THE UNIVERSITY OF MANITOBA

THE SYNTHESIS AND STUDY OF CYCLOURIDINE NUCLEOSIDES AND NUCLEOTIDES

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

WINNIPEG, MANITOBA

October, 1973

THE SYNTHESIS AND STUDY OF CYCLOURIDINE NUCLEOSIDES AND NUCLEOTIDES

By: Donald James Iwacha

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

To my wife

Clare

and my parents

John and Annie Iwacha

ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. Kelvin Kenneth Ogilvie for the guidance, inspiration and enthusiasm that he provided during the course of this work.

The financial assistance of the National Research Council and the University of Manitoba is gratefully acknowledged.

I wish to thank Edith and Bud Ogden for their help in typing and drafting the thesis and for their encouragement.

ABSTRACT

The purpose of this work was a) to investigate the use of O^2,2'-cyclouridine in the synthesis of O^2,2'-cyclouridine nucleotides as interesting analogs of natural nucleic acids, and b) to investigate the possibilities of the conversion of the O^2,2'-cyclouridine nucleotides into arabino- and ribonucleic acids.

Initially, the synthesis of suitably protected derivatives of O^2,2'-cyclouridine was investigated. 5'-O-Trityl-O^2,2'-cyclouridine was synthesized by treatment of 5'-O-trityluridine with diphenylcarbonate and sodium bicarbonate. The acidic conditions required to remove the trityl group resulted in hydrolysis of the anhydro linkage. To resolve the problem, the more sensitive acid-labile monomethoxytrityl group was used; thus, 5'-O-monomethoxytrityl-O^2,2'-cyclouridine was synthesized from O^2,2'-cyclouridine. Reaction of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine with β-benzoylpropionic acid or acetic anhydride followed by removal of the monomethoxytrityl group yielded 3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine and 3'-O-acetyl-O^2,2'-cyclouridine, respectively.

Phosphorylation of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine with β-cyanoethyl phosphate followed by condensation with 2',3'-O-isopropylideneuridine gave the cyanoethyl derivative of 5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (MMTr-cUp(CE)U-OIP). Removal of the monomethoxytrityl and cyanoethyl groups yielded O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine which was degraded by snake venom but not by spleen phosphodiesterase. The anhydro linkage was successfully hydrolysed by acid

to yield arabinouridylyl-(3'-5')-uridine. Removal of the monomethoxytrityl group from MMTr-cUp(CE)U-OIP yielded the cyanoethyl derivative of O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (cUp(CE)U-OIP). Attempts to displace the anhydro linkage with sodium benzoate and benzoic acid led to degradation of the cUp(CE)U-OIP.

3'-O-Acetyl-O^2,2'-cyclouridine was condensed with 5'-O-monomethoxytritylthymidine 3'-phosphate. Deblocking yielded thymidylyl-(3'-5')-O^2,2'-cyclouridine which was degraded by spleen enzyme but not by snake venom phosphodiesterase. The anhydro linkage was hydrolysed to yield thymidylyl-(3'-5')-arabinouridine which was degradable by enzymes.

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine and 3'-O-acetyl-O^2,2'-cyclouridine were condensed using methyl phosphorodichloridate to yield a mixture of the 3'-5' and 3'-3' isomers of O^2,2'-cyclouridylyl-O^2,2'-cyclouridine. Neither was degraded by the phosphodiesterases. Hydrolysis of the anhydro linkages led to the isomers of arabinouridylylarabinouridine. Only the 3'-5' isomer was degraded by the phosphodiesterases.

Synthesis of the β,β,β-trichloroethyl derivative of 5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (MMTr-cUp(TCE)U-OIP) was unsuccessful either by the use of β,β,β-trichloroethyl phosphorodichloridate or by the condensation of 5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine with β,β,β-trichloroethanol. MMTr-cUp(TCE)U-OIP was synthesized by phosphorylation of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine with

β,β,β-trichloroethyl phosphate followed by condensation with 2',3'-O-isopropylideneuridine. Deblocking the product yielded O^2,2'-cyclouridyly1-(3'-5')-2',3'-O-isopropylideneuridine. An attempt to displace the anhydro linkage of the β,β,β-trichloroethyl derivative of O^2,2'-cyclouridyly1-(3'-5')-2',3'-O-isopropylideneuridine with sodium benzoate and benzoic acid led to degradation of the product. An attempt to displace the anhydro linkage with potassium fluoride and acetic acid also led to degradation of the product.

The synthesis of the β,β,β-trichloroethyl and β-cyanoethyl triesters of dinucleoside monophosphates containing only the O^2,2'-cyclouridine moiety was unsuccessful.

The β,β,β-trichloroethyl derivative of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate was converted into 5'-O-monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate (MMTr-cUp) by treatment with copper/zinc in dimethylformamide. MMTr-cUp was condensed with 3'-O-acetyl-O^2,2'-cyclouridine to yield O^2,2'-cyclouridyly1-(3'-5')-O^2,2'-cyclouridine. Neither phosphodiesterase degraded the product. The anhydro linkage was hydrolysed by base to yield arabinouridyly1-(3'-5')-arabinouridine which was degraded by the phosphodiesterases.

ABBREVIATIONS

TsCl	p-toluenesulfonyl chloride
MS	2,4,6-trimethylbenzenesulfonyl chloride (mesitylenesulfonyl chloride)
TPS	2,4,6-triisopropylbenzenesulfonyl chloride (triisopropylbenzenesulfonyl chloride)
DCC	dicyclohexylcarbodiimide
βCEP	pyridinium β-cyanoethyl phosphate (β-cyanoethyl phosphate)
$TCEPCl_2$	β,β,β-trichloroethyl phosphorodichloridate
$MePCl_2$	methyl phosphorodichloridate
TCEP	dicyclohexylammonium salt of β,β,β-trichloroethyl phosphate (β,β,β-trichloroethyl phosphate)
Tr	triphenylmethyl (trityl)
MMTr	p-Anisyldiphenylmethyl (Monomethoxytrityl)
DMTr	di-p-Anisylphenylmethyl (dimethoxytrityl)
βB	β-benzoylpropionyl
Ac	acetyl
Bz	benzoyl
IP	isopropylidene
NaOBz	sodium benzoate
BzOH	benzoic acid
DMF	dimethylformamide
HOAc	acetic acid
R_m	mobility on electrophoresis relative to thymidine 3'-phosphate
R_f	mobility on chromatograms relative to the solvent front
Thy	thymine ring

Ura	uracil ring
T	thymidine
U	uridine
cU	O^2,2'-cyclouridine
aU	1-β-D-arabinofuranosyluracil (arabinouridine or arauridine)
Tr-cU	5'-O-trityl-O^2,2'-cyclouridine
MMTr-cU	5'-O-monomethoxytrityl-O^2,2'-cyclouridine
MMTr-cU-OβB	5'-O-monomethoxytrityl-3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine
cU-OβB	3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine
MMTr-cU-OAc	5'-O-monomethoxytrityl-3'-O-acetyl-O^2,2'-cyclouridine
cU-OAc	3'-O-acetyl-O^2,2'-cyclouridine
U-OIP	2',3'-O-isopropylideneuridine
MMTr-cUp(CE)	β-cyanoethyl ester of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate
MMTr-cUp(CE)U-OIP	β-cyanoethyl derivative of 5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine
MMTr-cUpU-OIP	5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine
cUp(CE)U-OIP	β-cyanoethyl derivative of O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine
cUpU-OIP	O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine
aUpU-OIP	arabinouridylyl-(3'-5')-2',3'-O-isopropylideneuridine
aUpU	arabinouridylyl-(3'-5')-uridine

MMTr-cUp(CE)cU-0βB	β-cyanoethyl derivative of 5'-0-monomethoxytrityl-0^2,2'-cyclouridylyl-(3'-5')-3'-0-(β-benzoylpropionyl)-0^2,2'-cyclouridine
TpcU-OAc	thymidylyl-(3'-5')-3'-0-acetyl-0^2,2'-cyclouridine
TpcU	thymidylyl-(3'-5')-0^2,2'-cyclouridine
TpaU	thymidylyl-(3'-5')-arabinouridine
MMTr-cUp(TCE)	β,β,β-trichloroethyl ester of 5'-0-monomethoxytrityl-0^2,2'-cyclouridine 3'-phosphate
MMTr-cUp(TCE)U-OIP	β,β,β-trichloroethyl ester of 5'-0-monomethoxytrityl-0^2,2'-cyclouridylyl-(3'-5')-2',3'-0-isopropylideneuridine
cUp(TCE)U-OIP	β,β,β-trichloroethyl ester of 0^2,2'-cyclouridylyl-(3'-5')-2',3'-0-isopropylideneuridine
MMTr-cUp	5'-0-monomethoxytrityl-0^2,2'-cyclouridine 3'-phosphate
cUpcU	0^2,2'-cyclouridylyl-(3'-5')-0^2,2'-cyclouridine
aUpaU	arabinouridylyl-(3'-5')-arabinouridine

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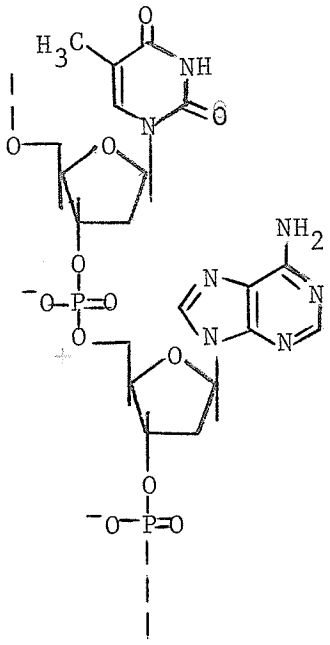
INTRODUCTION

Nucleic acids are among the most important molecules in nature. Deoxyribonucleic acid (DNA) stores and transmits the genetic make-up of living organisms. Ribonucleic acid (RNA) utilizes the information carried in the DNA to synthesize cellular proteins.

The structures of the two types of nucleic acids are similar. Both DNA(I) and RNA(II) are linear polymers of monomeric units called nucleotides.



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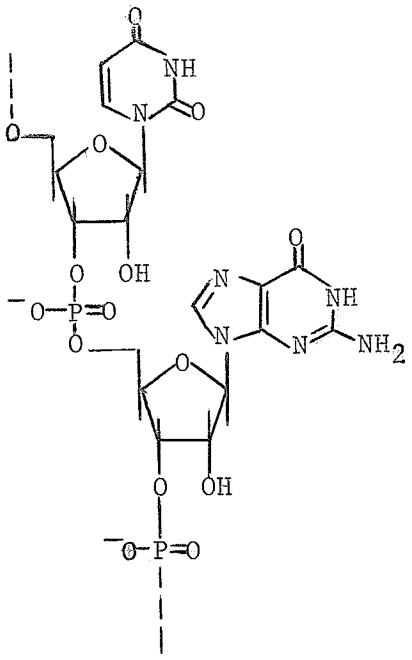
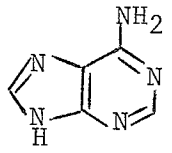


II

Nucleosides combine a heterocyclic base and a pentose sugar through a β-N-glycoside linkage. Nucleotides are the sugar-O-phosphate esters of the nucleosides. The nucleotides in nucleic acids are linked

by phosphodiester bridges between the sugars, forming polynucleotides possessing 3'-5' internucleotide linkages, i.e., the linkage is from the 3'-position of one nucleoside sugar to the 5'-position of the adjacent nucleoside sugar moiety. In DNA(I), the pentose sugar is D-2'-deoxyribose. The common bases are the purines adenine(III) and guanine(IV), and the pyrimidines thymine(V) and cytosine(VI). In RNA(II), the pentose sugar is D-ribose. The common bases are the purines adenine(III) and guanine(IV), and the pyrimidines cytosine(VI) and uracil(VII).



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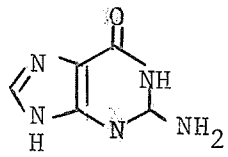

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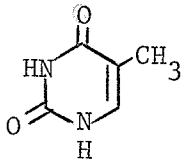

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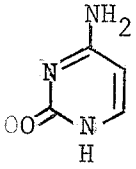

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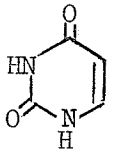
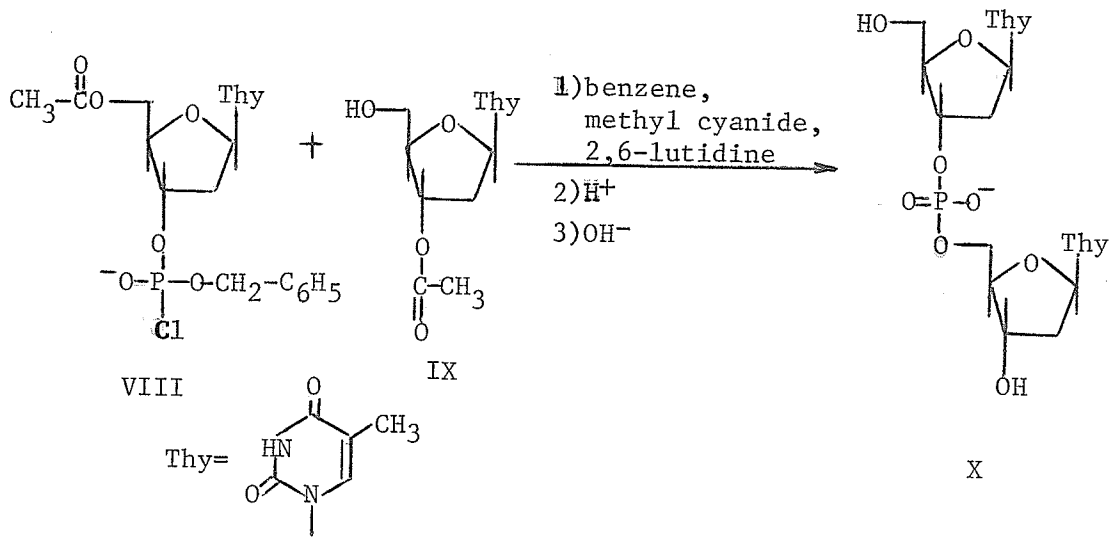


VII

To study the nucleic acids, the synthesis of nucleic acids and their components is of primary importance. An invaluable synthetic technique is organic chemical synthesis.

The first chemical synthesis of a molecule containing the 3'-5' internucleotide linkage found in nucleic acids was performed by Michelson and Todd[1] in 1955. Their synthesis involved the condensation of 5'-O-acetylthymidine 3'-(benzylphosphorochloridate) (VIII) with 3'-O-acetylthymidine(IX). The product of the condensation

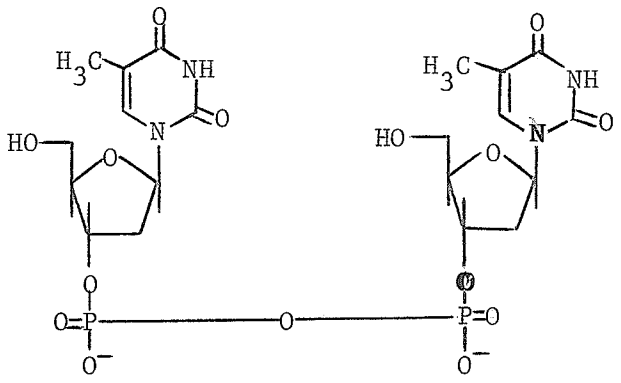
reaction, after removal of the hydroxyl blocking groups, was thymidylyl-(3'-5')-thymidine, TpT(X).



Two important aspects of their synthesis deserve comment. First, the specificity of the internucleotide linkage was predetermined by blocking those hydroxyl groups not taking part in forming the linkage by the use of the acetyl group. Second, the acetyl blocking group could be removed by base without affecting the rest of the dinucleoside monophosphate. These aspects of the specific blocking of hydroxyl groups and of the lability of the blocking groups are basic principles in the specific synthesis of the 3'-5' internucleotide linkage.

The condensation method unfortunately gave a low yield of the dinucleoside monophosphate product, TpT(25%). The phosphorochloridate (VIII) was prepared in situ from the corresponding phosphite by the

use of N-chlorosuccinimide, and, without purification, was condensed with added 3'-O-acetylthymidine. Also, the use of a phosphorochloridate in the presence of base (2,6-lutidine) led to substantial amounts of the pyrophosphate(XI). These problems resulted in a low yield of product and restrict the desirability of using this method in the synthesis of larger oligonucleotides.

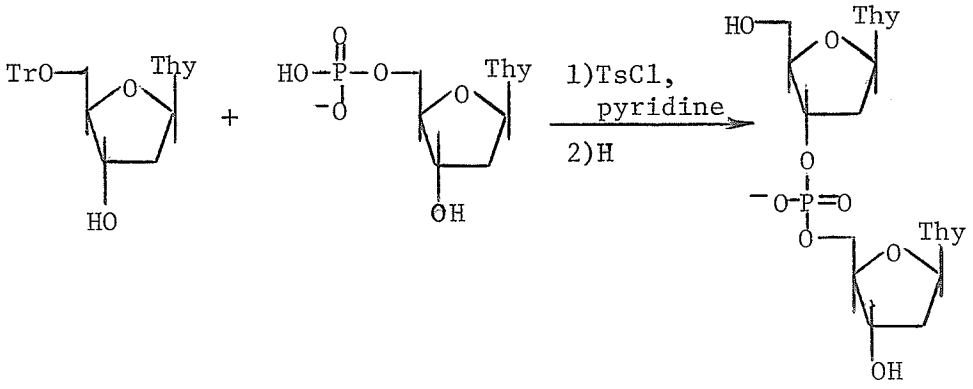


XI

A dramatic advance in the method of forming the 3'-5' internucleotide linkage was made by Khorana et al[2]. Condensation between a protected nucleoside and a nucleoside 5'-phosphate was achieved by using a condensing agent (p-toluenesulfonyl chloride, TsCl) to activate the phosphomonoester group in situ.

The yield of thymidylyl-(3'-5')-thymidine, TpT, was 45%. Through improving their technique the yield was increased to 66%[3], and finally 90%[4]. The 5'-hydroxyl group of the nucleoside was blocked using the triphenylmethyl (trityl or Tr) blocking group, leaving a free 3'-hydroxyl to form the required 3'-5' internucleotide

linkage with the nucleoside 5'-phosphate. The trityl group was removed by acid. To prevent self-condensation of the nucleoside 5'-phosphate, the base-labile acetyl blocking group was used to block the 3'-hydroxyl[3,4] in later work.



In addition to the introduction of arylsulfonyl condensing agents, Khorana et al[5] also introduced dicyclohexylcarbodiimide (DCC) as a condensing agent, In a comparison of the two activating agents[4], comparable yields (90%) were obtained using stoichiometric amounts of nucleoside and nucleotide. To achieve optimum yields, a greater excess of condensing agent to nucleotide was required for dicyclohexyl-carbodiimide (4-fold excess) than for arylsulfonyl chloride (1-fold excess). The rate of condensation was found to be faster for the arylsulfonyl chlorides (6 hours to completion of the reaction) than for DCC (24-48 hours to completion). Dicyclohexylcarbodiimide, however, gave a very clean reaction, the only DCC by-product being

dicyclohexylurea (DCU). The DCU is easily removed from the quenched reaction by filtration. The arylsulfonyl chlorides, on the other hand, gave sulfonylated derivatives of nucleosides by reaction of the chlorides with the free hydroxyls of the nucleosides. Though both methods gave similar yields, Khorana[3] preferred to use dicyclohexylcarbodiimide for the cleaner reactions which resulted.

Using these new condensation methods, the "diester synthesis" of oligonucleotides rapidly advanced. Dinucleoside phosphates were synthesized containing all the common bases present in DNA[3,6]. The chain length of oligonucleotides was increased using a stepwise approach[7], the length of the chain being increased by one nucleotide unit after each condensation.

In addition, the synthesis of oligonucleotides by the condensation of preformed "blocks" was developed[8,9]. The advantages of using blocks were twofold; first, the number of synthetic steps was reduced; and second, the greater charge difference between the starting and product oligonucleotides allowed better separation on ion-exchange columns[10].

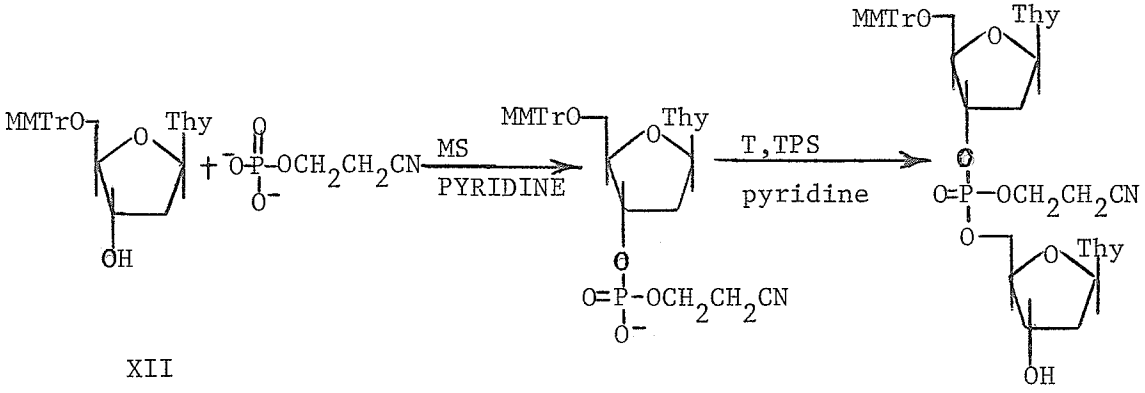
The "diester method", however, posed certain difficulties which rendered the method unsuitable to large-scale synthesis. These disadvantages were: i) the necessity of using DEAE-cellulose chromatography for the separation of the products, a time-consuming and low capacity technique[7], ii) the rapid falling off of yields with increasing chain length, a difficulty which could only be overcome by

employing increasingly large excesses of the 5'-phosphomonoester component[7,9], and iii) the occurrance of chain fission due to intra- and intermolecular condensation reactions involving the phosphodiester anions which produce undesirable 3'-3' phosphodiester linkages[7].

To overcome these difficulties, the "triester method" was developed by Letsinger and Ogilvie[11] and by Eckstein and Rizk[12]. The products of this method were uncharged phosphotriesters which could be separated by silica gel chromatography using organic solvents. Silica gel chromatography has a much larger capacity and greater flow rate than DEAE-cellulose[11].

Letsinger and Ogilvie synthesized oligothymidylates in a stepwise manner[11,13] and by the use of preformed "blocks"[14]. In a stepwise manner, 5'-O-monomethoxytritylthymidine, MMTr-T(XII) was phosphorylated using β-cyanoethyl phosphate (β-CEP)[15] and a condensing agent (mesitylenesulfonyl chloride, MS). The resulting phosphodiester was condensed with thymidine using triisopropylbenzenesulfonyl chloride (TPS) to yield the phosphotriester product(XIII) in 64% yield.

Removal of the blocking groups, monomethoxytrityl by acid and β-cyanoethyl by base, yielded thymidylyl-(3'-5')-thymidine, TpT. The sequence was extended to synthesize the fully blocked triesters of TpTpT and TpTpTpT, the yields being maintained (49% and 57% respectively). In each condensation step, the ratio of phosphate to nucleoside was constant at 2:1.



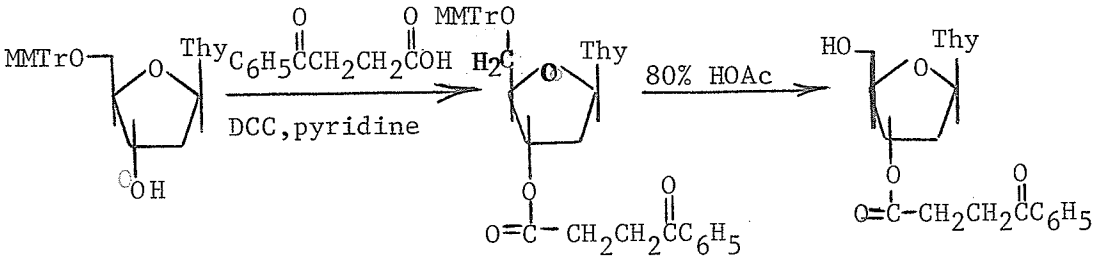
XII

XIII

The use of thymidine in the condensation led to the synthesis of the 3'-3' isomer of TpT. The yield of this undesirable isomer was 4% of the total product synthesized. To prevent the 3'-3' condensation, the thymidine must be selectively blocked at the 3'-position. To allow a stepwise synthesis of oligonucleotides, the choice of blocking group was crucial. The β-cyanoethyl (β-CE) group was base-labile. The desired 3'-blocking group must be labile to reagents which do not affect the other blocking groups. The β-benzoylpropionyl (βB) group was chosen as it was removed under conditions (hydrazine hydrate in pyridine-acetic acid) that did not affect either the β-cyanoethyl or the monomethoxytrityl blocking group[16].

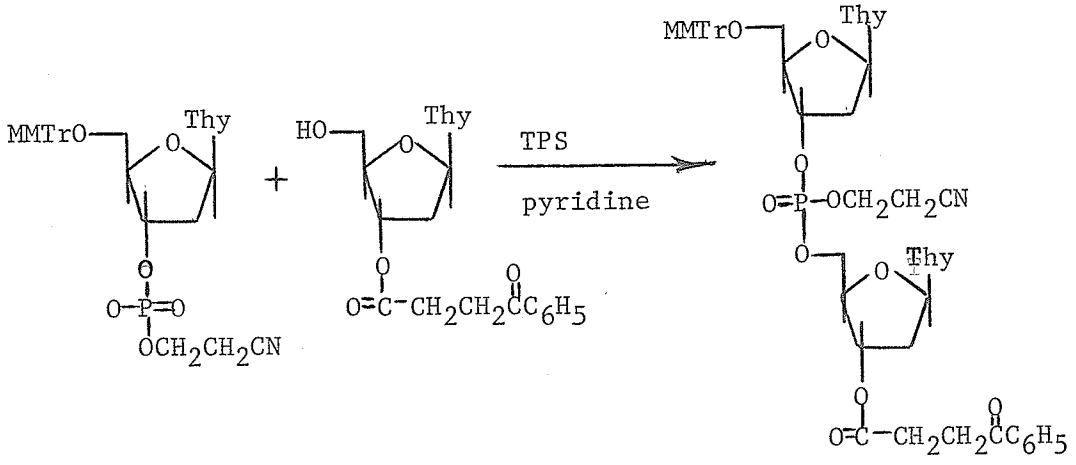
The β-benzoylpropionyl blocking group was employed in the triester synthesis of oligothymidylates possessing only the 3'-5'

internucleotide linkage[14]. The simplicity of the method used in obtaining the blocked starting materials is demonstrated. One reaction sequence gave both the protected nucleosides required, 5'-O-monomethoxytritylthymidine(XIV) having a free 3'-hydroxyl and 3'-O-(β-benzoylpropionyl)-thymidine, (XV) having a free 5'-hydroxyl.



XIV XV

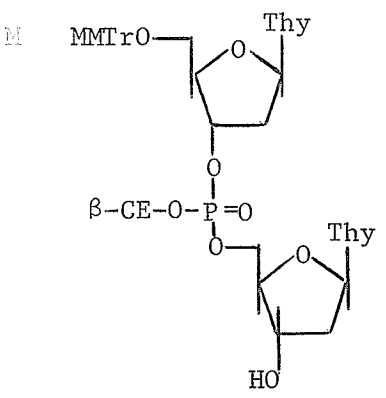
5'-O-Monomethoxytritylthymidine(XIV) was phosphorylated using β-cyanoethyl phosphate[15] and mesitylenesulfonyl chloride. The resulting diester was condensed with 3'-O-(β-benzoylpropionyl)-thymidine(XV) using triisopropylbenzenesulfonyl chloride as condensing agent to yield the fully blocked triester product(XVI) in 64% yield.



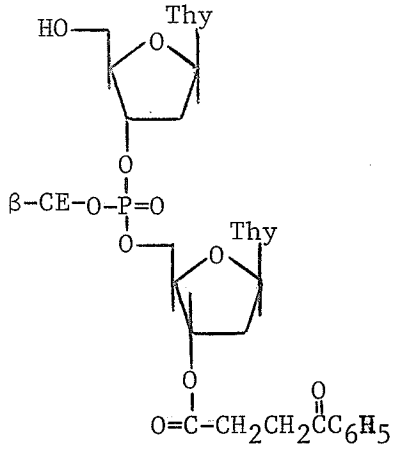
XVI

Removal of the blocking groups gave thymidylyl-(3'-5')-thymidine having only the desired 3'-5' internucleotide linkage. By removal of the β-benzoylpropionyl group from XVI with hydrazine hydrate and repetition of the phosphorylation and condensation procedures, the chain length was extended.

Oligonucleotide synthesis using preformed "blocks"[14] was also achieved from the materials obtained above. Thus, treatment of XVI with hydrazine hydrate led to the triester(XVII) having a free 3'-hydroxyl. Treatment of XVI with acid gave the triester(XVIII) having a free 5'-hydroxyl.



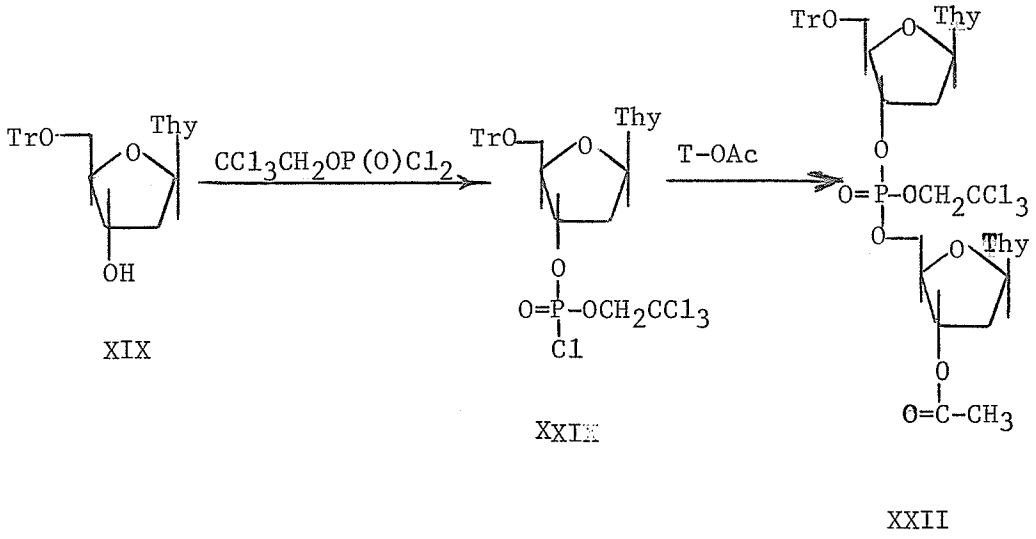
XVII



XVIII

Phosphorylation of XVII with β-cyanoethyl phosphate and mesitylenesulfonyl chloride, followed by condensation of the product with XVIII yielded the fully blocked triester derivative of TpTpTpT in 52% yield.

An alternate approach to the triester synthesis of oligodeoxyribonucleotides was developed by Eckstein and Rizk[12]. A condensing agent was not required to activate the phosphate due to the reactivity of the phosphorylating agent, β,β,β-trichloroethyl phosphorodichloridate(XX). By this method, the initial phosphorylation and subsequent condensation reactions were done without isolation of the intermediate phosphorylated product, the nucleoside 3'-phosphorochloridate(XXI).



XIX

XXII

The triester product(XXII) contained the β,β,β-trichloroethyl (TCE) blocking group for the phosphate. The TCE group was removed by Cu/Zn in dimethylformamide at 50° C. By removal of the trityl group from XXII with acid, and reaction with XXI, the chain length was extended. Here also, the yields and relative reaction amounts were maintained in increasing the chain length, an improvement over

the "diester method".

