THE UNIVERSITY OF MANITOBA

tert-BUTYLDIMETHYLSILYL PROTECTING GROUP IN THE SYNTHETIC CHEMISTRY OF RIBONUCLEOSIDES AND RIBONUCLEOTIDES

by

KRISHAN LAL SADANA

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THE SYNTHETIC CHEMISTRY OF RIBONUCLEOSIDES AND RIBONUCLEOTIDES

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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

Ravi

and our daughters

Anubha and Vanita

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ABSTRACT

A detailed study was made of the application of the tert.-butyldimethylsilyl (TBDMS) hydroxyl protecting group to the complex case of protecting a primary and two secondary vicinal hydroxyl groups in ribonucleosides.

The study of silylation of the model ribonucleoside uridine showed that the TBDMS group was very convenient for the synthesis of protected nucleosides and hence nucleotides. Direct silylation of uridine by tert.-butyldimethylsilyl chloride in the presence of imidazole and DMF was a rapid process and the four main products (5'-O-, 2',5'-di-O-3',5'-di-O- and 2',3',5'-tri-O-silyluridine) were cleanly separated from each other by silica gel chromatography (plates) employing non-polar developing solvents such as ether and ether-hexane. Economy of time and materials resulting from this protecting procedure was illustrated by the preparation of 2',5'-di-O-TBDMSuridine and 5'-O-monomethoxytrityl-2'-O-TBDMSuridine in 45% yield in both cases. Isomerisation in basic media (3'⇌2') proved useful in increasing the 'effective' yield of the 2',5'-di-O-silyl derivatives because of the ease of separation of the 2',5'-di-O- and 3',5'-di-O- isomers.

The removal of the TBDMS protecting groups was achieved with tetra-n-butylammonium fluoride (TBAF) as well as by acidic and basic conditions. The preferential acidic cleavage of the primary 5'-TBDMS group allowed preparation of 2'-O-, 3'-O- and 2',3'-di-O-TBDMSuridines. Another interesting finding, which could have important implications, was the selective removal of the 2'(3')-TBDMS group from the derivatives having a free vicinal hydroxyl groups by 7M NH_4OH at 70°C. There was no

isomerisation (2'⇋3') or cleavage of the TBDMS group(s) under phosphorylation and condensation conditions used for the synthesis of dinucleotides by the triester method (the phosphate was protected by β-cyanoethyl group).

The orientation of the TBDMS group(s) in the uridine molecule was determined by preparing known derivatives. The techniques of thin-layer chromatography, high performance liquid chromatography and mass spectrometry also proved very useful for this purpose. The general fragmentation patterns observed in the mass spectra of orientated uridine derivatives helped characterise the TBDMS derivatives of adenosine and cytidine.

Phosphorylation of 2',5'-di-O-TBDMSuridine and 5'-O-monomethoxytrityl-2'-O-TBDMSuridine by β-cyanoethyl phosphate in the presence of the activating agent triisopropylbenzenesulphonyl chloride (TPS) proceeded smoothly. An attempt was made to purify the resulting monophosphate on silica gel plates but the yield was not encouraging. Condensation of the β-cyanoethyl ester of 2',5'-di-O-TBDMSuridine-3'-phosphate with 2',3'-O-isopropylideneuridine and 2'-O-TBDMSuridine in the presence of TPS was successful. Yields were of the order of 25-30%, although no optimisation was attempted. It was indicated though that considerable sulphonation of the nucleoside component took place (46% in the case of 2',3'-O-isopropylideneuridine) and it was clear that the nature of the protecting groups at 2' and 3' positions of the nucleoside component determined the yield at the condensation stage. The β-cyanoethyl ester of 5'-O-monomethoxytrityl-2'-O-TBDMSuridylyl-(3'-5')-2',3'-O-isopropylideneuridine was also prepared in a similar manner and 5'-O-monomethoxytrityl group could be selectively removed indicating that the chain length could be extended from the 5'-end.

The 2'-TBDMS protecting group was found to be compatible with

other protecting groups provided these groups could be introduced in mild acidic conditions. The compatibility of the 5'-TBDMS group was limited because of its greater lability under acidic conditions.

The removal of the 2'-TBDMS group from a protected dinucleoside monophosphate (a triester) by TBAF was studied in detail because of initial difficulties in obtaining enzymatically degradable products. Conditions were found for deprotecting the dinucleotide so that it was fully degradable by ribonuclease, snake venom and spleen phosphodiesterases.

The success in selectively protecting secondary hydroxyl groups of uridine led to the study of the silylation of adenosine, cytidine and guanosine. Equally efficient procedures were developed for preparing the TBDMS derivatives of adenosine and cytidine.

It is hoped that this approach will be found to be generally applicable in the preparation of other nucleotides, including mixed nucleotides, using corresponding N-acyl adenosine, guanosine or cytidine derivatives.

ABBREVIATIONS

A	adenosine; solvent A
Ac	acetyl
Ac_2O	acetic anhydride
Ade	adenine
B	base residue
C	cytidine
β-CEP	β-cyanoethyl phosphate
CE	cyanoethyl
DCC	dicyclohexylcarbodiimide
DMTr	dimethoxytrityl
DMF	dimethylformamide
DMSO	dimethylsulphoxide
Em	electrophoretic mobility
EtOH	ethanol
G	guanosine
MeOH	methanol
MMTrCl	monomethoxytrityl chloride
Ph	phenyl
Pv	pivaloyl
TBDMSCl	tert - butyldimethylsilyl chloride
TBAF	tetra-n-butylammonium fluoride
THF	tetrahydrofuran
THP	tetrahydropyranyl
TPS	triisopropylbenzenesulphonyl chloride

tlc	thin layer chromatography
Ts	toluenesulphonyl
Rf	relative mobility
U	uridine
Ura	uracil base
Up	uridine-3'-phosphate
pU	uridine-5'-phosphate.
U-OIP	2',3'-O-isopropylidene uridine
UpU	uridylyl-(3'-5')-uridine
UpU-OIP	uridylyl-(3'-5')-2',3'-O-isopropylideneuridine
UpU-2'-O-TBDMS	uridylyl-(3'-5')-2'-O-TBDMSuridine.

