f : high R_f = 60 : 40) indicating the approach of dihydropyran to the 2' hydroxyl must favor one side over the other.

Only one of these diasterioisomers is capable of forming a hydrogen bond with the 3' hydroxyl (Fig. 8). This locks its conformation in this most energetically stable form and provides

different physical properties for the two diasterioisomers. Analogous properties have been described for the 2'-O-tetrahydropyranyl derivatives of adenosine, uridine, and cytidine (50, 60, 61).

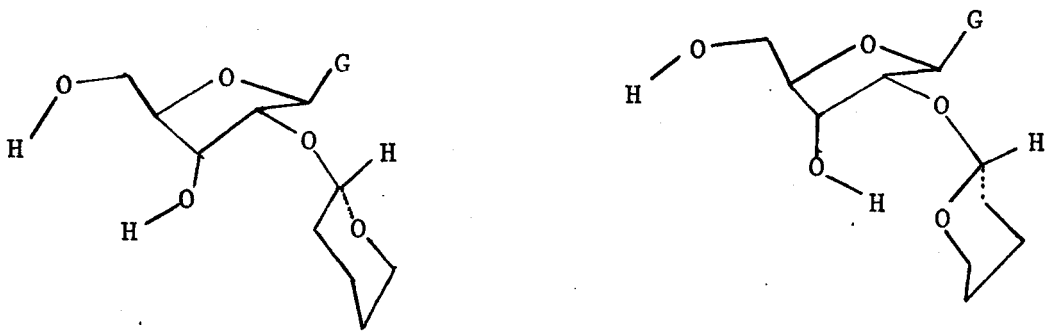


Figure 8: Chiral center of 2'-O-tetrahydropyranyl attachment

The p.m.r. spectra of these derivatives reveal differences in chemical shifts of H(1') and H(2') between the isomers; coupling constants ($J_{1'-2'}$) differ (Table III). These indicate differences in riboside conformation of the diasterioisomers. The effect of substitution at the 2'- hydroxyl can be noted in the lower field position of H(1') for these derivatives compared with 3'-O-acetate derivative III or N^2-benzoylguanosine (I) (δ= 6.15 for VI vs. δ= 6.00 for III, δ= 5.95 for I, table III).

The isolation of these derivatives as crystalline compounds ensured pure protected guanosine derivatives necessary for oligoribonucleotide synthesis.

3. SYNTHESIS AND CHARACTERIZATION OF N^2-BENZOYL-2'-O-TETRAHYDROPYRANYL-5'-O-TRIPHENYLMETHOXYACETYLGUANOSINE (VIII)

Reaction of VI (low R_f isomer) with TPS-activated sodium triphenylmethoxyacetate was attempted under conditions used for the analogous reaction with the uridine series (42); viz. 1.1 equivalents of tri-isopropylbenzenesulfonyl chloride (TPS) and 2.0 equivalents of sodium triphenylmethoxyacetate in anhydrous pyridine. Reaction, as followed by tlc. on silica gel in 10% methanol-methylene chloride, proceeded to 10% completion after 16 hours. No further reaction was noted after a further 2 days at room temperature in the dark. Further additions of TPS were necessary to drive the reaction to completion. Purification by column chromatography on silica gel revealed a 40% yield of the di-triphenylmethoxyacetylated derivative (VII), on elution with 1% methanol in methylene chloride. Elution with 2.5% methanol in methylene chloride gave a pure fraction (R_f = 0.66 in 10% methanol-methylene chloride) of VIII in 40% yield (eq. 9).

$$\underset{\text{VI}}{\text{HO-G}^{bz}\text{(thp)-OH}} \xrightarrow[\text{anhydrous pyridine}]{\text{trOac}^-\text{Na}^+ \ / \ \text{TPS}} \underset{\begin{array}{l}\text{VII: R = trOac}\\ \text{VIII: R = H}\end{array}}{\text{trOac-G}^{bz}\text{(thp)-OR}} \qquad (9)$$

P.m.r. spectra of VIII, one-spot material on tlc, revealed an impurity which was suspected to be the 5'-O-tri-isopropylbenzenesulfonated derivative IX (eq. 10); it showed proton signals at δ = 1.2,

the position where the tri-isopropyl protons of IX would appear*. (The presence of this impurity is of common occurence when the literature conditions are used. However, the impurity has a slightly different R_f from the nucleoside product in other cases and can be separated from the product by chromatography (62)).

$$\underset{}{\text{TPS}} + \underset{\text{VI}}{\text{HO-G}^{bz}\text{(thp)-OH}} \xrightarrow[\text{pyridine}]{} \underset{\text{IX}}{\text{TPS-O-G}^{bz}\text{(thp)-OH}} \qquad (10)$$

By recrystallization of VIII from methylene chloride-hexane the amount of impurity IX could be reduced from ca. 10% to less than 3%. However, recrystallization also leads to material loss.

The following modifications maintained a good yield of VIII with no hint of impurity IX in the product. 1.5 equivalents of sodium triphenylmethoxyacetate was activated with 0.75 equivalent of TPS in anhydrous pyridine as before (eq. 9). After 2 hours, 0.5 ml of molten dicylcohexylcarbodiimide (DCC) was added and the reaction left overnight (16 h.). (DCC will not react with the salt, sodium triphenylmethoxyacetate, since its reactions are acid catalyzed. After 1 hour of TPS-activated reaction, enough acid is liberated to

---

*An estimated detection limit is 0.5 proton in this region (δ= 1.2) of the spectrum. The impurity IX has 15 tri-isopropyl protons absorbing here, so theoretical limit of detection is 0.5(1/15)(100) ~ 3%.

enable DCC to activate efficiently. TPS would also react with any strongly basic aliphatic amines present as impurities in pyridine, which can hinder reactions of DCC in this medium (63)). This procedure gave quantitative substitution of the free hydroxyls of N^2-benzoyl-2'-O-tetrahydropyranylguanosine (VI) during 16 hours of reaction. P.m.r. spectrum of VIII isolated from this preparation by silica gel chromatography in methanol-methylene chloride gave no propyl signals corresponding to IX.

A comparison of the p.m.r. spectra of VII and VIII is shown in table II.

TABLE II

P.m.r. data of guanosine derivatives VIII and VII

Compound	chemical shift(ppm.)			Integration ratio	
	H(1')(J Hz)	H(2')	H(3')	region δ(ppm.) 8.3-7.0	region δ(ppm.) 2.0-1.0
trOac-G^{bz}(thp)-OH (VIII)	6.10(6.0)	5.40	4.75	21	6
trOac-G^{bz}(thp)-trOac (VII)	6.1 (7)	5.5	5.0	36	6

The problem of the existence of the isostere 3'-O-triphenylmethoxyacetyl derivative as an impurity occuring with the 5'-O-triphenylmethoxyacetyl derivative, VIII, was next considered. Although a primary, 5' hydroxyl is expected to esterify much faster than a secondary, 3' hydroxyl on a chemical kinetic basis, the 3' hydroxyl is not so hindered as to prevent reaction, clearly shown by isolation of VII. However, a

primary hydroxyl stands a much better chance of being specifically esterified in equimolar conditions and this factor would keep the unwanted isostere, 3'-O-triphenylmethoxyacetate derivative, to a minimum. A very sharp guanine H(8) singlet resonance for VIII with no shoulders indicated the absence of isosteric impurities. The absence of any H(3') resonance at $\delta = 5.0$ (as in table II for VII) supports this.

Alkaline lability of the triphenylmethoxyacetyl group was studied on the 3', 5'-O-di-triphenylmethoxyacetyl derivative (VII). Conditions necessary for selective removal of the 5'-O-triphenylmethoxyacetate without removal at N^2-benzoate were established using tlc. monitor. In general, primary hydroxylic esters hydrolyze faster than secondary esters, and so the first substituent to be removed from the above derivative (VII) is expected to be from the 5' hydroxyl. Complete removal of 5'-O-triphenylmethoxyacetate occurred in 30 min. (R_f: 0.9 0.65 in 10% methanol-methylene chloride) in 1/10 saturated (at 0 °C) methanolic ammonia. Complete de-O-triphenylmethoxyacetylation was observed by 90 minutes of reaction ($R_f = 0.30$ in 10% methanol-methylene chloride for major product VI). At this time traces of de-N^2-benzoylated material began to appear ($R_f = 0.2$ in 10% methanol-methylene chloride). However, a concommitant run with N^2-benzoyl-2'-O-tetrahydropyranylguanosine (low R_f isomer) (VI) under identical conditions, revealed no trace on tlc. of de-N^2-benzoylated material 5 hours of hydrolysis. The time for complete removal of the N^2 benzamido group was 36 hours.