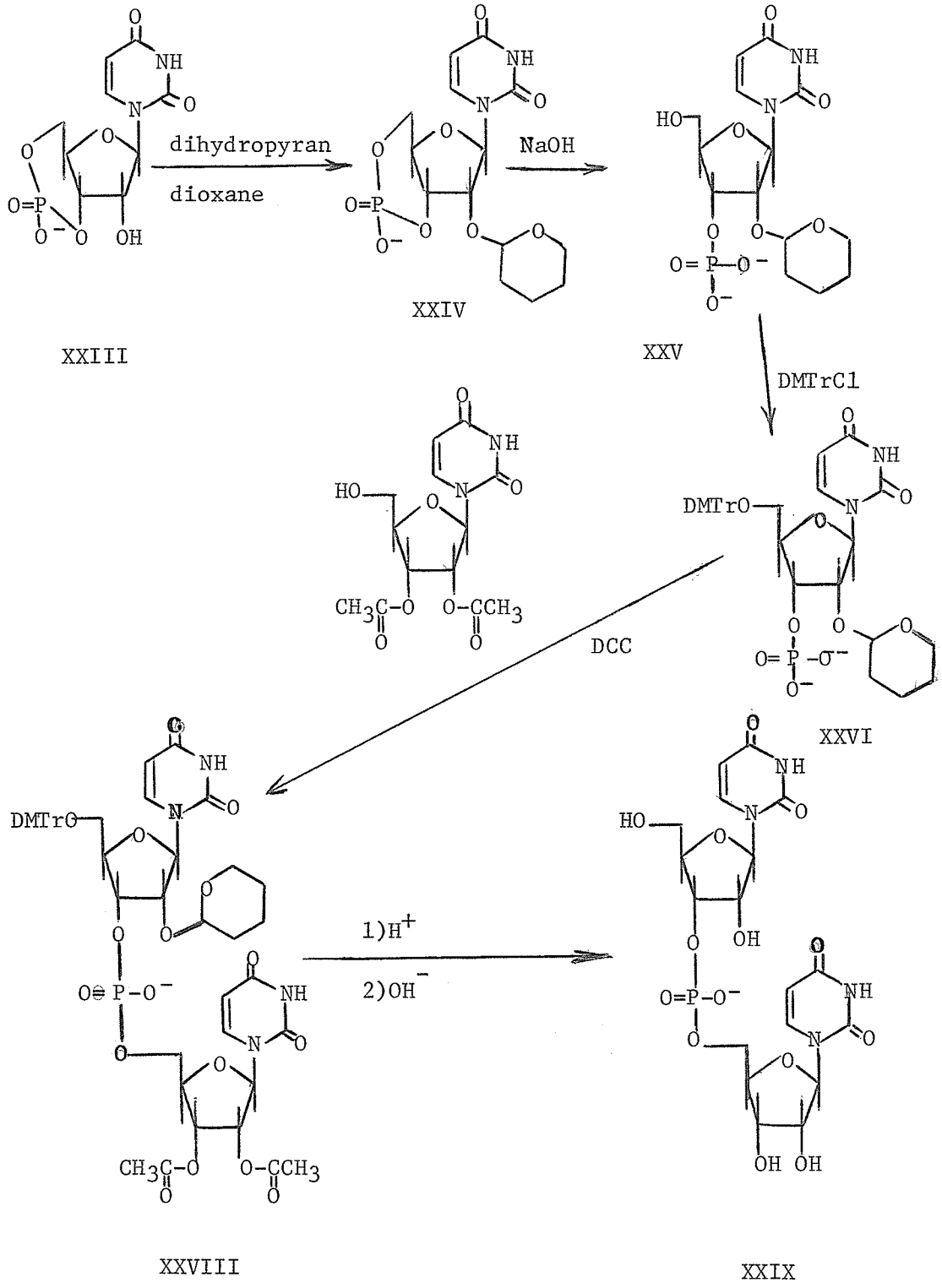
Using the deoxyribonucleosides, the organic synthesis of nucleic acids has been investigated and developed to a high degree. Indeed, a combination of chemical and enzymic methods has led to the synthesis of the most dramatic deoxyribonucleotide segment yet obtained, the double-stranded DNA corresponding to the gene for an alanine transfer ribonucleic acid from yeast[17].

Chemical synthetic investigations into RNA have not advanced to the same degree as with DNA. In DNA(I), the sugar moiety is D-2'-deoxyribose; hence, the 3'-5' internucleotide linkage makes use of both the hydroxyl groups present in the sugar. In RNA(II), the sugar moiety is D-ribose, possessing a cis-2',3'-diol. The presence of the 2'-hydroxyl complicates the synthetic procedures as the 2'-hydroxyl must be selectively blocked leaving the 3'-hydroxyl free to form the 3'-5' inter-ribonucleotide linkage.

The first successful chemical synthesis of a 3'-5' inter-ribonucleotide linkage in the ribose series was achieved by Smith and Khorana[18,19] (Scheme I).

Uridine 3',5'-cyclic phosphate(XXIII), bearing a free 2'-hydroxyl, was converted into the 2'-O-tetrahydropyranyl (THP) derivative(XXIV) by treatment with dihydropyran in dioxane. The cyclic phosphate was then opened by base to give a mixture of the 3'-phosphate(XXV) and 5'-phosphate (ratio 9:1). The 3'-phosphate alone reacted with dimethoxytrityl chloride (DMTrCl) to yield

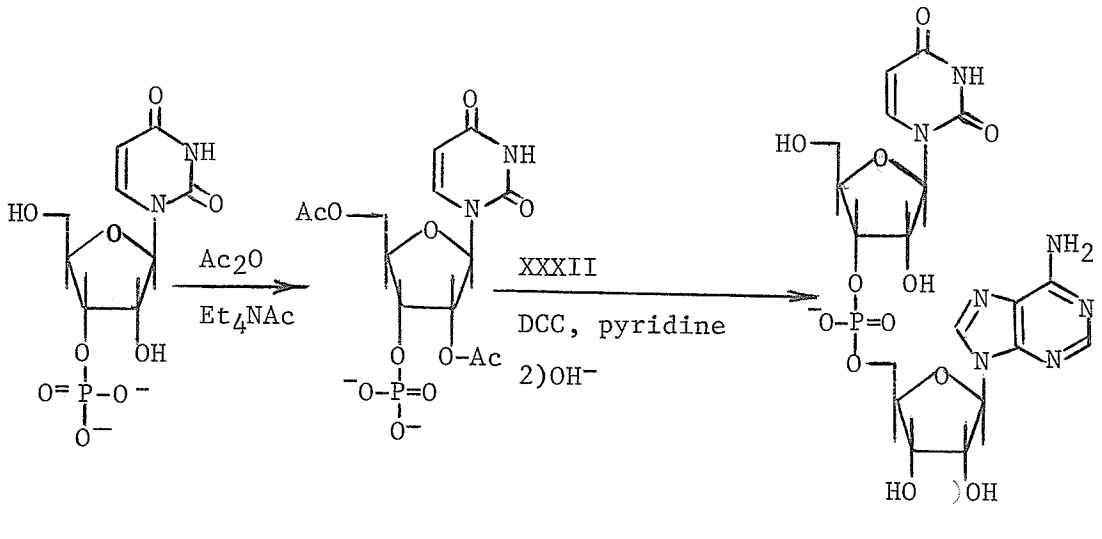
Scheme I. Synthesis of UpU



5'-O-dimethoxytrityl-2'-O-tetrahydropyranyluridine 3'-phosphate(XXVI). XXVI was easily separated from the unreacted 5'-phosphate by chromatography. Condensation with 2',3'-O-acetyluridine(XXVII) using dicyclohexylcarbodiimide as condensing agent yielded the blocked diribonucleoside monophosphate, XXVIII. Removal of the dimethoxytrityl and tetrahydropyranyl blocking groups by acid and the acetyl by base gave uridylyl-(3'-5')-uridine(XXIX) in 50% yield.

The rather low yield was attributed to incomplete removal of the acid-labile blocking groups. Treatment with acid for a longer period of time (10 hours vs. 4 hours) increased the yield to 69%. Unfortunately, extended exposure to acid caused detectable isomerization to yield the 2'-5' phosphate isomer (1%). To overcome this problem, the use of alkali-labile groups for the protection of the 2'-hydroxyl groups in ribonucleoside 3'-phosphates was developed[20]. No phosphate isomerization occurred under the alkaline conditions of hydrolysis which were used.

In the method developed[20], treatment of uridine 3'-phosphate (XXX) with acetic anhydride (Ac_2O) and tetraethylammonium acetate (Et_4NAc) yielded 2',5'-di-O-acetyluridine 3'-phosphate(XXXI). Condensation with N,N,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine(XXXII) using DCC, followed by treatment with concentrated ammonium hydroxide gave uridylyl-(3'-5')-adenosine(XXXIII) in 84% yield (no 2'-5' isomer detectable).

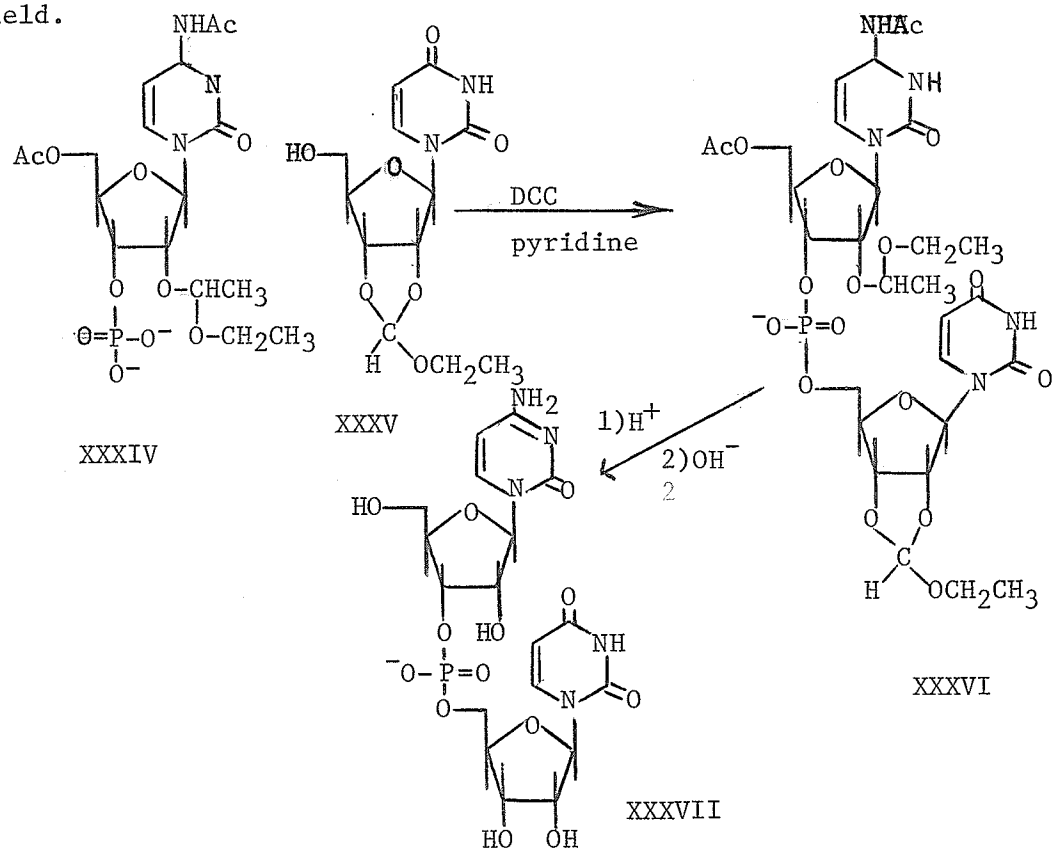


XXX XXXI XXXIII

Replacing the base-labile acetyl group at the 5'-position of the nucleoside 3'-phosphate with an acid-labile dimethoxytrityl group allowed extension of the method to the synthesis of triribonucleoside diphosphates[21]. Yields were based on unrecovered dinucleoside phosphate; thus, a true indication of the extent of reaction was not indicated.

Holy and Smrt[22] reinvestigated the use of acid-labile ether blocking groups in the synthesis of ribonucleic acids. The blocking groups chosen were the 1-ethoxyethyl group for the 2'-hydroxyl of the nucleoside 3'-phosphate and ethoxymethylene group for the cis-2',3'-diol of the ribonucleoside. These blocking groups were much more sensitive to acid than the tetrahydropyranyl group used previously[18,19]. No migration of the phosphate occurred on acid hydrolysis. To illustrate,

N^4,$O^{5'}$-diacetyl-2'-O-(1-ethoxyethyl)cytidine 3'-phosphate(XXXIV) was condensed with 2',3'-O-ethoxymethyleneuridine(XXXV) using DCC as condensing agent to give the diester product XXXVI. Hydrolysis of the blocking groups gave cytidylyl-(3'-5')-uridine(XXXVII) in 50% yield.



The method was extended to synthesize triribonucleoside diphosphates as base treatment of XXXVII yields a product with a free 5'-hydroxyl suitable for condensation with a nucleoside 3'-phosphate[22]. Yields were in the range 12-25%.

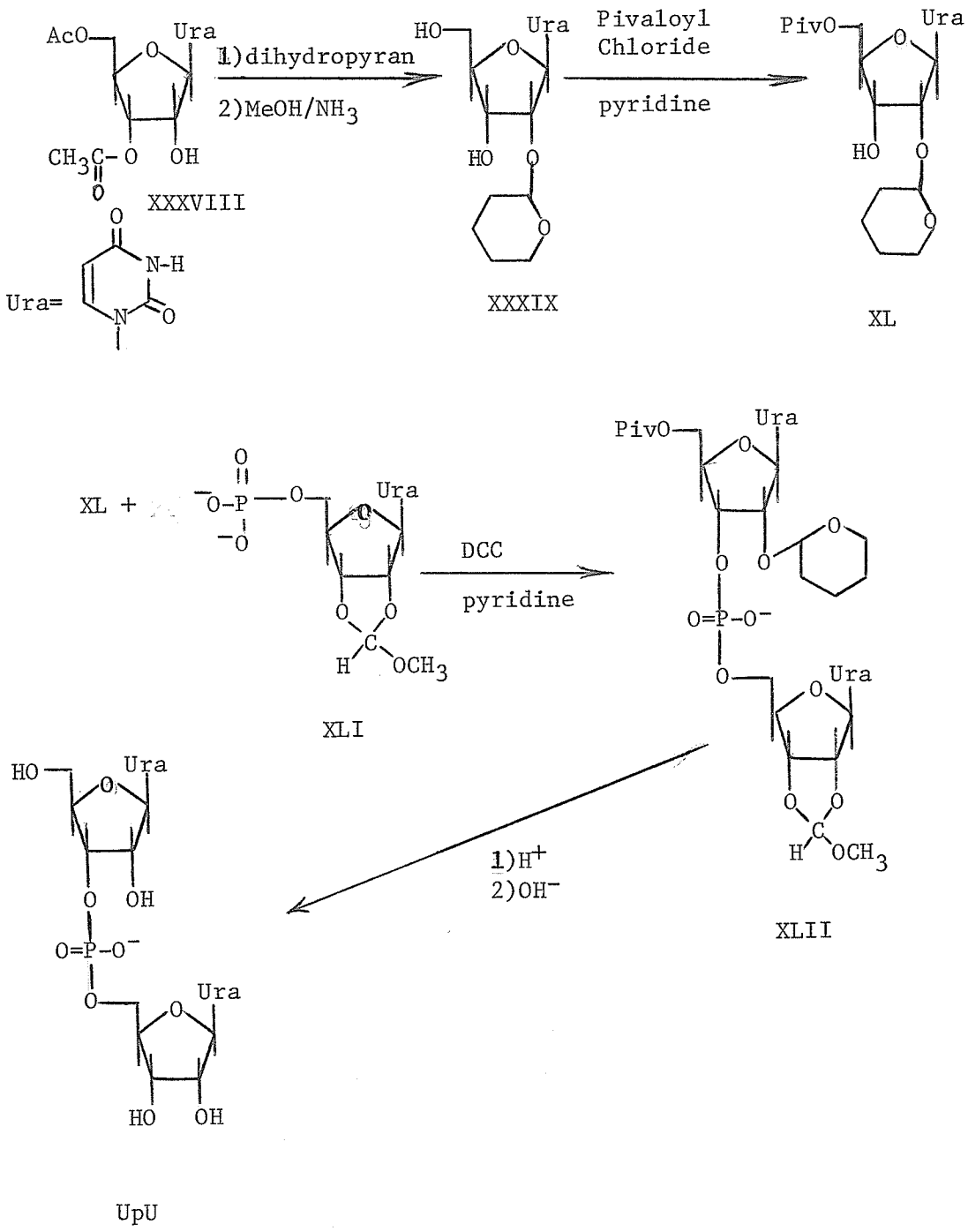
The method developed above[20,22] condensed a protected nucleoside 3'-phosphate with a nucleoside having a free 5'-hydroxyl.

The opposite approach, condensing a nucleoside 5'-phosphate with a nucleoside having a free 3'-hydroxyl was investigated[23]. The acyl 2'-hydroxyl blocking group of the nucleoside was found to migrate to the 3'-position during phosphorylation with β-cyanoethyl phosphate, resulting in a significant yield (18%) of the undesirable 2'-phosphate. The migration of acyl groups greatly restricted the use of this approach to the synthesis of oligoribonucleotides when a base-labile 2'-O-acyl nucleoside was involved.

Griffin and Reese[24,25] were able to synthesize 3'-5' diribonucleoside monophosphates using the approach discarded by Rammler and Khorana[23], provided that the nucleoside having a free 3'-hydroxyl was blocked at the 2'-position by an acid-labile ether blocking group. The tetrahydropyranyl group was used. Two diastereoisomers were produced and the higher melting isomer was used in the nucleotide work. The reaction scheme is outlined in Scheme II.

To selectively block the 5'-hydroxyl of XXXIX, the pivaloyl group was used. This bulky group reacted preferentially with the primary 5'-hydroxyl group to give XL, having a free 3'-hydroxyl. Reaction with the nucleoside 5'-phosphate XLI gave the blocked diribonucleoside monophosphate XLII. Removal of the blocking groups by acid (tetrahydropyranyl and methoxymethylene) and base (pivaloyl) gave uridylyl-(3'-5')-uridine in 80% yield. Removal of the pivaloyl group alone gave a dinucleoside monophosphate having a free 5'-hydroxyl.

Scheme II. Synthesis of UpU



Phosphorylation of this product followed by condensation with a nucleoside having a free 3'-hydroxyl afforded triribonucleoside diphosphates[25]. The yields were 54-57% for the products, UpUpU, UpApU and ApApU.

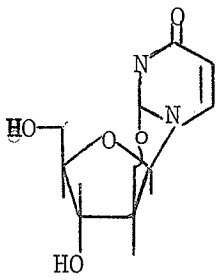
The methods used to synthesize oligoribonucleotides required lengthy procedures to arrive at suitably blocked starting materials. The overall yields, thus, are low making extended syntheses difficult and inefficient.

The "triester" approach to the synthesis of ribonucleic acids has made no significant advances in terms of selectively blocking the 2'-hydroxyl[26,27]. The only advantages in this approach are the synthesis of a neutral product which is more easily isolated by silica gel chromatography and higher yields in the condensation steps.

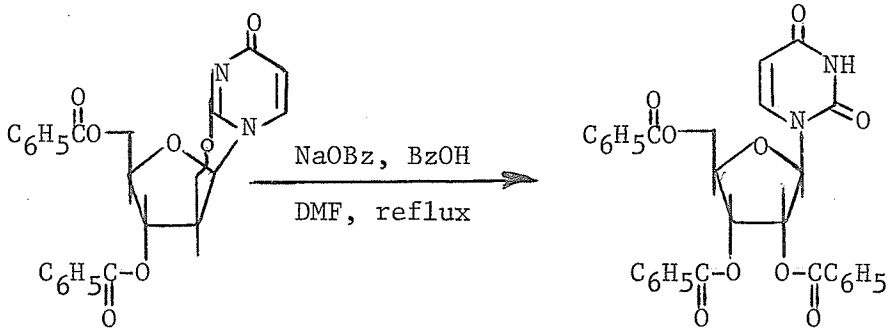
The difficulties involved in overcoming the presence of the 2'-hydroxyl in ribonucleic acid synthesis have not been satisfactorily solved as yet. For this reason, the chemical methods available would appear inadequate in the synthesis of a true ribonucleic acid, such as a transfer RNA. Thus, alternate routes to effectively protect the 2'-hydroxyl need to be developed. A possible solution might be the use of cyclonucleosides.

Cyclonucleosides are nucleosides containing a covalent linkage between a carbon in the sugar moiety and a carbon or nitrogen in the base moiety. This linkage is in addition to the normal glycoside

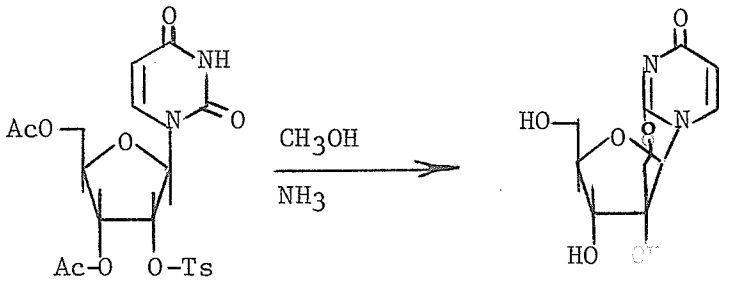
linkage and can be direct or through another atom. An example of a cyclonucleoside is O^2,2'-anhydro-1-β-D-arabinofuranosyluracil (O^2,2'-cyclouridine or cU).



The O^2,2'-cyclouridine (formed by loss of water) has the 2'-carbon of the sugar moiety covalently linked to the 2'-carbon of the base through an oxygen atom. There is no longer a free 2'-hydroxyl; indeed, the O^2,2'-cyclouridine can be likened to a deoxyribose analog. This would appear to offer an effective method of selectively blocking the 2'-hydroxyl to be used in the synthesis of ribonucleotides, provided the ribose sugar could be regenerated from the O^2,2'-cyclouridine. Displacement of the anhydro linkage of an O^2,2'-cyclouridine derivative has been accomplished to give a uridine derivative[28].

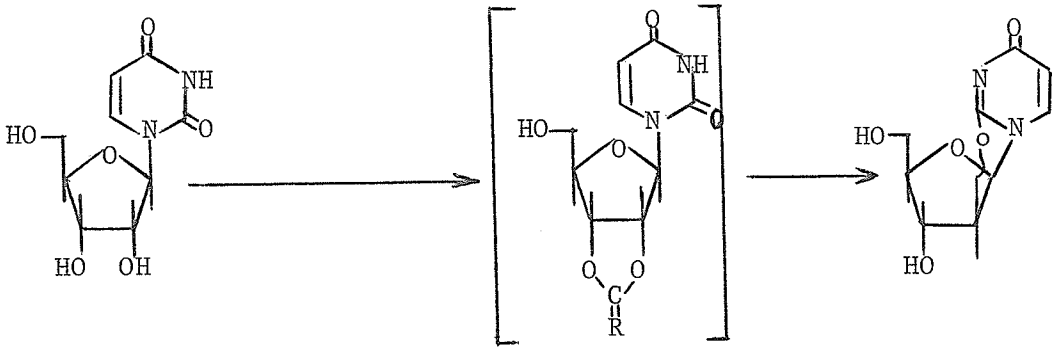


The first synthesis of a cyclonucleoside from a pyrimidine ribonucleoside was done by Brown[29]. The synthesis involved treatment of 3',5'-di-O-acetyl-2'-O-toluenesulfonyluridine(XLIII) with ammonia in methanol. The product was O^2,2'-cyclouridine.



XLIII

Direct syntheses of O^2,2'-cyclouridine from uridine were achieved by Fox and Wempen[30] and by Hampton and Nichol[31]. The Fox method involved treating uridine with thiocarbonyldiimidazole in refluxing toluene (conditions A). The reaction proceeded through a cyclic carbonate (R=S) to yield cU (40%). The Hampton method involved treating uridine with diphenyl carbonate and sodium benzoate (NaOBz) in dimethylformamide (DMF) at 150°C (conditions B). The reaction again proceeded through a cyclic carbonate (R=O) to yield cU (59%).

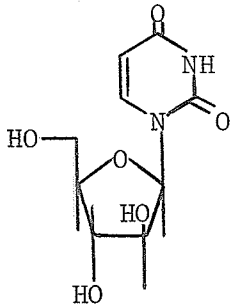


A)R=S

B)R=O

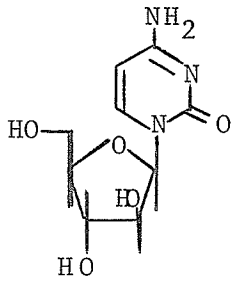
Thus O^2,2'-cyclouridine can be synthesized in good yield directly from uridine. The 2'-hydroxyl has been effectively blocked without going through the involved procedures detailed previously for the synthesis of 2'-blocked nucleosides and nucleotides. The simple synthesis of O^2,2'-cyclouridine and the displacement of the anhydro linkage[28] to give a uridine derivative would appear to favour the use of O^2,2'-cyclouridine in the synthesis of oligoribonucleic acids.

In addition to the displacement of the anhydro linkage to yield a uridine derivative, the anhydro linkage has also been hydrolysed by acid[29] and base[30] to yield 1-β-D-arabinofuranosyl-uracil (arabinouridine or aU), XLIV.



XLIV

These hydrolysis procedures could be used to synthesize arabinonucleotides from nucleotides containing cyclonucleoside moieties. The importance of arabinonucleosides and -nucleotides has been demonstrated by 1-β-D-arabinofuranosylcytosine (arabinocytidine or aC).



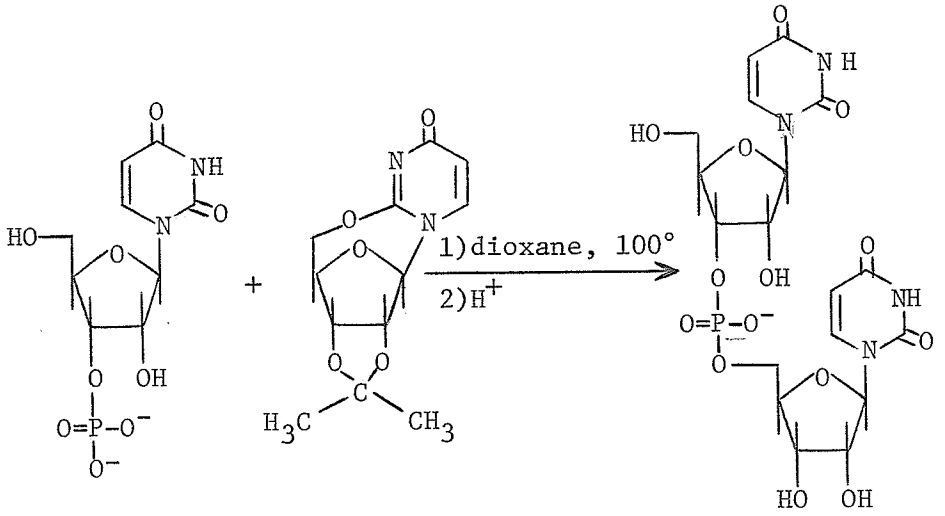
Arabinocytidine was found to be cytotoxic[32], inhibiting the reduction of cytidine 5'-phosphate to 2'-deoxycytidine 5'-phosphate. Anti-neoplastic activity in humans was found by Carey and Ellison[33] for a low level of arabinocytidine. The toxicity to the patients was low, encouraging more study of arabinocytidine and its derivatives.

The interesting results with arabinocytidine suggested that the synthesis of nucleotides containing the arabinose sugar might be of importance. Wechter[34] has synthesized nucleotides and dinucleoside monophosphates containing arabinocytidine by condensing nucleosides and nucleoside 5'-phosphates having an arabinocytidine as part of at least one of the starting materials. The products were 3'-5' and 2'-5' dinucleoside monophosphates. Separation of the isomers was done by either DEAE-cellulose chromatography or continuous-flow film

electrophoresis. These arabinonucleotides were found to possess biological activity, being cytotoxic to carcinoma cells[35] and possessing antiviral activity *in vitro* and *in vivo*[36]. Arabinocytidine and arabinouridine nucleotides have been shown to be excellent inhibitors of pancreatic ribonuclease[37].

These important biological results provide further support for the synthesis of nucleotides containing cyclonucleosides. Hydrolysis would yield the corresponding arabinonucleotides.

Cyclonucleosides and cyclonucleotides have been used to synthesize nucleic acids. Zemlicka and Smrt[38] reported the synthesis of a mixture of the 3'-5' and 2'-5' isomers of uridylyluridine by condensation of uridine 3'-phosphate with 2',3'-O-isopropylidene-O^2,5'-cyclouridine in dioxane at 100°C.

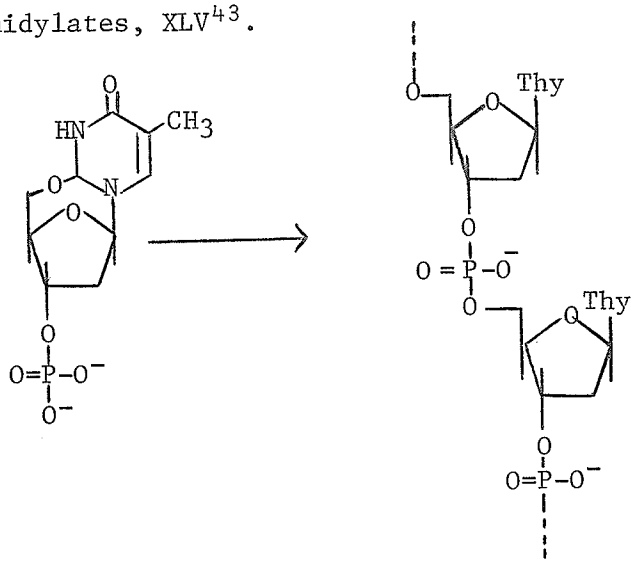


+ 2'-5' isomer

The mixture of isomers was due to phosphate migration during the reaction.

Agarwal and Dhar[39] reported the synthesis of thymidylyl-(3'-5')-adenosine by condensation of 5'-O-trityl-O^2,3'-cyclothymidine and adenosine 5'-phosphate in refluxing dimethylformamide. Nagyvary[40,41] condensed uridine 5'-phosphate with 2',3'-O-isopropylidene-O^2,5'-cyclouridine to yield the 5'-5' isomer of UpU.

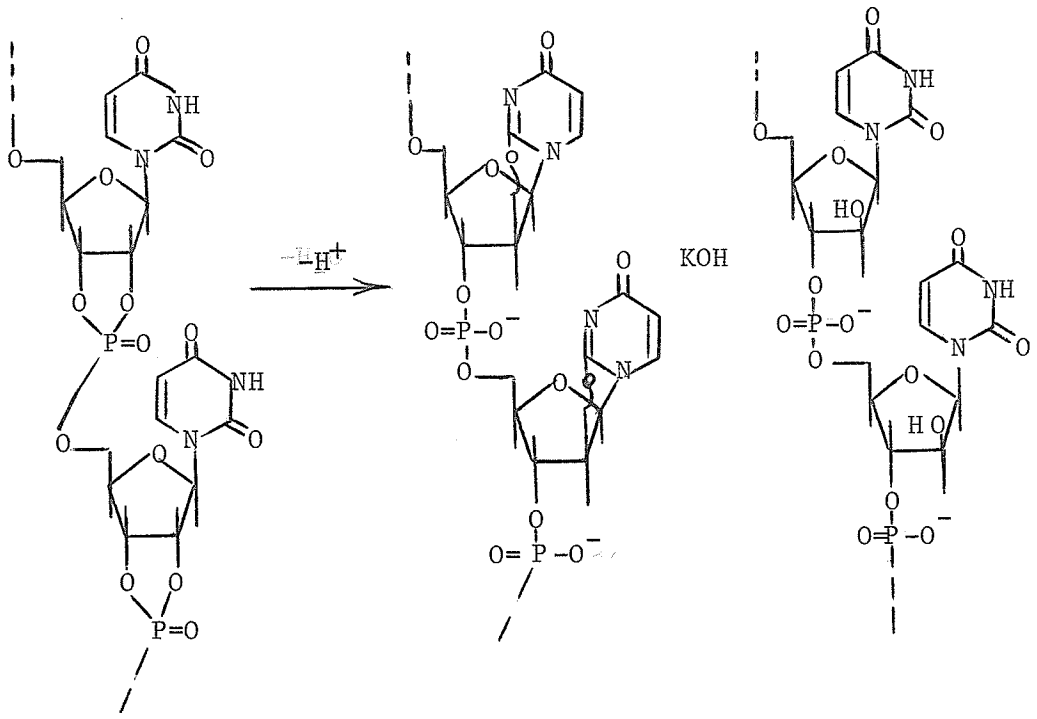
Nagyvary demonstrated that polymerization of O^2,5'-cyclouridine 2',3'-cyclic phosphate[42] gave polyarabinouridylic acid, while polymerization of O^2,5'-cyclothymidine 3'-phosphate gave oligothymidylates, XLV[43].