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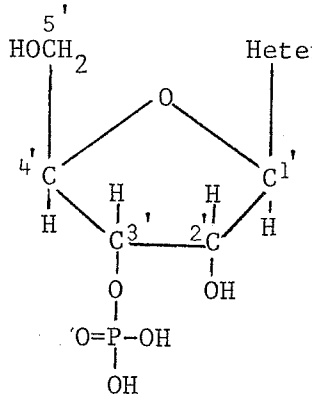
INTRODUCTION

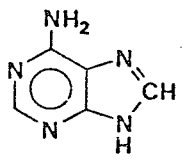
General. Nucleic acids are a class of biopolymers (macromolecules) which, like proteins and polysaccharides, are vital components of living materials. In fact, of all the biopolymers known, the study of nucleic acids is the most important as they are a key to understanding the nature of life.

These macromolecules bear the same relation to their repeating units, the nucleotides, as proteins do to the amino acids. A nucleotide is made up of three components linked together: a basic heterocyclic ring compound (purine or pyrimidine); a pentose sugar (ribose or deoxyribose) and an orthophosphate unit.

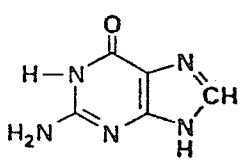


a ribonucleotide

The common bases that occur in nucleic acids are the purines, adenine (I) and guanine (II) and the pyrimidines, cytosine (III), thymine (IV) and uracil (V).



I



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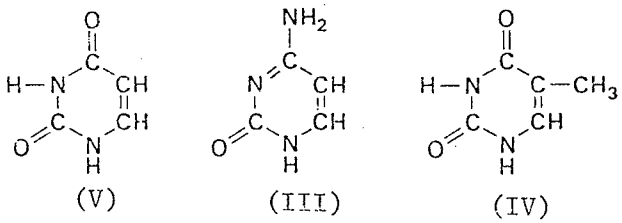
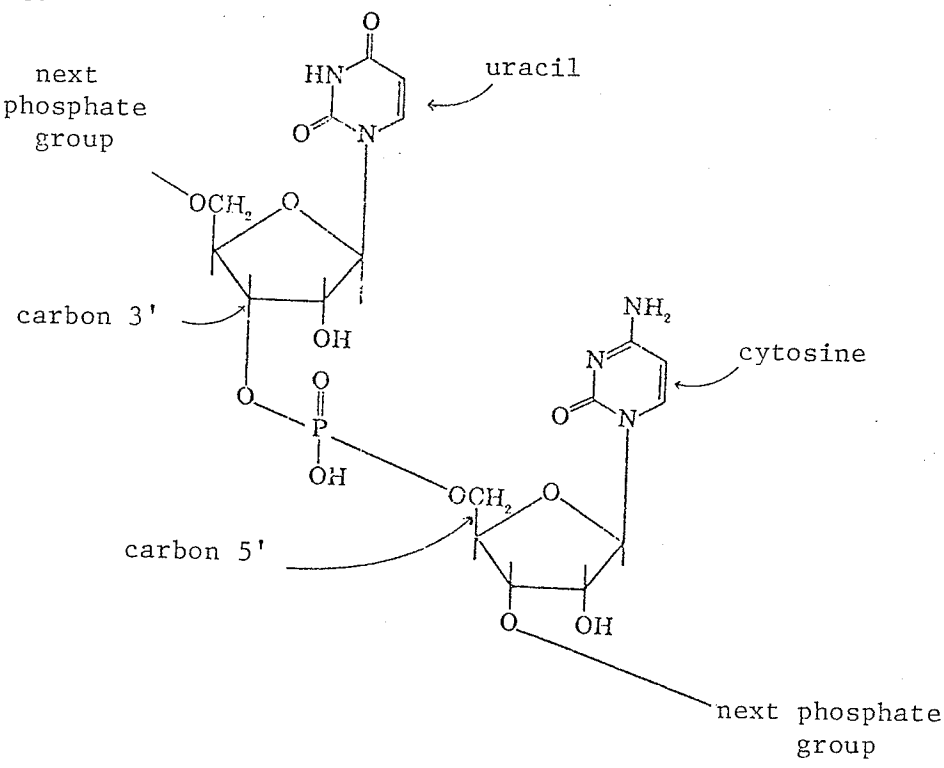


(V) (III) (IV)

One nucleotide is linked to another through the phosphate group which is covalently linked to carbon atom 3' of the pentose ring of one nucleotide and to carbon atom 5' of the pentose ring of the adjacent nucleotide.



A segment of an RNA strand

Nucleic acids are of two types: ribonucleic acid (RNA) in which the sugar of the component nucleotides is ribose; and deoxyribonucleic acid

(DNA) in which the sugar is deoxyribose. The substituent OH at carbon atom 2' is replaced by a hydrogen atom in the deoxyribose. DNA contains the bases adenine(I), guanine(II), cytosine(III) and thymine(IV), whereas in RNA the last base thymine is replaced by uracil(V). Nucleosides contain only a nitrogen base and a sugar. DNA is present in the nucleus of the cell and is the repository of the hereditary information. RNA occurs, in most organisms, mainly in ribosomes (granules of protein and RNA), transfer RNA and messenger RNA, and in all these forms it is mainly concerned in transfer of information contained in the structure of DNA molecules.