Apparent discrepancy in the rates for de-N^2-benzoylation may be explained on the basis of a change of mechanism of benzamide ammonlysis in the presence of water (64), introduced as water of crystallization in the derivative VI. Perhaps greater discrimination in hydrolytic stability of ester *vs.* amide may be achieved if 'wet' methanolic ammonia was used.

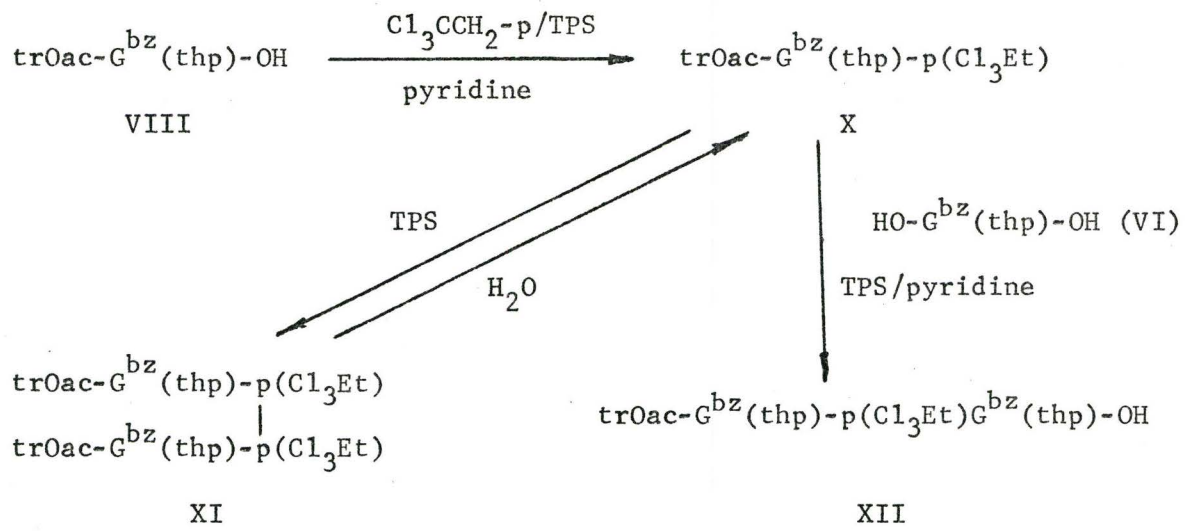
The difference in the rates of ammonolysis of 5'-O-triphenylmethoxyacetyl *vs.* N^2-benzoyl group should be sufficient to enable selective deblocking of the 5'- protection. This information will be invaluable in working out partial de-blocking conditions for oligoribonucleotides to be used in block synthesis.

4. THE SYNTHESIS OF BLOCKED GUANYLYL(3'-5')GUANOSINE (XII)

5'-O-triphenylmethoxyacetate derivative VIII was reacted with TPS-activated 2,2,2-trichloroethyl phosphate in anhydrous pyridine. It is essential that all water be removed as traces of water gave marked inhibition of this phosphorylation. Under these conditions 3' hydroxyls are usually phosphorylated in 1-2 days (42); however, this reaction went more slowly and it required additional amounts of TPS (0.1 equivalent/da.) to drive it to *ca.* 75% completion within one week.

Usually, phosphorylation is efficient (>95%) leaving only traces of starting material; consequently, the problem of separating starting nucleoside derivatives from protected dinucleoside phosphates (to be made in the next step) never arises. However, in this instance, a quantity of unreacted nucleoside, VIII, (R_f = 0.65 in 10% methanol-methylene chloride)

remained, and to avoid a difficult separation of VIII from protected GpG (XII, expected R_f = 0.4-0.6 in 10% methanol-methylene chloride), isolation of protected Gp (X, R_f = 0.0-0.1 in 10% methanol-methylene chloride) from VIII was carried out. The removal of unreacted VIII was accomplished using a deactivated silica gel column (10% H_2O) where VIII eluted with 1% methanol-methylene chloride and X eluted with 4% methanol-methylene chloride.



SCHEME V

The pyrophosphate, XI, might be an expected side product in the first reaction (scheme V). However, it would be a tetra-ester pyrophosphate which is very labile to hydrolysis. Any pyrophosphate (XI) formed would be expected to hydrolyze to X on work-up of the reaction in aqueous solution.

The phosphorylated derivative (X) was activated with TPS in anhydrous pyridine and an anhydrous solution of VI in pyridine was added (scheme V). The presence of any new material containing the trityl group at the expected R_f of 0.45-0.60 was slow to appear. (Such coupling reactions usually take 3 days to 1 week for completion (65)). Continued addition of TPS (0.1 equivalent/day) for 2 weeks caused the appearance of a new, yellow-staining product of R_f = 0.35-0.45 in 10% methanol-methylene chloride, partially obscurred by VI (R_f = 0.34 in 10% methanol-methylene chloride). Column chromatography of the reaction mixture on silica gel in methanol-methylene chloride gave a fraction containing XII and VI. This situation had been encountered with the other nucleoside couplings; the reaction of the contaminant, 2'-O-tetrahydropyranyl nucleoside, with p-anisylchlorodiphenylmethane gave its 5'-O-monomethoxytrityl ether derivative, a much more lipophilic compound (higher R_f), which could be readily separated from other blocked nucleotide products (42). The monomethoxytrityl ether derivatives appear as orange spots on tlc when sprayed with ceric sulfate-sulfuric acid and heated to 400 °C; these could be easily distinguished from the desired product (yellow spot).

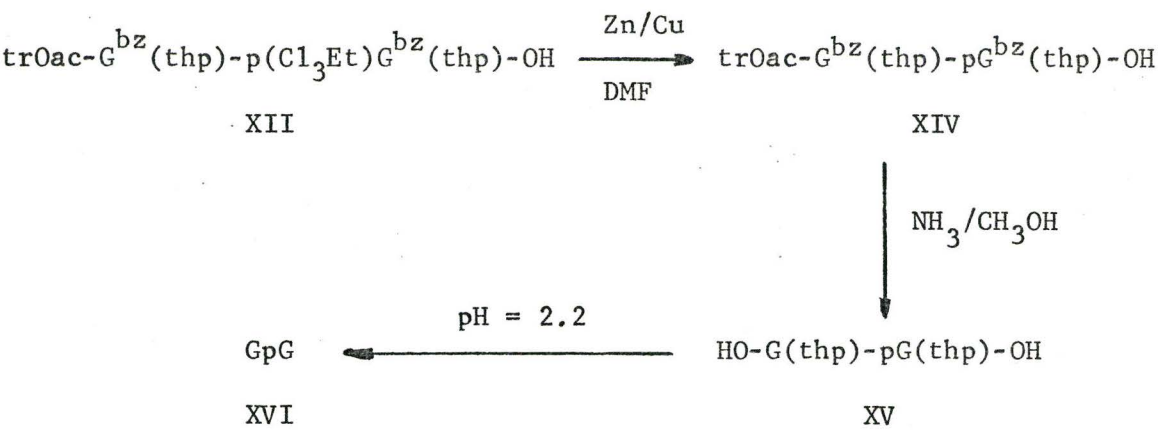
Thus, back tritylation with p-anisylchlorodiphenylmethane in anhydrous pyridine converted VI into XIII (R_f = 0.67 in 10% methanol-methylene chloride). Any 3'-3' dinucleotide phosphotriesters formed would also tritylate at the free 5' hydroxyls under these conditions. Free secondary hydroxyls survive this treatment.