XLV

The above syntheses used pyrimidine cyclonucleosides and cyclonucleotides in the synthesis of nucleic acids; however, the products did not contain the cyclonucleoside moieties as the anhydro linkages were opened by attack of the phosphate anion.

Nucleic acids containing the O^2,2'-cyclouridine moiety have been synthesized by Nagyvary[44,45]. The synthesis was accomplished by converting oligo- and polynucleotide cyclic phosphates(XLVI) into oligo- and polynucleotides(XLVII) containing the 3'-5' internucleotide linkage and cyclouridine moieties. These intermediates were then hydrolysed to yield the arabinonucleic acids(XLVIII).



XLVI XLVII XLVIII

While this work was in progress, the stepwise synthesis of nucleic acids containing cyclopurine moieties was reported[46,47]. No stepwise synthesis involving pyrimidine cyclonucleosides has been reported.

The purpose of my work is a) to investigate the use of O^2,2'-

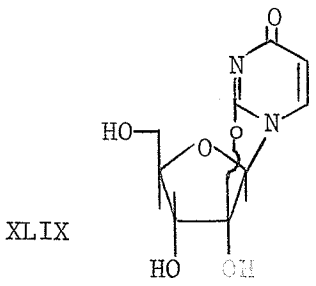
cyclouridine in the synthesis of cyclouridine nucleotides as interesting analogs of natural nucleic acids, and b) to investigate the possibilities of the conversion of the cyclouridine nucleotides into arabinonucleic and ribonucleic acids. The anhydro linkage of O^2,2'-cyclouridine is considered to be an effective and selective blocking technique for the 2'-hydroxyl function of the sugar moiety. The synthesis of derivatives of O^2,2'-cyclouridine is described. Various "diester" and "triester" organic synthetic approaches to the synthesis of dinucleoside monophosphates containing O^2,2'-cyclouridine are investigated. Studies on the displacement and hydrolysis of the anhydro linkage are reported.

DISCUSSION AND RESULTS

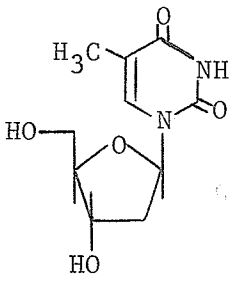
I. Approach to the Synthesis of Dinucleoside Monophosphates Containing O^2,2'-Cyclouridine

The chemical synthesis of ribonucleic acids is complicated by the presence of the cis-2',3'-diol in the sugar moiety of ribonucleosides. To direct phosphorylation or condensation reactions solely to the 3'-position, the 2'-hydroxyl must be selectively blocked leaving the 3'-hydroxyl free.

The O^2,2'-cyclonucleosides are viewed as a solution to the problem of selectively blocking the 2'-hydroxyl. O^2,2'-Cyclouridine (cU) XLIX, resembles the 2'-deoxyribonucleoside thymidine(L).



XLIX



L

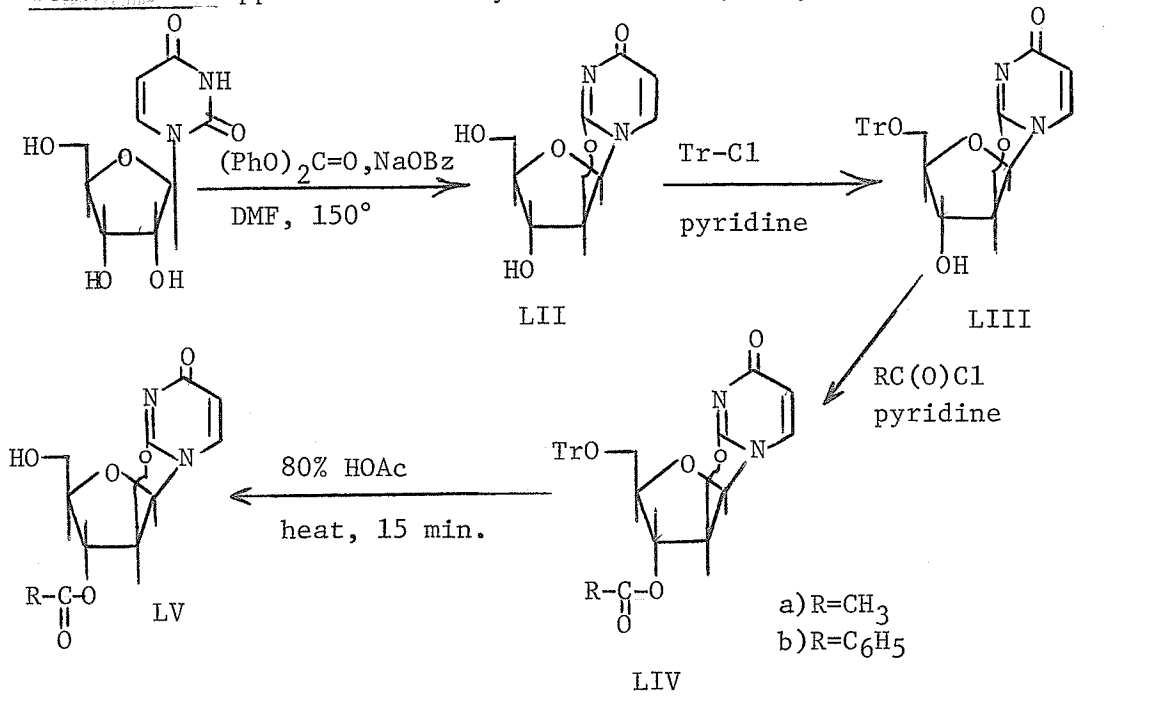
The anhydro linkage in XLIX masks the 2'-hydroxyl and cU appears as a deoxyribonucleoside analogue, having only the 5'- and 3'-hydroxyls present. Methods developed for the synthesis of deoxyribonucleic acids should be applicable to the synthesis of nucleic acid analogues containing O^2,2'-cyclouridine.

An elegant, yet simple approach to the synthesis of triesters of oligodeoxyribonucleotides was demonstrated by Letsinger[14]. By this approach, one reaction sequence presented both the protected

nucleosides necessary for the synthesis of triesters of dinucleoside monophosphates having only 3'-5' internucleotide linkages. 5'-O-Monomethoxytritylthymidine (XIV), having a free 3'-hydroxyl, was synthesized from thymidine and monomethoxytrityl chloride. 3'-O-β-Benzoylpropionylthymidine (XV), having a free 5'-hydroxyl, was synthesized from XIV by condensation with β-benzoylpropionic acid, followed by removal of the monomethoxytrityl group by acid. Phosphorylation of XIV, followed by condensation of the product with XV gave the fully blocked triester derivative (XVI) of TpT.

A similar approach was envisioned for the synthesis of dinucleoside monophosphates containing O^2,2'-cyclouridine. The scheme for the preparation of the required partially blocked O^2,2'-cyclouridine derivatives by one simple reaction sequence is outlined in Scheme III.

Scheme III. Approach to the Synthesis of O^2,2'-Cyclouridine Derivatives



O^2,2'-Cyclouridine(LII) would be synthesized from uridine by the method of Hampton and Nichol[31]. Treatment of LII with a triphenylmethyl chloride (trityl chloride) in refluxing pryidine[48] should yield a 5'-O-trityl-O^2,2'-cyclouridine(LIII) having an acid-labile trityl group at the 5'-position and a free 3'-hydroxyl. Treatment of LXIII with either acetic anhydride[3] or benzoyl chloride[48] in dry pyridine should yield the fully blocked nucleoside LIV, the 3'-blocking group being base-labile [either acetyl($R=CH_3$) or benzoyl($R=C_6H_5$)]. 3'-O-Acyl-O^2,2'-cyclouridine(LV), having a free 5'-hydroxyl, should result from treatment of LIV with acid to remove the trityl group.

The above reaction sequence would yield the nucleoside derivatives (LIII, and LV) to be used in the synthesis of dinucleoside monophosphates containing O^2,2'-cyclouridine by the "triester method"[11]. The simplicity of the synthetic route to O^2,2'-cyclouridine analogues of uridylyl-(3'-5')-uridine combined with the conversion of the O^2,2'-cyclouridine moiety to arabinouridine[29,30] or uridine[28] clearly would establish the value of the approach to the synthesis of ribonucleic acid analogues.

II. Synthesis of O^2,2'-Cyclouridine Derivatives

O^2,2'-Cyclouridine was easily synthesized (in 82.6% yield) from uridine by the method of Hampton and Nichol[31]. The formation of the anhydro linkage caused dramatic changes in the spectral and

chromatographic properties of O^2,2'-cyclouridine relative to uridine. The changes allowed easy identification of molecules containing the anhydro linkage. The infrared carbonyl absorption shifted from 5.9μ in uridine to 6.1μ in O^2,2'-cyclouridine, while the ultraviolet absorption maximum underwent a hypsochromic shift from 262nm to 249nm with the appearance of a second absorption maximum at 223nm. Chromatographically, the thin-layer R_f value in tetrahydrofuran (THF) changed from 0.56 for uridine to 0.05 for O^2,2'-cyclouridine. Indeed, R_f values were lower, in general, for O^2,2'-cyclouridine derivatives compared to the corresponding uridine derivatives (Tables I and II).

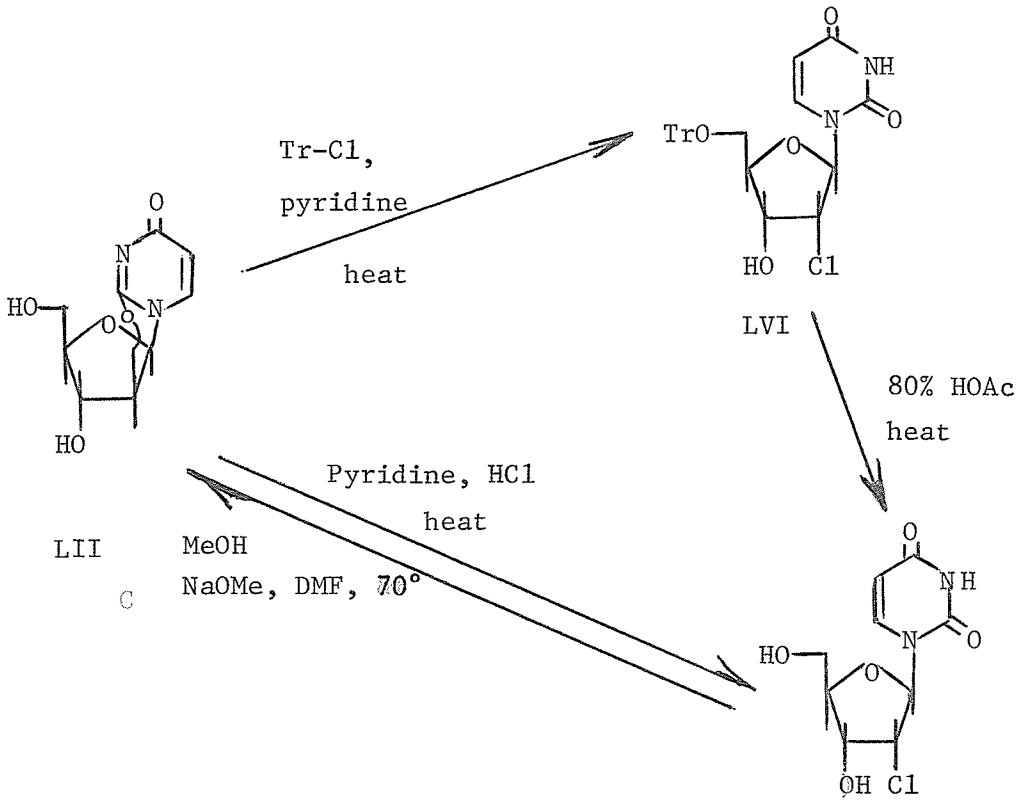
Treatment of O^2,2'-cyclouridine with trityl chloride in dry pyridine at reflux for 2 hours[48], did not yield the expected product, LIII. A new compound was produced containing the trityl group but having uridine-like spectral properties (infrared carbonyl absorption at 5.9μ and ultraviolet absorption maximum at 259nm). The anhydro linkage had been hydrolysed during the reaction.

To determine the nature of this new product, it was detritylated by heating in 80% acetic acid for 15 minutes[48] and compared chromatographically with uridine and arabinouridine. The arabinouridine was prepared by hydrolysis of O^2,2'-cyclouridine with 1N sodium hydroxide[30]. No correlation was found between the detritylated unknown and either uridine or arabinouridine. The detritylated unknown moved faster than both uridine and arabinouridine on thin-layer chromatograms in tetrahydrofuran and ethanol. The new product, thus,

was not formed by hydrolysis or displacement of the anhydro linkage to give either 5'-O-trityluridine or 5'-O-tritylarabinouridine.

Before examining the new products further, the reaction conditions for the tritylation of O^2,2'-cyclouridine were investigated. Refluxing O^2,2'-cyclouridine in pyridine for 2 hours caused no change in the nucleoside. Heating O^2,2'-cyclouridine in pyridine, to which a few drops of hydrochloric acid had been added, converted O^2,2'-cyclouridine into 2'-chloro-2'-deoxyuridine, LVII (mp 200 - 206°, lit mp 205 - 206°[71] and 207 - 212°[49]). Chloride ions had displaced the anhydro linkage under these conditions. A similar result could have occurred from the trityl chloride treatment of O^2,2'-cyclouridine, the chloride ions displacing the anhydro linkage of the tritylated product to yield 5'-O-trityl-2'-deoxy-2'-chlorouridine(LVI). Comparison of 2'-deoxy-2'-chlorouridine with the detritylated LVI gave identical results by chromatography. In addition, a mixed melting point of the two 2'-deoxy-2'-chlorouridines showed no depression (mp 198 - 205°, lit. mp 205 - 206°[71]). Thus, the tritylation reaction conditions converted O^2,2'-cyclouridine into LVI.

The identity of the 2'-chloro-2'-deoxyuridine was confirmed by conversion of LVII into O^2,2'-cyclouridine by the method of Codington[50]. The O^2,2'-cyclouridine produced was identical to authentic O^2,2'-cyclouridine chromatographically and spectrophotometrically (infrared).



The above studies indicated that a modification to Scheme III was necessary. Rather than synthesize 0^2,2'-cyclouridine first, the conversion of uridine into 5'-0-trityluridine(LVIII) was done[48]. The synthesis of Tr-cU from LVIII was achieved by the method of Hampton and Nichol[31]. Scheme III was followed and the 3'-0-acyl derivatives LIV were synthesized[3,48] and characterized. Treatment of 5'-0-trityl-3'-0-benzoyl-0^2,2'-cyclouridine(LIV, R=C_6H_5) with 80% HOAc on a steam bath for fifteen minutes, not only removed the acid-

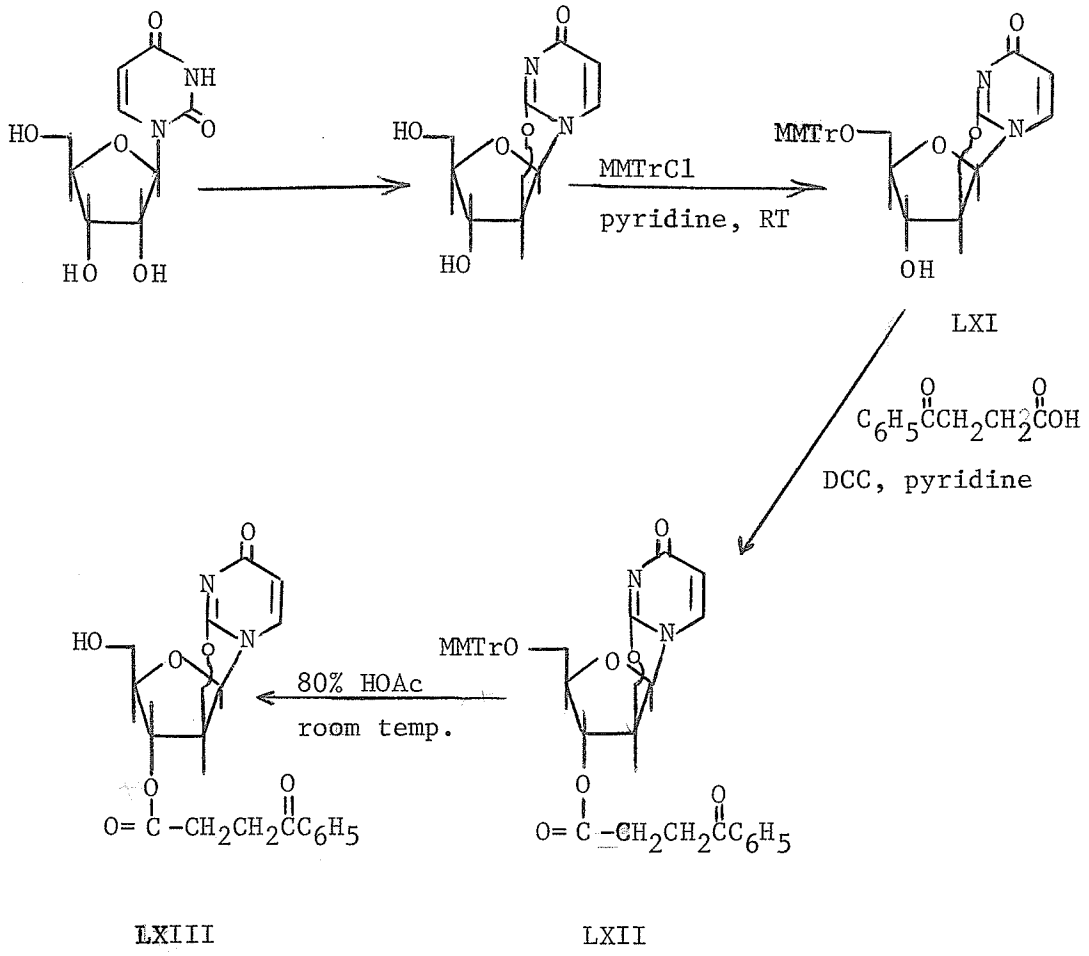
labile trityl blocking group, but also partially hydrolysed the anhydro linkage to yield a mixture of 3'-O-benzoyl-O^2,2'-cyclouridine (LX) and 1-(3'-O-benzoyl-β-D-arabinofuranosyl)uracil(LIX). The presence of these two compounds which did not contain the trityl group was observed by thin-layer chromatography. Only one compound (LX) was expected. The trityl group is detected by spraying the chromatogram with 10% perchloric acid, followed by drying in a stream of warm air to produce a yellow-coloured spot showing the presence of the trityl group.

The hydrolysis of the anhydro linkage by 80% acetic acid on a steam bath posed problems in the detritylation of 5'-O-trityl-O^2,2'-cyclouridine derivatives. Other reaction conditions were studied to find a solution. Treatment of LIII with 10% acetic acid on a steam bath for fifteen minutes resulted in incomplete removal of the trityl group as well as some hydrolysis of the anhydro linkage. Treatment of LIII with 80% acetic acid at room temperature for 22 hours gave similar results.

The hydrolysis of the anhydro linkage under the acidic conditions necessary to remove the trityl group indicated that one of the more sensitive acid-labile trityl blocking groups was required for the 5'-hydroxyl. The monomethoxytrityl group was chosen as complete removal of the monomethoxytrityl group from 5'-O-monomethoxytritylthymidine occurred in 1.5 hours with 80% acetic acid at room temperature[51].

It was also decided to follow more closely the reaction sequence of Letsinger[14]. Thus, in addition to using the monomethoxytrityl group to block the 5'-hydroxyl, the β-benzoylpropionyl group was chosen to block the 3'-hydroxyl to yield protected nucleosides more closely resembling those of Letsinger. The new reaction sequence for the synthesis of protected O^2,2'-cyclouridine nucleosides is detailed in Scheme IV.

Scheme IV. Synthesis of O^2,2'-Cyclouridine Derivatives



O^2,2'-Cyclouridine was converted into 5'-O-monomethoxytrityl-O^2,2'-cyclouridine (MMTr-cU), LXI, in 60% yield, by treatment with monomethoxytrityl chloride (MMTrCl) in dry pyridine at room temperature for 4 days[51]. No hydrolysis or displacement of the anhydro linkage occurred as the infrared carbonyl absorption at 6.06μ indicated an O^2,2'-cyclouridine compound. In addition, the chromatographic R_f values were less than for 5'-O-monomethoxytrityl-uridine prepared by the method of Smith[19]. The chromatographic results were as expected for a monomethoxytrityl derivative of O^2,2'-cyclouridine which, itself, moves slower than uridine in tetrahydrofuran. A check of LXI was made by treating 5'-O-monomethoxytrityluridine under the conditions of Hampton and Nichol[31] to produce LXI. The 5'-O-monomethoxytrityl-O^2,2'-cyclouridines prepared by the two methods were shown to be identical by infrared spectroscopy. A mixed melting point showed no melting point depression, further indication that the 5'-O-monomethoxytrityl-O^2,2'-cyclouridine produced by both methods were the same compound.

The fully blocked derivative, LXII, was synthesized by treatment of MMTr-cU with β-benzoylpropionic acid and DCC in dry pyridine[16]. The product, 5'-O-monomethoxytrityl-3'-O-(β-benzoyl-propionyl)-O^2,2'-cyclouridine (MMTr-cU-OβB), was isolated in 82.5% yield and characterized by chromatography, elemental analysis and infrared and ultraviolet spectroscopy.

The removal of the monomethoxytrityl group from MMTr-cU-OβB

with 80% acetic acid at room temperature[51] was monitored by thin-layer chromatography in tetrahydrofuran. After 4.5 hours, detritylation was complete. Work-up of the reaction yielded 3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine (cU-OβB), LXIII, in 85% yield. The product was fully characterized by chromatography, elemental analysis and infrared and ultraviolet spectroscopy.

The chromatographic and spectroscopic data for the nucleosides above are reported in Table I, II and III.

The desired derivatives of O^2,2'-cyclouridine have been synthesized in good yield by one reaction sequence (Scheme IV). Initial studies in the phosphorylation of MMTr-cU and in the synthesis of dinucleoside monophosphates containing O^2,2'-cyclouridine are discussed in Section III.

III. Phosphorylation of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine and Synthesis of a Dinucleoside Monophosphate Containing O^2,2'-Cyclouridine

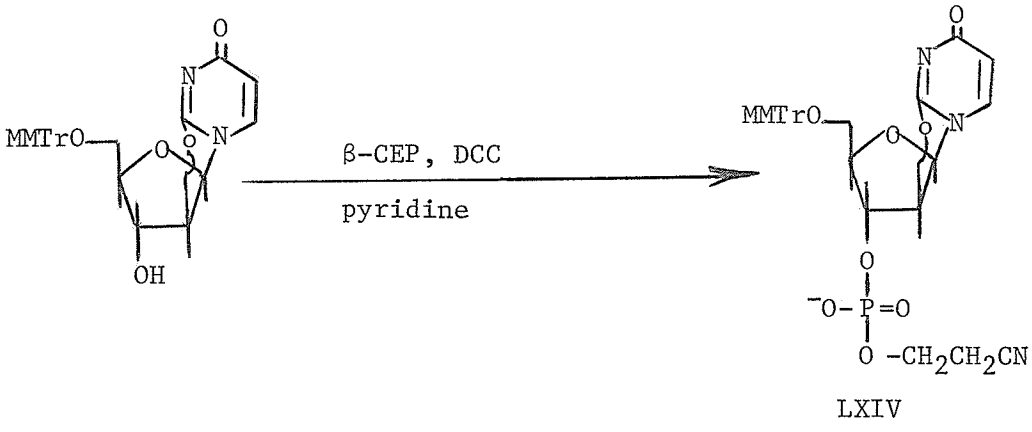
For the initial studies on the synthesis of dinucleoside monophosphates containing O^2,2'-cyclouridine, the "triester method"[11,13] was chosen, the reason being that the neutral character of the phosphotriester product would allow rapid separation by silica gel chromatography using organic solvents[13].

In a small-scale reaction, 5'-O-monomethoxytrityl-O^2,2'-cyclouridine (25 mg, 0.05 mmole) was treated with β-cyanoethyl phosphate (0.1 mmole) in dry pyridine using mesitylenesulfonyl chloride (0.2 mmole) as the condensing agent[13]. After 6 hours, the reaction was quenched by the addition of water. The mixture was stirred for 16 hours to break up any pyrophosphates formed[52]. The absence of starting material was determined by thin-layer chromatography, indicating complete phosphorylation. Spraying with 10% perchloric acid, however, revealed the presence of a large amount of monomethoxytritanol, indicating extensive detritylation. The acidic character of the sulfonic acids present in the mixture was considered to be the cause of the detritylation. Later however, the heat caused by addition of water to pyridine, coupled with the presence of the arylsulfonic acids was determined to be the cause of the detritylation.

Dicyclohexylcarbodiimide (DCC) is also used as a condensing agent in nucleotide synthesis. Although the rate of phosphorylation using dicyclohexylcarbodiimide is slower than that of arylsulfonyl chlorides, the yields are comparable[4]. More important to this work, the weakly basic nature of dicyclohexylcarbodiimide and its by-product, dicyclohexylurea (DCU), should not contribute to the detritylation of compounds having the monomethoxytrityl blocking group.

In a small-scale reaction, 5'-O-monomethoxytrityl-O^2,2'-cyclouridine (50 mg, 0.1 mmole) was treated with β-cyanoethyl phosphate (0.4 mmole) and dicyclohexylcarbodiimide (1.6 mmole) in pyridine for

three days[53]. An electrophoresis of the reaction mixture showed the major material of the reaction as a spot moving as fast as the phosphodiester, TpT. Spraying this major spot with 10% perchloric acid and heating gave an orange colour, indicating that the major product contained the monomethoxytrityl group. 5'-O-monomethoxytrityl-O^2,2'-cyclouridine is neutral; hence, it will not move from the origin on electrophoresis. The charged, monomethoxytrityl product corresponding to the major spot moving as fast as TpT on electrophoresis was the β-cyanoethyl derivative of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate (MMTr-cUp(CE)), LXIV.



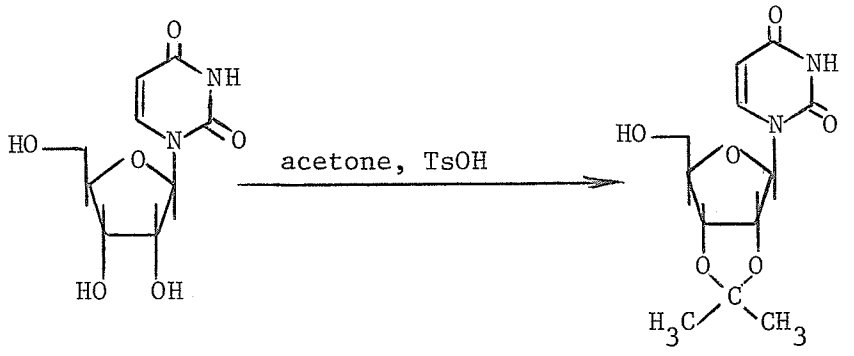
LXIV

The mixture was cooled in an ice bath and the reaction quenched by the addition of cold water to prevent any heat of mixing from contributing to detritylation or hydrolysis of the anhydro linkage. After stirring for 16 hours to precipitate dicyclohexylurea and to break up pyrophosphates, the mixture was filtered to remove DCU. The product (85.5% yield, as the ammonium salt) was isolated by preparative paper chromatography. An infrared spectrum gave a

carbonyl absorption at 6.06μ, indicating the presence of a cU derivative. In addition, the weak nitrile absorption at 4.4μ indicated the presence of the β-cyanoethyl group. Infrared spectroscopy thus confirmed the product as MMTr-cUp(CE).

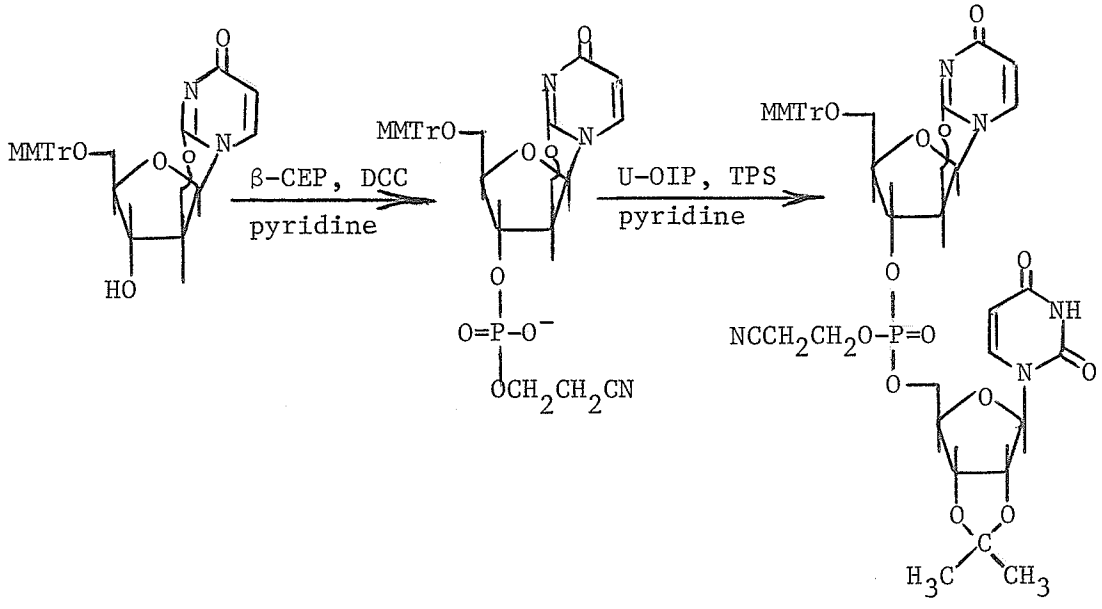
Though efficient in the synthesis of phosphodiesters, dicyclohexylcarbodiimide is not effective in the synthesis of phosphotriesters[53]. The arylsulfonyl chloride, 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) has been effectively used in the synthesis of phosphotriesters[11].

To examine the effects of triisopropylbenzenesulfonyl chloride with respect to the lability of the monomethoxytrityl and O^2,2'-cyclouridine moieties, and to study the effect of the O^2,2'-cyclouridine moiety on the synthesis of a dinucleoside monophosphate, a uridine derivative having a free 5'-hydroxyl, rather than 3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine was used. Only one O^2,2'-cyclouridine moiety would then have to be considered in examining the results of the condensation reactions. 2',3'-O-Isopropylideneuridine (U-OIP), LXV, was readily synthesized from uridine by treatment with p-toluenesulfonic acid (TsOH) in dry acetone at room temperature . This uridine derivative has a free 5'-hydroxyl and a small, acid-labile isopropylidene group blocking the cis-2',3'-diol.



LXV

The scheme involving the triester condensation of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine with 2',3'-O-isopropylidene-uridine is outlined below.



LXVI

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (1g, 2 mmoles) was phosphorylated by β-cyanoethyl phosphate (8 mmoles) and dicyclohexylcarbodiimide (32 mmoles) in dry pyridine to yield MMTr-cUp(CE). After quenching the reaction by the addition of cold water to the solution cooled in an ice bath, the mixture was stirred for 16 hours. A thin-layer chromatogram in tetrahydrofuran revealed complete phosphorylation as shown by the absence of any 5'-O-monomethoxytrityl-O^2,2'-cyclouridine. In addition, no detritylation of the product was observed as indicated by the absence of monomethoxytritanol. After removing dicyclohexylurea by filtration, the filtrate was extracted with chloroform-ethanol (∿5:1). The limited solubility of MMTr-cUp(CE) in chloroform meant that a small amount of ethanol must be used to aid in dissolving the product in the organic layer, thus separating the product from the excess β-cyanoethyl phospate which remains in the aqueous layer. After separation of the layers, a thin-layer chromatogram in tetrahydrofuran showed the product in the organic layer, not in the aqueous layer. The organic layer was extracted with saturated aqueous sodium chloride, and then concentrated under reduced pressure. Dry pyridine was added and the MMTr-cUp(CE) dissolved. The mixture was filtered to remove undissolved salts and dicyclohexylurea.