The nucleosides and nucleotides are attracting considerable attention not only because they are the building blocks of the nucleic acids but also for various other reasons. For example, they are cofactors and allosteric effectors for many of the fundamental enzyme reactions. Analogs of natural purine and pyrimidine nucleosides have proved to be quite effective as antiviral, antibacterial and anticancer or antitumor agents[1].

Role of Synthetic Oligo- and Polynucleotides

The study of various aspects of nucleic acids, as their chemistry, physical chemistry, molecular biology and enzymology has been greatly facilitated due to the availability of defined nucleotide sequences. For example,the precise elucidation of the genetic code was possible because trinucleotides could be synthesised[2].

More recently, Khorana and co-workers[3,4] have focussed their attention on developing methods for the synthesis of macromolecular

bihelical DNA of defined sequence, and in particular, of genes for certain transfer RNAs including the adjacent DNA regions which signify the initiation and termination of their transcription. Further, synthetic deoxyribopolynucleotides have formed the basis of a general approach for the determination of nucleotide sequences of the above control regions in DNA. It is apparent that future studies of the biological functions of the nucleic acids in gene structure and function relationships, mechanisms of the protein-nucleic acid interactions and the control of genetic expression will be greatly aided by the availability of synthetic DNA chains of predetermined sequence.

The role of transfer RNAs in protein biosynthesis is well-established. The ribonucleoside sequence of several transfer RNAs has now been elucidated and correlations between the structure and function are being actively investigated. In this connection, some of the ways in which synthetic ribonucleotides could be used are: firstly, they may be reconstituted with fragments obtained by the enzymatic digestion of natural tranfer RNAs to form biologically active nucleic acids; secondly, the total synthesis of a transfer RNA molecule by joining such oligoribonucleotides might be important in its own right; thirdly, the chemical synthesis of unnatural transfer RNA like polynucleotides may open avenues to a more penetrating understanding of protein biosynthesis. The recent findings[5,6] that RNA ligase can join oligonucleotides as short as trimers, provide various uses of short chain oligonucleotides. RNA ligase has the advantage of not requiring the complementary strand, unlike DNA ligase, thereby simplifying the synthetic task.

Chemical Synthesis of Oligo- and Polynucleotides

Perhaps the most brilliant success in the field of deoxypolynucleotides is the synthesis of a gene for transfer RNA by Khorana and co-workers[3,4]. This and other important syntheses of similar nature were accomplished by a combination of chemical synthesis of deoxyribooligonucleotides and the joining of these sequences by DNA ligase.

In this combined chemical-enzymatic approach chemical synthesis of short deoxypolynucleotide segments (up to 20 units) continues to determine the progress in the synthesis of the required defined DNA molecule[7], and despite intensive efforts made to date[8,9], chemical synthesis is still a time-consuming process. Efforts are being made in Khorana's[7] and other[10] laboratories to enhance the rapidity and efficiency of chemical procedures. A significant improvment in efficiency would result from the ready availability of protected mono- and oligonucleotide blocks carrying 5' phosphate groups.