The separation of XIII from XII (R_f = 0.40 in 10% methanol-methylene chloride) on silica gel column occurred cleanly and the compound, XII, was isolated as a dry foam in 10% yield based on VIII.

No attempt was made to optimize conditions for this coupling reaction, but it is likely that yields in the range 60-80% should be obtainable here in analogy with coupling yields of the other nucleosides.

5. GUANYLYL(3'-5')GUANOSINE

The blocked dinucleoside phosphate derivative (XII) was deblocked using the reported method (scheme VI) (42).



SCHEME VI

The reactions were followed initially by tlc on silica gel in 10% methanol-methylene chloride. When the 2,2,2-trichloroethyl group is removed in the first step, the material becomes ionic and R_f drops from 0.4 to 0.0 as shown by yellow coloration (XIV). Treatment with

methanolic ammonia then releases the triphenylmethoxyacetic acid to leave a brown coloration at R_f = 0.0 for the partially deblocked dinucleoside phosphate (XV).

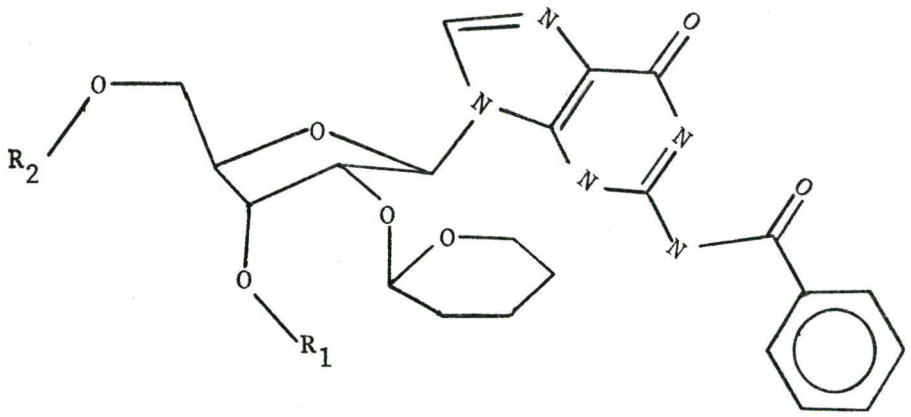
Acid hydrolysis of XV was followed by tlc on cellulose plates (fluorescent background). Compound XV has R_{Up} = 1.0 in solvent system A (see Experimental). After 1 day at pH = 2.2, free GpG (XVI) (R_{Up} = 0.79) appeared. Paper chromatography in system A showed R_{Up} = 0.81 (lit.(31): R_{Up} of GpG = 0.82 in system A).

Purification of GpG was performed first on Whatman No. 3 MM paper using descending solvent system A, and then on Whatman No. 40 (ashless) paper with the same solvent. Ultraviolet spectra of GpG at acid, neutral, and basic pH are shown in Figure 11. Values of R_{Up} of this product in several solvent systems are tabulated in Table IV.

Enzymatic degradation with snake venom phosphodiesterase gave complete hydrolysis to the expected products (G and pG) in the correct ratio (see Experimental).

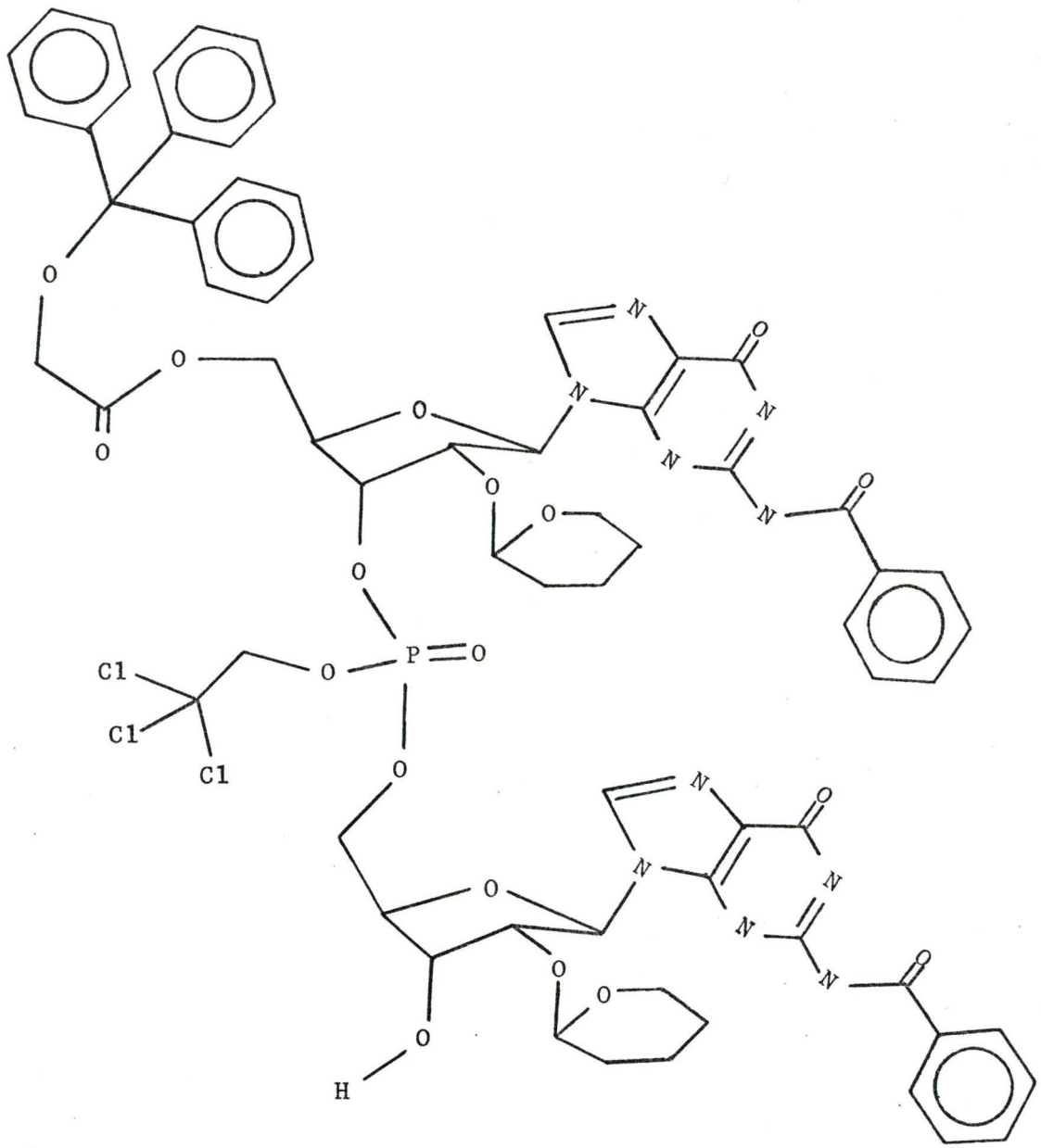
6. CONCLUDING REMARKS

This work has sucessfully provided a synthesis and characterization of the derivatives, N^2-benzoyl-2'-O-tetrahydropyranylguanosine and N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyl-guanosine. The synthesis of guanylyl(3'-5')guanosine using these derivatives provides correlative evidence with p.m.r. data for the position of the tetrahydropyranyl group as on the 2' hydroxyl.