The product was dried and condensed with 2',3'-O-isopropylidene-uridine (3 mmoles) using triisopropylbenzenesulfonyl chloride (4 mmoles) as the condensing agent. The reaction was quenched after 18 hours by

the addition of cold water to the cooled reaction. After stirring for 1 hour, the product was extracted into chloroform, the layers separated, the organic layer concentrated to a small volume, and the products precipitated with ether. The residue was dissolved in tetrahydrofuran and applied to thick-layer plates. The plates were developed first in ethyl acetate, then in tetrahydrofuran. The band with R_f (THF) 0.23 was eluted with tetrahydrofuran to give 480mg (27%) of a product identified as the β-cyanoethyl derivative of 5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine, LXVI (MMTr-cUp(CE)U-OIP). The product was characterized by thin-layer and paper chromatography, elemental analysis, and by infrared and ultraviolet spectroscopy. The studies involving this analog of a natural dinucleoside monophosphate are reported below and also serve to characterize the product. The synthesis of LXVI represents the first stepwise chemical synthesis of a dinucleoside monophosphate containing a cyclouridine moiety.

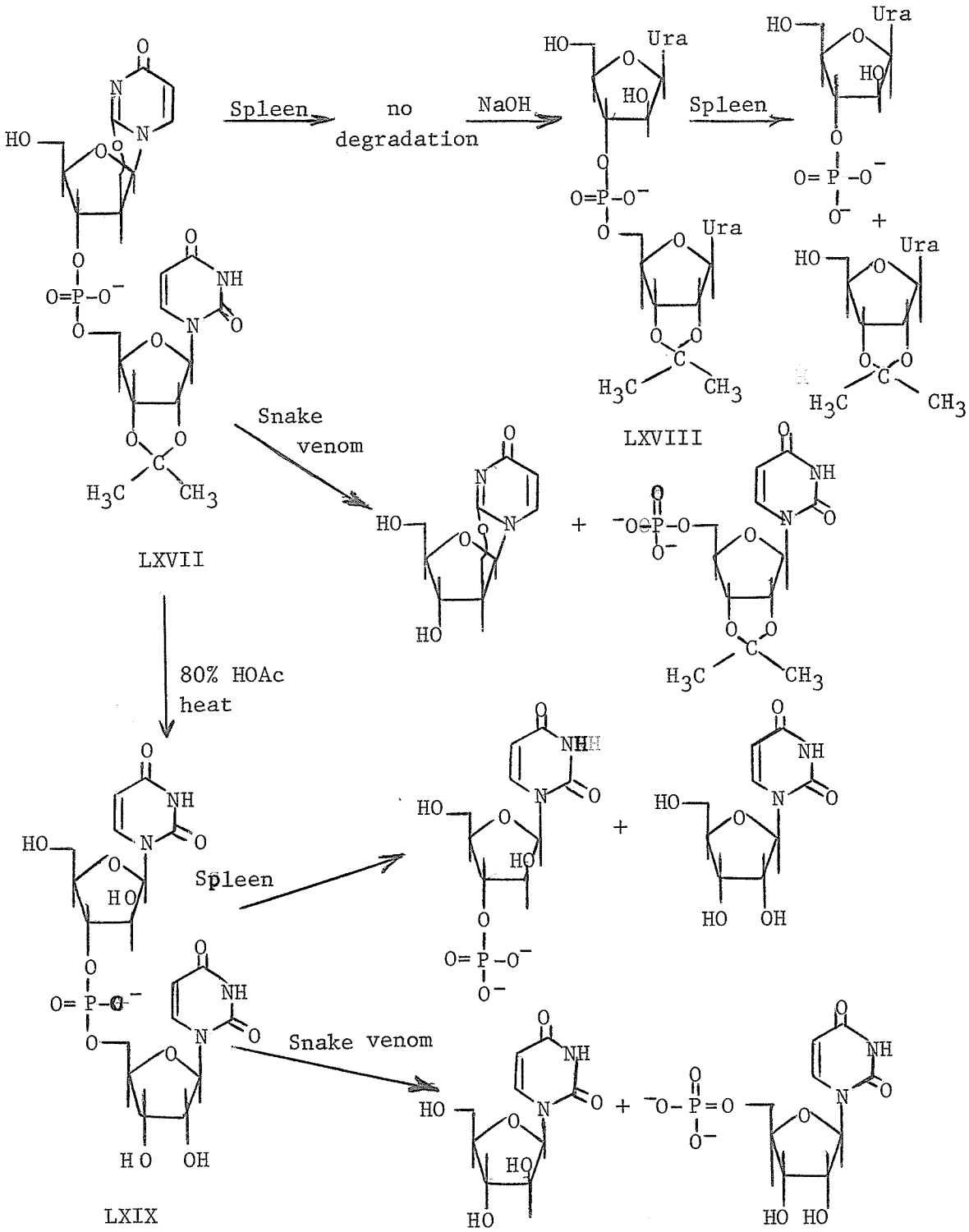
IV. Studies on MMTr-cUp(CE)U-OIP and its Derivatives

To prepare the phosphodiester O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (cUpU-OIP), LXVI was treated with 80% acetic acid at room temperature for 2 hours to remove the monomethoxytrityl group. The solvents were removed under reduced pressure. The residue was treated with a weakly alkaline solution[6], pyridine-ammonium hydroxide (5:2), for 30 minutes to remove the cyanoethyl group. The product, cUpU-OIP

(LXVII) was isolated by paper chromatography followed by preparative electrophoresis. An ultraviolet spectrum of cUpU-OIP gave an absorption maximum (λmax) of 255nm, a result expected from the combination of an O^2,2'-cyclouridine moiety (λmax 249nm) and a uridine moiety (λmax 262nm). The isopropylidene group was not removed as the acidic conditions required (80% acetic acid at 100°C for 90 minutes)[55] were found previously to result in the hydrolysis of the anhydro linkage.

cUpU-OIP was treated with snake venom and spleen phosphodiesterases. Snake venom requires the nucleotide to have a free 3'-hydroxyl in order to act. cUpU-OIP has an isopropylidene group blocking the 3'-hydroxyl; however, the small group did not affect the ability of the enzyme to recognize a non-phosphorylated 3'hydroxyl. Complete degradation to O^2,2'-cyclouridine and to 2',3'-O-isopropylideneuridine 5'-phosphate (pU-OIP) occurred on treatment of cUpU-OIP with snake venom. The products were isolated by paper chromatography and characterized by ultraviolet spectroscopy (λmax for cU of 250nm and λmax for pU-OIP of 262nm). Spleen enzyme requires the nucleotide to have a free 5'-hydroxyl. Although cUpU-OIP had a free 5'-hydroxyl, no enzyme degradation of the dinucleoside monophosphate occurred (cf. 44,46). To determine whether the inactivity was a result of the O^2,2'-cyclouridine moiety or perhaps a faulty enzyme preparation, the undegraded cUpU-OIP from the spleen enzyme assay was isolated and treated with 1N sodium

Scheme V. Enzyme Studies on cUpU-OIP and aUpU.



hydroxide to hydrolyse the anhydro linkage. The product arabino-uridylyl-(3'-5')-2',3'-O-isopropylideneuridine, aUpU-OIP (LXVIII) was isolated by paper chromatography. Treatment with spleen phosphodiesterase gave complete degradation to arabinouridine 3'-phosphate (aUp) and 2',3'-O-isopropylideneuridine. The inactivity of the spleen enzyme on cUpU-OIP was, in fact, due to the presence of the O^2,2'-cyclouridine moiety (Scheme V).

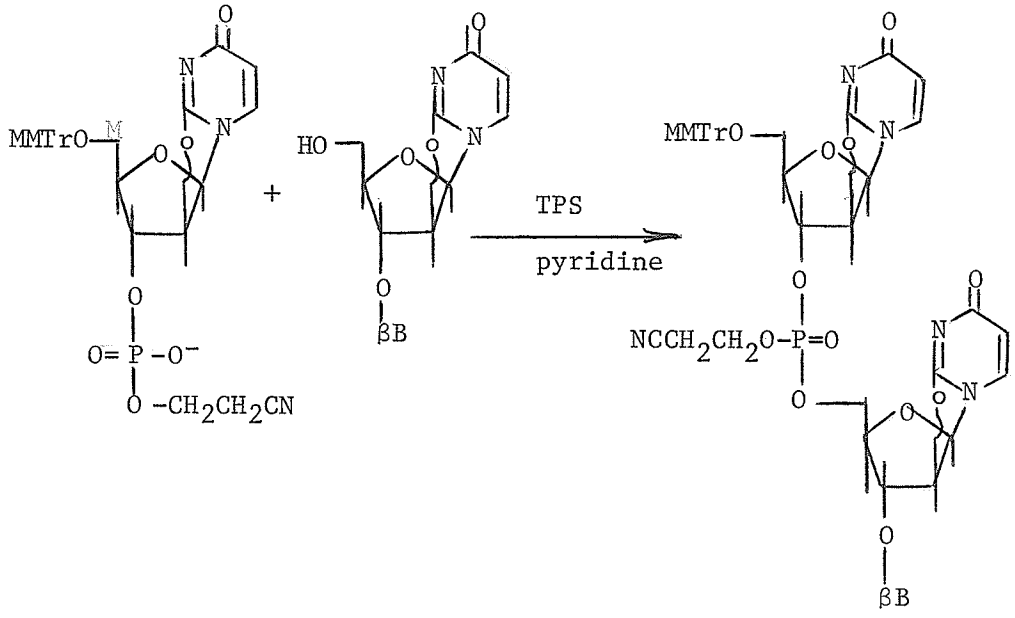
To further characterize the triester product, the acid-labile blocking groups were removed and the anhydro linkage hydrolysed by treatment with 80% acetic acid at reflux for 3 hours. After removal of the cyanoethyl group by base, the phosphodiester arabino-uridylyl-(3'-5')-uridine, aUpU (LXIX) was isolated by paper chromatography and characterized by electrophoresis, paper chromatography and ultraviolet spectroscopy (absorption maximum of 260nm indicating the absence of the cU moiety). Treatment with snake venom phosphodiesterase gave complete degradation to arabinouridine (aU) and uridine 5'-phosphate. Treatment with spleen phosphodiesterase gave complete degradation to arabinouridine 3'-phosphate (aUp) and uridine. The products of the enzyme assays were characterized by paper chromatography and ultraviolet spectroscopy (Scheme V).

Finally, the conversion of cUpU-OIP to aUpU was accomplished by refluxing in 80% acetic acid. Comparison with aUpU prepared from LXVI showed identical compounds by paper chromatography and ultraviolet spectroscopy. The characterization of the triester MMTr-cUp(CE)U-OIP

and its derivatives by chemical and enzymic studies was a further proof that a dinucleoside monophosphate triester containing the O^2,2'-cyclouridine moiety was synthesized by chemical means.

V. Attempted Synthesis of MMTr-cUp(CE)cU-OβB

The successful synthesis of MMTr-cUp(CE)U-OIP encouraged attempts to synthesize the β-cyanoethyl ester of 5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-3'-O-(β-benzoyl-propionyl)-O^2,2'-cyclouridine, MMTr-cUp(CE)cU-OβB (LXX), a dinucleoside monophosphate containing only the O^2,2'-cyclouridine moiety.



LXX

MMTr-cUp(CE) (LXIV) was synthesized by phosphorylation of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine with β-cyanoethyl phosphate (8 mmoles) and dicyclohexylcarbodiimide (32 mmoles). After the work-up described previously, LXIV was treated with 3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine (LXIII) and triisopropylbenzenesulfonyl chloride in dry pyridine. Thin-layer chromatography showed no condensation to have occurred.

To make certain LXIII was dry, the compound was dissolved in tetrahydrofuran, precipitated with hexane, and dried in a drying pistol over P_2O_5. LXIII (7mg, 2 equiv) was mixed with MMTr-cUp(CE)(6mg, 1 equiv) obtained as a lyophilized powder from the small-scale phosphorylation. Triisopropylbenzenesulfonyl chloride (2 equiv) and dry pyridine were added. After 24 hours, no condensation occurred. No condensation occurred in a third attempt using a procedure similar to the first method.

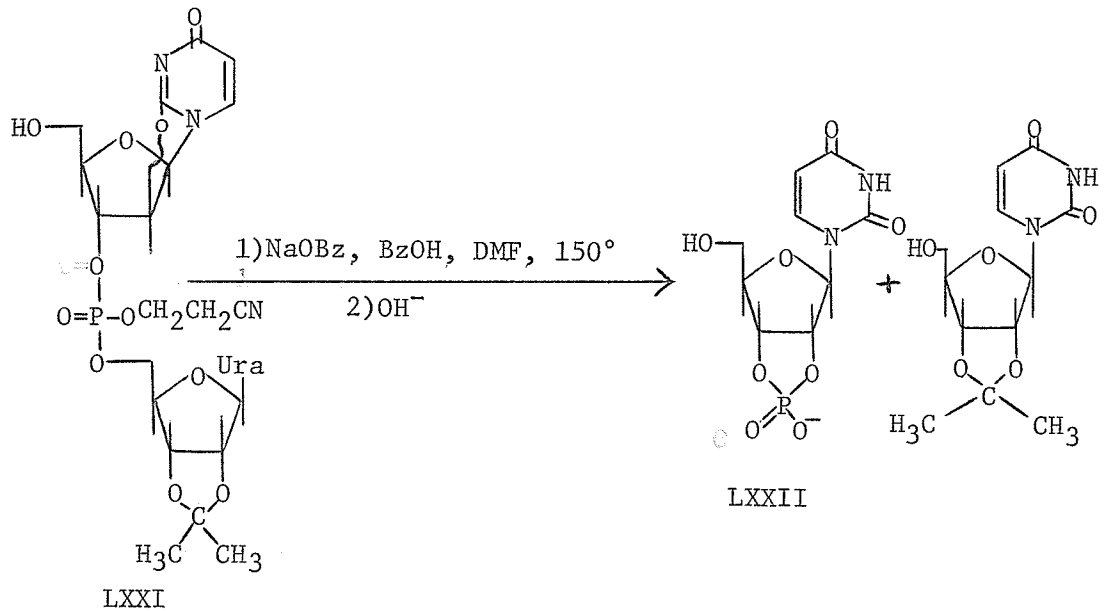
The inability to condense two O^2,2'-cyclouridine moieties by the "triester method" was disturbing; however, the results of the displacement studies on MMTr-cUp(CE)U-OIP reported below made further attempts to synthesize LXX unnecessary.

VI. Studies on Displacement of the Anhydro Linkage in MMTr-cUp(CE)U-OIP

Having successfully hydrolysed the anhydro linkage to yield the arabino derivative aUpU, the displacement of the anhydro linkage to the corresponding ribonucleic acid was investigated.

MMTr-cUp(CE)U-OIP was treated with 80% acetic acid at room temperature to remove the monomethoxytrityl group. After removal of

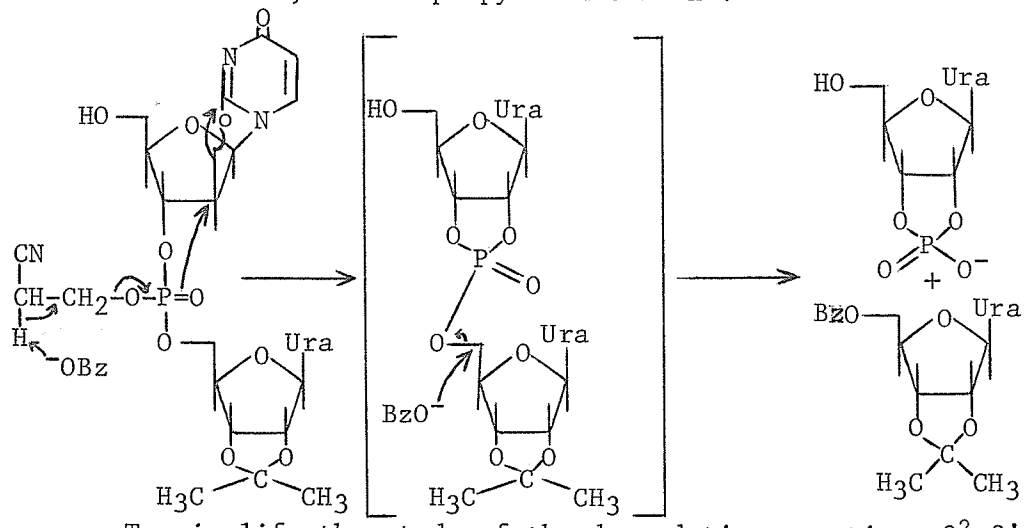
temperature to remove the monomethoxytrityl group. After removal of the solvents under reduced pressure and treatment of the residue with pentane to remove monomethoxytritanol, the product obtained was the β-cyanoethyl ester of O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine, cUp(CE)U-OIP (LXXI). To displace the anhydro linkage and produce the uridine derivative, uridylyl-(3'-5')-2',3'-O-isopropylideneuridine (UpU-OIP), cUp(CE)U-OIP was treated with sodium benzoate (NaOBz) and benzoic acid (BzOH) in dimethylformamide at reflux for 3 hours[28], followed by basic treatment to remove the base-labile β-cyanoethyl and benzoyl groups. Two products were isolated by paper chromatography, i) a neutral product shown to be 2',3'-O-isopropylideneuridine by comparison with authentic U-OIP, and ii) an anionic product identified as uridine 2',3'-cyclic phosphate, Ucp (LXXII) by comparison with authentic Ucp on paper chromatography and electrophoresis.



LXXI

LXXII

A possible mechanism to explain the degradation of the triester involved β-elimination of the cyanoethyl group followed by phosphate anion displacement of the anhydro linkage to yield the cyclic intermediate LXXIII. Benzoate ion attack at the 5'-carbon of the 2',3'-O-isopropylideneuridine moiety gave the products, uridine 2',3'-cyclic phosphate and 5'-O-benzoyl-2',3'-isopropylideneuridine (BzU-OIP). In the work-up, treatment with base converted the 5'-O-benzoyl derivative into 2',3'-O-isopropylideneuridine.



To simplify the study of the degradation reaction, O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (cUpU-OIP) was used. Treatment of cUpU-OIP with sodium benzoate and benzoic acid in dimethylformamide led to the formation of uridine 2',3'-cyclic phosphate and 5'-O-benzoyl-2',3'-O-isopropylideneuridine (by comparison with authentic BzU-OIP on thin-layer chromatography and by mass spectrometry). The mechanism of phosphate anion displacement of the anhydro linkage under the reaction conditions above appeared to be confirmed.

To investigate the displacement of the anhydro linkage by the benzoate ion under milder reaction conditions, O^2,2'-cyclouridine was treated with sodium benzoate and benzoic acid in dimethylformamide at 50° and at 100° for 3 hours. Little change in the O^2,2'-cyclouridine was observed. Treatment of O^2,2'-cyclouridine with sodium benzoate and benzoic acid in refluxing ethanol for 6 hours also showed little change in the nucleoside. Obviously, milder conditions did not work; the more vigorous conditions of Fox[28] were necessary to displace the anhydro linkage.

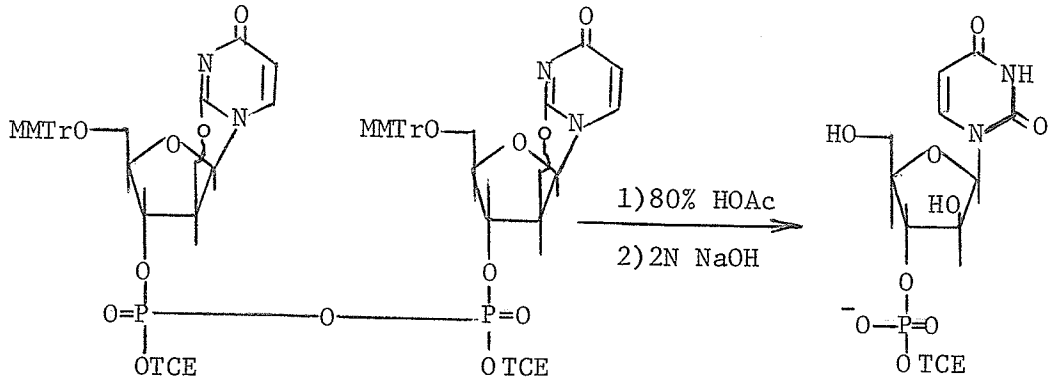
The β-cyanoethyl group of cUp(CE)U-OIP readily underwent a β-elimination reaction when exposed to the conditions required for the displacement of the anhydro linkage. To resolve the problem and prevent degradation of the O^2,2'-cyclouridine nucleotide, a phosphate blocking group which would not undergo such elimination was required. The β,β,β-trichloroethyl group[12] was considered as the alternative to the cyanoethyl group as the trichloroethyl group could not undergo β-elimination.

Initial Studies in the Use of the β,β,β-Trichloroethyl Phosphate Blocking Group

Eckstein[12] used β,β,β-trichloroethyl phosphorodichloridate ($TCEPCl_2$) as the phosphorylating agent in the synthesis of triesters of nucleic acids. The high reactivity of the phosphorodichloridate permitted phosphorylation and condensation reactions without the aid of a condensing agent.

The synthesis of $TCEPCl_2$ was achieved by the method of Eckstein[56]. To study the effects of the phosphorochloridate on the O^2,2'-cyclouridine moiety, the synthesis of the β,β,β-trichloroethyl ester of MMTr-cUpU-OIP was investigated. As the β,β,β-trichloroethyl triester of MMTr-cUpU-OIP was not expected to differ greatly from the β-cyanoethyl triester product MMTr-cUp(CE)U-OIP with respect to physical properties, the analysis of the phosphorodichloridate reaction would be aided by reference to the cyanoethyl triester previously synthesized.

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (100mg, 0.2 mmole) in dry pyridine (1.2ml) was slowly dropped into a solution of $TCEPCl_2$ (0.2 mmole) in dry pyridine (0.8ml)[56]. After stirring for 17 hours, a thin-layer chromatogram in tetrahydrofuran showed complete conversion of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine. To this solution 2',3'-O-isopropylideneuridine (0.1 mmole) was added. After 24 hours, the product containing the monomethoxytrityl group was isolated by thick-layer chromatography. The monomethoxytrityl and trichloroethyl groups were removed by treatment with 80% acetic acid and zinc dust for 3 hours[56]. Preparative electrophoresis gave a product band moving like TpT, indicating a monoanionic species (expected to be cUpU-OIP). The ultraviolet spectrum gave an absorption maximum of 250nm, characteristic of O^2,2'-cyclouridine, not 255nm, characteristic of cUpU-OIP. Snake venom phosphodiesterase, which degraded cUpU-OIP, did not degrade the new product. It appeared that a new product was formed in the initial phosphorylation. This product contained only the cU moiety.



LXXIV

To determine the identity of the new product, 5'-O-monomethoxytrityl-O^2,2'-cyclouridine was treated with $TCEPCl_2$. The product of the reaction was identical chromatographically to the new product formed above. After isolation by thick-layer chromatography, the monomethoxytrityl group was removed by treatment with 80% acetic acid. The solvents were removed and the residue treated with 2N sodium hydroxide for 4 minutes. The product was isolated by preparative paper chromatography. An electrophoresis showed the product to be monoanionic. Snake venom gave no degradation, while spleen phosphodiesterase gave a dianionic product, in addition to undegraded starting material. The results indicated a pyrophosphate product, LXXIV. The base treatment hydrolysed the detritylated pyrophosphate to the β,β,β-trichloroethyl ester of arauridine 3'-phosphate (aUp(TCE)). Spleen degraded the aUp(TCE) to yield arauridine 3'-phosphate. As aUp(TCE) had no free 3'-hydroxyl, snake

venom was unable to degrade the product.

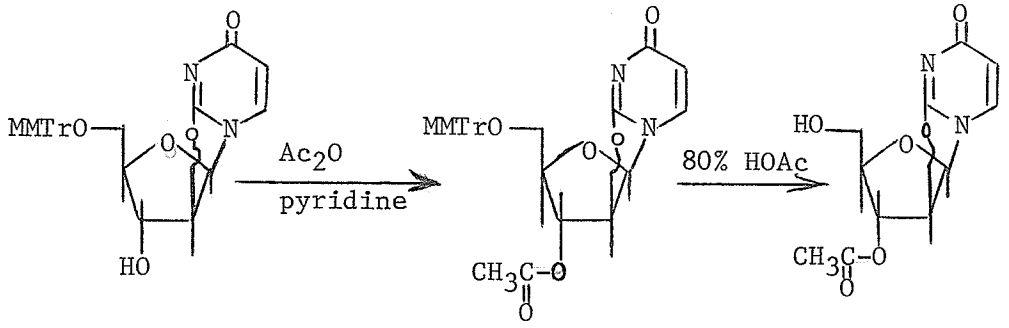
The above results indicated that the $TCEPCl_2$ might in fact be another active phosphorylating agent. Before investigating the phosphorodichloridate reaction further, the important spleen enzyme result with cUpU-OIP indicated that the synthesis of a dinucleoside monophosphate containing a cU moiety at the 3'-terminus with a free 3'-hydroxyl was of primary importance. The ability of phosphodiesterase, particularly snake venom, to degrade such a dinucleoside monophosphate would be of interest.

Synthesis of Thymidylyl-(3'-5')-3'-O-acetyl-O^2,2'-cyclouridine and Enzyme Studies

The unsuccessful synthesis of the triester, MMTr-cUp(CE)cU-OβB, may have been due to interference of the phosphodiester attack on the 5'-hydroxyl of 3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine by a combination of the rigid nucleoside conformation (caused by the anhydro linkage) and the benzoylpropionyl group attached to the 3'-position. To minimize any interference in the phosphodiester condensation to the free 5'-hydroxyl of a O^2,2'-cyclouridine derivative, a "diester" synthetic route, condensing a phosphomonoester to a O^2,2'-cyclouridine, was investigated. The benzoylpropionyl group was replaced by the smaller acetyl group to give 3'-O-acetyl-O^2,2'-cyclouridine. As the unsuitable base-labile cyanoethyl phosphate blocking group was to be replaced by the trichloroethyl group in triester syntheses, 3'-O-acetyl-O^2,2'-cyclouridine, having a base-

labile acetyl group, would then replace 3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine in triester synthetic schemes to synthesize dinucleoside monophosphate derivatives containing only the O^2,2'-cyclouridine moiety.

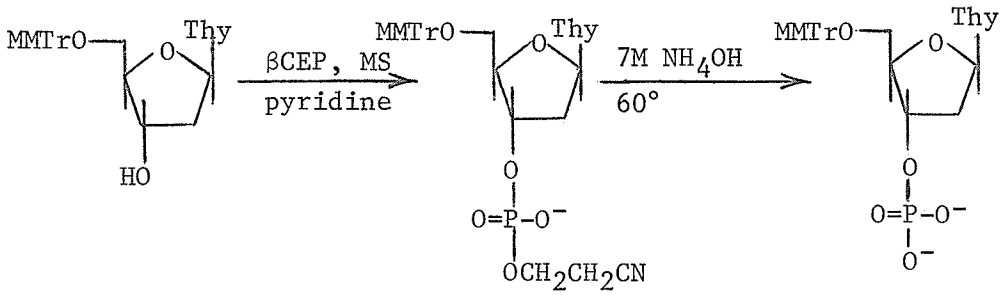
5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (2.5g, 5 mmole) was treated with acetic anhydride in dry pyridine to yield[3] 5'-O-monomethoxytrityl-3'-O-acetyl-O^2,2'-cyclouridine, LXXV (2.57g, 95%). The product was fully characterized. Treatment of LXXV with 80% acetic acid at room temperature yielded 3'-O-acetyl-O^2,2'-cyclouridine, LXXVI (78% yield). The product was fully characterized by paper chromatography, elemental analysis and infrared and ultraviolet spectroscopy.



LXXV LXXVI

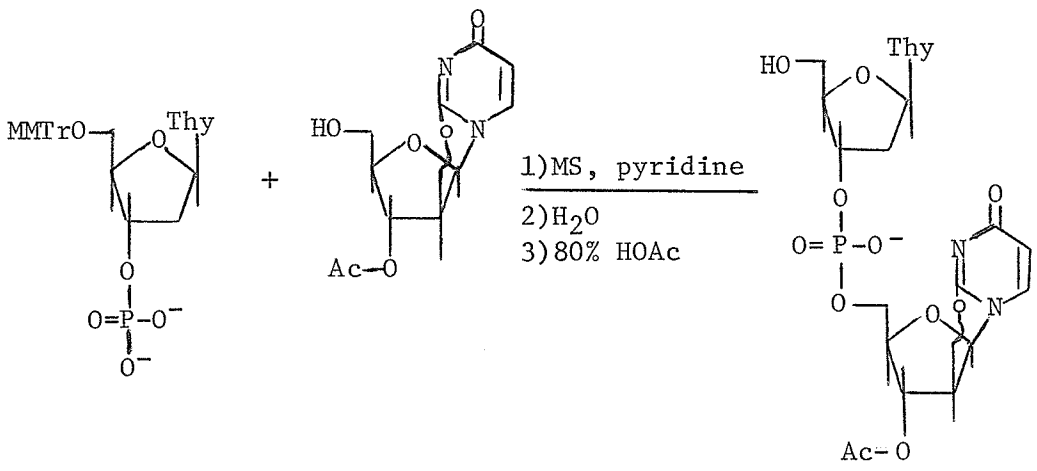
To determine whether an O^2,2'-cyclouridine moiety having a free 5'-hydroxyl could be condensed with a nucleoside 3'-phosphate in a "diester synthesis", 5'-O-monomethoxytritylthymidine 3'-phosphate (MMTr-Tp, LXVII) was chosen as the nucleoside 3'-phosphate for two reasons. First, the product of the condensation reaction would contain only one O^2,2'-cyclouridine moiety allowing easy analysis of

the reaction. Second, MMTr-Tp could be readily synthesized[13,57]. 5'-O-Monomethoxytritylthymidine (80mg, 0.15 mmole) was phosphorylated[13] to yield the β-cyanoethyl ester of 5'-O-monomethoxytritylthymidine 3'-phosphate. The cyanoethyl group was removed by treatment with 7M ammonium hydroxide[57] at 60°C to yield LXXVII. The product was isolated by paper chromatography.