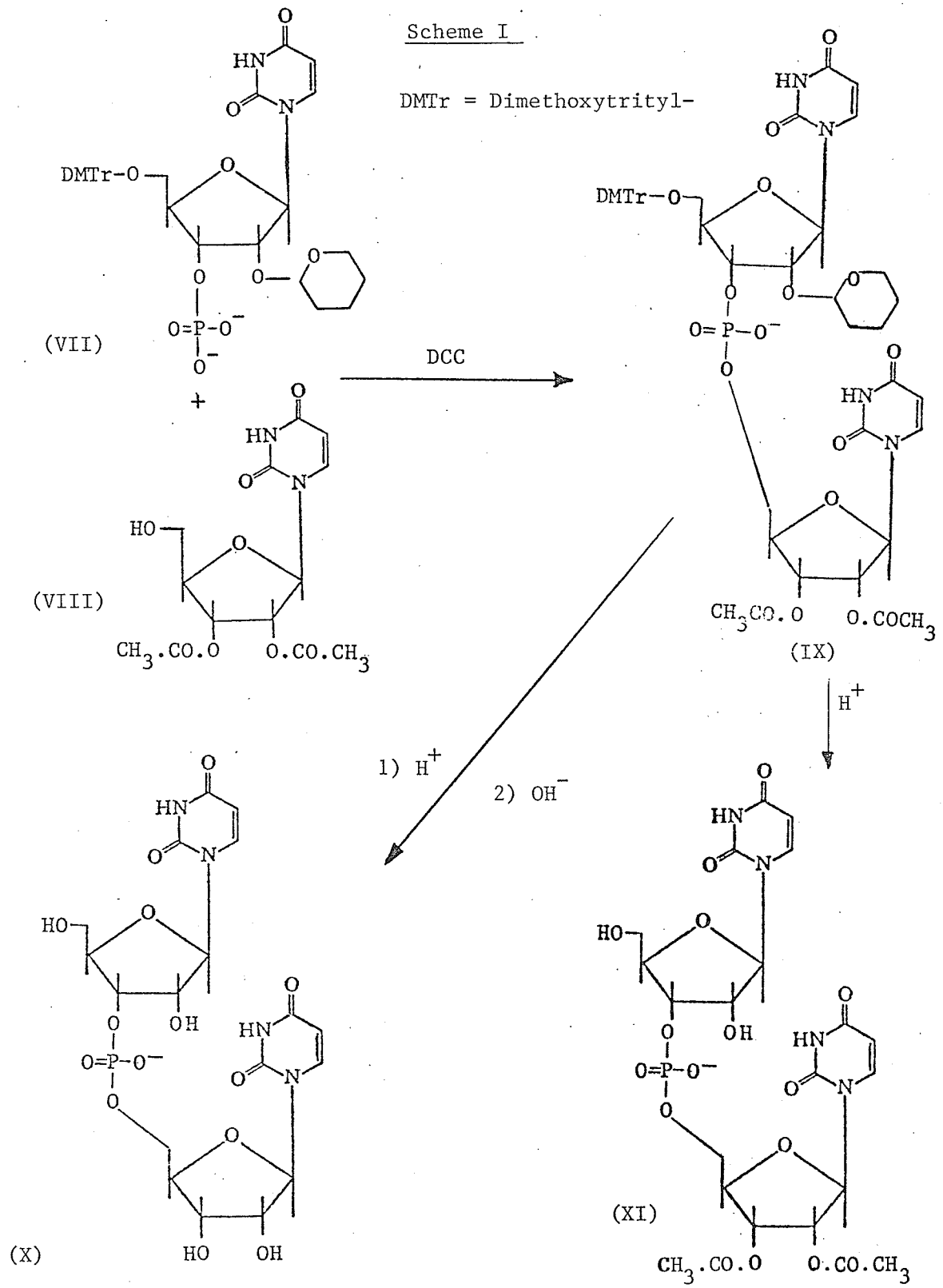
Methods of chemical synthesis are much less developed in the case of oligoribonucleotides due to the presence of an additional 2'-hydroxyl group in each carbohydrate moiety, which causes principally the high lability of the internucleotide bond of ribonucleotides in an alkaline medium and the ability to undergo isomerisation in an acid medium. Additionally difficulties arise because of the need to protect the 2'-hydroxyl group of the nucleosides and nucleotides used in the reaction. Yields are relatively low in the condensation step that joins nucleotide units, presumably due to steric hindrance.

The usual approach to oligoribonucleotide synthesis is by the stepwise increase in the length of the oligonucleotide chain from the 5'-end.

To synthesise dinucleoside phosphates, 2',5'-protected ribonucleoside-3'-phosphates are usually used as the nucleotide component and 2',3'-protected

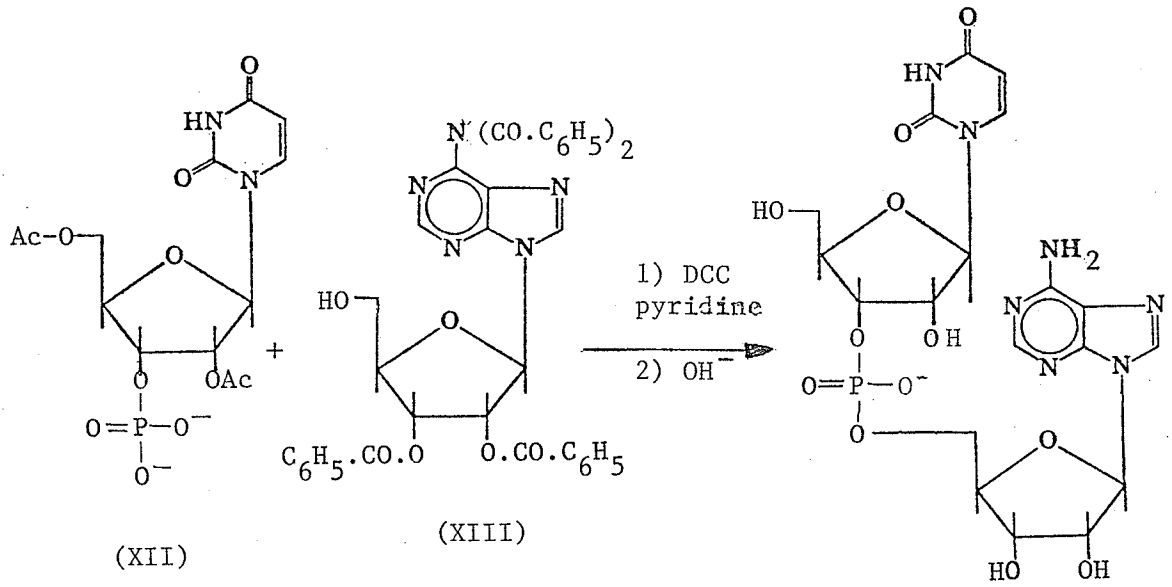
Scheme I

DMTr = Dimethoxytrityl-



ribonucleosides as the nucleoside component (Route A). This approach is illustrated in Scheme I.