	R_1	R_2
VI	H	H
VII	triphenylmethoxyacetyl	triphenylmethoxyacetyl
VIII	H	triphenylmethoxyacetyl
IX	H	tri-isopropylbenzenesulfonyl
X	$-P(=O)(O^-)-OCH_2CCl_3$	triphenylmethoxyacetyl
XIII	H	monomethoxytriphenylmethyl

Figure 9: Guanosine derivatives



XII

Figure 10: Diagram of trOac-G^{bz}(thp)-p(Cl_3Et)G^{bz}(thp)-OH (XII)

TABLE III

P.m.r. data of guanosine derivatives

Derivative	Chemical Shift (ppm)				
	H(8)	H(1')(J hz)[a]	H(2')(J hz)[a]	H(3')	other
HO-G^{bz}(OH)-OH	8.35	5.95(5.5 hz)	4.55(5.5 hz)	4.25	
bz-G^{bz}(OH)-ac	8.25	6.00(6.0 hz)	5.05(6.0 hz)	5.50	$CH_3C(O)$- 2.20
bz-G^{bz}(ac)-OH[b]	8.25	6.20(4.0 hz)	5.75(4.0 hz)		2.18
meOac-G^{bz}(OH)-ac[b]	8.18	5.95(7 hz)	4.95(7 hz)	5.2	2.10
meOac-G^{bz}(ac)-OH[b]	8.18	6.05(5 hz)	5.60(5 hz)		2.05
bz-G^{bz}(thp)-ac	8.25	6.20(8.0 hz)	5.25(8 hz)	5.7	2.20
Lo R_f HO-G^{bz}(thp)-OH	8.35	6.15(8.0 hz)	5.20	4.80	thp- 1.5
Hi R_f HO-G^{bz}(thp)-OH	8.40	6.12(7.0 hz)	5.05	4.70	1.5
Lo R_f trOac-G^{bz}(thp)-OH	8.25	6.15(6.0 hz)	5.4	4.75	$-CH_2C(O)$- 3.8
trOac-G^{bz}(thp)-trOac	8.20	6.0(7 hz)	5.5	5.0	3.8

a: H(1') occurs as a doublet resonance; H(2') is a triplet resonance where J is given. b: Data is from a mixture of the 2'- and 3'-O-acetate isomers.

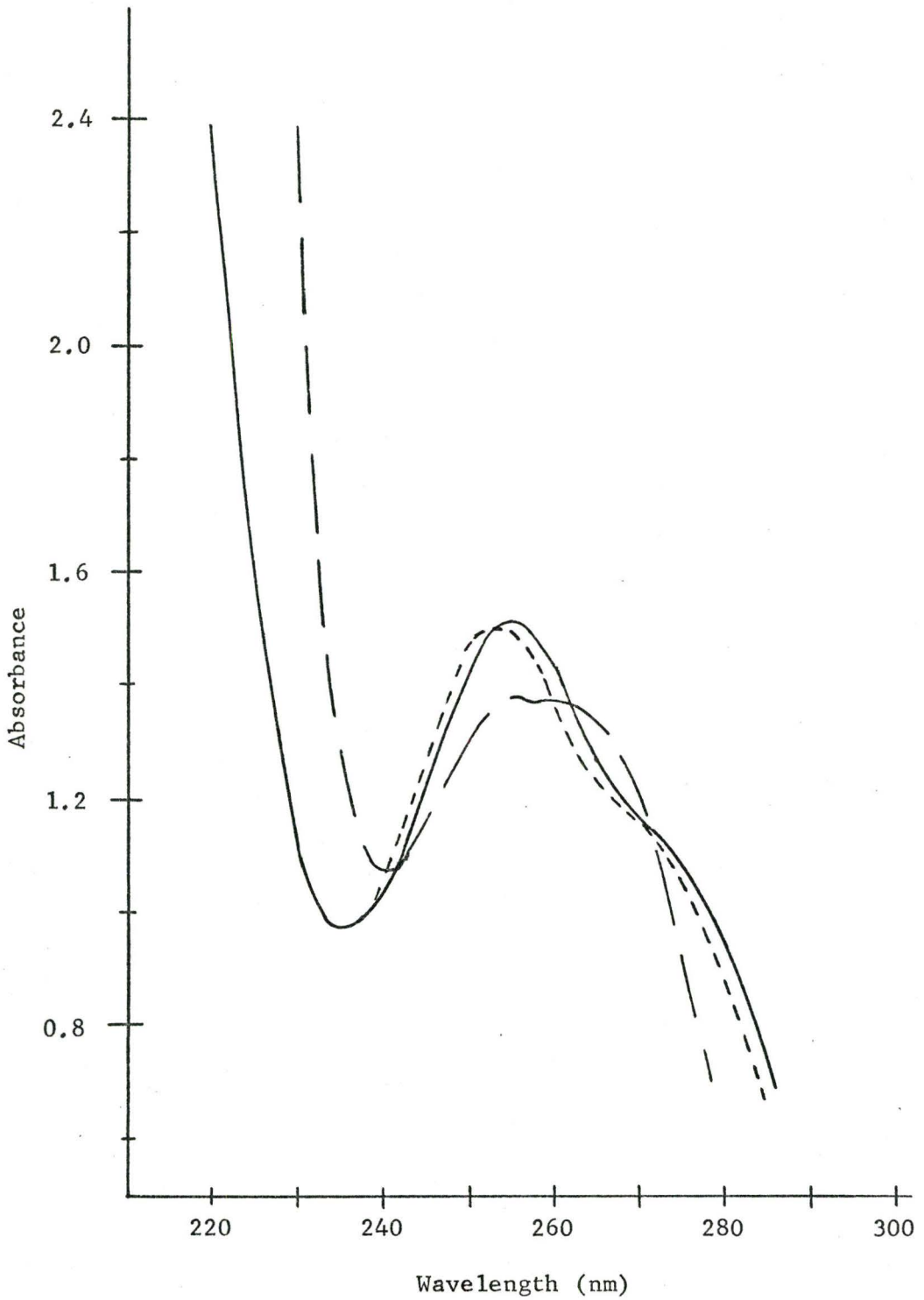


Figure 11: U.v. spectra of guanylyl(3'-5')guanosine (XVI)
(—— pH = 1.0; - - - pH = 6.5; —— — pH = 12.5)

TABLE IV

Paper chromatography of guanosine derivatives (R_{Up}'s)

Compound	Solvent*				
	A		B		C
	Exp.	Lit.	Exp.	Lit.	Exp.
Up	1.00		1.00		1.00
Gp	0.50		0.78	0.81(66)	0.63
pG			0.66		
G			1.09		
GpG	0.81	0.82(31) 0.90(67)**	0.70	0.75(31) 0.70(67)**	0.15

*Solvent systems were: A, ethanol-1M ammonium acetate (pH = 7.5) (7 : 3 v/v); B, 1-propanol-conc. ammonium hydroxide-water (55 : 10 : 35 v/v); C, 1-butanol-glacial acetic acid-water (5 : 3 : 2 v/v).

**Khorana reports diverse values for these R_{Up}'s.

TABLE V

Chromatography of guanosine derivatives on tlc. cellulose (R_f's)

Compound	Solvent*	
	A	C
Up	0.15	0.18
Gp	0.055	0.090
pG		0.087
G	0.44	0.28
GpG	0.104	0.042

*Solvent systems were: A, ethanol-1M ammonium acetate (pH = 7.5) (7 : 3 v/v); C, 1-butanol-glacial acetic acid-water (5 : 3 : 2 v/v).

These compounds, then, complete the set of precursor nucleosides necessary for a chemical synthesis of the oligoribonucleotide duplex sequences of the acceptor stem region of yeast $tRNA^{ala}$.

EXPERIMENTAL

1. MATERIALS AND PHYSICAL METHODS

Reagents and solvents used were of the best commercially available. Solvents that were required to be anhydrous, such as pyridine and N,N-dimethylformamide, were stored over molecular seives.

Analtech prescored silica gel G plates (250 microns thickness) were used for qualitative tlc. analysis of reaction mixtures. These plates were developed in 10% methanol-methylene chloride solvent system. Detection was effected by spraying with a ceric sulfate-sulfuric acid spray (1 g. $H_4Ce(SO_4)_4$ per 100 g. 10% aqueous sulfuric acid solution) and heating to 300-400 ^{o}C. Compounds containing a trityl group develop as yellow spots, while others appear as brown colorations.

Silica gel (Baker analyzed, 40-140 mesh) columns were prepared from a slurry in the stated solvent.

Cellulose tlc. plates (Avicel F, fluorescent background, 250 microns thickness) were used to detect deblocked nucleotides. The development of these plates was done in a closed chamber which had been equilibrated 4 h. with the stated solvent. A short wave u.v. lamp was used to visualize the materials.