LXXVII

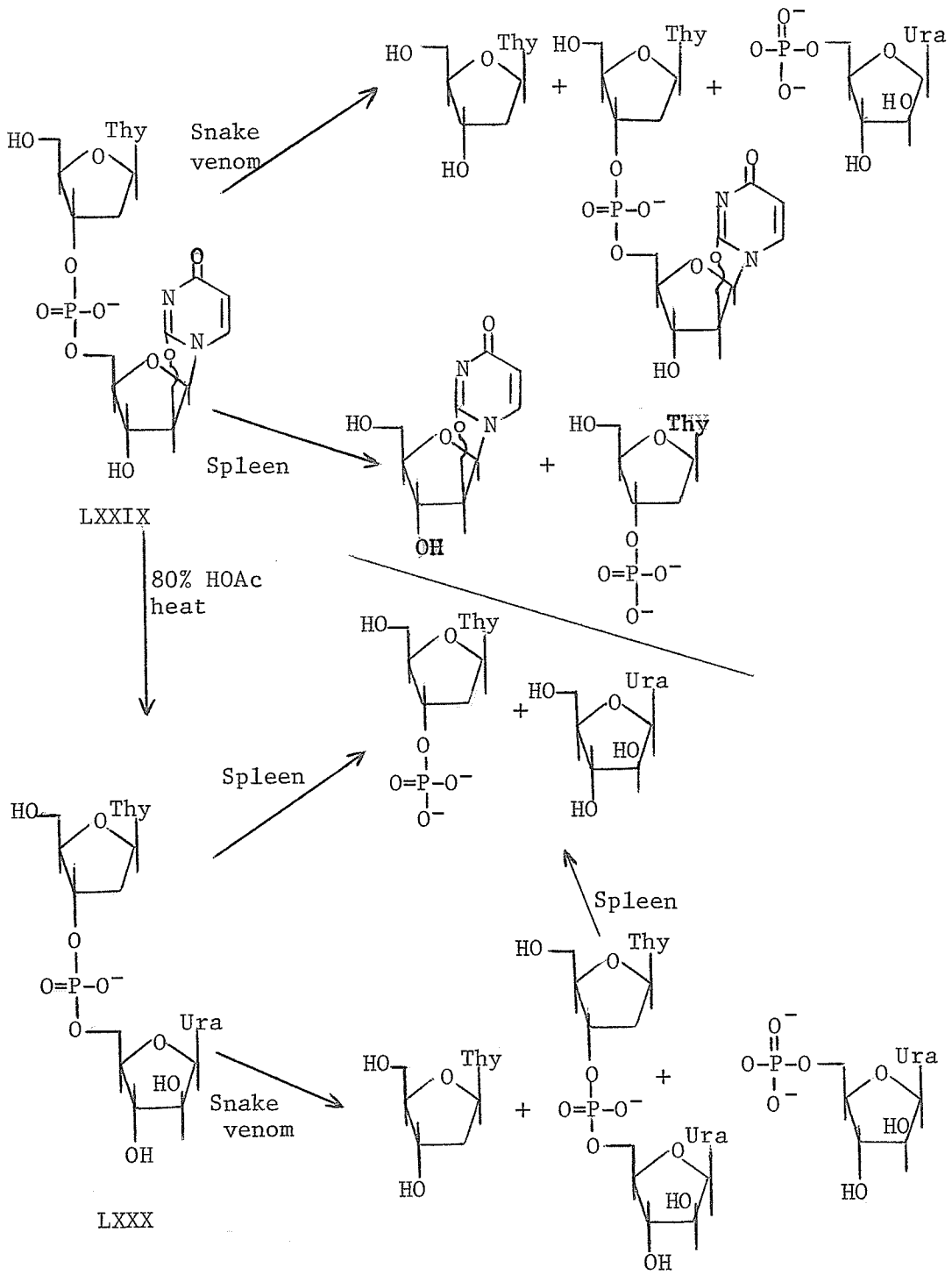
LXXVII was condensed with 3'-O-acetyl-O^2,2'-cyclouridine (20mg, 0.075 mmole) in dry pyridine with mesitylenesulfonyl chloride as condensing agent. The reaction was quenched after 6.5 hours by the addition of cold water. The solvents were removed and the residue treated with 80% acetic acid for 2.5 hours. The product, thymidylyl-(3'-5')-3'-O-acetyl-O^2,2'-cyclouridine, LXXVIII (TpcU-OAc) was isolated by preparative paper chromatography followed by preparative electrophoresis. The yield was spectrophotometrically determined to be 13%. The ultraviolet spectrum gave an absorption maximum of 260nm, a result expected from the combination of a thymidine moiety (λmax 267) and an O^2,2'-cyclouridine moiety (λmax 249nm).



LXXVIII

To characterize the product, TpcU-OAc was treated with 15% ammonium hydroxide - ethanol for 90 minutes to remove the acetyl group. The product, thymidylyl-(3'-5')-O^2,2'-cyclouridine, TpcU(LXXIX) was isolated by paper chromatography. The ultraviolet spectrum gave an absorption of 260nm, indicating no hydrolysis of the anhydro linkage on removal of the acetyl group. TpcU was treated with spleen phosphodiesterase for the usual 5 hours and also for 24 hours. Complete degradation occurred after 5 hours to yield thymidine 3'-phosphate (Tp) and O^2,2'-cyclouridine. After 24 hours, in addition to complete degradation, significant conversion of Tp to thymidine was observed due to the phosphomonesterase activity present in the spleen enzyme preparation. Treatment of TpcU with snake venom for 7 hours gave no degradation of TpcU. However, the basic buffer (pH 9.0) caused 7% hydrolysis of the anhydro linkage to yield TpaU which was degraded

Scheme VI. Enzyme Studies on TpcU and TpaU.



to yield thymidine (T) and arabinouridine 5'-phosphate (paU). After 24 hours treatment with snake venom, TpcU was hydrolysed to TpaU to the extent of 35%. The TpaU degraded to T and paU while the TpcU was undegraded (Scheme VI).

To further characterize the product, TpcU was converted into TpaU (LXXX) by 80% acetic acid and heat. Treatment of TpaU with spleen enzyme gave complete degradation to thymidine 3'-phosphate and arabinouridine. Treatment of TpaU with snake venom gave 35% degradation to thymidine and arabinouridine 5'-phosphate after seven hours, and 75% degradation after 24 hours. The undegraded TpaU from the snake venom assays was treated with spleen enzyme. Complete degradation of the product occurred (Scheme VI).

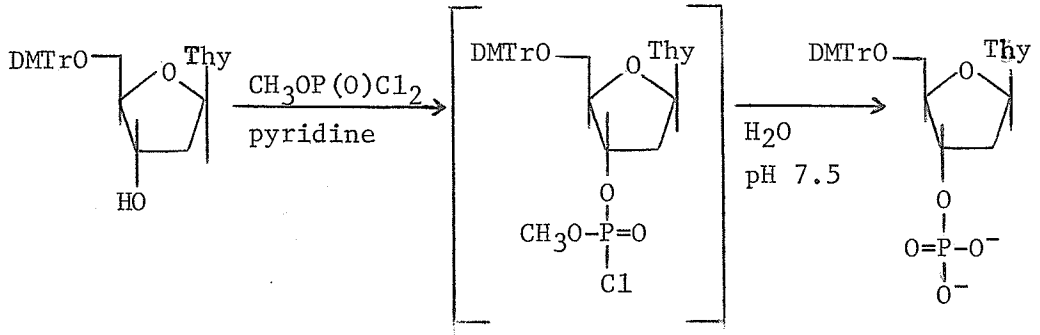
Spleen phosphodiesterase, which degrades from the 5'-hydroxyl end of a nucleic acid, did not degrade cUpU-OIP, the 5'-hydroxyl nucleoside moiety being O^2,2'-cyclouridine. Snake venom, which degrades from the 3'-hydroxyl end of a nucleic acid, did not degrade TpcU, the 3'-hydroxyl nucleoside moiety being O^2,2'-cyclouridine. In addition, the rate of degradation of the arabino derivative TpaU, was slower than for a dinucleoside monophosphate containing uridine in place of arabinouridine (Wechter[34] found the dinucleoside monophosphates containing arabinocytidine to require more enzyme and longer incubation times than the corresponding ribocytidine nucleotides in order to achieve complete degradation).

The synthesis of dinucleoside monophosphates having only one O^2,2'-cyclouridine moiety, cUpU-OIP and TpcU, yielded interesting

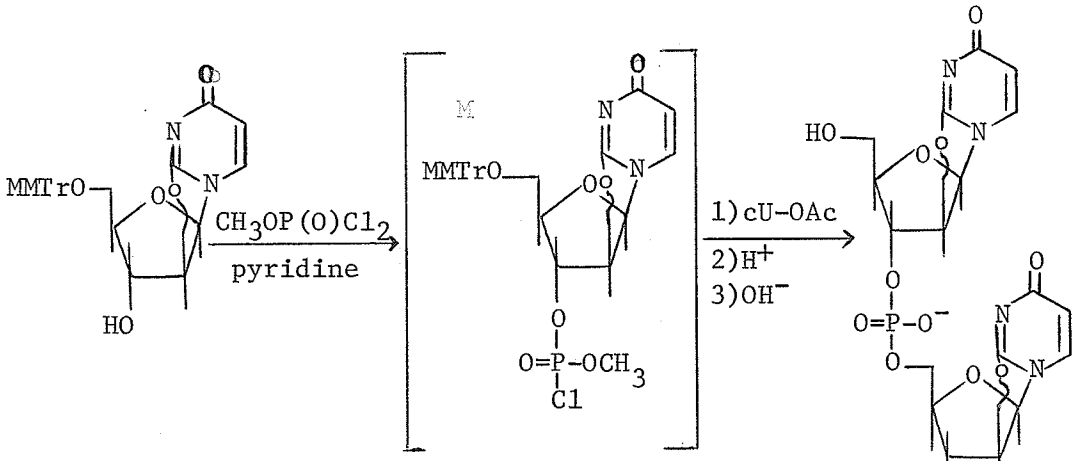
phosphodiesterase results. To complete the series, the synthesis of a dinucleoside monophosphate containing only O^2,2'-cyclouridine moieties was investigated.

Synthesis of the 3'-5' and 3'-3' Isomers of O^2,2'-Cyclouridylyl-O^2,2'-cyclouridine (cUpcU)

The β,β,β-trichloroethyl phosphorodichloridate "triester method" was considered the preferred route in the synthesis of cUpcU. The triester product would have the base-stable trichloroethyl blocking group on the phosphate allowing displacement of the anhydro linkage without degradation of the dinucleoside monophosphate. As problems arose in the use of $TCEPCl_2$, however, the synthesis of cUpcU using methyl phosphorodichloridate ($MepCl_2$)[58] was investigated. The phosphorylation of 5'-O-dimethoxytritylthymidine by $MePCl_2$ to yield 5'-O-dimethoxytritylthymidine 3'-phosphate, LXXXI, was reported[59]. The methyl group, from the intermediate diester, was easily removed by treatment with weak base (pH 7.5).



The synthesis of cUpcU by the method Eckstein[12], using methyl phosphorodichloridate, was investigated. 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (0.5 mmole) in pyridine was slowly dropped into a solution of methyl phosphorodichloridate (0.5 mmole) in pyridine. After stirring for five hours, 3'-O-acetyl-O^2,2'-cyclouridine (0.25 mmole) was added. Cold water was added after 16 hours to quench the reaction. An impure monomethoxytrityl containing product was isolated by thick-layer plates. The blocking groups were removed by treatment with 80% acetic acid followed by 15% ammonium hydroxide-ethanol and the product "cUpcU" (LXXXII, 7.6%) was isolated by preparative paper chromatography.



LXXXI

+

3'-3' isomer

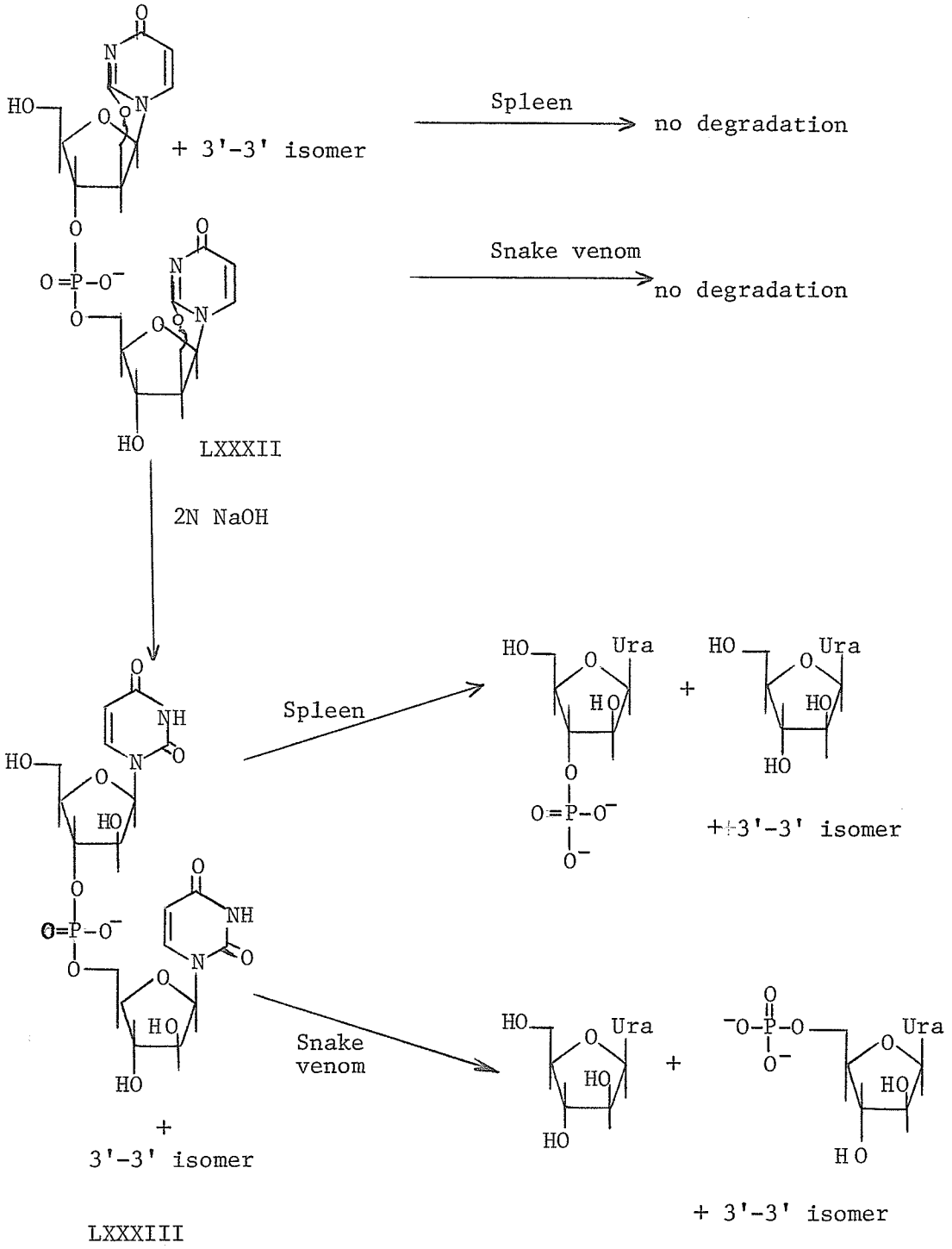
LXXXII

The absorption maxima (250nm and 223nm) and electrophoretic mobility (R_m 0.28) indicated that a diester containing only the O^2,2'-cyclouridine moiety had been synthesized.

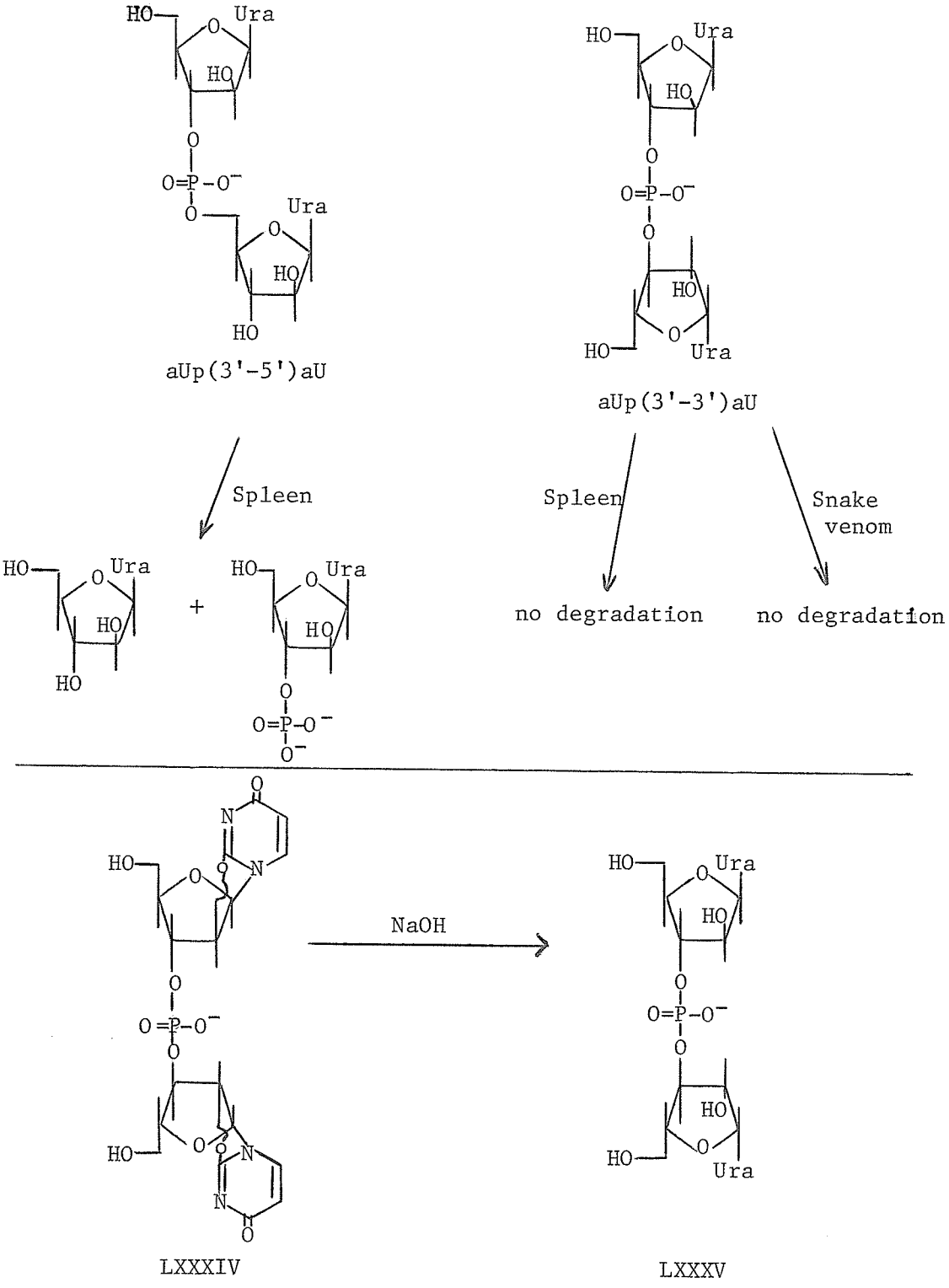
LXXXII was not degraded by snake venom or spleen phosphodiesterase. The anhydro linkages were hydrolysed by treatment with 2N sodium hydroxide to yield "aUpaU", LXXXIII (λmax 262nm). Spleen enzyme gave only 36% degradation of LXXXIII to arabinouridine 3'-phosphate and arabinouridine, even after incubation for 24 hours. Spleen enzyme did not degrade the isolated, undegraded product in a separate enzyme assay. Snake venom gave 24% degradation to arabinouridine 5'-phosphate and arabinouridine after incubation for 7 hours, and 35% degradation after 24 hours. Obviously, ∿65% of the product believed to be O^2,2'-cyclouridylyl-(3'-5')-O^2,2'-cyclouridine was, in fact, another compound (Scheme VII).

LXXXII was treated with 2N sodium hydroxide. Paper chromatography in solvent C for 48 hours yielded two products, both arabinouridine derivatives (λmax 262nm). The slower moving product was completely degraded by spleen enzyme to yield aUp and aU, this product being aUp(3'-5')aU. The faster moving product was undegraded by either snake venom or spleen enzyme. The stability of the impurity to concentrated base, coupled to its stability to phosphodiesterases suggested that the impurity was the 3'-3' phosphate isomer LXXXV, derived from O^2,2'-cyclouridylyl-(3'-3')-O^2,2'-cyclouridine, LXXXIV (Scheme VIII).

Scheme VII. Enzyme Studies on "cUpcU" and "aUpaU".



Scheme VIII. Enzyme Studies on 3'-5' and 3'-3' isomers of aUpaU



The 3'-3' isomer was probably synthesized during the initial phosphorylation by the attack of LXXXI at the 3'- position of a second MMTr-cU. To test the hypothesis, the phosphorylation reaction was repeated. The reaction was quenched with water after 4.5 hours and the monomethoxytrityl group removed by treatment with 80% acetic acid. The product was then treated with 2N sodium hydroxide to remove the methyl group and hydrolyse the anhydro linkage. Paper chromatograms revealed the presence of a monoanionic species (LXXXV) in addition to the expected arabinouridine 3'-phospate.

It is interesting to note that Smrt and Catlin[59] were able to remove the methyl group from the methyl ester of 5'-O-dimethoxytritylthymidine 3'-phosphate by slightly basic conditions of pH 7.5. Using ethyl phosphorodichloridate and following the same procedures, these workers isolated both the 5'-O-dimethoxytritylthymidine 3'-phosphate and the ethyl ester. (Apparently the ethyl ester was more stable to the hydrolysis conditions than the methyl ester). Ts'O and co-workers[60;61], however, synthesized the methyl and ethyl triester derivatives of TpT and d-ApA and found the ethyl triester to be stable to 2N sodium hydroxide for 10 minutes at room temperature. No stability study of the methyl triester to base was reported. One would expect the methyl or ethyl groups in a triester to be more sensitive to base than in the diester; however, no comment was made by Ts'O *et al*[60] whose synthesis appeared in the literature approximately one year later.

No other comment on these results will be attempted except to state that work presented in this thesis gave satisfactory removal of the methyl group from the triester to yield the mixture of the 3'-5' and 3'-3' isomers of cUpcU and aUpaU.

Although the 3'-3' isomer was synthesized and isolated as an impurity, cUp(3'-5')cU, in fact, was synthesized. The product was not degraded by the phosphodiesterases. The lability of the methyl group with base limited the usefullness of the methyl triester in displacement studies. The synthesis of a triester containing the apparently base-stable trichloroethyl group by the "phosphorodichloridate method" was investigated.

Attempted Synthesis of MMTr-cUp(TCE)U-OIP (LXXXVI) using β,β,β-Trichloroethyl Phosphorodichloridate

The β,β,β-trichloroethyl phosphorodichloridate ($TCEPCl_2$) used in the following experiments was purchased, hence, it was not the phosphorylating agent used in the previous "phosphorodichloridate" reactions.

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (0.1 mmole) in pyridine was slowly dropped into a solution of β,β,β-trichloroethyl phosphorodichloridate (0.1 mmole) in pyridine over a period of 6 hours[12]. After stirring for 20 hours, the products were isolated by paper chromatography. The nucleotide band with R_f^C 0.48 was eluted and lyophilized. The product (R_m 0.20 and

λmax 230nm, indicative of MMTr-cUp(TCE)) was treated with 2',3'-O-isopropylideneuridine and triisopropylbenzenesulfonyl chloride. No condensation had occurred.

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (0.2 mmole) was phosphorylated in the usual manner with $TCEPCl_2$. After 16 hours, triisopropylbenzenesulfonyl chloride was added, followed by 2',3'-O-isopropylideneuridine. After 21 hours, the monomethoxytrityl and trichloroethyl groups were removed by zinc and 80% acetic acid[56]. The products, isolated by preparative paper chromatography, all had arabinouridine or uridine ultraviolet spectra (λmax 260-262nm). The anhydro linkage had been hydrolyzed, probably during the concentration of the pyridine and acetic acid solutions.

To resolve this problem, the reaction was repeated and quenched by pouring the pyridine solution into ether to precipitate the product. After treatment with zinc and acetic acid, the solution was lyophilized. The products isolated by paper chromatography, again were uridine-like, indicating anhydro linkage hydrolysis.

O^2,2'-Cyclouridine was treated with 80% acetic acid and zinc to test the stability of the anhydro linkage. No hydrolysis of O^2,2'-cyclouridine to arabinouridine occurred after 4 hours.

To determine whether the anhydro linkage was hydrolysed during the initial phosphorylation reaction, 5'-O-monomethoxytrityl-O^2,2'-cyclouridine (0.2 mmole) was phosphorylated in the usual manner with $TCEPCl_2$. After 16 hours, a thin-layer

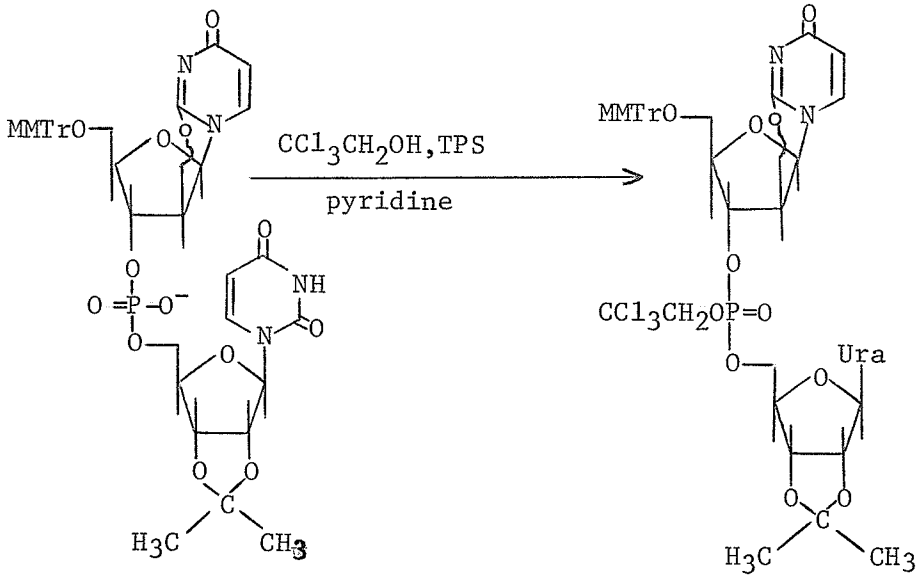
chromatogram showed no MMTr-cU present; however, noticeable detritylation had occurred. The solution was poured into ether and preparative paper chromatography yielded a charged (R_m 0.24), monomethoxytrityl containing product having an ultraviolet spectrum similar to that of MMTr-cU (λmax 230nm, (sh) 255nm), indicative of MMTr-cUp(TCE). Hydrolysis, of the anhydro linkage had not occurred during the initial phosphorylation to any great extent.

The phosphorylation of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine was repeated. After 16 hours, only 2',3'-O-isopropylideneuridine (not triisopropylbenzenesulfonyl chloride as well) was added. Thin-layer chromatograms after 23 hours showed no condensation. The products, isolated in the usual way, were characterized by uridine-like ultraviolet spectra, indicating hydrolysis of the anhydro linkage. The lack of condensation, extensive hydrolysis of the anhydro linkage and the occurrence of appreciable detritylation indicated that further investigation into the use of β,β,β-trichloroethyl phosphorodichloridate in the synthesis O^2,2'-cyclouridine nucleotides would be of little value.

Although efforts to synthesize trichlorethyl esters of O^2,2'-cyclouridine nucleotides were unsuccessful, the potential value of the trichloroethyl blocking group encouraged investigations into other synthetic routes.

Conversion of the β-Cyanoethyl Ester of MMTr-cUpU-OIP into the β,β,β-Trichloroethyl Ester

The lability of the β-cyanoethyl group in a triester to base[13] allowed the synthesis of 5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (MMTr-cUpU-OIP, LXXXVII). The approach was to consider MMTr-cUpU-OIP as a phosphodiester and to condense the phosphodiester with the alcohol β,β,β-trichloroethanol to form the triester product, MMTr-cUp(TCE)U-OIP[60].



LXXXVII LXXXVI

MMTr-cUp(CE)U-OIP (40mg, 0.045 mmole) was treated with pyridine-ammonium hydroxide to remove the cyanoethyl group. LXXXVII was isolated by paper chromatography and characterized by its electrophoetic mobility (R_m 0.30) and ultraviolet spectrum (λmax (sh)251 and 230nm). Treatment with freshly distilled β,β,β-trichloroethanol (0.09 mmole) and triisopropylbenzenesulfonyl

chloride (0.09 mmole) in pyridine for 12 hours yielded no triester product, LXXXVI. The only products were the starting material, and its detritylated diester, cUpU-OIP.

The experiment was repeated. After stirring for 8.5 hours, more triisopropylbenzenesulfonyl chloride was added and stirring was continued for 20 hours. No condensation to yield the triester, LXXXVI, occurred.

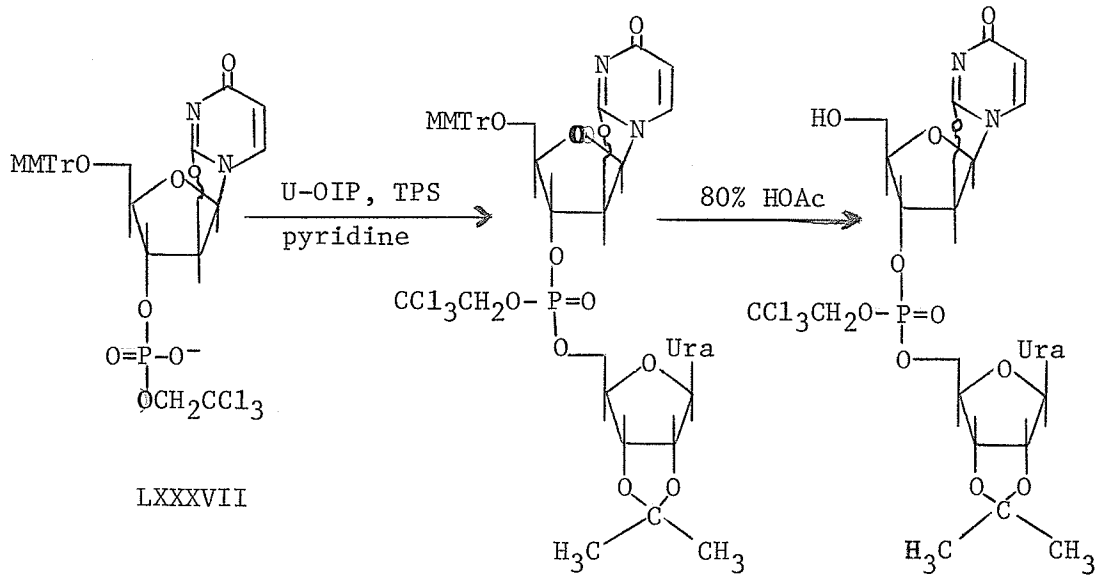
Neither the "phosphorodichloridate method" or the "trichloroethanol condensation" yielded the desired product, MMTr-cUp(TCE)U-OIP. The final method to be investigated involved the conversion of $TCEPCl_2$ into an amine salt followed by the usual "triester synthetic method" procedures.

Synthesis of the β,β,β-Trichloroethyl Ester of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (MMTr-cUp(TCE)U-OIP, LXXXVI)

The dicyclohexylammonium salt of β,β,β-trichloroethyl phosphate[62] was synthesized from the corresponding dichloridate. The procedure followed was similar to that of Neilson[63] and involved converting the dicyclohexylammonium salt to the pyridinium salt prior to the initial phosphorylation reaction.

To test the method, a small-scale synthesis was carried out. 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (0.05 mmole) was phosphorylated with β,β,β-trichloroethyl phosphate (0.10 mmole) using triisopropylbenzenesulfonyl chloride as the condensing agent to yield

the trichloroethyl ester of 5'-O-monomethoxytrityl-O^2,2'cyclouridine 3'-phosphate (MMTr-cUp(TCE), LXXXVII).



LXXXVII LXXXVI LXXXVIII

LXXXVII was condensed with 2',3'-O-isopropylideneuridine (0.10 mmole) using TPS as the condensing agent. The product was extracted into methylene chloride and applied to thick-layer plates. The impure product, LXXXVI, was eluted from the plates and treated with 80% acetic acid to remove the monomethoxytrityl group. Paper chromatography yielded 4.6mg (13%) of the β,β,β-trichloroethyl ester of O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (cUp(TCE)U-OIP, LXXXVIII). The absorption maximum at 255nm was characteristic of the combination of O^2,2'-cyclouridine and uridine moieties.

Treatment with zinc and 80% acetic acid removed the trichloroethyl group from LXXXVIII to yield cUpU-OIP. As expected, spleen enzyme did not degrade cUpU-OIP. Snake venom enzyme degraded

cUpU-OIP to O^2,2'-cyclouridine and 2',3'-O-isopropylideneuridine 5'-phosphate (pU-OIP).

Slow hydrolysis of the cUpU-OIP occurred in aqueous solution to yield aUpU-OIP. This product was completely degraded by spleen enzyme to yield arabinouridine 3'-phosphate and 2',3'-O-isopropylideneuridine and by snake venom enzyme to yield arabinouridine and pU-OIP.

The above enzyme studies parallel those of the derivatives of MMTr-cUp(CE)U-OIP and confirm the synthesis of cUp(TCE)U-OIP (hence, cUpU-OIP and aUpU-OIP).