The above example is the first ever successful synthesis of the 3'→5' inter-ribonucleotide linkage, by Smith and Khorana[11,12]. Initially the yield of X was 50%, but this was increased to 69% by increasing the time for acid treatment from 4 to 10 hours. As the prolonged reaction time caused isomerisation (1%) to the 2'→5' linked dinucleotide, alternative protective groups were used. The one chosen was 2',5'-di-O-acetyluridine 3'-phosphate (XII) as the nucleotide component, which when condensed with N,N,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (XIII) gave the dinucleoside phosphate (XIV) in 84% yield[13] without any isomerisation of the internucleo--tide linkage. (Scheme II)



(XII) (XIII) (XIV)

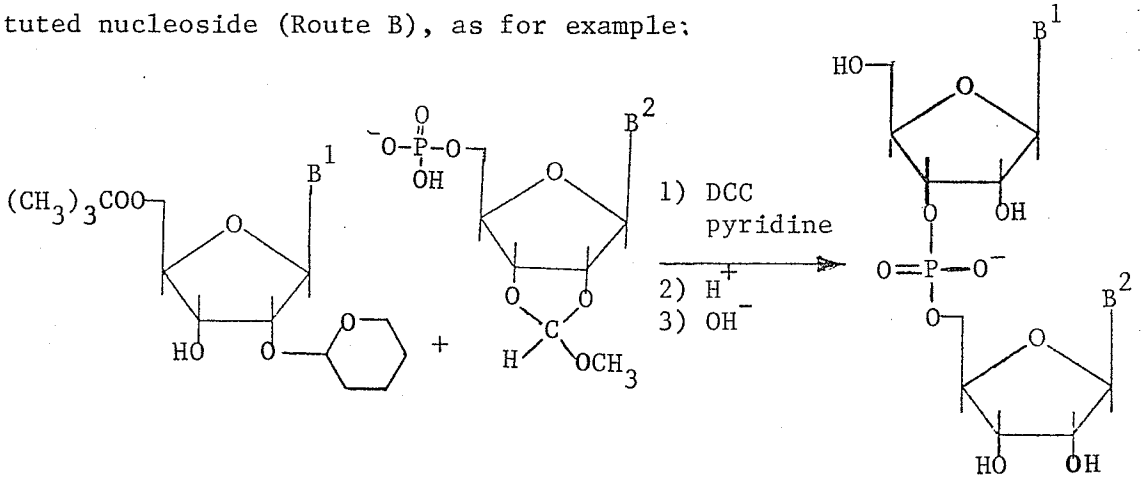
Scheme II.

The most successful approach has been based on the use of 5'-O-mono-(or di-)methoxytrityl-2'-O-acetylnucleoside-3'-phosphate as the nucleotide component and 2',3'-di-O-acetyl-(or benzoyl)nucleosides as the nucleoside component (the amino groups of the heterocyclic bases of both components also must be protected).

In this procedure the chain is lengthened by deblocking of the 5'-hydroxyl group in the diribonucleoside phosphate formed and performing the condensation with a protected ribonucleoside-3'-phosphate. In this way all 64 possible trinucleoside diphosphates, composed of the ordinary nucleotides found in RNA, have been obtained by Khorana and co-workers[14].

Several modifications of the above have been investigated in the laboratories of Holy and Smrt, Cramer and Reese in which the 5'-hydroxyl is protected by alkali labile groups (acetyl, benzoyl, triphenylacetyl), while the 2'-hydroxyl is protected by acid labile groups (tetrahydro--pyranyl, 1-ethoxyethyl). These procedures apparently gave lower yields compared to those reported in reference 14. However, the discrepancy seems to be due to the way of calculating the results[15].

Another approach to the preparation of dinucleoside monophosphates has also been suggested[42,43,119]. This is based on the condensation of a 2',3'-di-O-substituted nucleoside-5'-phosphate and a 2',5'-di-O-substituted nucleoside (Route B), as for example:



Scheme III. Synthesis of a Dinucleoside Monophosphate (Route B).

This approach has not been used widely in the ribonucleotide field due to steric reasons, particularly when employing the triester method.