Analytical paper chromatography was done on Whatman No. 1 MM paper. The development (descending) was done in a closed chamber that had been equilibrated with the stated solvent for 8 h. U.v. absorbing compounds could be visualized under short wave u.v. lamp.

Preparative paper chromatography was done on Whatman No. 3 MM and Whatman No. 40 (ashless) paper.

The solvent systems used for tlc on cellulose or paper chromatography were as follows: System A, ethanol-1 M ammonium acetate (pH = 7.5)(7 : 3 v/v); system B, 1-propanol-conc. ammonia-water (55 : 10 : 35 v/v); system C, 1-butanol-glacial acetic acid-water (5 : 3 : 2 v/v).

Elemental analysis was done by Galbraith Laboratories, Knoxville Tennesse.

Melting points were recorded, uncorrected, from a Gallenkamp hotblock apparatus.

U.v. spectra were obtained on a Beckman, Acta IV, recording spectrophotomer operating on double-beam mode using 1.0 cm. quartz cells.

P.m.r. spectra were recorded on a Varian Associates T-60 spectrometer. The positions of the signals are reported on the δ scale in ppm. downfield of the signal of tetramethylsilane(TMS) as internal standard. The spectra were run in deuterated dimethylsulfoxide (DMSO-d_6) (99.5% d_6, Stohler Isotope Chemicals) as a solvent with a concentration of 10-15% w/w of compound. In cases where the DMSO-d_6 proton peak was used as a reference, values of chemical shift were corrected to the δ scale by the correction factor 155 hz. upfield ($\Delta\delta$ = 2.55).

Phosphorylation reactions required anhydrous conditions. This was achieved by evaporation <u>in vacuo</u> of reactants from anhydrous pyridine several times, and then concentrating to one-half volume in a final operation. In all cases, pressure was restored in the system using dry nitrogen gas.

All *in vacuo* evaporations were done using water aspirator Buchi Rotovapor at 35-40 °C.

Last traces of pyridine could be removed from compounds by azeotropic evaporation *in vacuo* with toluene. Evaporation of N,N-dimethylformamide (DMF) could be accomplished by azeotropic evaporation *in vacuo* with water.

Emulsions, which were sometimes encountered in extractions of aqueous pyridine with methylene chloride, were broken by the addition of a few mililiters of saturated sodium chloride solution.

Dowex 50W-X8 resin was recycled. Used resin was treated one week with 2 N hydrochloric acid (H^+ cycle). Dowex 50 W-X8 (pyridinium cycle) was prepared by treating resin (H^+ cycle) one week with 50% aqueous pyridine. Dowex 50W-X8 (NH_4^+ cycle) was prepared from resin (H^+ cycle) by a 16 h. treatment with 2 N ammonium hydroxide.

The literature preparation of sodium triphenylmethoxyacetate(42) was modified to include a final crystallization of the compound from methyl ethyl ketone to give a sample of m.p. 296-298 °C.

2. PREPARATION OF TETRABENZOYLGUANOSINE

(a) This compound was prepared on a 10 g. scale in 80% yield as described (51).

(b) Guanosine (30 g. 0.11 mole) was suspended in pyridine (250 ml) and cooled to 0 °C on an ice bath. Benzoyl chloride (96 ml, 0.81 mole) was added dropwise to the stirred solution over a 2 h. period. The course of the reaction was followed by tlc. on SiO_2 in 10% MeOH-CH_2Cl_2. Tlc. at this point indicated complete reaction (R_f: 0.0 → 0.9). Ice (ca. 10 g.) was added to the yellow solution and stirring continued for one-half hour. The solution was then poured into ice-water (500 ml) and the product extracted by partitioning with methylene chloride (3 x 100 ml). The combined organic layers were washed with saturated $NaHCO_3$ solution (300 ml), followed by water (2 x 100 ml), and evaporated to dryness in vacuo. The yellow-orange color and benzoic acid were triturated from the product using CH_2Cl_2 (2 x 50 ml). Yield of crude tetrabenzoylguanosine was 68.3 g. (92%).

A small sample of this material was crystallized from methyl ethyl ketone. It had a melting point of 162-165 °C (Lit. (51): m.p. of tetrabenzoylguanosine 162.5-165 °C).

3. PREPARATION OF N^2-BENZOYLGUANOSINE (I)

(a) A 5 g. scale preparation was done as described (51).

(b) The above crude tetrabenzoylguanosine (68.3 g, 0.098 mole) was suspended in methanol (150 ml), dioxane (250 ml), chloroform (250 ml)

and cooled to 0 °C on an ice bath. Sodium methoxide (25 g, 0.46 mole) was suspended in methanol (200 ml) and added dropwise to the above stirred solution over a 1 1/2 h. period. The suspension thickened initially and stirring had to be effected mechanically. The course of the reaction was followed by tlc. on SiO_2 in 10% $MeOH/CH_2Cl_2$. After 2 h. of reaction, tlc. indicated complete conversion to a less mobile product (R_f: 0.9 → 0.06). Products of intermediate mobility could be detected at earlier times during the reaction.

The reaction was halted by pouring the mixture into 0.1 N HCl (1000 ml) followed by the addition of Dowex 50W-X8 (pyridinium cycle) (350 ml). Acetone (500 ml) was added to dissolve all white materials in the near neutral solution (pH ca. 6). The resin was filtered off and washed with 50% aqueous methanol. The two phase system in the filtrate was partitioned and the water layer extracted with methylene chloride (3 x 200 ml) until a white precipitate began to form in the water layer. This was then set aside to allow completion of precipitation of product. The combined organic layers were washed with water (3 x 100 ml) and these aqueous layers were added to the above precipitating solution.

Filtration and drying of the white precipitate afforded 31.6 g. (83%) of N^2-benzoylguanosine (I) of m.p. 242-244 °C (Lit. (51) m.p. 246-248 °C of analytical sample of N^2-benzoylguanosine).

The p.m.r. spectrum in DMSO-d_6 included the following signals (δ (TMS), ppm.): 8.35(1 H, singlet, H(8)); 8.1-7.6(5 H, multiplet, $C_6H_5C(O)$- protons); 5.95(1 H, doublet(J = 5.5 hz), H(1')); 4.55(1 H, triplet(J = 5 hz), H(2')); 4.25(1 H, multiplet, H(3')); 4.00(1 H, multiplet, H(4')); 3.65(2 H, multiplet, H(5')).

4. PREPARATION OF N^2-BENZOYL-2', 3'-O-METHOXYETHYLIDINEGUANOSINE (II)

N^2-benzoylguanosine (I) (4.0 g, 10.3 mmoles) was suspended in DMF (10 ml) and trimethylorthoacetate (10 ml). p-Toluenesulfonic acid monohydrate (1.5 g, 7.9 mmoles) was dissolved in DMF (5 ml) and this was added dropwise to the above suspension with stirring at room temperature over 1.5 h. period. By this time the suspension had cleared; the course of the reaction proceeded to a major product (R_f: 0.06 → 0.5 in 10% MeOH-CH_2Cl_2) judged to be the N^2-benzoyl-2', 3'-O-methoxyethylidineguanosine (II). The reaction was halted at 1.5 h. by the addition of pyridine (10 ml) and a few drops of conc. ammonia. The mixture was evaporated *in vacuo* to one-half volume and used immediately in the next preparation.