Having achieved the synthesis of the trichloroethyl ester of cUpU-OIP, the reaction was scaled-up in order to isolate MMTr-cUp(TCE)U-OIP. The procedure above was followed. 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (0.5 mmole) was phosphorylated with β,β,β-trichloroethyl phosphate and the product was condensed with 2',3'-O-isopropylideneuridine (1.0 mmole) to yield MMTr-cUp(TCE)U-OIP (111mg, 13%). The product was isolated by thick-layer chromatography and characterized by its chromatographic and electrophoretic properties, elemental analysis, and ultraviolet spectrum.

Studies on MMTr-cUp(TCE)U-OIP and its Derivatives

Treatment of MMTr-cUp(TCE)U-OIP with 80% acetic acid removed the monomethoxytrityl group to yield cUp(TCE)U-OIP. Treatment of cUp(TCE)U-OIP with 80% acetic on a steam bath yielded

aUp(TCE)U which was converted to aUpU by treatment with zinc and 80% acetic acid.

The displacement of the anhydro linkage was investigated. The studies also were a test of the stability of the trichloroethyl blocking group.

cUp(TCE)U-OIP was treated with sodium benzoate and benzoic acid in dimethylformamide at reflux[28]. Degradation occurred to yield uridine 3'-phosphate, uridine 2',3'-cyclic phosphate and 5'-O-benzoyl-2',3'-O-isopropylideneuridine. The reaction conditions were too drastic even for the trichloroethyl group and caused degradation.

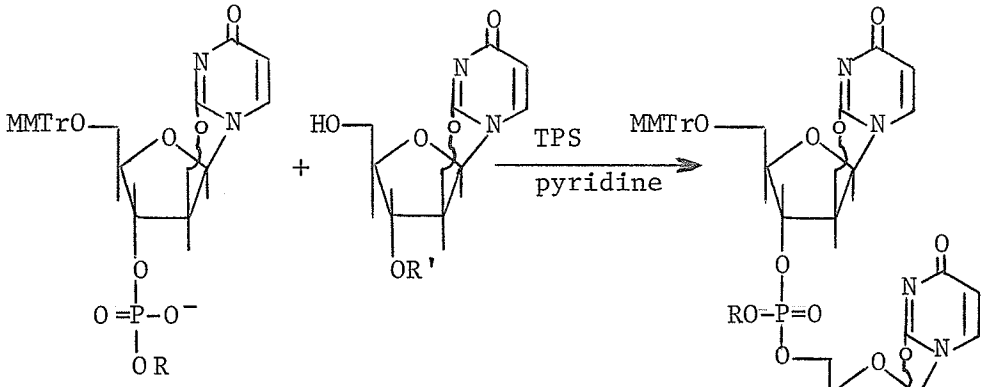
A second displacement reaction was investigated, using the method of Etzold et al[64]. cUp(TCE)U-OIP was treated with potassium fluoride and glacial acetic acid in acetonylacetone at 190°. After treatment with base and then with Cu/Zn in dimethylformamide to deblock the products, an electrophoresis showed no charged product (UpU-OIP). The products isolated by paper chromatography were not nucleosides as determined by mass spectroscopy. Total degradation of the cUp(TCE)U-OIP had occurred.

The conversion of nucleotides containing the O^2,2'-cyclo-uridine moiety into the corresponding ribonucleotides could not be accomplished using the vigorous reaction conditions required[28,64]. The degradation of the nucleotides indicated that milder displacement conditions must be found to prevent the degradation while allowing the displacement of the anhydro linkage.

Attempted Synthesis of the β,β,β-Trichloroethyl and β-Cyanoethyl Esters of O^2,2'-Cyclouridylyl-(3'-5')-O^2,2'-Cyclouridine

Having successfully synthesized dinucleoside monophosphates containing the O^2,2'-cyclouridine moiety (cUpU-OIP and TpcU), and having synthesized a mixture of the 3'-5' and 3'-3' isomers of O^2,2'-cyclouridylyl-O^2,2'-cyclouridine, the unambiguous synthesis of O^2,2'-cyclouridylyl-(3'-5')-O^2,2'-cyclouridine by the "triester method" was investigated.

The reaction scheme is given below. A phosphate ester of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate was to be condensed with an O^2,2'-cyclouridine derivative.



R= $-CH_2CCl_3$; R'= Ac
R= $-CH_2CH_2CN$; R'= Ac
R= " ; R'= H

In the first method investigated, MMTr-cUp(TCE) (R = $-CH_2-CCl_3$) was reacted with 3'-O-acetyl-O^2,2'-cyclouridine (R^1 = $-C(O)-CH_3$). MMTr-cUp(TCE) was synthesized by the phosphorylation of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine (0.05 mmole) with β,β,β-trichloroethyl phosphate as described previously. The product was treated with 3'-O-acetyl-O^2,2'-cyclouridine (0.05 mmole) and triisopropylbenzenesulfonyl chloride in pyridine. After 3 days, thin-layer chromatograms showed no condensation to have occurred. To check, the solvent was evaporated and the residue was treated with 80% acetic acid and heat to remove the monomethoxytrityl group and hydrolyse the anhydro linkages, followed by zinc and acetic acid to remove the trichloroethyl group. An electrophoresis showed no spot corresponding to aUpaU-OAc, confirming that no condensation had occurred.

A second condensation was attempted on a larger scale (0.5 mmole). Thick-layer plates were used to isolate the monomethoxytrityl-containing product. After treatment with zinc and 80% acetic acid, an electrophoresis showed no monoanionic product corresponding to cUpcU-OAc. Other similar attempts at condensation also failed.

To test whether the trichloroethyl group hindered the condensation reaction, MMTr-cUp(CE) (R = $-CH_2-CH_2-CN$) was treated with 3'-O-acetyl-O^2,2'-cyclouridine and triisopropylbenzenesulfonyl chloride. After 3 days, no condensation had occurred as the only

monomethoxytrityl containing products observed on thick-layer plates were MMTr-cUp(CE) and monomethoxytritanol.

To test whether the acetyl group hindered the condensation reaction, MMTr-cUp(CE) (1.0 mmole) was treated with O^2,2'-cyclouridine (2.0 mmoles) and triisopropylbenzenesulfonyl chloride. After 3.5 days, thin-layer chromatograms showed no condensation to have occurred.

The synthesis of an O^2,2'-cyclouridylyl-(3'-5')-O^2,2'-cyclouridine derivative by the "triester method" was unsuccessful. The experiments above were representative of the many condensation reactions attempted. The reason for the failure of the "triester" condensation is not known; however, the approach of the phosphodiester to the free 5'-hydroxyl of an O^2,2'-cyclouridine derivative may be hindered by phosphodiester interaction with the rigid cyclouridine system.

The alternative to the "triester method" was the "diester method" involving condensation between a phosphomonoester and a nucleoside derivative. This alternative route is explored below.

Synthesis of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate (MMTr-cUp, LXXXIX)

The "diester method" required the synthesis of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate, a phosphomonoester. Two phosphodiesters, MMTr-cUp(CE) and MMTR-cUp(TCE) were readily available. Removal of the cyanoethyl group from MMTr-cUp(CE) or the trichloroethyl group from MMTr-cUp(TCE) would yield MMTr-cUp.

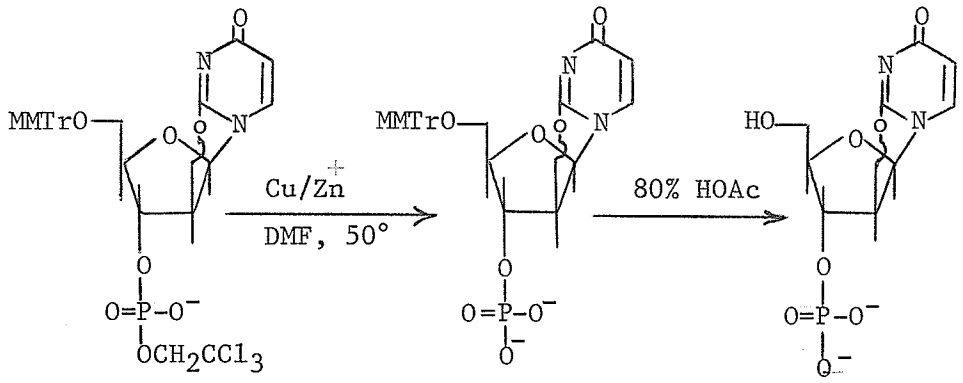
The conditions normally used to remove the cyanoethyl group

from a diester (7M ammonium hydroxide at 60° for 90 minutes[57]) would hydrolyse the anhydro linkage in MMTr-cUp(CE). Torrence and Witkop[65] reported that the cyanoethyl group could be removed from a phosphodiester by reflux in methanol-pyrrolidine (9:1) for 4 hours. To test these reaction conditions, MMTr-cUp(CE) was refluxed in methanol-pyrrolidine for 4 hours. Electrophoresis showed that the cyanoethyl group was removed. Isolation of the products by paper chromatography followed by ultraviolet spectrum revealed complete hydrolysis of the anhydro linkage as the products all exhibited an arabinouridine-like spectrum (λmax 261nm). The conversion of MMTr-cUp(CE) to MMTr-cUp, thus, would not be possible without accompanying hydrolysis of the anhydro linkage.

The conditions used in previous experiments with cUp(TCE)-U-OIP to remove the trichloroethyl group (zinc and 80% acetic acid[56]) would convert MMTr-cUp(TCE) to O^2,2'-cyclouridine 3'-phosphate. The monomethoxytrityl group must be maintained to block the 5'-hydroxyl and prevent self-condensation of cUp. Eckstein[56] also reported that the trichloroethyl group was removed by treatment with a copper/zinc (Cu/Zn) complex in dimethylformamide at 50°.

MMTr-cUp(TCE) was treated with Cu/Zn[66] in dimethylformamide at 50°. An electrophoresis after 30 minutes showed complete conversion of MMTr-cUp(TCE) to MMTr-cUp. To check the anhydro linkage, MMTr-cUp was treated with 80% acetic acid to remove the monomethoxytrityl group. Preparative electrophoresis yielded O^2,2'-cyclouridine

3'-phosphate, XC (λmax 250nm).



LXXXIX XC

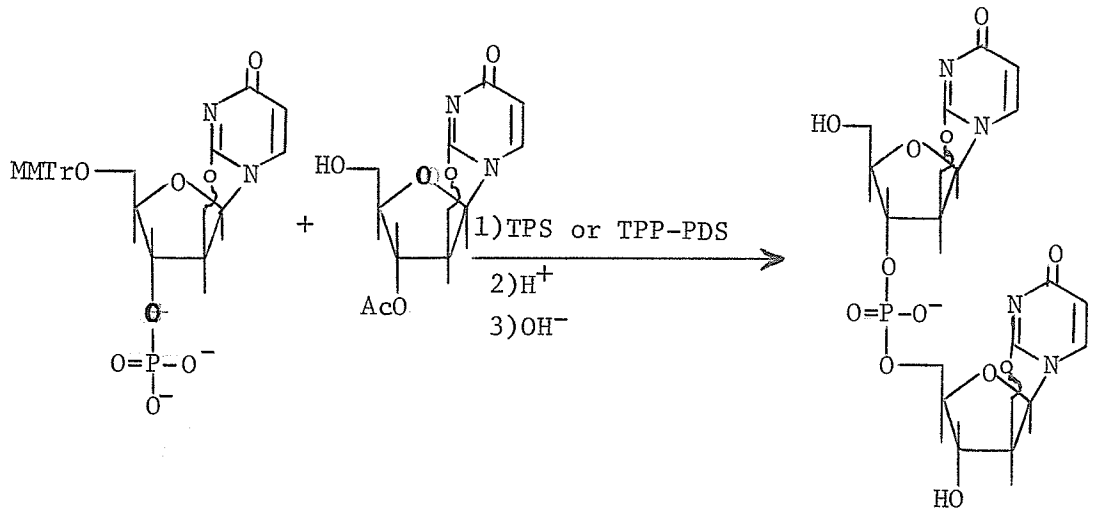
MMTr-cUp was synthesized without hydrolysing the anhydro linkage. The use of MMTr-cUp in the "diester synthesis" of O^2,2'-cyclouridylyl-O^2,2'-cyclouridine is discussed below.

Synthesis of O^2,2'-Cyclouridylyl-(3'-5')-O^2,2'-Cyclouridine (cUpcU)

The reaction scheme for the synthesis of O^2,2'-cyclouridylyl-(3'-5')-O^2,2'-cyclouridine involved condensation of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate with 3'-O-acetyl-O^2,2'-cyclouridine, followed by removal of the blocking groups.

In the first synthesis, dicyclohexylcarbodiimide was the condensing agent. 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (0.5 mmole) was phosphorylated with β,β,β-trichloroethyl phosphate in the usual way to yield MMTr-cUp(TCE). The trichloroethyl group was removed by treatment with Cu/Zn in dimethylformamide and the product, MMTr-cUp, was condensed with 3'-O-acetyl-O^2,2'-cyclouridine (0.46 mmole)

using dicyclohexylcarbodiimide as the condensing agent. After 11 days[46] the product was treated with 80% acetic acid to remove the monomethoxytrityl group and then 25% ammonium hydroxide-ethanol to remove the acetyl group. The product was isolated by paper chromatography to yield 93mg (39%) of cUpcU, XCI.



XCI

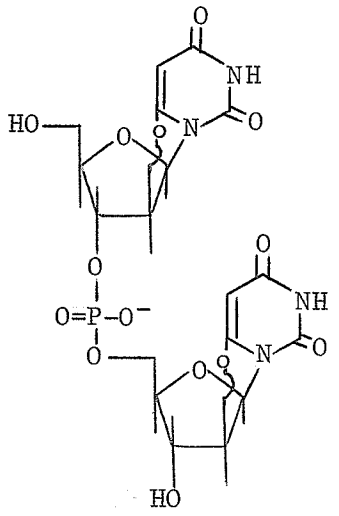
The product was not degraded by snake venom nor by spleen phosphodiesterase. cUpcU was hydrolysed to arabinouridylyl-(3'-5')-arabinouridine, aUpaU (XCII) by treatment with base. Spleen enzyme degraded aUpaU to yield arabinouridine 3'-phosphate (aUp) and arabinouridine. Snake venom enzyme only partially hydrolysed (32%) aUpaU to yield arabinouridine 5'-phosphate (paU) and arabinouridine. The slower rate of degradation with snake venom enzyme for arabino-nucleotides was not unexpected as it had occurred before in the molecules prepared in this work (TpaU) and was noted by Wechter[34].

In the second synthesis, triphenylphosphine and 2,2'-dithiodi-

pyridine[69] were the condensing agents used in the reaction between MMTr-cUp (0.5 mmole) and 3'-O-acetyl-O^2,2'-cyclouridine (1.0 mmole). The blocking groups were removed as described previously and preparative paper chromatography yielded 118mg (46%) of cUpcU. As above, the product was degraded by neither spleen enzyme nor snake venom enzyme. Treatment of cUpcU with base yielded aUpaU which was completely degraded by spleen enzyme.

Conformational Requirement of Enzymes

While this thesis was in preparation, Ikehara and Tezuka[67] reported the synthesis of O^6,2'-cyclouridylyl-(3'-5')-O^6,2'cyclouridine (XCIII). They reported that XCIII was completely degraded by snake venom phosphodiesterase. The nucleoside bases in XCIII are fixed in the "anti" conformation range[68], i.e., the C_2 keto group points away from the sugar ring.



XCIII

The bases in the undegradable cUpcU (XCI) are close to the "syn" conformation range, i.e., the C_2-O- lies over the sugar ring. The results with these molecules indicate that snake venom phosphodiesterase has a conformational requirement for the bases of the mucleoside moieties to be in the "anti" conformation. The requirement may apply only to pyrimidine derivatives, however, as thioanhydropurines[46,47], although constrained in the "anti" conformation, were not degraded by the phosphodiesterases.

A conformational requirement has been exhibited by other enzymes. Holy and Bald[71] found that substitution of a methyl group at the 6-position of a uracil moiety in uridine 2',3'-cyclic phosphate resulted in complete resistance of the phosphodiester to hydrolysis by pancreatic ribonuclease. The substitution of a methyl group for a hydrogen converted the uracil base from the "anti" conformation to the "syn" conformation. Ribonuclease apparently required the nucleoside moiety to be in the "anti" conformation. Pollard and Nagyvary[37] found that cytidine 2',3'-cyclic phosphate hydrolysis by pancreatic ribonuclease A was inhibited more by arabinocytidine derivatives than by ribocytidine derivatives. They concluded that the arabino configuration of the sugar decreased the rotational freedom in the nucleoside fixing the base in the "anti" conformation and thereby increasing the binding power of the arabinocytidine inhibitors to ribonuclease A. In addition, they observed that O^2,2'-cyclocytidine and O^2,2'-cyclouridine derivatives, having the "syn" conformation, did not inhibit the hydrolysis of cytidine 2',3'-cyclic phosphate.

2',3'-cyclic phosphate.

Adenosine deaminase is another enzyme that appears to have the "anti" conformational requirement for its substrates. Simon et al[72] found that bulky substituents in the 8-position of adenosine prevented these derivatives (e.g., 8-bromoadenosine and 8-thioadenosine) from being substrates for adenosine deaminase. The bulky groups constrained the nucleosides into the "syn" conformation. Ogilvie et al[73] found that 8,2'-thioanhydroadenosine, having the purine ring constrained in the "anti" conformation range, was a substrate. Hampton[74,75] obtained similar results with 9,5'-cycloadenosine and its 5'-phosphate. Again the purine ring is constrained in the "anti" conformation range for these derivatives.

Hampton[75] also found that 8,5'-cycloadenosine 5'-phosphate was a substrate for snake venom 5'-nucleotidase and AMP kinase suggesting that these enzymes also require substrates (in nature, adenosine 5'-phosphate) with "anti" conformation.

The "anti" conformation requirement for the substrate is being observed for an increasing number of enzymes although studies are far from complete even where the conformation requirement has been observed. The enzyme results found for the cyclouridine nucleotides synthesized in this work contribute to knowledge being acquired about the function of enzymes.

EXPERIMENTAL

General Methods:

Paper chromatography was carried out using the descending technique on Whatman 3MM paper. The solvent systems used were: Solvent A, isopropyl alcohol-concentrated ammonium hydroxide-water (7:1:2); Solvent B, ethanol-water (7:3); Solvent C, n-butanol-ethanol-water (4:1:5, organic phase); Solvent D, n-butanol-water (86:14). The solvents were prepared on a volume basis. Thin-layer chromatography was carried out using the ascending technique in closed jars which were not coated with absorbant paper. All thin-layer chromatography (TLC) was run on Eastman Chromagram Sheets 6060, silica gel with fluorescent indicator, on strips 10cm x 2cm. Thick-layer chromatography was carried out on glass plates (20cm x 20cm) coated with a 2mm thick layer of silica gel DSF-5 (Mondray Chemicals Limited). Paper electrophoresis was performed using Whatman 3MM paper in a Savant Flat Plate electrophoretic chamber with a Savant Model HV power supply operated at 2000 volts for 1 hour. A triethylammonium bicarbonate buffer (0.05M, pH7.5) was used, prepared by making 15.15g triethylamine up to 3ℓ volume with water followed by bubbling carbon dioxide (20g) through the solution . Nucleosides and their derivatives were detected on paper chromatograms, thin-layer sheets and thick-layer plates using an ultraviolet light source (Mineralite, output ∿254nm). Compounds containing the trityl or monomethoxytrityl groups were detected by spraying the papers or thin-layer sheets with

10% perchloric acid and drying them in a stream of warm air. Trityl containing compounds appear yellow and monomethoxytrityl containing compounds appear yellow-orange.

Column chromatography was accomplished using silica gel (60-200 mesh) from Fisher Scientific. A slurry of silica gel was allowed to settle under its own weight at the desired flow rate. The eluant was monitored by thin-layer chromatography.

Infrared spectra were obtained on a Perkin-Elmer 337 recording spectrophotometer. Samples were prepared in KBr disks. Ultraviolet spectra were obtained on a Cary Model 14 recording spectrophotometer. Water or 95% ethanol was used for neutral solutions. Extinction coefficients at absorption maxima used in determining yields and enzyme rations were obtained from the literature. For aqueous solutions containing nucleosides, the absorption maxima and corresponding extinction coefficients used were: $O^2,2'$-cyclouridine (250nm, 7820)[30]; uridine (262nm, 10,100)[76]; arabinouridine (263nm, 10,500)[78]; thymidine (267nm, 9650)[76]. The term O.D. unit refers to the extinction of a nucleotidic solution at neutral pH in 1ml of solution using a 1cm light path quartz cell. A number in superscript refers to the wavelength used in determination of the O.D. units. Mass spectra were obtained on a Finnigan 1015 mass spectrometer.

Melting points were determined on a Fischer-Johns melting point apparatus and are reported uncorrected. Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Illinois and by Galbreith Laboratories Inc., Knoxville, Tennessee. Samples submitted

to them were prepared by crystallization or precipitation from tetrahydrofuran with hexane followed by heating in a drying pistol over P_2O_5.

Reagents and Chemicals

Reagent grade pyridine was distilled from p-toluenesulfonyl chloride, redistilled from calcium hydride, and stored over Linde Molecular Sieves. Reagent grade acetic anhydride was distilled from phthalic anhydride and stored in the dark. Reagent grade acetone was dried over sodium sulfate and distilled into the reaction flask. Pyridinium β-cyanoethyl phosphate was prepared from the barium salt by passage over a column of Dowex 50W-X8 resin (pyridinium form). The clear solution containing the pyridinium salt was first concentrated to a small volume and then lyophilized to a gum. The gum was dissolved in pyridine, diluted to a known volume and stored in a sealed flask under refrigeration. β,β,β-Trichloroethyl phosphorodichloridate was purchased from Eastman. The dicyclohexylammonium salt of β,β,β-trichloroethyl phosphate was prepared by dropping the phosphorodichloridate into a cooled solution of water-dioxane (1:1). After removal of solvents and dissolving the residue in ethanol-dioxane (1:1), cyclohexylamine was added. The precipitated product was recrystallized from ethanol-dioxane (mp 194 - 198°)[62]. Methyl phosphorodichloridate was purchased from Aldrich.

Mesitylenesulfonyl chloride and 2,4,6-triisopropylbenzene-

sulfonyl chloride were recrystallized from pentane and stored under pentane in the dark. Aldrithiol-2 and triphenyl phosphine were purchased from Aldrich. Dicyclohexylcarbodiimide was purchased from Aldrich and stored under refrigeration.

Uridine was purchased from Sigma Chemical Company. O^2,2'-Cyclouridine (mp 244 - 248°, 83%)[31], 5'-O-trityluridine (mp 114.5 - 116°, 89%)[48], 5'-O-monomethoxytrityluridine (mp 98 - 102°, 76%)[19], 2',3'-O-isopropylideneuridine (mp 161 - 162.5°, 69%)[54], and 5'-O-benzoyl-2',3'-O-isopropylideneuridine (mp 139 - 141°, 52%)[77] were prepared by established procedures. 5'-O-Monomethoxytritylthymidine was kindly donated by Dr. K.K. Ogilvie.

Enzyme Assays:

a) Spleen Phosphodiesterase

Lyophilized spleen phosphodiesterase (10 - 15 units) obtained from Nutritional Biochemicals Corp. was dissolved in 1ml of 0.01M sodium pyrophosphate buffer (adjusted to pH6.5 with phosphoric acid). An aliquot (0.1ml) of this solution and 0.2ml of 0.5M ammonium acetate (adjusted to pH 6.5 with acetic acid) were added to the nucleotide (0.1 to 1mg) in a small test tube. The solution was incubated at 37° for 5 hours (occasionally 24 hours) and applied to Whatman papers as a band 5cm wide. The paper was developed in either Solvents A, B or C depending on the nature of the nucleoside moieties (Solvent A not being used for O^2,2'-cyclouridines as its basic nature partially hydrolyses the anhydro linkage). Nucleoside and

nucleotide bands were cut out, eluted with water and diluted to 10ml. Absorbances were recorded against blanks cut from the paper directly opposite the nucleotide bands. The blanks were eluted with water and diluted to 10ml.

b) Snake Venom Phosphodiesterase

Two hundred units of lyophilized venom phosphodiesterase obtained from Calbiochem were dissolved in 1ml of tris-(hydroxy-methyl)-aminoethane buffer (adjusted to pH 9.2 with 0.1N hydrochloric acid). An aliquot (0.1ml) of the enzyme solution was added to the nucleotide (0.1 to 1mg) and incubated at 37° for 7 hours (occasionally 24 hours). The solution was then worked up as described for the spleen enzyme.

General Procedures

All reactions of more than 0.2 mmole of the limiting reagent were carried out in tightly stoppered ground glass joint flasks. Reactions of less than 0.2 mmole of the limiting reagent were carried out in pyrex test tubes (10ml) stoppered with a serum cap. Reactants were dried by the evaporation of pyridine at reduced pressure, air being readmitted to the sample through a column (30 x 2cm) of anhydrous magnesium perchlorate. All reactions were run at room temperature unless otherwise noted. Phosphorylation and condensation reactions were run in the dark.

Synthetic Methods

5'-O-Trityl-2'-chloro-2'-deoxyuridine (Tr-dU-Cl, LVI)

O^2,2'-Cyclouridine (3.4g, 15 mmoles) and trityl chloride (4.6g, 16.1 mmoles) were dissolved in pyridine (50ml) and the solution was refluxed for 2 hours. The solution was poured into ice-water (500ml) with stirring. The precipitate was collected by filtration and washed with petroleum ether (40ml). The precipitate was dissolved in chloroform and dried over anhydrous sodium sulfate. The solvents were removed under reduced pressure and the residue was crystallized from ethyl alcohol to yield 3.9g (52%) of LVI (mp 164 - 166.5°). Chromatographic data is listed in Table I while spectral properties (u.v. and i.r.) are recorded in Table III.

2'-Chloro-2'-deoxyuridine (dU-Cl, LVII) from Tr-dU-Cl

LVI (200mg, 0.4 mmoles) was dissolved in 80% acetic acid and the solution heated on a steam bath for 20 minutes. The solvents were removed at reduced pressure and the last traces of acetic acid were removed by evaporation of ethanol. The residue was crystallized from ethanol (EtOH) to yield 69mg (66%) of LVII (mp 200 - 206°, lit. mp 205 - 206°[70]). Chromatographic data is listed in Tables I and II.

2'-Chloro-2'-deoxyuridine (dU-Cl) from cU

O^2,2'-Cyclouridine (50mg, 0.22 mmoles) was dissolved in pyridine (4ml). One drop of concentrated hydrochloric acid was added

and the solution was heated near reflux for 2 hours. The solvents were removed at reduced pressure and the residue was crystallized from ethyl acetate to yield 5mg (8.6%) of LVII (mp 198 - 201°). A mixed melting point with dU-Cl prepared from Tr-dU-Cl gave mp 198 - 205°. Chromatographic data is listed in Tables I and II.

O^2,2'-Cyclouridine (cU) from dU-Cl

LVII (25mg, 0.1 mmole) and methanol (0.3ml) containing sodium methoxide (8.1mg) were dissolved in dimethylformamide and the solution was heated at 70 - 75° for 2 hours. The cooled solution was triturated with ether-petroleum ether (2:3, 50ml). The ethers were decanted and the residue crystallized from ethanol to yield 5.2mg (24%) of cU (mp 225 - 235°). Chromatographic data is listed in Table I and spectral properties (i.r.) are recorded in Table III.

5'-O-Trityl-O^2,2'-cyclouridine (Tr-cU, LIII)

5'-O-Tritylуridine (670mg, 1.36 mmoles) was dissolved in dimethylformamide (1.5ml) and treated with diphenyl carbonate (390mg, 1.8 mmoles) and sodium bicarbonate (10mg). The mixture was heated at 150° for 30 minutes and then was poured into ether. The precipated gum was crystallized from ethanol to yield 400mg (62.5%) of LVIII (mp 213 -218°, lit. mp 217 - 219°[30]). Chromatographic data is listed in Tables I and II and spectral properties (u.v. and i.r.) are recorded in Table III.

5'-O-Trityl-3'-O-acetyl-O^2,2'-cyclouridine (Tr-cU-OAc, LIV(a))

LVIII (230mg, 0.5 mmole) was dissolved in pyridine (2.5ml) by warming. After cooling, acetic anhydride (0.071ml, 0.75 mmole) was added and the solution was stirred for 18 hours. The solution was poured into ice-water (150ml) with stirring. The precipitate was collected by filtration, dissolved in chloroform, and dried over anhydrous sodium sulfate. The solution was concentrated to a gum and the gum was dissolved in tetrahydrofuran. The product was precipitated upon addition of hexane to yield 210mg (80.5%) of LIV(a) (mp 98 - 104°). Chromatographic data is listed in Tables I and II and spectral properties (i.r.) are recorded in Table III.

5'-O-Trityl-3'-O-benzoyl-O^2,2'-cyclouridine (Tr-cU-OBz, LIV(b))

LVIII (230mg, 0.5 mmole) was dissolved in pyridine (2.5ml) by warming. After cooling, benzoyl chloride (0.08ml, 0.75 mmole) was added and the solution was stirred for 18 hours. The solution was poured into ice-water (150ml) with stirring. The precipitate was collected by filtration, dissolved in chloroform, and dried over anhydrous sodium sulfate. The chloroform was removed at reduced pressure and the residue dissolved in tetrahydrofuran. The product was precipitated upon addition of hexane to yield 210mg (70%) of LIV(b) (mp 220 - 224°). Chromatographic data is listed in Tables I and II and spectral properties (i.r.) are recorded in Table III.

Attempted Synthesis of 3'-O-Benzoyl-O^2,2'-cyclouridine (LV(b))

LIV(b) (100mg, 0.17 mmoles) was dissolved in 80% acetic acid (2ml) and the solution was heated on a steam bath for 15 minutes. The solvents were removed at reduced pressure and the residue was dissolved in ethanol. Thin-layer chromatograms in tetrahydrofuran (THF) showed the presence of 3'-O-benzoyl-O^2,2'-cyclouridine (R_f^{THF}0.20) as a minor nucleoside component, 3'-O-benzoylarabinouridine (R_f^{THF}0.67) as the major nucleoside component, and tritanol (R_f^{THF}0.75). Crystallization occurred to yield 62mg of impure material containing predominantly 3'-O-benzoylarabinouridine. The product was not purified; however an ultraviolet spectrum gave an absorption maximum of 258nm, indicating a predominance of the arabinouridine derivative.

Stability of 5'-O-Trityl-O^2,2'-cyclouridine to Acidic Conditions

The products of the following reactions to test the trityl lability versus the susceptibility of the anhydro linkage to hydrolysis were characterized by thin-layer chromatography in tetrahydrofuran (THF).

A. 10% Acetic Acid on a Steam Bath: Tr-cU (10mg) and 10% acetic acid (1ml) were heated on a steam bath for 10 minutes. In addition to unreacted Tr-cU (R_f^{THF}0.29), two non-trityl containing nucleosides were present; cU (R_f^{THF}0.07) and arabinouridine (R_f^{THF}0.54) in a ratio of approximately 2:1 (cU:aU).

B. 80% Acetic Acid at Room Temperature: Tr-cU (10mg) was dissolved in 80% acetic acid (1ml) and the solution was stirred for 21 hours. In addition to unreacted Tr-cU (R_f^{THF}0.31), cU (R_f^{THF}0.04) and arabino-

uridine (R_f^{THF}0.52) were present in the ratio of approximately 7:1 (cU:aU).

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (MMTr-cU, LXI)

O^2,2'-Cyclouridine (4.35g, 19.2 mmoles) and monomethoxytrityl chloride (6.0g, 19.5 mmoles) were stirred in pyridine (100 ml) for 4 days. The solvent was removed at reduced pressure and the residue was dissolved in a mixture of chloroform-water (600ml, 1:1). The chloroform layer was separated and washed with water (2x50ml). The organic solution was dried over anhydrous sodium sulfate and then was concentrated. Crystallization occurred to yield 5.70g (60% of LXI (mp 154 - 158°). Chromatographic data is listed in Tables I and II and spectral properties (u.v. and i.r.) are recorded in Table III.