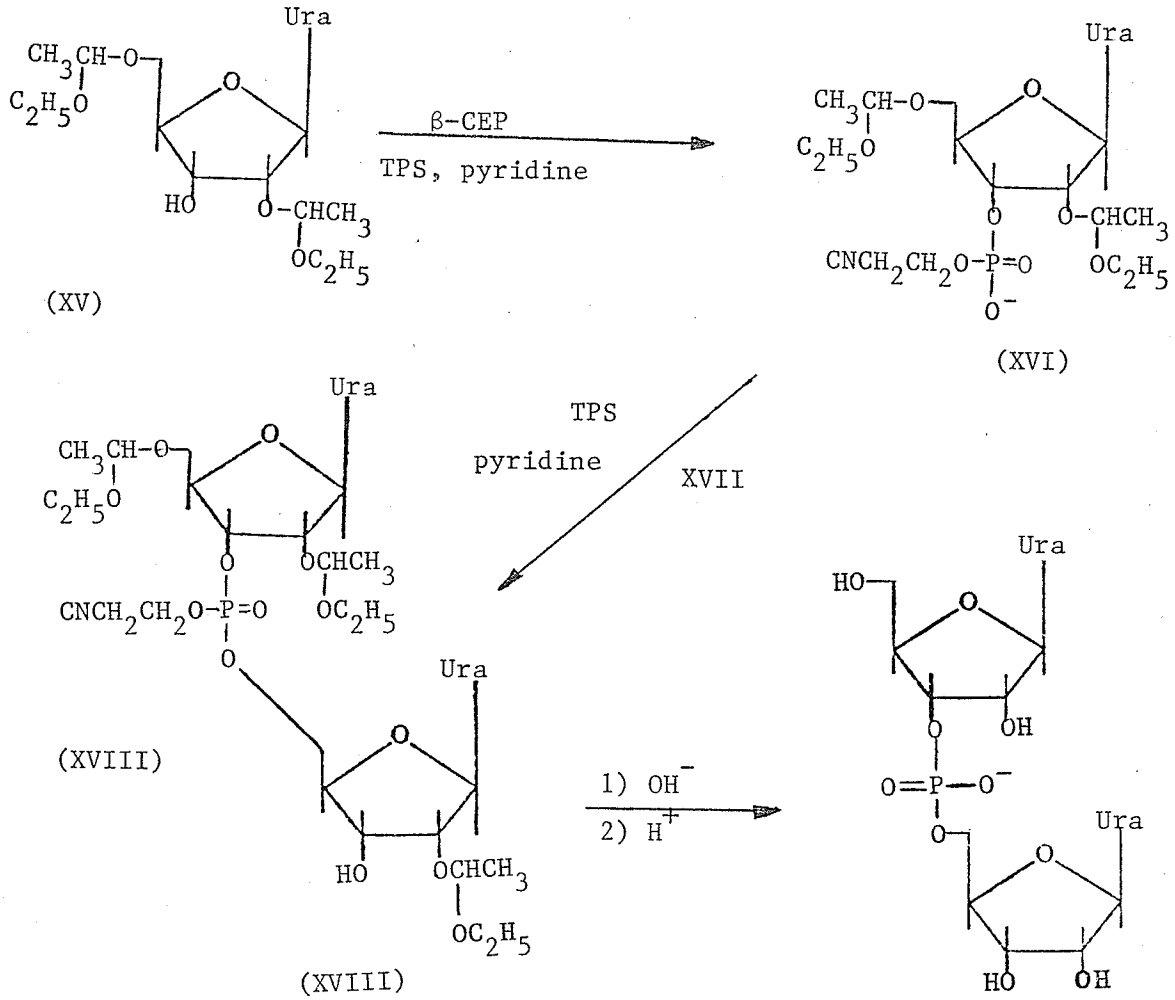
The phosphodiester bond as formed in routes A and B described above is known to undergo activation under certain conditions so as to react further with hydroxyl containing compounds[17]. This unfavourable effect of the acidic function of the phosphodiester bond was eliminated in the triester synthesis of the internucleotide bond as described by Letsinger and Ogilvie[18] and Eckstein and Rizk[19] for deoxynucleosides. In this new approach to oligonucleotide synthesis, the phosphate residues are blocked by protecting groups as β-cyanoethyl[18,20,21], β,β,β-trichloroethyl[19], phenyl[23] or o-chlorophenyl[23,24,25] esters. The criteria for selecting this esterifying function are: firstly, it must be selectively and readily removable from the phosphotriester functions, and secondly, it must remain intact under the conditions of acidic and basic hydrolysis which are necessary for the removal of common protecting groups from the sugar hydroxyl functions.

The β-cyanoethyl group has been employed by Letsinger and Ogilvie in synthesising oligothymidylates in a stepwise manner[18] and by the use of preformed blocks[20]. In a stepwise manner, fully blocked triesters of TpT, TpTpT and TpTpTpT are obtained in 64%, 49% and 57% yields respectively using a constant nucleotide to nucleoside ratio of 1:2. As the nucleoside component thymidine is not protected at the 3'-hydroxyl, about 4% of the 3'→3' isomer is found in the case of TpT, but this is completly prevented by using β-benzoylpropionyl group for protecting the 3'-hydroxyl group. The fully blocked triester of TpT is obtained in 64% yield. TpTpTpT is obtained from preformed blocks[20] in 52% yield in a similar manner.

Letsinger and Grams[21] also used the β-cyanoethyl group in the

triester synthesis of oligoribonucleotide. For example, when 2',5'-di--O-(1-ethoxyethyl)uridine (XV) is treated with a mixture of pyridinium β-cyanoethyl phosphate and TPS for 16 hours, β-cyanoethyl 2',5'-di-O-(1-ethoxyethyl)uridine-3'-phosphate (XVI) is produced in 92% yield (spectrophotometric analysis). Condensation of 2'-O-(1-ethoxyethyl)-uridine (XVII) with XVI gives the β-cyanoethyl phosphate of 2',5'-di-O-(1-ethoxyethyl)uridylyl-(3'-5')-2'-O-(1-ethoxyethyl)uridine (XVIII) as shown in Scheme IV.