5. PREPARATION OF 3'-O-ACETYL-N^2, 5'-O-DIBENZOYLGUANOSINE (III)

Pyridine (30 ml) was added to the above solution of N^2-benzoyl-2', 3'-O-methoxyethylidineguanosine (10.3 mmoles) and the mixture cooled to 0 °C on an ice bath. Benzoyl chloride (5ml, 43 mmoles) was added dropwise over a 2 h. period. A more mobile product began to appear (R_f: 0.5 → 0.85 in 10% MeOH-CH_2Cl_2) and after 2 h. reaction was complete. Ice (*ca.* 5 g.) was stirred into the amber solution for 30 min. The solution was then poured into ice-water (50 ml) and partitioned with methylene chloride (3 x 25 ml). The combined organic extracts were washed with saturated $NaHCO_3$ solution (50 ml), followed by water (2 x 25 ml), and evaporated *in vacuo* to a syrup. 10% acetic acid (25 ml) was added and the heterogeneous mixture stirred for 16 h. at room temperature. Tlc. indicated complete hydrolytic opening of the

2', 3'-O-methoxyethylidine ring had occurred (R_f: 0.85 → 0.63 in 10% MeOH/CH_2Cl_2). The product (III) was extracted with methylene chloride (3 x 25 ml) and the combined organic layers washed with saturated $NaHCO_3$ solution (50 ml). Evaporation left a foam which was column chromatographed on 100 g. of SiO_2 in methylene chloride. Elution with 3 and 3.5% MeOH/CH_2Cl_2 mixtures (1000 ml, 1000 ml, respectively) yielded 4.5 g. (80%) of crude product (R_f: 0.64 in 10% MeOH/CH_2Cl_2). Crystallization from absolute ethanol was effected by warming (60 °C) a suspension of the product until dissolution and allowing the solution to cool. The first crop of crystals yielded 2.8 g. (50%) of 3'-O-acetyl-N^2, 5'-O-dibenzoylguanosine of melting point 208-212 °C. A second crop of crystals could be had by evaporating the mother liquor to dryness in vacuo and repeating the crystallization procedure. This crop could contain some 2'-O-acetate isomer (p.m.r. spectrum).

A small sample was recrystallized from absolute ethanol. It had a m.p. of 214-216 °C.

Elemental analysis: Found: %C = 58.80, %H = 4.27, %N = 13.14. Calculated for $C_{26}H_{23}N_5O_8$ (mol. wt. 533.50): %C = 58.54, %H = 4.35, %N = 13.13.

The p.m.r. spectrum in DMSO-d_6-3 drops CD_3COOD (1 M, D_2O) included the following signals (δ (TMS), ppm.): 8.25(1 H, singlet, H(8)); 8.2-7.4(5 H, multiplet, $C_6H_5C(O)$- protons); 6.00(1 H, doublet (J = 6.0 hz), H(1')); 5.5(1 H, multiplet, H(3')); 5.05(1 H, triplet (J = 6.0 hz), H(2')); 2.20(3 H, singlet, $CH_3C(O)$- protons); (the $CH_3C(O)$- protons of 2'-O-acetate isomer occur at 2.15δ).

6. PREPARATION OF N^2-BENZOYL-2'-O-TETRAHYDROPYRANYLGUANOSINE (VI)

A solution of p-toluenesulfonic acid monohydrate (0.80 g, 4.2 mmoles) in dry dioxane (20 ml) containing molecular seives (0.4 g.) was cooled on an ice bath until solid dioxane coated the inside of the flask. Dihydropyran (7.37 g, 8 ml, 0.088 mole) was then added dropwise with stirring. When a bright red color had developed (ca. 10 min.), this solution was added dropwise over one-half hour period to a suspension of 3'-O-acetyl-N^2, 5'-O-dibenzoylguanosine (III) (2.13 g, 4.0 mmoles) in dry dioxane (20 ml) at room temperature. The course of the reaction was followed by tlc. on SiO_2 in 10% MeOH-CH_2Cl_2 by monitoring the disappearance of starting material (III) (R_f = 0.64). The appearance of more mobile products was obscured by a dihydropyran-derived splotch at R_f = 1.0. After 1 h. of reaction, tlc. indicated ca. 80% conversion to products; the suspension had cleared. A further portion of dihydropyran (1 ml) was added to the clear solution to ensure complete reaction. After 1/2 h. conc. NH_4OH was added (0.5 ml) and the precipitated ammonium p-toluenesulfonate collected and washed with CH_2Cl_2. Evaporation of the filtrate in vacuo left a syrup.

Pyridine (10 ml), absolute ethanol (20 ml) and 2 N-50% ethanolic sodium hydroxide (20 ml) was added. Tlc. showed the immediate appearance of products (R_f: 0.33 and 0.30 in 10% MeOH-CH_2Cl_2). After 10 min. (the optimum time for this reaction) Dowex 50W-X8 (pyridinium form) (100 ml) was added and stirred in the reaction mixture for 30 min. The resin was collected and washed well with CH_2Cl_2-EtOH. Evaporation of the filtrate in vacuo afforded a syrup. This was

adsorbed to silica gel (5 g.) by evaporation in vacuo of a methylene chloride slurry of the syrup. This was then put on a column of silica gel (60 g.) (1.5 cm x 100 cm) which had been prepared with 2% MeOH-CH_2Cl_2. This procedure reduced 'cracking' of the column by local heating effects when the first band (a hydrolysis product of dihydropyran) was washed through the column by elution with 2% MeOH-CH_2Cl_2. Continued elution with 2.5% MeOH-CH_2Cl_2 (500 ml) gave some high R_f impurity; and then, high R_f diasterioisomer (0.30 g, 16%, R_f = 0.34 in 10% MeOH-CH_2Cl_2). A mixture of diasterioisomers (0.43 g.) and finally pure low R_f diasterioisomer (0.94 g, 50%, R_f = 0.30 in 10% MeOH-CH_2Cl_2) was eluted with 3.0, 3.5, 4.0% MeOH-CH_2Cl_2 mixtures (500 ml, 500 ml, 500 ml, respectively). The overall yield of tetrahydropyranyl derivative (VI) was then 0.30 g. + 0.43 g. + 0.94 g. = 1.67 g. (90%).

High R_f N^2-benzoyl-2'-O-tetrahydropyranylguanosine was crystallized from ethyl acetate-(trace) methanol to give an analytical sample of m.p. 152-154 °C.

Elemental analysis: Found: %C = 53.95, %H = 5.58, %N = 14.12. Calculated for $C_{22}H_{25}N_5O_7.H_2O$ (mol. wt. 489.49): %C = 53.98, %H = 5.56, %N = 14.31.

P.m.r. spectra in DMSO-d_6 included the following signals (δ(TMS), ppm.): 8.40(1 H, singlet, H(8)); 8.2-7.4(5 H, multiplet, $C_6H_5C(O)$- protons); 6.18(1 H, doublet(J = 7 hz), H(1')); 5.00(1 H, multiplet, H(2')); a broad peak at 1.50(6 H, multiplet, thp methylene protons).

Crystallization of low R_f N^2-benzoyl-2'-O-tetrahydropyranyl-guanosine from ethyl acetate-methanol (2 : 1) gave an analytical sample of m.p. 192-194 °C.

Elemental analysis: Found: %C = 54.12, %H = 5.54, %N = 14.27. Calculated for $C_{22}H_{25}N_5O_7.H_2O$ (mol. wt. 489.49): %C = 53.95, %H = 5.56, %N = 14.31.

P.m.r. spectra in DMSO-d_6 included the following signals (δ (TMS), ppm.): 8.38(1 H, singlet, H(8)); 8.2-7.4(5 H, multiplet, $C_6H_5C(O)$- protons); 6.15(1 H, doublet(J = 8 hz), H(1')); 5.20(1 H, multiplet, H(2')); a broad peak at 1.50(6 H, multiplet, thp methylene protons).

7. PREPARATION OF N^2-BENZOYL-2'-O-TETRAHYDROPYRANYL-5'-O-TRIPHENYLMETHOXYACETYLGUANOSINE (VIII)

(a) Sodium triphenylmethoxyacetate (42) (0.686 g, 2 mmoles) was evaporated *in vacuo* from anhydrous pyridine (3 x 10 ml) and then brought to *ca*. 5 ml and sealed under dry N_2. Tri-isopropylbenzenesulfonyl chloride (0.325 g, 1.1 mmoles) was added and the solution set aside in the dark at room temperature for 2 h.