Anal. Calcd. for $C_{29}H_{26}N_2O_6$: C, 69.87; H, 5.26; N, 5.62

Found: C, 70.23; H, 5.28; N, 5.53

MMTr-cU (LXI) from 5'-O-Monomethoxytrityluridine

5'-O-Monomethoxytrityluridine (730mg, 1.41 mmole) was dissolved in dimethylformamide (0.7ml) and was treated with diphenyl carbonate (392mg, 1.82 mmoles) and sodium bicarbonate (7mg). The mixture was heated at 150° for 30 minutes. After cooling, the mixture was poured into ether. The precipitated gum was crystallized from ethanol to yield 91mg (13%) of MMTr-cU (mp 162 - 168°). A mixed melting point with LXI gave no melting point depression (mp 160 - 168°). The infrared spectrum was identical to LXI and showed principal bands at 6.06, 6.13, 8.04 and 14.08μ.

5'-O-Monomethoxytrityl-3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine (MMTr-cU-OβB, LXII)

LXI (4.0g, 9.0 mmoles), 3-benzoylpropionic acid (4.28g, 24.0 mmoles) and dicyclohexylcarbodiimide (6.6g, 32.0 mmoles) were dissolved in pyridine (80ml) and the solution was stirred for thirteen hours. Cold water (24ml) was added and the mixture was stirred for 6 hours. The insoluble dicyclohexylurea was removed by filtration and was washed with pyridine-water (1:1, 50ml). The filtrate was extracted with chloroform (3x200ml) and the aqueous layer discarded. The organic layer was extracted with saturated sodium bicarbonate solution (2x150ml), then washed once with water. The solution was concentrated to a small volume and applied to a column (3.5x46cm) in ether. The column was eluted first with ether (4.5ℓ) followed by tetrahydrofuran (3.5ℓ). Pure MMTr-cU-OβB was obtained in the tetrahydrofuran fractions. Concentration of the solution followed by precipitation of the product with hexane yielded 4.3g (82.5%) of LXII (mp 92 - 97°). Chromatographic data is listed in Tables I and II and spectral properties (u.v. and i.r.) are recorded in Table III.

Anal. Calcd. for $C_{39}H_{34}N_2O_8$: C, 71.11; H, 5.20; N, 4.25

Found: C, 70.68; H, 5.27; N, 4.42

TABLE I Thin-layer Chromatographic Properties of Nucleosides

COMPOUND	Tetrahydrofuran	Ethyl Acetate	Ethanol
U	0.56	0.02	0.55
cU	0.05	0.02	0.55
aU	0.48	----	0.60
Tr-dU-Cl (LVI)	0.88	0.70	0.87
dU-Cl (LVII, from LVI)	0.69	0.22	0.79
dU-Cl (LVII, from cU)	0.71	0.21	0.80
cU (from LVII)	0.04	0.02	0.64
TrU (LVIII)	0.81	0.39	0.75
Tr-cU (LIII)	0.33	0.04	0.77
Tr-cU-OAc (LIV(a))	0.41	0.05	0.81
Tr-cU-OBz (LIV(b))	0.46	0.13	0.83
MMTr-cU (LXI)	0.26	0.05	0.64
MMTr-cU-OβB (LXII)	0.42	0.06	0.70
cU -OβB (LXIII)	0.16	0.00	0.60
U-OIP (LXV)	0.78	0.25	0.68
MMTr-cU-OAc (LXXV)	0.36	0.07	0.65
cU-OAc (LXXVI)	0.13	0.00	0.50

TABLE II Paper Chromatographic Properties of Nuclesoides

COMPOUND	Solvent A	Solvent B	Solvent C	Solvent D
U	0.50	0.70	0.31	0.16
cU	0.54	0.72	0.30	0.16
aU	0.54	0.73	0.41	0.25
dU-Cl (LVII, from LVI)	0.64	0.81	0.63	0.50
dU-Cl (from cU)	0.63	0.80	0.62	0.48
TrU (LVIII)	0.85	0.88	0.86	0.88
Tr-cU (LIII)	0.88	0.86	0.84	0.84
Tr-cU-OAc (LIV(a))	0.90	0.90	0.88	0.87
Tr-cU-OBz (LIV(b))	0.92	0.91	0.90	0.91
MMTr-cU (LXI)	0.88	0.88	0.85	0.86
MMTr-cU-OβB (LXII)	0.92	0.91	0.90	0.91
cU-OβB (LXIII)	0.80	0.85	0.60	0.44
U-OIP (LXV)	0.70	0.83	0.75	0.70
MMTr-cU-OAc (LXXV)	----	0.88	0.87	0.89
cU-OAc (LXXVI)	----	0.74	0.40	0.23

TABLE III Ultraviolet (U.V.) and Infrared (.I.R.) Spectral Properties of Nucleosides

	U.V. Spectra*		I.R. Spectra°
COMPOUND	λmax,nm( ) U.V. Spectra*	λmin,nm( )	μ
cU	248(7820),223(9730)	236.5(6690),210(6170)	6.10,8.00,8.10,12.00
cU (from LVII)			6.10,8.00,8.08,12.50
Tr-dU-Cl (LVI)	259(10,700),233(sh)	241(5980)	
Tr-cU (LIII)	249(6950)sh,221(20,900)sh		6.12,8.06,9.62,14.25
Tr-cU-OAc (LIV(a))	250(6560)sh,223(17,800)sh		5.70,6.10,8.23,14.25
Tr-cU-OBz (LIV(b))	255(7960)sh,228(30,000)	219,(27,000)	5.80,6.12,7.84,14.20
MMTr-cU (LXI)	229(16,800)	223(16,300)	6.06,6.13,8.05,14.20
MMTr-cU-OβB (LXII)	236(29,000)	221,(22,000)	5.71,5.93,6.06,8.67,14.20
cU-OβB (LXIII)	244(18,700)	215(8,000)	5.72,5.92,6.17,8.62,13.29
MMTr-cU-OAc (LXXV)	230(19,000)	223(18,300)	5.70,6.08,12.10,14.20
cU-OAc (LXXVI)	249(8220),224(9600)	237(7330),210(6480)	5.73,6.10,8.20,12.20

* 95% Ethanol as Solvent

° Principal Bands

3'-O-(β-Benzoylpropionyl)-O^2,2'-cyclouridine (cU-OβB, LXIII)

LXII (4.02g, 6.1 mmoles) was dissolved in 80% acetic acid and the solution was stirred for 4.5 hours. The solvent was removed at reduced pressure and the last trace of acetic acid was removed by evaporation of ethanol. The residue was dissolved in ethanol and applied to fifteen thick-layer plates. The plates were developed in ethyl acetate and in tetrahydrofuran. The nucleoside band at R_f 0.15 was eluted from the plates with tetrahydrofuran. Concentration of the solution followed by addition of hexane to precipitate the product yielded 2.0g (85%) of LXIII (mp 177.5 - 180°). Chromatographic data is listed in Tables I and II and spectral properties (u.v. and i.r.) are recorded in Table III.

Anal. Calcd. for $C_{19}H_{18}N_2O_7 \cdot 1/2H_2O$: C, 57.72; H, 4.84; N, 7.08

Found : C, 57.61; H, 4.70; N, 7.03

The β-Cyanoethyl Derivative of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate (MMTr-cUp(CE), LXIV)

Method A: 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (25mg, 0.05 mmole), β-cyanoethyl phosphate (0.1 mmole) and mesitylenesulfonyl chloride (44mg, 0.2 mmole) were dried by evaporation of pyridine (4x1ml). Pyridine (1ml) was added and the solution was concentrated to 0.2ml. After stirring for 6 hours, water (0.2ml) was added and the stirring was continued for 16 hours. A thin-layer chromatogram in tetrahydrofuran showed monomethoxytrityl containing compounds with R_f^{THF} 0.01 (MMTr-cUpCE) and 0.85 (MMTrOH). The detritylation was

extensive as evidenced by the large amount of monomethoxytritanol relative to the product.

Method B: 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (50mg, 0.1 mmole) and β-cyanoethyl phosphate (0.4 mmoles) were dried by evaporation of pyridine (3x1ml). Dicyclohexylcarbodiimide (330mg, 1.6 mmole) and pyridine (2ml) were added and the mixture was stirred in the dark for 3 days. After the addition of cold water (2ml) to the cooled solution, stirring was continued for 16 hours. The dicyclohexylurea was removed by filtration and the solvents were removed at reduced pressure. The residue was dissolved in chloroform-ethanol and the solution was chromatographed on Whatman paper in Solvent A. The product band at R_f 0.71 was eluted with water and lyophilized yielding MMTr-cUp(CE) (55.7mg, 88.5%) as the ammonium salt. Chromatographic and electrophoretic data are listed in Table IV and spectral properties (u.v. and i.r.) are reported in Table V.)

The β-Cyanoethyl Derivative of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridylyl-(3'→5')-2',3'-O-isopropylideneuridine (MMTr-cUp(CE)U-OIP, LXVI)

β-Cyanoethyl phosphate (8.0 mmoles) was dried by evaporation of pyridine (2x10ml). 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (1.0g, 2.0 mmoles), dicyclohexylcarbodiimide (6.6g, 32 mmoles) and pyridine (10ml) were added and the mixture was stirred for 3 days. After the addition of cold water (14ml), stirring was continued for 16 hours. The mixture was filtered to remove dicyclohexylurea and

the residue was washed with pyridine-water (1:1, 35ml). The combined filtrates were extracted with chloroform-ethanol (5:1, 400ml). After separation of the layers, the organic layer was concentrated to a volume of ∿75ml. The organic layer was extracted with saturated aqueous sodium chloride (10ml) and then concentrated at reduced pressure. Pyridine (10ml) was added to dissolve the MMTr-cUp(CE). The mixture was filtered to remove undissolved salts and dicyclohexylurea and the solution was concentrated to a gum at reduced pressure.

The gum was dried by evaporation of pyridine (2x10ml). 2',3'-O-Isopropylideneuridine (900mg, 3.16 mmoles) was added and the mixture was dried by evaporation of pyridine (2x10ml). Triisopropylbenzenesulfonyl chloride (1.2g, 4.0 mmoles) and pyridine (10ml) were added and the solution was concentrated to a volume of ∿5ml. The solution was stirred for 18 hours. Pyridine (7ml) was added to increase the volume of the solution. After the addition of cold water (8ml), stirring was continued for 1 hour. The solution was extracted with chloroform and the layers were separated. The organic layer was concentrated to a small volume and the solution was poured into ether with stirring. The residue was dissolved in tetrahydrofuran and applied to twenty thick-layer plates. The plates were developed in ethyl acetate and tetrahydrofuran. The nucleotide band with R_f 0.23 was eluted with tetrahydrofuran and ethanol followed by concentration and addition of hexane to yield 480mg (27%) of

MMTr-cUp(CE)-OIP (mp 140 - 150°, softening at 134°). Chromatographic and electrophoretic data is listed in Table IV and spectral properties (u.v. and i.r.) are recorded in Table V.

Anal. Calcd for $C_{44}H_{44}N_5O_{14}P$: C, 58.86; H, 4.94; N, 7.80

Found : C, 58.63; H, 5.03; N, 7.75

O^2,2'-Cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (cUpU-OIP, LXVII)

MMTr-cUp(CE)U-OIP (30mg) was dissolved in 80% acetic acid (0.5ml) and the solution was stirred for 2 hours. The solvent was removed at reduced pressure. The residue was dissolved in pyridine-ammonium hydroxide (5:2) and the solution was stirred for 30 minutes. The solution was applied to Whatman papers and developed in Solvent B. The product cUpU-OIP (R_f 0.69) was eluted with water and applied to Whatman electrophoretic paper. The product (R_m 0.33) was eluted with water and identified through its paper chromatographic and electrophoretic properties (Table IV) and its ultraviolet spectrum (Table V). Snake venom enzyme degraded the cUpU-OIP to cU(R_f^B 0.71, 2.80 $O.D._{250}$units) and pU-OIP (R_f^B 0.60, 3.65 $O.D._{262}$units) with pU-OIP/cU = 1.01. Spleen enzyme did not degrade cUpU-OIP.

Arabinouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (aUpU-OIP, LXVIII)

cUpU-OIP (undegraded by spleen enzyme, 4.80 $O.D._{255}$units) was dissolved in 1N sodium hydroxide and the solution was stirred for 1 hour. The solution was neutralized with Dowex 50W-X8 resin. The filtered solution was applied to Whatman paper and developed in

Solvent A. The product (R_f 0.28) was eluted with water and characterized by its paper chromatographic and electrophoretic properties (Table IV) and its hydrolysis by spleen phosphodiesterase. Spleen enzyme degraded the aUpU-OIP to aUp(R_f^A 0.03, 2.45 O.D.$_{263}$units) and U-OIP(R_f^A 0.72, 2.17 O.D.$_{262}$units) with aUp/U-OIP = 1.1.

Arabinouridylyl-(3'-5')-uridine (aUpU, LXIX)

Method A: MMTr-cUp(CE)U-OIP (16mg) was dissolved in 80% acetic acid (5ml) and the solution was refluxed for 2 hours. The solvent was removed at reduced pressure. The residue was dissolved in pyridine-ammonium hydroxide (5:2, 2ml), applied to Whatman paper, and developed in Solvent A. Due to the ammonia content in the solvent as well as in Solvent A, the cyanoethyl group was immediately hydrolyzed from aUp(CE)U. The product aUpU was eluted from the paper with water and characterized by its chromatographic and electrophoretic properties (Table IV) and by its ultraviolet spectrum (Table V). Snake venom degraded aUpU to aU(R_f^A 0.49, 5.24 O.D.$_{263}$units) and pU(R_f^A 0.04, 5.79 O.D.$_{262}$units) with pU/aU = 1.1.

Method B: cUpU-OIP (1mg) was dissolved in 80% acetic acid (0.5ml) and the solution was heated on a steam bath for 2.5 hours. The product aUpU was characterized by its chromatographic and electrophoretic properties (Table IV) against cUpU-OIP and aUpU (Method A).

Attempted Synthesis of the β-Cyanoethyl Derivative of 5'-O-monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-3'-O-(β-benzoylpropionyl)-O^2,2'-cyclouridine (MMTr-cUp(CE)cU-OβB, LXX)

Method A: β-Cyanoethyl phosphate (8.0 mmoles) was dried by evaporation of pyridine (2x10ml). MMTr-cU (1.0g, 2.0 mmoles), dicyclohexylcarbodiimide (6.6g, 32 mmoles) and pyridine (10ml) were added and the mixture was stirred for 3 days. After the addition of cold water (11ml), the mixture was stirred for 16 hours. The mixture was filtered and the residue was washed with pyridine-water (1:1, 35ml). The combined filtrates were extracted with chloroform-water and the layers were separated. The organic layer was concentrated to ∿75ml volume and extracted with saturated aqueous sodium chloride (10ml). The organic layer was concentrated at reduced pressure. Pyridine (10ml) was added to dissolve the product, MMTr-cUp(CE). The mixture was filtered and the solvent was removed at reduced pressure.

The residue was dried by evaporation of pyridine (2x10ml). 3'-O-(β-Benzoylpropionyl)-O^2,2'-cyclouridine (1.16g, 3.0 mmoles) was added and the mixture was dried by evaporation of pyridine (2x10ml). Triisopropylbenzenesulfonyl chloride (1.2g, 4.0 mmoles) and pyridine (10ml) were added and the solution was concentrated to a small volume of 5ml. The solution was stirred for 15 hours. Thin-layer chromatograms in tetrahydrofuran showed no condensation as the only trityl containing material present was MMTr-cUp(CE) (R_f^{THF} 0.03).

Method B: 3'-O-(β-Benzoylpropionyl)-O^2,2'-cyclouridine (7mg, .018 mmole), was dissolved in tetrahydrofuran, precipitated with hexane, filtered and dried in a drying pistol over P_2O_5. MMTr-cUp(CE) (6mg, 0.01 mmole), isolated from the small-scale phosphorylation of MMTr-cU with β-CEP using DCC, triisopropylbenzenesulfonyl chloride (6mg, 0.02 mmole) and pyridine (5 drops) were added. The solution was stirred for 48 hours. Thin-layer chromatograms in tetrahydrofuran and chloroform-ethanol (7:3) showed the only trityl product to be the starting material, MMTr-cUp(CE) (R_f^{THF} 0.00 and $R_f^{CHCl_3-EtOH}$ 0.00).

The β-Cyanoethyl Derivative of O^2,2'-Cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (cUp(CE)U-OIP, LXXI)

MMTr-cUp(CE)U-OIP (18mg) was dissolved in 80% acetic acid (3.5ml) and the solution was stirred for 2 hours. The solvent was removed at reduced pressure and the residue was treated with pentane to remove tritanol. The product, cUp(CE)U-OIP (12mg) was tested in the displacement reaction described below.

Stability of cUp(CE)U-OIP and cUpU-OIP to Sodium Benzoate-Benzoic Acid in Dimethylformamide

cUp(CE)U-OIP: cUp(CE)U-OIP (12mg, 0.02 mmoles), sodium benzoate (16mg, 0.11 mmole), and benzoic acid (5mg, 0.04 mmole), were dissolved in dimethylformamide (1ml) and the solution was refluxed for 3 hours. The cooled mixture was filtered, the residue washed with dimethylformamide and the solvent was removed at reduced pressure. The residue was dissolved in concentrated ammonium hydroxide-ethanol (4:1, 10ml) and the solution was stirred for 30 minutes. The solution was applied to

Whatman papers and the papers were developed in Solvent A. Two nucleoside bands (R_f^A 0.22 and 0.65) were eluted with water and lyophilized. The product with R_f^A 0.22 was found to be identical with uridine 2',3'-cyclic phosphate on electrophoresis (R_m 0.64, cf. aUpU-OIP, R_m 0.38). Both nucleotides on elution in Solvent A yielded a slower moving product (R_f^A 0.07), uridine 3'(2')-phosphate on basic hydrolysis of the cyclic phosphate. The product with R_f^A 0.65 was identical to U-OIP on electrophoresis (R_m -0.14) and on a paper chromatogram in Solvent A (R_f 0.67).

cUpU-OIP: cUpU-OIP (1mg), sodium benzoate (1.1mg) and benzoic acid (0.34mg) were dissolved in dimethylformamide and the solution was heated at 150° for 90 minutes. The product was degraded to uridine 2',3'-cyclic phosphate and 5'-O-benzoyl-2',3'-O-isopropylideneuridine.

Stability of O^2,2'-Cyclouridine to Various Displacement Conditions

The products of the following reactions were characterized by thin-layer and paper chromatography.

A. Sodium Benzoate-Benzoic Acid in DMF at 50°: cU (2mg), sodium benzoate (5.8mg) and benzoic acid (1.5mg) were dissolved in DMF (0.5ml) and the solution was heated at 50° for 3 hours. The starting material was unchanged.

B. Sodium Benzoate-Benzoic Acid in DMF at 100°: cU (2mg), sodium benzoate (5.8mg) and benzoic acid (1.5mg) were dissolved in DMF (0.5ml) and the solution was heated at 100° for 3 hours. The starting material was unchanged at the end of this time.

C. Sodium Benzoate-Benzoic Acid in Refluxing Ethanol: cU (10mg),

sodium benzoate (28mg) and benzoic acid (8mg) were dissolved in ethanol (5ml) and the solution was refluxed for 6 hours. The starting material was unchanged.

D. Sodium Benzoate-Benzoic Acid in DMF at Reflux: cU (5mg), sodium benzoate (15mg) and benzoic acid (4mg) were dissolved in dimethylformamide (3ml) and the solution was refluxed for 10 hours. cU was approximately 50% converted to 2'-O-benzoyluridine[28].

Attempted Synthesis of the β,β,β-Trichloroethyl Derivative of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (MMTr-cUp(TCE)U-OIP)

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (100mg, 0.2 mmole) in pyridine (1.2ml) was slowly dropped into a solution of β,β,β-trichloroethyl phosphorodichloridate (0.2 mmole) (synthesized according to the procedure of Eckstein[56]) in pyridine (0.8ml). The solution was stirred for 17 hours. 2',3'-O-Isopropylideneuridine (30mg, 0.1 mmole) was added and the solution was stirred for 24 hours. Ether was added to the solution to precipitate the products. The residue was dissolved in ethanol and applied to two thick-layer plates. The plates were developed in ether and n-butanol-acetone-water (4:2:1). The nucleotide band with R_f 0.42 was eluted with ethanol. The solvent was removed at reduced pressure. The residue was dissolved in 80% acetic acid, zinc dust was added and the mixture was stirred for 3 hours. After filtration, the solvent was removed at reduced pressure. The residue was dissolved in ethanol-water (1:1) and applied to Whatman paper. A preparative electrophoresis yielded a product with R_m 0.35. The product

was eluted with water and an ultraviolet spectrum gave an absorption maximum of 250nm, characteristic of an O^2,2'-cyclouridine derivative.

Identification of the New Product from the Attempted Synthesis of MMTr-cUp(TCE)U-OIP

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (50mg, 0.1 mmole) in pyridine (0.6ml) was slowly dropped into a solution of β,β,β-trichloroethyl phosphorodichloridate (0.1 mmole) in pyridine (0.4ml). After stirring for 12 hours, the solution was poured into ether with stirring. The residue was dissolved in ethanol and applied to a thick-layer plate. The plate was developed in ether and n-butanol-acetone-water (4:2:1). The nucleotide band with R_f 0.44 (cf. new product, R_f 0.42) was eluted with ethanol and the solvent was removed at reduced pressure. The residue was dissolved in 80% acetic acid and the solution was heated on a steam bath for 15 minutes. The solvent was removed at reduced pressure and the residue was dissolved in 2N sodium hydroxide. The solution was stirred for 4 minutes, neutralized with Dowex 50W-X8 resin and the solution was applied to Whatman paper. The paper was developed in Solvent A. The band with R_f 0.54 was eluted with water and lyophilized. Snake venom gave no degradation of the product (R_f^A 0.58, R_m 0.50, λmax 262nm). Spleen enzyme partially degraded the unknown to aUp (R_f^A 0.04, R_m 1.15, λmax 262nm). The product was assigned as aUp(TCE) and the new product from the attempted synthesis of MMTr-cUp(TCE)U-OIP was the 3'-3' pyrophosphate, MMTr-cUp(TCE)-(3'-3')-p(TCE)MMTr-cU.

5'-O-Monomethoxytrityl-3'-O-acetyl-O^2,2'-cyclouridine (MMTr-cU-OAc, LXXV)

MMTr-cU (2.5g, 5.0 mmoles) and acetic anhydride (0.8ml, 8.5 mmoles) were dissolved in pyridine (15ml) and the solution was stirred for 18 hours. The solution was poured into ice-water (500ml) with stirring. The precipitated product was collected by filtration and dissolved in chloroform. The solution was dried over anhydrous sodium sulfate. Concentration of the solution to a small volume followed by the addition of hexane yielded 2.57g (95%) of LXXV (mp 100 - 107°). Chromatographic data is listed in Tables I and II and spectral properties (u.v. and i.r.) are recorded in Table III.

Anal. Calcd. for $C_{31}H_{28}N_2O_7$: C, 68.88; H, 5.22; N, 5.18

Found : C, 68.78; H, 5.14: N, 5.08

3'-O-Acetyl-O^2,2'-cyclouridine (cU-OAc, LXXVI)

MMTr-cU-OAc (21.7g, 40 mmoles) was dissolved in 80% acetic acid and the solution was stirred for 4 hours. The solvent was removed at reduced pressure and the last trace of acetic acid was removed by evaporation of ethanol. The residue was dissolved in ethanol-chloroform and the solution was poured into ether. The precipitate was filtered to yield 8.35g (83%) of LXXVI (mp 204 - 209). Chromatographic data is listed in Tables I and II and spectral properties (u.v. and i.r.) are recorded in Table III.

Anal. Calcd. for $C_{11}H_{12}N_2O_6$: C, 49.25; H, 4.51; N, 10.44

Found : C, 49.10; H, 4.50; N, 10.26

Thymidylyl-(3'-5')-3'-O-acetyl-O^2,2'-cyclouridine (TpcU-OAc, LXXVIII)

5'-O-Monomethoxytritylthymidine (80mg, 0.15 mmole) and β-cyanoethyl phosphate (0.30 mmole) were dried by evaporation of pyridine (4x2ml). Mesitylenesulfonyl chloride (130mg, 0.6 mmole) and pyridine (0.6ml) were added and the solution was stirred for 8 hours. After the addition of cold water (0.6ml), the solution was stirred for 16 hours. The solvents were removed at reduced pressure and the residue was dissolved in chloroform (4.0ml). After extraction with water (2x0.7ml), the chloroform was removed at reduced pressure. The residue was dissolved in 7M ammonium hydroxide and the solution was heated at 70° for 1.5 hours. The solution was applied to Whatman papers and developed in Solvent A. The band with R_f 0.41 was eluted with water and lyophilized to yield 5'-O-monomethoxtrityl-thymidine 3'-phosphate (MMTr-Tp, R_f^{THF} 0.00, R_m 0.63).

3'-O-Acetyl-O^2,2'-cyclouridine (20mg, 0.075 mmole) was added to the MMTr-Tp and the compounds were dried by evaporation of pyridine (4x2ml). Mesitylenesulfonyl chloride (65mg, 0.30 mmole) and pyridine (0.7ml) were added and the solution was stirred for 6.5 hours. After the addition of cold water (0.7ml), stirring was continued for 16 hours. The solvents were removed at reduced pressure. The residue was dissolved in chloroform (4ml) and extracted with water (3x1ml). The solvents were removed at reduced pressure and the residue was dissolved in 80% acetic acid (2ml). The solution was stirred for 2.5 hours and the solvent was removed at reduced pressure. The residue was dissolved in ethanol-water (1:1, 2ml) and the solution was

chromatographed on Whatman paper in Solvent B. The nucleotide band with R_f 0.52 was eluted with water, applied to Whatman electrophoresis paper, and run under preparative electrophoresis. Product bands appeared at R_m 1.00 (Tp) and R_m 0.30 (TpcU-OAc). The TpcU-OAc band was eluted with water, diluted to 10ml and used as a stock solution. The yield (13%) based on the starting amount of cU-OAc was determined spectroscopically. The spectral properties (u.v.) are recorded in Table V.

Thymidylyl-(3'-5')-0^2,2'-cyclouridine (TpcU, LXXIX)

Four milliliters of the stock solution were evaporated at reduced pressure. The residue was dissolved in 15% ammonium hydroxide-ethanol and the solution was stirred for 90 minutes. The solution was chromatographed on Whatman paper in Solvent B. The nucleotide band with R_f 0.55 was eluted with water to yield TpcU(LXXIX). The spectral properties (recorded in Table V) are identical with those of TpcU-OAc. Incubation with spleen enzyme for 5 hours degraded the TpcU to Tp (R_f^B 0.58, R_m 1.00, 7.75 O.D.$_{267}$units) and cU (R_f^B 0.69, R_m -0.13, 6.28 O.D.$_{250}$units) with Tp/cU = 1.01. Incubation with spleen enzyme for 24 hours degraded the TpcU to Tp (R_f^B 0.57, 3.3 O.D.$_{267}$units), cU (R_f^B 0.69, 5.5 O.D.$_{250}$units), and T (R_f^B 0.75, 4.1 O.D.$_{267}$units) with (Tp+T)/cU = 1.09. Incubation with snake venom enzyme for 7 hours partially hydrolysed the TpcU to TpaU and yielded TpcU (R_f^B 0.61, 8.2 O.D.$_{260}$units, 93%), T (R_f^B 0.75, 0.3 O.D.$_{267}$units) and paU(R_f^B 0.44, 0.4 O.D.$_{263}$units) with paU/T = 1.23. Incubation with snake venom enzyme for 24 hours partially hydrolysed the TpcU to TpaU and yielded

TpcU (R_f^B 0.61, 4.84 O.D.$_{260}$units, 65%), T (R_f^B 0.77, 1.96 O.D.$_{267}$units) and paU (R_f^B 0.39, 1.83 O.D.$_{263}$ units) with paU/T = 0.86.

Thymidylyl-(3'-5')-arabinouridine (TpaU, LXXX)

Three milliliters of the stock solution were evaporated at reduced pressure. The residue was dissolved in 80% acetic acid and the solution was heated on a steam bath for 2 hours. The solvent was removed at reduced pressure and the residue was dissolved in 15% ammonium hydroxide-ethanol. The solution was stirred for 90 minutes and chromatographed on Whatman paper in Solvent A. The nucleotide band with R_f 0.14 was eluted with water to yield TpaU. The spectral properties are recorded in Table V. Spleen enzyme degraded the TpaU to Tp (R_f^A 0.05, 4.87 O.D.$_{267}$units) and aU (R_f^A 0.52, 4.80 O.D.$_{263}$units) with Tp/aU = 1.11. Snake venom enzyme in 7 hours degraded the TpaU to paU (R_f^A 0.02, 1.2 O.D.$_{263}$units), T (R_f^A 0.63, 1.23 O.D.$_{267}$units) and TpaU (R_f^A 0.11, 4.77 O.D.$_{264}$units, 66%) with paU/T = 0.99. Spleen enzyme degraded the TpaU (undegraded by snake venom) to yield Tp (R_f^A 0.07, 1.15 O.D.$_{267}$units) and aU(R_f^A 0.55, 1.22 O.D.$_{263}$units) with Tp/aU = 1.02. Snake venom enzyme in 24 hours degraded the TpaU to paU (R_f^A 0.04, 4.05 O.D.$_{263}$units), T (R_f^A 0.66, O.D. 4.15 O.D.$_{267}$units) and TpaU (R_f^A 0.16, 3.0 O.D.$_{264}$units, 28%) with paU/T = 0.90.

O^2,2'-Cyclouridylyl-O^2,2'-cyclouridine (3'-5' and 3'-3' Isomers) (LXXXII)

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (250mg, 0.5 mmole) in pyridine (9.0ml) was slowly dropped into a solution of methyl phosphorodichloridate (0.5 mmole) in pyridine (6.0ml). After stirring for 5 hours, 3'-O-acetyl-O^2,2'-cyclouridine (67mg, 0.25 mmole) in pyridine (1.5ml) was added. The solution was stirred for 16 hours. Cold water (6ml) was added and the solution was stirred for 20 hours. The solution was concentrated and applied to three thick-layer plates. The plates were developed in ether and tetrahydrofuran and the nucleotide band with R_f 0.15 was eluted with ethanol. The solvent was removed at reduced pressure, dissolved in tetrahydrofuran, and precipitated with hexane to yield 91mg of highly impure product. The precipitate was dissolved in 80% acetic acid and the solution was stirred for 3 hours. The solvent was removed at reduced pressure, the last trace of acetic acid being removed by evaporation of ethanol. The residue was dissolved in 15% ammonium hydroxide-ethanol and the solution was stirred for 1.5 hours. The solution was applied to Whatman papers and developed in Solvent C for 40 hours. The band with R_f 0.08 was eluted with water, diluted to 10ml and used as a stock solution. The yield (7.6%) based on the starting amount of cU-OAc was determined spectroscopically. The product was characterized by absorption maxima of 250nm and 223nm and by electrophoretic mobility (R_m 0.28[Tp 1.00, TpT 0.28]). Snake venom and spleen enzyme did not degrade the "cUpcU".

Arabinouridylylarabinouridine (3'-5' and 3'-3' isomers) (LXXXIII)

Two milliliters of stock solution were evaporated at reduced pressure. The residue was dissolved in 2N sodium hydroxide and the solution was stirred for 30 minutes. After neutralization with Dowex 50W-X8 resin, the solution was chromatographed on Whatman papers in Solvent A. The band with R_f 0.25 was eluted to yield "aUpaU" (λmax 262nm and λmin 231nm, 45 O.D.$_{263}$units). The solution was divided into four roughly equal parts and the solvent lyophilized. Incubation with spleen enzyme for 5 hours gave aUp (R_f^A 0.10, 2.07 O.D.$_{263}$units), aU (R_f^A 0.57, 2.04 O.D.$_{263}$units) and "aUpaU" (R_f^A 0.22, 7.99 O.D.$_{263}$ units, 66%) with aUp/aU = 1.02. Incubation with spleen enzyme for 24 hours gave aUp (R_f^A 0.09, 2.08 O.D.$_{263}$units), aU (R_f^A 0.53, 2.18 O.D.$_{263}$units), and "aUpaU" (R_f^A 0.20, 7.53 O.D.$_{263}$units, 64%) with aUp/aU = 0.95. Incubation of the "aUpaU" (undegraded by spleen enzyme) with spleen enzyme gave no degradation. Snake venom enzyme in 7 hours gave paU (R_f^A 0.07, 1.25 O.D.$_{263}$units), aU (R_f^A 0.49, 1.43 O.D.$_{263}$units) and "aUpaU" (R_f^A 0.22, 8.46 O.D.$_{263}$units, 76%) with paU/aU = 0.88. Snake venom enzyme in 24 hours gave paU (R_f^A 0.06, 1.47 O.D.$_{263}$units), aU (R_f^A 0.50, 1.73 O.D.$_{263}$units) and "aUpaU" (R_f^A 0.21, 5.83 O.D.$_{263}$units, 65%) with paU/aU = 0.85.