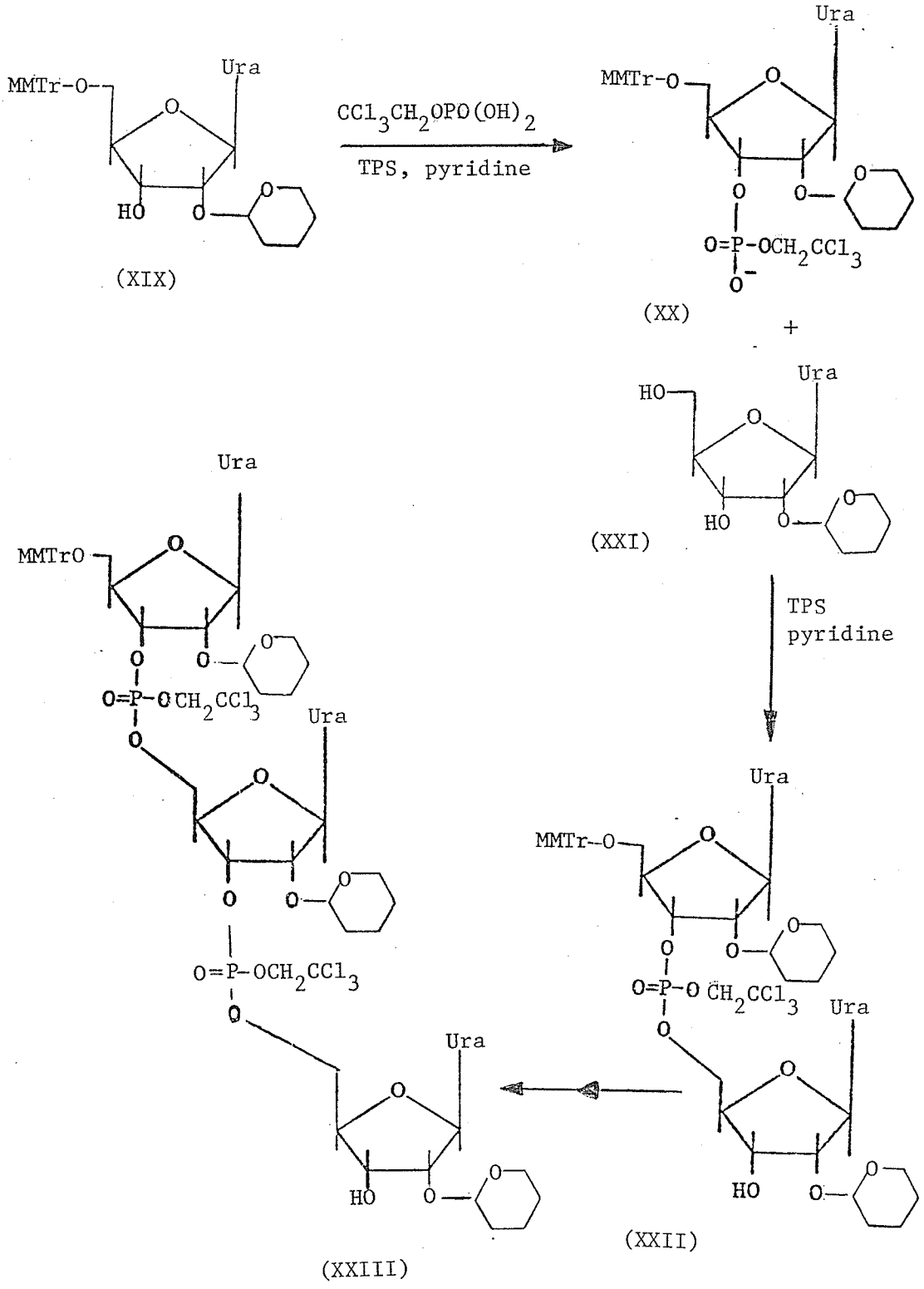
Scheme IV. Synthesis of UpU



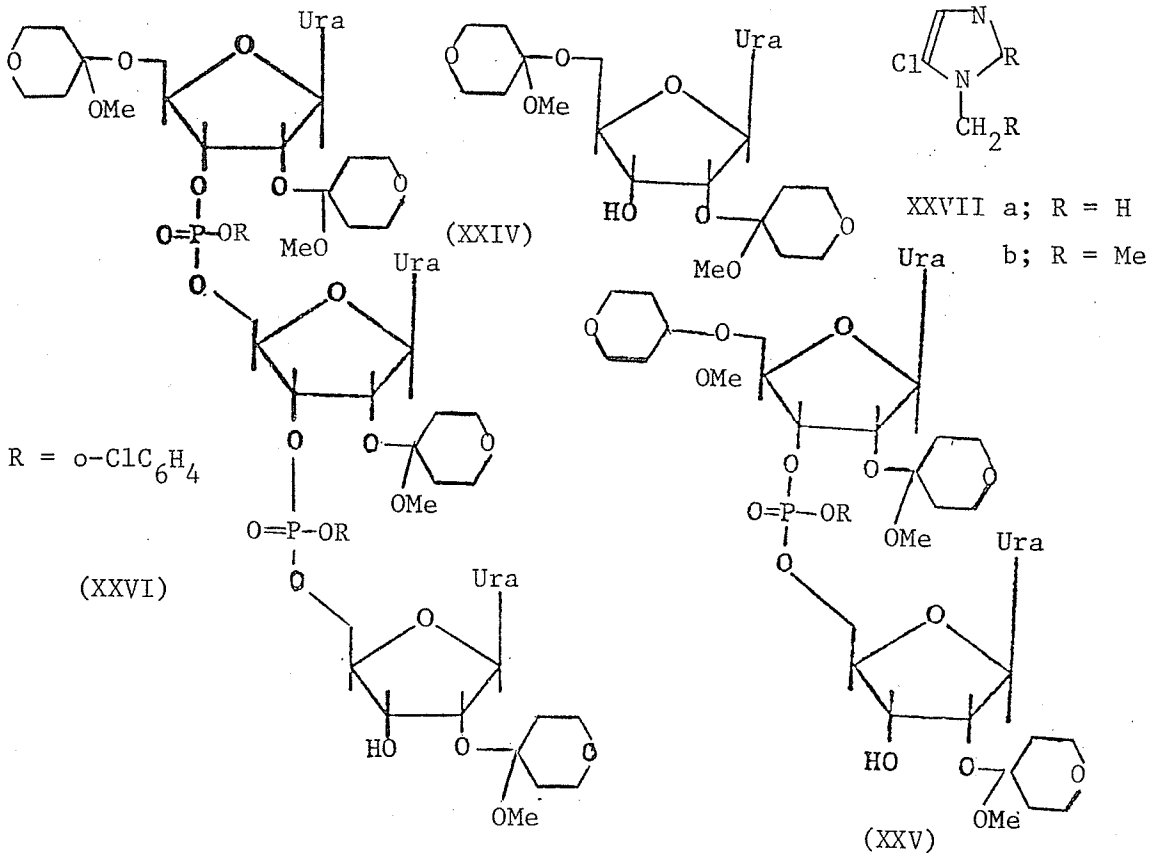
The total conversion as seen by removing the protecting groups directly is 54% (on the basis of XV). However, isolation by silica gel chromatography gave XVIII in 33% yield. There are also found dinucleoside monophosphates without one or more ethoxyethyl groups, to the extent of 16%.