The low R_f diasterioisomer of N^2-benzoyl-2'-O-tetrahydropyranyl-guanosine (VI) (0.420 g, 0.89 mmole) was evaporated *in vacuo* from anhydrous pyridine (3 x 10 ml) and its volume reduced to *ca*. 5 ml and sealed under dry N_2. This solution was then quickly added to the above activated triphenylmethoxyacetate and this amber solution set aside at room temperature in the dark. The course of the reaction could be

followed by tlc. on SiO_2 in 10% MeOH-CH_2Cl_2 (R_f: 0.30 → 0.66). A more mobile 'yellow' staining product appeared after one day of reaction. After 2 da. of reaction, the proportions (10%), as estimated visually, of product to reactant had changed very little. TPS (0.1 g, 0.3 mmole) was then added; one day later, tlc. indicated ca. 50% reaction. TPS (0.1 g, 0.3 mmole) was again added; one day later, tlc. indicated ca. 75% reaction. TPS (0.1 g, 0.3 mmole) was again added and on the following or fifth day, the reaction was judged to be almost complete.

Ice (ca. 1 g.) was added, and the solution stirred for 2 h. The amber mixture was then poured into water (40 ml) and partitioned with methylene chloride (3 x 10 ml). The combined organic layers were washed with water (20 ml) and evaporated in vacuo to a foam. This was column chromatographed on SiO_2 (20 g.) in methylene chloride.

Elution with 1.0% MeOH-CH_2Cl_2 (500 ml) yielded some 'yellow' staining material (R_f = 0.85 in 10% MeOH-CH_2Cl_2). This was later shown to be di-triphenylmethoxyacetate derivative (VII).

Elution with 2.0, 2.5% MeOH-CH_2Cl_2 mixtures (250 ml, 500 ml, respectively) gave 0.290 g. (42%) of N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanosine (VIII). P.m.r. of this material, one-spot on tlc. (R_f = 0.66 in 10% MeOH-CH_2Cl_2), revealed 5'-O-tri-isopropylbenzenesulfonate (IX) as a 10% impurity. Crystallization was attempted. The product was dissolved in CH_2Cl_2 (ca. 5 ml) and hexane added until the solution became cloudy. A few drops of CH_2Cl_2 were then added to give a clear solution. This mixture was sealed and cooled to 0 °C overnight. Recrystallization was performed on the first crop of

amorphous precipitate obtained above to give a sample of melting point 110 °C (decomp.) and less than 3% impurity by p.m.r. in DMSO-d_6 (15% w/v). The spectrum included the following signals (δ (TMS), ppm.): 8.25(1 H, singlet, H(8)); 8.2-7.1(20 H, multiplet, $C_6H_5C(O)$- and C_6H_5- protons); 6.14(1 H, doublet(J = 6 hz), H(1')); 5.45(1 H, multiplet, H(2')); 4.75(1 H, multiplet, H(3')); 3.8(2 H, singlet, -$OCH_2C(O)$- protons); a broad peak at 1.5(6 H, multiplet, thp methylene protons).

Elemental analysis: Found: %C = 67.03, %H = 5.46, %N = 8.98. Calculated for $C_{43}H_{41}N_5O_9$ (mol. wt. 771.83): %C = 66.92, %H = 5.35, %N = 9.07.

(b) A modified preparation of X was carried out as follows. Sodium triphenylmethoxyacetate (42) (0.525 g, 1.53 mmoles) was dissolved in pyridine (25 ml) and evaporated in vacuo to ca. 5 ml. TPS (0.234 g, 0.775 mmole) was added and the solution set aside for 1 h. in the dark at room temperature.

Low R_f diasterioisomer of N^2-benzoyl-2'-O-tetrahydropyranyl-guanosine (VI) (0.50 g, 1.03 mmoles) was dissolved in pyridine (25 ml) and evaporated in vacuo to ca. 10 ml. This solution was quickly added to the above activated triphenylmethoxyacetate and the amber solution set aside in the dark at room temperature. After 2 h. ca. 0.5 ml of dicyclohexylcarbodiimide (DCC) was added (DCC was warmed to a viscous fluid before handling). The course of reaction was followed by tlc. on SiO_2 gel in 10% MeOH/CH_2Cl_2. After 3 h, an estimated 50% reaction had occurred; after 5 h, the more mobile 'yellow' staining product (R_f = 0.66) composed an estimated 70% of the reaction material. After 16 h. the reaction was judged complete.

Ice (ca. 1 g.) was added and solution stirred for 2 h. White precipitate was filtered off and washed with CH_2Cl_2; the filtrate and washings were added to ice-water (35 ml) and repeatedly extracted with methylene chloride (5 x 10 ml). The combined extracts were washed with H_2O (20 ml) and evaporated in vacuo to a yellow-orange glass. This was put on a SiO_2 gel column (15 g.) in methylene chloride.

Elution with 1% MeOH-CH_2Cl_2 (250 ml) gave some impure di-triphenylmethoxyacetate derivative (VII). Elution with 2.0, 3.0% MeOH-CH_2Cl_2 mixtures (250 ml, 250 ml, respectively) gave 0.6 g. (75%) of product VIII (R_f = 0.66 in 10% MeOH-CH_2Cl_2). P.m.r. spectra of this material was identical to that prepared above, (a), except no impurity, IX, could be detected.

8. PURIFICATION OF N^2-BENZOYL-2'-O-TETRAHYDROPYRANYL-3', 5'-O-DI-TRIPHENYLMETHOXYACETYLGUANOSINE (VII)

The 1% MeOH-CH_2Cl_2 eluate fractions from the preparation of VIII described above were combined to give 0.7 g. of material, which was put on a silica gel column (10 g.) in 70% hexane-methylene chloride. Elution with 70%, 30% hexane-methylene chloride mixtures (100 ml, 100 ml, respectively) removed some impurity (ca. 0.2 g.). Elution with methylene chloride (250 ml) gave 0.50 g. of VII as one-spot material on tlc. (R_f = 0.85 in 10% MeOH-CH_2Cl_2). Crystallization could not be effected from methylene chloride-hexane mixtures.

P.m.r. spectra of a sample in DMSO-d_6 included the following signals (δ (TMS), ppm.): 8.20(1 H, singlet, H(8)); 8.2-7.4(35 H,

multiplet, $C_6H_5C(O)$- and C_6H_5- protons); 6.00(1 H, doublet(J = 7 hz), H(1')); 5.5(1 H, multiplet, H(2')); 5.1(1 H, multiplet, H(3')); 3.8(4 H, singlet, $-OCH_2C(O)$- protons); a broad peak at 1.5(6 H, multiplet, thp methylene protons).

9. HYDROLYSIS STUDY ON N^2-BENZOYL-2'-O-TETRAHYDROPYRANYL-3', 5'-O-DI-TRIPHENYLMETHOXYACETYLGUANOSINE (VII)

3 mg. of sample (VII or VI) was dissolved in 1 ml of 1/10 saturated (at 0 °C) methanolic ammonia at room temperature and sealed (Methanolic ammonia was prepared by bubbling NH_3 (gas) through a cooled (0 °C) solution of methanol for one-half hour, followed by dilution with cooled methanol (0 °C).). Samples were taken periodically and spotted on tlc. plates and developed in 10% MeOH-CH_2Cl_2. Proportions of products in the reaction were estimated visually upon development of the plates. The results are discussed in Results and Discussion, Section 3.

10. PREPARATION OF N^2-BENZOYL-2'-O-TETRAHYDROPYRANYL-5'-O-TRIPHENYL-METHOXYACETYLGUANOSINE-3'-2,2,2-TRICHLOROETHYL PHOSPHATE (X)

Bis-cyclohexylamine salt (0.36 g, 0.81 mmole) of mono-2,2,2-trichloroethyl phosphate monohydrate (48) was dissolved under gentle reflux in anhydrous pyridine (20 ml) and converted to its pyridinium salt by repeatative evaporation in vacuo and redissolution in pyridine (5 x 20 ml). Finally, the volume was brought to ca. 10 ml, sealed under dry N_2, and TPS (0.494 g, 1.62 mmoles) was added. The activation was allowed to proceed for 1 h. at room temperature in the dark.