Arabinouridylyl-(3'-5')-arabinouridine and Arabinouridylyl-(3'-3')-arabinouridine

One milliliter of the stock solution was evaporated at reduced pressure. The residue was dissolved in 2N sodium hydroxide and the solution was stirred for 12 minutes. Following neutralization

with Dowex 50W-X8 resin, the solution was chromatographed on Whatman papers in Solvent C for 2 days. The nucleotide bands with R_f 0.14 and 0.18 were eluted with water. Both compounds gave ultraviolet spectra with λmax 262nm and λmin 233nm.

Incubation of the slower moving product (R_f 0.14) with spleen enzyme gave complete degradation to aUp (R_f^A 0.07, 3.98 O.D.$_{263}$units) and aU (R_f^A 0.55, 3.61 O.D.$_{263}$units) with aUp/aU = 1.1. Incubation of R_f^C 0.14 with snake venom enzyme gave paU (R_f^A 0.05, 2.22 O.D.$_{263}$units), aU (R_f^A 0.48, 2.50 O.D.$_{263}$units) and aUpaU (R_f^A 0.14, 4.16 O.D.$_{263}$units, 47%) with paU/aU = 0.89. The slow-moving product (R_f^C 0.14) was arabinouridylyl-(3'-5')-arabinouridine.

The faster moving product (R_f^C 0.18) was not degraded either by snake venom enzyme or by spleen enzyme.

Study of the Phosphorylation of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine with Methyl Phosphorodichloridate

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (25mg, 0.05 mmole) in pyridine (0.9ml) was slowly dropped into a solution of methyl phosphorodichloridate (0.05 mmole) in pyridine (0.6ml). The solution was stirred for 4.5 hours. After the addition of cold water (1ml), the solution was stirred for 16 hours. The solvents were removed at reduced pressure and the residue was dissolved in 80% acetic acid (1ml). The solvent was removed at reduced pressure. The residue was dissolved in 2N sodium hydroxide and the solution was stirred for 10 minutes. The solution was chromatographed on Whatman papers in Solvent A. The

bands with R_f 0.07 and 0.14 were eluted with water; R_f 0.07 being aUp (R_m 1.00, λmax 263nm) and R_f 0.14 being aUp(3'-3')aU (R_m 0.34, λmax 262nm).

Attempted Synthesis of the β,β,β-Trichloroethyl Ester of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylidene-uridine (MMTr-cUp(TCE)U-OIP, LXXXVI)

Method A: 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (50mg, 0.1 mmole) in pyridine (1.8ml) was slowly dropped over a period of 6 hours into a solution of β,β,β-trichloroethyl phosphorodichloridate (0.1 mmole) in pyridine (1.2ml). After stirring for 20 hours, the volume was reduced and the solution was applied to Whatman papers. The papers were developed in Solvent C and the nucleotide band with R_f 0.48 was eluted and lyophilized to yield the trichloroethyl ester of 5'-O-monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate.

The product and 2',3'-O-isopropylideneuridine (16mg, 0.07 mmole) were dried by evaporation of pyridine (3x1ml). Triisopropylbenzenesulfonyl chloride (30mg, 0.1 mmole) and pyridine (0.5ml) were added and the solution was stirred for 24 hours. No condensation occurred as the only products from paper chromatography of the solution were the starting compounds.

Method B: MMTr-cU (100mg, 0.2 mmole) was phosphorylated as described above with $TCEPCl_2$ (0.2 mmole). After stirring for 16 hours, triisopropylbenzenesulfonyl chloride (60mg, 0.2 mmole) was added and stirring was continued for 2 hours. 2',3'-O-Isopropylideneuridine

(33mg, 0.12 mmole) was added and the solution was stirred for 21 hours. The solvents were removed at reduced pressure. The residue was dissolved in zinc and 80% acetic acid and the mixture was stirred for 4 hours. The mixture was filtered and the solvent was removed at reduced pressure. The residue was dissolved in ethanol-water and chromatographed on Whatman papers in Solvent C. The nucleotide bands were eluted with water. All the products were uridine-like in nature (ultraviolet absorption maximum 260-261nm) indicating hydrolysis of the anhydro linkage.

Method C: The phosphorylation and condensation reaction was repeated as in Method B. After stirring for 22 hours, the solution was poured into ether with stirring. The residue was dissolved in zinc and 80% acetic acid and the mixture was stirred for 4 hours. After filtration, water was added and the solution was lyophilized to remove the solvents. The residue was dissolved in ethanol-water and chromatographed on Whatman papers in Solvent C. As above, the products, on elution with water, all had uridine-like ultraviolet spectra (λmax 260nm) indicating hydrolysis of the anhydro linkage.

Method D: The phosphorylation of MMTr-cU (100mg, 0.2 mmole) was repeated as described in Method A. 2',3'-O-Isopropylideneuridine (33mg, 0.12 mmole) was added and the solution was stirred for 23 hours. The volume was reduced to 1ml and the solution was poured into ether (250ml) with stirring. The precipitated gum was washed with ether (2x25ml). The gum was dissolved in chloroform-ethanol (3:1)

and made up to a volume of 10ml. A one milliliter aliquot was chromatographed on Whatman papers in Solvent C. The products, eluted with water, all had uridine-like ultraviolet spectra (λmax 259-263nm) except cUp(TCE) (R_m 0.44, λmax 250nm).

Stability of O^2,2'-Cyclouridine to Zinc and 80% Acetic acid

O^2,2'-Cyclouridine (5mg) was dissolved in 80% acetic acid. Zinc was added and the mixture was stirred for 4 hours. A paper chromatogram in Solvent C showed no conversion to arabinouridine.

Phosphorylation of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine with β,β,β-Trichloroethyl Phosphorodichloridate

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (100mg, 0.2 mmole) was phosphorylated with β,β,β-trichloroethyl phosphorodichloridate (0.2 mmole) in the usual manner. After stirring for 16 hours, the solution was concentrated to half volume and poured into ether with stirring. The reside was dissolved in ethanol and diluted to a volume of 10ml. A one milliliter aliquot was applied to Whatman papers and developed in Solvent C. The nucleotide band with R_f 0.49 was eluted with water to yield MMTr-cUp(TCE) (R_m 0.24, λmax 230nm and 255(sh)nm).

Attempted Synthesis of MMTr-cUp(TCE)U-OIP(LXXXVI) from MMTr-cUp(CE)U-OIP (LXVI)

Method A: MMTr-cUp(CE)U-OIP (40mg, 0.045mmole) was dissolved in pyridine-ammonium hydroxide (2:5, 3ml) and the solution was stirred for 30 minutes. The solution was applied to Whatman papers and developed in Solvent C. The nucleotide band with R_f 0.32 was eluted with water and lyophilized to yield MMTr-cUpU-OIP (R_m 0.30, λmax(sh)251nm and (sh)230nm).

MMTr-cUpU-OIP was dried by evaporation of pyridine (2x1ml). Triisopropylbenzenesulfonyl chloride (27mg, 0.09 mmole) and pyridine (1ml) were added and the volume was reduced to ∿0.5ml. β,β,β-Trichloroethanol (0.09 mmole) was added and the solution was stirred for 12 hours. After the addition of cold water (1ml), the solution was stirred for 5 hours. The solvents were removed at reduced pressure. The residue was dissolved in ethanol-water and applied to Whatman papers. The papers were developed in Solvent C. Two nucleotide bands, R_f 0.10 and 0.34, were observed and eluted with water. The products were cUpU-OIP (R_f^C 0.10, R_m 0.40, λmax 256nm) and MMTr-cUpU-OIP (R_f^C 0.34, R_m 0.26, λmax(sh)250nm).

Method B: MMTr-cUp(CE)U-OIP (20mg, 0.02 mmole) was treated with pyridine-ammonium hydroxide and the work-up followed that described in Method A. MMTr-cUpU-OIP was reacted with triisopropylbenzenesulfonyl chloride (14mg, 0.045 mmole) and trichlorethanol (0.045 mmole) as described above. After stirring for 8.5 hours,

additional triisopropylbenzenesulfonyl chloride (5mg) was added and the solution was stirred for 20 hours. Work-up as in Method A yielded cUpU-OIP and MMTr-cUpU-OIP only.

The β,β,β-Trichloroethyl Ester of O^2,2'-Cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (cUp(TCE)U-OIP, LXXXVIII)

The dicyclohexylammonium salt of β,β,β-trichloroethyl phosphate (42mg, 0.10 mmole) was dissolved in pyridine (3ml) and converted to the pyridinium salt by evaporation of pyridine (4x2.5ml) at reduced pressure. Triisopropylbenzenesulfonyl chloride (64mg, 0.2 mmole) and pyridine (2.5ml) were added and the solution was stirred for 30 minutes. 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (25mg, 0.05 mmole) was added and the solution was stirred for 40 hours. After the addition of cold water (0.5ml), the solution was stirred for 1 hour and then poured into ice-water (5ml). The solution was extracted with methylene chloride (4x2.5ml) and the organic extracts were washed with water (4x2.5ml). The solvents were removed from the organic layer by evaporation at reduced pressure and the residual gum was dried by evaporation of pyridine (4x2ml).

Triisopropylbenzenesulfonyl chloride (32mg, 0.10 mmole) and pyridine (3ml) were added and the solution was stirred for 30 minutes. 2',3'-O-Isopropylideneuridine (28mg, 0.10 mmole) was added and the solution was stirred for 65 hours. After the addition of cold water (0.5ml), the solution was stirred for 30 minutes and then poured into ice-water (2.5ml). The solution was extracted with methylene chloride

(4x2.5ml) and the organic extracts washed with water (4x2.5ml). The solvents were removed from the organic layer by evaporation at reduced pressure and the residue was dissolved in methylene chloride. The solution was applied to a thick-layer plate and developed in ether. The nucleotide band with R_f 0.68 was eluted with ethanol and the solvent was removed at reduced pressure. The residue was dissolved in 80% acetic acid and the solution was stirred for 4 hours. The solution was chromatographed on Whatman papers (developed in Solvent C) to yield 4.6mg (13%) of cUp(TCE)U-OIP (R_f^C 0.62, R_f^{THF} 0.19). The spectral properties (u.v.) are recorded in Table V.

O^2,2'-Cyclouridylyl-(3'-5')-2',3'-O-Isopropylideneuridine

cUp(TCE)U-OIP (4.6mg) was dissolved in 80% acetic acid (0.5ml). Zinc (5mg) was added and the mixture was stirred for one hour. After filtration, the solvent was evaporated at reduced pressure. The residue was dissolved in ethanol-water and applied to Whatman electrophoresis paper. The nucleotide band with R_m 0.28 was eluted with water to yield cUpU-OIP (R_m 0.41, λmax 255nm). Half the solution was taken and lyophilized to obtain product for enzyme studies. Spleen enzyme did not degrade the cUpU-OIP. Snake venom enzyme degraded the cUpU-OIP to yield cU(R_f^B 0.75, 3.5 O.D.$_{250}$units) and pU-OIP (R_f^B 0.54, 4.5 O.D.$_{262}$units) with pU-OIP/cU = 0.99.

Arabinouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (aUpU-OIP, LXVII)

The aqueous solution of cUpU-OIP was let stand for 7 days and chromatographed on Whatman paper in Solvent C. The nucleotide band with R_f^B 0.15 was eluted with water to yield aUpU-OIP (λmax 262nm). Spleen enzyme degraded the aUpU-OIP to yield aUp (R_f^B 0.59, 4.5 $O.D._{262}$ units) and U-OIP (R_f^B 0.87, 4.5 $O.D._{262}$units) with aUp/U-OIP = 0.96. Snake venom enzyme degraded the aUpU-OIP to yield aU (R_f^B 0.73, 5.75 $O.D._{263}$ units) and pU-OIP (R_f^B 0.52, 5.55 $O.D._{262}$ units) with pU-OIP/aU = 1.00.

The β,β,β-Trichloroethyl Ester of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (MMTr-cUp(TCE)U-OIP, LXXXVI)

β,β,β-Trichloroethyl phosphate (420mg, 1.0 mmole) was dissolved in dry pyridine (25ml) and converted to the pyridinium salt by evaporation of pyridine (2 x 25ml). Triisopropylbenzenesulfonyl chloride (450mg, 1.5 mmole) and pyridine (25ml) were added and the solution was stirred for 30 minutes. 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (250mg, 0.5 mmole) was added and the solution was stirred for 4 hours. After the addition of more TPS (225mg, 0.75 mmole), the solution was stirred for 17 hours. To the cooled solution, cold water (5ml) was added and the solution was stirred for 30 minutes and then poured into ice-water (50ml). The solution was extracted with methylene chloride (4 x 25ml) and the combined methylene chloride extracts were washed with water (4 x 25ml) and concentrated to a gum at reduced pressure.

The gum was dried by evaporation of pyridine at reduced pressure (2x25ml). Triisopropylbenzenesulfonyl chloride (225mg, 0.75 mmole) and pyridine (25ml) were added and the solution was stirred for 30 minutes. 2',3'-O-Isopropylideneuridine (280mg, 1.0 mmole) was added and the solution was stirred for 6 hours. After the addition of more TPS (100mg, 0.30 mmole), stirring was continued for 19 hours. Cold water (5ml) was added and after stirring for 30 minutes the solution was poured into ice-water (50ml). After extraction with methylene chloride (4x25ml), the combined methylene chloride extracts were washed with water (4x25ml). The solvents were evaporated at reduced pressure and the residue was dissolved in methylene chloride. The solution was applied to ten thick-layer plates and developed in ethyl acetate and tetrahydrofuran. The band with R_f 0.43 was eluted with tetrahydrofuran. The solution was concentrated and upon the addition of hexane, precipitation occurred to yield 111mg (23%) of MMTr-cUp(TCE)U-OIP (mp 140--150°). The chromatographic and electrophoretic properties are listed in Table IV and the spectral properties (u.v.) are recorded in Table V.

Anal. Calcd for $C_{43}H_{42}Cl_3N_4O_{14}P$: C, 52.91; H, 4.34; N, 5.74

Found : C, 53.06; H, 4.46; N, 5.61

The β,β,β-Trichloroethyl ester of O^2,2'-Cyclouridylyl-(3'-5')-2',3'-O-isopropylideneuridine (cUp(TCE)U-OIP, LXXXVIII)

MMTr-cUp(TCE)U-OIP (48mg) was dissolved in 80% acetic acid and the solution was stirred for 4.5 hours. The solvent was evaporated

at reduced pressure and the residue was dissolved in ethanol. The solution was applied to Whatman papers and developed in Solvent C. The nucleotide band with R_f 0.62 was eluted with water and lyophilized to yield 30mg (86%) of cUp(TCE)U-OIP (R_f^C 0.62, R_f^{THF} 0.20). The spectral properties (u.v.) are recorded in Table V.

Stability of cUp(TCE)U-OIP to Displacement Conditions

The products of the following reactions were characterized by thin-layer and paper chromatography.

A. Sodium Benzoate-Benzoic Acid in DMF: cUp(TCE)U-OIP (5 mg), sodium benzoate (5 mg), and benzoic acid (1.3 mg) were dissolved in dimethylformamide (0.5 ml) and the solution was refluxed for 90 minutes. The product was degraded to uridine 3'-phosphate, uridine 2',3'-cyclic phosphate and 5'-O-benzoyl-2',3'-O-isopropylideneuridine.

B. Potassium Fluoride-Acetic Acid in Acetonylacetone: cUp(TCE)U-OIP (5 mg), dry potassium fluoride (5 mg), and glacial acetic acid (7 µl) were dissolved in acetonylacetone (0.35 ml) and the solution was heated at 190-194° for 90 minutes. The solvents were removed at reduced pressure and the residue was dissolved in concentrated ammonium hydroxide-ethanol (1:3). The solution was stirred for 90 minutes, the solvents were removed at reduced pressure, and the residue was treated with Cu/Zn in dimethylformamide at 50° for 45 minutes. An electrophoresis showed no charged products. The products isolated by paper chromatography were shown by mass spectroscopy not to be nucleosidic in character.

Attempted Synthesis of the β,β,β-Trichloroethyl Ester of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-3'-O-acetyl-O^2,2'-cyclouridine

Method A: The dicyclohexylammonium salt of β,β,β-trichloroethyl phosphate (0.10 mmole) was converted into the pyridinium salt by evaporation of pyridine (3x2ml). Triisopropylbenzenesulfonyl chloride (60mg, 0.20 mmole) and pyridine (2ml) were added and the solution was stirred for 30 minutes. 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (25mg, 0.05 mmole) was added and the solution was stirred for 17 hours. After the addition of cold water (0.5ml), the solution was stirred for 30 minutes and then poured into ice-water (5ml). The solution was extracted with methylene chloride (4x2.5ml) and the combined organic extracts were washed with water (4x2.5ml). The solvents were removed by evaporation at reduced pressure to yield a gum.

The gum was dried by evaporation of pyridine (2x2ml). 3'-O-Acetyl-O^2,2'-cyclouridine (13.5mg, 0.05 mmole) was added and the compounds were dried by evaporation of pyridine (3x2ml). Triisopropylbenzenesulfonyl chloride (60mg, 0.20 mmole) and pyridine (2ml) were added and the solution was stirred for 3 days.

The solvents were removed by evaporation at reduced pressure and the residue was dissolved in tetrahydrofuran and diluted to a volume of 5ml. An aliquot (1.5ml) was evaporated at reduced pressure and the residue was dissolved in 80% acetic acid (2ml). The solution was heated on a steam bath for 2 hours. Zinc dust was added and the

mixture was stirred for 1 hour. After filtration, the solvent was removed by evaporation at reduced pressure and the residue was dissolved in ethanol water. An electrophoresis showed aUp (R_m 0.99) as the only anionic product present.

Method B: 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (250mg, 0.5 mmole) was phosphorylated by β,β,β-trichloroethyl phosphate (1.0 mmole) and triisopropylbenzenesulfonyl chloride (2.0 mmoles) in pyridine (25ml) by the method described above. After the addition of water (5ml), the solution was stirred for 30 minutes followed by pouring into ice-water (50ml). The product was extracted with methylene chloride (4x25ml) and the combined organic extracts were washed with water (4x25ml). The solvents were removed by evaporation at reduced pressure.

The gum was dried by evaporation of pyridine (2x10ml). Triisopropylbenzenesulfonyl chloride (225mg, 0.75 mmole) and pyridine (25ml) were added and the solution was stirred for 30 minutes. 3'-O-Acetyl-O^2,2'-cyclouridine (268mg, 1 mmole) was added and the solution was stirred for 6 hours. After the addition of further triisopropylbenzenesulfonyl chloride (0.75 mmole), stirring was continued for 36 hours. Cold water (5ml) was added the reaction was worked-up in the same manner as for the phosphorylation reaction. The solvents were removed by evaporation at reduced pressure and the gum was dissolved in ethanol-pyridine (10:1). The solution was applied to five thick-layer plates and developed in ethyl acetate and tetrahydrofuran. The nucleotide bands with R_f 0.10 and 0.48 were eluted

with ethanol. The solvents from both elutions were removed by evaporation at reduced pressure and the residue was dissolved in 80% acetic acid. Zinc was added and the mixtures were stirred for four hours. An electrophoresis showed the product with R_f 0.10 to yield cUp (R_m 1.00) on treatment with zinc and acetic acid, while the product with R_f 0.48 yielded cU (R_m -0.02).

Attempted Synthesis of the β-Cyanoethyl Ester of 5'-O-Monomethoxytrityl-O^2,2'-cyclouridylyl-(3'-5')-O^2,2'-cyclouridine

Method A: β-Cyanoethyl phosphate (0.2 mmole) was dried by evaporation of pyridine (3x2ml). 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (50mg, 0.1 mmole), triisopropylbenzenesulfonyl chloride (0.4 mmole) and pyridine (1ml) were added. The volume was concentrated to 0.5ml and the solution was stirred for 8 hours. After the addition of cold water (0.5ml), stirring was continued for 12 hours. The product was extracted into methylene chloride (4x2.5ml) and the combined organic extracts were washed with water (4x2.5ml). The solvents were removed by evaporation at reduced pressure.

3'-O-Acetyl-O^2,2'-cyclouridine (40mg, 0.15 mmole) was added and the compounds were dried by evaporation of pyridine (2x2ml). Triisopropylbenzenesulfonyl chloride (0.2 mmole) and pyridine (2ml) were added. After concentration of the volume to 0.75ml, the solution was stirred for 12 hours. Cold water (1ml) was added and the solution was stirred for 12 hours. The products were extracted into methylene chloride (4x2.5ml) and washed with water (4x2.5ml). The solvents were removed by evaporation at reduced

pressure and the residue was dissolved in ethanol. The solution was applied to a thick-layer plate and developed in ethyl acetate and ethanol. Spraying an edge of the plate with 10% perchloric acid and drying in a stream of warm air revealed the presence of MMTr-cUp(CE) and monomethoxytritanol as the only monomethoxytrityl containing compounds.

Method B: 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (500mg, 1.0 mmole) was phosphorylated using β-cyanoethyl phosphate (2.0 mmoles) and triisopropylbenzenesulfonyl chloride (4 mmoles) in pyridine (20ml) by the method described in Method A. The product was extracted into methylene chloride (4x20ml) and the combined extracts were washed with water (4x20ml). The solvents were removed by evaporation at reduced pressure.

O^2,2'-Cyclouridine (450mg, 2.0 mmoles) was added and the compounds were dried by evaporation of pyridine (3x5ml). Triisopropyl-benzenesulfonyl chloride (2.0 mmoles) and pyridine (25ml) were added and the solution was stirred for 24 hours. Additional TPS (0.5 mmole) was added and stirring was continued for 2.5 days. Thin-layer chromatograms in chloroform-methanol (7:3) showed the presence of only MMTr-cUp(CE) (R_f 0.06), cU (R_f 0.23) and monomethoxytritanol (R_f 0.91).

Stability of MMTr-cUp(CE) to Refluxing Methanol-Pyrrolidine (9:1)

β-Cyanoethyl phosphate (0.2 mmole) was dried by evaporation of pyridine (3x2ml). 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (25mn, 0.05 mmole), dicyclohexylcarbodiimide (0.8 mmole) and pyridine (1ml) were added and the mixture was stirred for 3 days. After the addition

of cold water (1ml), stirring was continued for 16 hours. The mixture was filtered and the solution was extracted with methylene chloride (4x2ml). The combined organic extracts were washed with water (4x1ml) and the solvents were removed by evaporation at reduced pressure.

The residue, MMTr-cUp(CE) was dissolved in methanol-pyrrolidine (9:1, 1.5ml) and the solution was gently refluxed for 4 hours. The solution was applied to Whatman paper and developed in Solvent C. The products with R_f 0.03 and 0.36 were eluted with water to yield arabinouridine 3'-phosphate (R_f^C 0.03, R_m 1.00, max 262nm) and 5'-O-monomethoxytritylarabinouridine 3'-phosphate (R_f^C 0.36, R_m 0.51, λmax 263nm).

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate (MMTr-cUp, LXXXIX)

5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (100mg, 0.2 mmole) was phosphorylated using β,β,β-trichloroethyl phosphate (0.4 mmole) and triisopropylbenzenesulfonyl chloride (0.8 mmole) in pyridine (10ml) as described previously. After the addition of cold water (2ml), the solution was stirred for 30 minutes and then poured into ice-water (20ml). The product was extracted into methylene chloride (4x10ml), washed with water (4x10ml), and the solvents were removed by evaporation at reduced pressure. The residue was dissolved in dimethylformamide and Cu/Zn was added. The mixture was heated for 30 minutes at 50°, cooled, filtered and spotted on electrophoresis paper. The electrophoresis paper showed complete conversion of MMTr-cUp(TCE) (R_m 0.13) to MMTr-cUp (R_m 0.55).

The solvent was removed by evaporation at reduced pressure and the residue was dissolved in 80% acetic acid. The solution was stirred for 3 hours and the solvent was removed by evaporation at reduced pressure, the last trace of acetic acid being removed by evaporation of ethanol. The residue was dissolved ethanol-water and applied to Whatman electrophoresis paper. A preparative electrophoresis yielded a nucleotide band with R_m 1.00. The band was eluted with water to yield O^2,2'-cyclouridine 3'-phosphate (λmax 250nm and λmin 239nm).

Synthesis of O^2,2'-Cyclouridylyl-(3'-5')-O^2,2'-cyclouridine (cUpcU, XCI)

Method A; 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine (250mg, 0.5 mmole) was phosphorylated with β,β,β-trichloroethyl phosphate (1.0 mmole) using triisopropylbenzenesulfonyl chloride (2.0 mmoles) in pyridine (25ml) in the usual manner as described previously. After the addition of cold water (5ml), pouring into ice-water (50ml), extraction into methylene chloride (4x25ml), and washing with water (4x25ml), the solvents were removed by evaporation at reduced pressure. The residue was dissolved in dimethylformamide (10ml), Cu/Zn was added, and the mixture was heated, with stirring, at 50° for thirty minutes. After filtration, the residue was washed with 50% aqueous pyridine (25ml). The combined filtrates were applied to a Dowex 50W-X8 column and the product, MMTr-cUp, was eluted with 50% aqueous pyridine (200ml). The solution was concentrated to a small volume and dropped into rapidly stirring ether (500ml). The ether was decanted and the residue was dissolved in pyridine (10ml).

The solvent was removed by evaporation at reduced pressure and the gum was dried by evaporation of pyridine (3x10ml). 3'-O-Acetyl-O^2,2'-cyclouridine (125mg, 0.46 mmole) was added and the compounds were dried by evaporation of pyridine (1x10ml). Dicyclohexyl-carbodiimide (10 mmoles) and pyridine (10ml) were added and the mixture was stirred for 11 days. Cold water (10ml) was added and stirring was continued for 12 hours. After filtration, the residue was washed with 50% aqueous pyridine (10ml) and the combined filtrates were evaporated at reduced pressure. The residue was dissolved in 80% acetic acid and the solution was stirred for 4 hours. The solvent was removed by evaporation at reduced pressure and the residue was dissolved in ammonium hydroxide-ethanol (1:3). After, stirring for 1 hour, the solution was applied to Whatman papers and developed in Solvent C for 3 days. The nucleotide band with R_f 0.16 was eluted with water and lyophilized to yield 93mg (39%) of cUpcU (λmax 251nm and 223nm, and λmin 235nm and 213nm). The product was not degraded by spleen or snake venom phosphodiesterase.

Method B: 5'-O-Monomethoxytrityl-O^2,2'-cyclouridine 3'-phosphate was synthesized by phosphorylation of 5'-O-monomethoxy-trityl-O^2,2'-cyclouridine (250mn, 0.5 mmole) with trichloroethyl phosphate (1.0 mmole) followed by treatment with Cu/Zn in dimethyl-formamide at 50° in the manner described in Method A.

MMTr-cUp was dried by evaporation of pyridine (3x10ml), 3'-O-acetyl-O^2,2'-cyclouridine (270mg, 1.0 mmole) was added and the mixture was dried by evaporation of pyridine (1x10ml). Triphenyl-

phosphine and 2,2'-dithiodipyridine (2.5 mmoles each) were added with pyridine (5ml) and the solution was stirred for 12 hours. Cold water (2ml) was added to the cooled solution and stirring was continued for 2 hours. The solution was dropped into ether with stirring and the precipitate was dissolved in 80% acetic acid. The solution was stirred for 4 hours, the solvent was removed by evaporation at reduced pressure, and the residue was dissolved in ammonium hydroxide-ethanol (1:3). After stirring for 1 hour, the solution was chromatographed on Whatman paper in Solvent C for 3 days. The nucleotide band with R_f 0.12 was eluted with water and lyophilized to yield 118mg (46%) of cUpcU (λmax 250nm and 223nm, and λmin 240nm and 214nm). The product was not degraded by snake venom or spleen phosphodiesterase.

Arabinouridylyl-(3'-5')-arabinouridine (aUpaU, XCII)

From Method A: cUpcU (4mg) was dissolved in 2N sodium hydroxide (1ml) and the solution was stirred for 20 minutes. After neutralization with Dowex 50W-X8 resin and filtration, the solution was applied to Whatman papers and developed in Solvent A. The band with R_f 0.15 was eluted with water to yield aUpaU (λmax 263nm, 20 O.D.$_{263}$units). Incubation of aUpaU (10 O.D. units) with spleen enzyme gave complete degradation to aUp (R_f^A 0.09, 5.01 O.D.$_{263}$units) and aU (R_f^A 0.59, 4.84 O.D.$_{263}$units) with aUp/aU = 1.03. Incubation with snake venom enzyme gave paU (R_f^A 0.07, 1.63 O.D.$_{263}$units), aU (R_f^A 0.55, 1.71 O.D.$_{263}$units) and aUpaU (R_f^A 0.16, 6.76 O.D.$_{263}$units, 68%) with paU/aU = 0.95.

From Method B: cUpcU (4mg) was dissolved in 2N sodium hydroxide (1ml) and the solution was stirred for 20 minutes. The work-up is the same as described above. The nucleotide band with R_f 0.14 was eluted with water to yield aUpaU (max 263nm, 18 O.D.$_{263}$ units). Incubation of aUpaU (9 O.D. units) with spleen enzyme gave degradation to aUp (R_f^A 0.09, 4.40 O.D.$_{263}$units) and aU (R_f^A 0.58, 4.33 O.D.$_{263}$units) with aUp/aU = 1.02.

TABLE IV Paper Chromatographic and Electrophoretic Properties of Mononucleotides and Dinucleoside Monophosphates

	R_f Values		R_m *
COMPOUND	Solvent A	Solvent B	
Tp	0.085	0.59	1.00
TpT	0.34	0.69	0.35
Ucp	0.24	0.65	0.66
pU	0.04	----	1.00
aUp	0.07	0.59	1.00
paU	0.06	0.44	1.00
pU-OIP	0.11	0.62	0.99
MMTr-cUp(CE) (LXIV)	0.75	----	0.34
cUp(CE)	0.38	----	0.60
MMTr-cUp(CE)U-OIP (LXVI)	----	0.94	0.00
cUpU-OIP (LXVII)	0.30	0.66	0.35
aUpU-OIP (LXVIII)	0.28	0.66	0.38
aUpU((LXIX, from LXVI)	0.15	0.60	0.38
aUpU (LXIX, from LXVII)	0.17	0.60	0.37

* Electrophoretic Mobility Relative to Thymidine 3'-phosphate (Tp) in Triethylammonium Bicarbonate Buffer (pH 7.5)

TABLE V Ultraviolet (U.V.) and Infrared (IR) Spectral Properties of Mononucleotides and Dinucleoside Monophosphates

		U.V. Spectra		I.R. Spectra
COMPOUND	Solvent	λmax,nm( )	λmin,nm( )	μ
MMTr-cUp(CE) (LXIV)	H_2O	226(sh)		4.40,6.06,8.00,11.97,14.20
MMTr-cUp(CE)U-OIP (LXVI)	95% EtOH	232(25,500),250(sh)	226(25,100)	4.42,5.89,6.05,8.00,14.10
cUpU-OIP (LXVII)	H_2O	255	236	
aUpU (LXIX)	H_2O	260	232	
TpcU-OAc (LXXVIII)	H_2O	260	234	
TpcU (LXXIX)	H_2O	260	235	
TpaU (LXXX)	H_2O	264	235	
MMTr-cUp(TCE)U-OIP (LXXXVI)	95% EtOH	230(22,400),251(14,900)sh	226(22,300)	
cUp(TCE)U-OIP (LXXXVIII)	H_2O	255	239	
cUpcU (XCI)	H_2O	250,223	240,214	
aUpaU (XCI)	H_2O	263	235	

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