An anhydrous pyridine solution (prepared by evaporation from pyridine (3 x 10 ml) and finally reducing the volume to ca. 5 ml) of N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanosine (VIII) (0.314 g, 0.41 mmole) was added to the activated phosphate, and the yellow reaction mixture sealed under dry N_2 and set aside at room temperature in the dark. The course of the reaction was followed by tlc. on SiO_2 in 10% MeOH-CH_2Cl_2. A less mobile yellow-staining product began to appear after 4 da. of reaction (R_f: 0.65 → 0.0-0.2). The addition of TPS (0.1 g, 0.33 mmole) once a day for the next 5 days, drove the reaction to ca. 80% completion. Ice (ca. 1 g.) was stirred into the solution for 2 h. The mixture was then poured into ice-water (150 ml) and repeatedly extracted with methylene chloride (6 x 50 ml). The combined organic extracts were washed with distilled water (3 x 40 ml) and evaporated in vacuo to a brown glass.

The phosphorylated product (X) was removed from the more mobile unreacted starting material (VIII) by column chromatography on deactivated silica gel (15 g, 10% H_2O) in methylene chloride. Elution with 2% MeOH-CH_2Cl_2 (500 ml) removed high R_f yellow-staining material (R_f > 0.5). Elution with 4, 5% MeOH-CH_2Cl_2 mixtures (250 ml, 250 ml, respectively) gave 0.3 g. of product (R_f = 0.0-0.2 in 10% MeOH-CH_2Cl_2) as a dark brown foam. This was dissolved in anhydrous pyridine and used immediately in the preparation of blocked dinucleoside phosphates.

11. PREPARATION OF N^2-BENZOYL-2'-O-TETRAHYDROPYRANYL-5'-O-TRIPHENYLMETHOXYACETYLGUANYLYL(3'-2,2,2-TRICHLOROETHYL-5')N^2-BENZOYL-2'-O-TETRAHYDROPYRANYLGUANOSINE (XII)

TPS (0.135 g, 0.45 mmole) was added to the blocked guanylate derivative X (0.41 mmole, theoretical) in anhydrous pyridine (prepared by evaporation from anhydrous pyridine (3 x 20 ml) and volume finally reduced to ca. 5 ml) and activation allowed to proceed for 30 min. in the dark at room temperature. High R_f diasterioisomer of N^2-benzoyl-2'-O-tetrahydropyranylguanosine (VI) (0.300 g, 0.61 mmole) dissolved in anhydrous pyridine (2 ml) was added and the reaction mixture sealed under dry N_2 and set aside at room temperature in the dark.

The course of the reaction was followed by tlc. on SiO_2 in 10% MeOH-CH_2Cl_2 (R_f of X = 0.0-0.2, R_f of VI = 0.33). Very little reaction occurred and TPS (0.04 g, 0.13 mmole) was added each day. After 1 wk. a new 'yellow'-staining product appeared at R_f = 0.4, partially obscurred by VI (R_f = 0.34). Continued additions of TPS, the addition of high R_f VI (0.050 g, 0.10 mmole) on the tenth day, increased the product. On the fifteenth day, ice (ca. 1 g.) was stirred into the dark brown mixture for 15 min. The solution was added to iced saturated $NaHCO_3$ solution (20 ml) and stirred for 30 min. (pH = 7). The solution was then added to ice water (100 ml) and extracted with methylene chloride (6 x 30 ml). The combined organic extracts were washed with water (30 ml) and evaporated in vacuo to dryness. This material was put on SiO_2 gel column (20 g.) in methylene chloride. Elution

with 1% MeOH-CH_2Cl_2 (250 ml) eluted dark red impurities (R_f = 0.0 in 10% MeOH-CH_2Cl_2). Elution with 3%, 3.5% MeOH-CH_2Cl_2 mixtures (250 ml, 250 ml, respectively) gave 0.240 g. of blocked GpG (XII) contaminated with VI, estimated to be in ca. 1 : 1 ratio (R_f of XII = 0.40, R_f of VI = 0.33 in 10% MeOH-CH_2Cl_2).

This mixture was treated with an excess of p-anisylchlorodiphenylmethan (0.24 g, 0.8 mmole; ca. 2 equivalents based on 1 : 1 ratio of XII : VI in the fraction) in anhydrous pyridine (3 ml). The reaction was set aside in the dark at room temperature for 16 h. Tlc. then showed complete conversion of VI into N^2-benzoyl-5'-O-p-methoxytrityl-2'-O-tetrahydropyranylguanosine (XIII) (R_f: 0.34 → 0.82 in 10% MeOH-CH_2Cl_2). Ice (ca. 5 g.) was stirred into the solution for 30 min; the mixture was added to a cold saturated $NaHCO_3$ solution (75 ml) and extracted with methylene chloride (6 x 15 ml). The combined organic extracts were washed with water (20 ml) and evaporated in vacuo to a yellow foam.

This mixture was put on a SiO_2 column (10 g.) in methylene chloride. Elution with CH_2Cl_2 (100 ml) gave trityl alcohol; elution with 1%, 2% MeOH-CH_2Cl_2 mixtures (100 ml, 100 ml, respectively) gave 5'-O-p-methoxytrityl ether derivative (XIII) (0.2 g, R_f = 0.80 in 10% MeOH-CH_2Cl_2). Elution with 3%, 3.5% MeOH-CH_2Cl_2 mixtures (100 ml, 100 ml, respectively) gave a 0.025 g. fraction of XII with a trace of impurity, and a 0.035 g. pure fraction of XII that had R_f = 0.40 in 10% MeOH-CH_2Cl_2. Yield of product XII was 0.025 g. + 0.035 g. = 0.060 g. (10%). No p.m.r. spectra were run on XII.

12. PREPARATION OF GUANYLYL(3'-5')GUANOSINE (XVI)

N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyl-guanylyl(3'-2,2,2-trichloroethyl-5')N^2-benzoyl-2'-O-tetrahydropyranyl-guanosine (XII) (5 mg, 4.2 μmoles) was stirred in a solution of DMF with a suspension of Cu-Zn couple (ca. 20 mg.) at 50 °C. After 2 da, tlc. on SiO_2 in 10% MeOH-CH_2Cl_2 indicated complete conversion to ionic product (XIV) (R_f: 0.40 → 0.0). Half-saturated (at 0 °C) methanolic ammonia (1 ml) was then added and the reaction mixture sealed and set aside at room temperature for 2 days. N-ammonia solution (1 ml) was added, and the Cu-Zn couple filtered off and washed well with dil. ammonia. The combined filtrate and washings were carefully concentrated in vacuo and the solution pH brought to 8-10. Dowex 50W-X8 (NH_4^+ cycle) (ca. 1 g.) was stirred into the mixture for 15 min. The resin was filtered off and washed well with water. The combined filtrate and washings were concentrated in vacuo to ca. 5 ml. The pH of the solution was brought to 2.20 with 0.1 N HCl solution and the reaction set aside, sealed, at room temperature for 2 days.

The course of hydrolysis reaction was followed by tlc. on cellulose (Avicel F) in solvent A. After 1 da. a new product appeared at R_{Up} = 0.79; a product at R_{Up} = 1.01 had dissappeared. GpG (40 $O.D._{260}$ units, 3.2 μmoles, 76%; R_{Up} = 0.81 on p.c. in system A) was isolated by preparative paper chromatography on Whatman No. 3 MM paper and purified on Whatman No. 40 (ashless) paper by development with system A. Tlc. on cellulose and p.c. indicated that this product was guanylyl(3'-5')guanosine (GpG) (XVI) (Tables IV and V).

13. SNAKE VENOM DIESTERASE DIGESTION OF GUANYLYL(3'-5')GUANOSINE

A solution of the ammonium salt of GpG (20 $O.D._{260}$) in 0.2 ml of tris buffer (0.1 M, pH = 8.0) was digested completely at 37 °C by 100 λ of snake venom diesterase (E.C. 3.1.4.1.) (Worthington, 1 mg./ml) in 16 h. Paper chromatography in system B showed expected products (G + pG) of R_{Up}: 1.07, 0.62, respectively, in correct ratio. Tlc. on cellulose (Avicel F) in system C showed expected products (G + pG) of R_f: 0.27, 0.086, respectively, in correct ratio. No undigested material at R_f = 0.042 could be detected (< 1%).

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