The β,β,β-trichloroethyl, originally introduced for deoxynucleotides, has also been used extensively by Neilson and co-workers[22,117,118] for synthesising oligoribonucleotides. It can be removed selectively by reduction with zinc or zinc/copper. The triesters may be synthesised either by stepwise addition of the two properly protected nucleosides β,β,β-trichloroethyl phosphodichloridate or by condensation of a β,β,β-trichloroethyl ester of a nucleoside with a nucleoside using an arylsulphonyl chloride as a condensing agent. The second nucleoside should bear the 5'- hydroxyl group for ester formation since reversal of the sequence leads to a drastic decrease in yield in either synthesis. One example of the use of this protecting group for synthesising a triribonucleotide[22] is described. 5'-O-Monomethoxytrityl-2'-O-tetrahydropyranyluridine (XIX) is phosphorylated with the pyridinium salt of β,β,β -trichloroethyl phosphate and TPS to give XX, which is then condensed with 2'-O-tetrahydropyranyluridine (XXI) in the presence of TPS to give a dinucleotide derivative XXII (62% yield over two steps), which is purified by silica gel column chromatography. Repetition of the phosphorylation procedure on XXII with subsequent coupling with XXI gives a trinucleotide derivative XXIII in 67% yield. The 3'→3' linked compounds are not detectable. Deblocking of XXIII by Zn-Cu couple in DMF and then treatment with 0.01 M HCl gives UpUpU in 59% yield. (Scheme V)

Scheme V. Synthesis of a triribonucleotide.



The application of the o-chlorophenyl protecting group in the synthesis of a triribonucleoside diphosphate[24] is described in the following example. 2',5'-Di-O-methoxytetrahydropyranyluridine (XXIV) (1 mmol) was phosphorylated with o-chlorophenyl phosphate (1.1 mmol) and TPS (2.2 mmol) in pyridine solution. After five hours, 2'-O-methoxytetrahydropyranyluridine (1.2 mmol) and more TPS (0.55 mmol) was added and the reaction continued for a further 16 hours. Work-up and chromatography on silica gel of the products gave the partially protected dinucleoside phosphate (XXV) in 75-80% yield based on XXIV. Phosphorylation of XXV with o-chlorophenyl phosphate and TPS followed after 7 hours, by the addition of 2'-O-methoxytetrahydropyranyluridine gave XXVI which was isolated in 73-76% yield.



When XXV and XXVI were treated with an excess of 0.1 M NaOH-aqueous dioxan (4:1, v/v) to remove the aryl protecting groups (for 90 minutes and 180 minutes respectively) and then submitted to acidic hydrolysis (6 hrs., pH 2) UpU and UpUpU respectively were obtained as the sole nucleotide products. There was no evidence that either product was contaminated with material containing 3'→3' internucleotidic linkages. A similar synthesis using phenyl phosphate was also reported[24].

Phenyl phosphodichloridate has been used successfully as the phosphorylating agent in the deoxy series[23]. However, in the ribo series, no success is achieved with this reagent in the presence of either 2,6-lutidine or pyridine[134] but a partially protected dinucleotide phosphate is obtained using phenyl phosphodichloridate in the presence of 5-chloro-1-methylimidazole (XXVIIa) in acetonitrile solution[134]. Though both phosphorylation steps are complete in 190 minutes, the product is contaminated with a hard-to-separate non-nucleotidic impurity. When 5-chloro-1-ethyl-2-methylimidazole XXVIIb is used instead of XXVIIa, the product is pure but the yield is lower and both phosphorylation steps are slower[24].

Some disadvantages of the triester approach have also been mentioned. In the ribo series, the presence of the bulky protecting groups in the phosphodiester component has been shown to lower considerably the reactivity of this component for the formation of the triester[26]. For the same reason route B synthesis can not be employed. Another problem is the susceptibility of the phosphotriesters to nucleophilic attack. Some triesters are partially hydrolysed even by aqueous pyridine[27].

The largest oligoribonucleotide synthesised by the phosphotriester method[28] is the nonaribonucleotide GCmUCAUAAC which corresponds to the anti-codon loop of transfer RNA_f^{Met} from E. coli. More recently, oligoribonucleotide sequences corresponding to those found in the double-stranded region adjacent to the dihydrouridine loop of several transfer RNAs have also been synthesised[135].

Another interesting large molecule is the nonaribonucleotide CGUCCACCA. This fragment which forms the 3' end of yeast alanine transfer RNA, has been synthesised by the phosphodiester method[29,30].

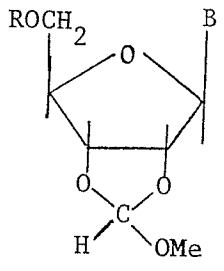
Hydroxyl Protection of Ribonucleosides and Ribonucleotides

Preparation of suitably protected ribonucleosides and ribonucleotides is much more difficult than the protection of corresponding deoxy derivatives, as the ribose ring has two secondary hydroxyl groups (2'-OH and 3'-OH) while deoxyribose has only one secondary hydroxyl function (3'-OH).

A. Nucleoside Components

A very common requirement in the synthesis of oligonucleotides is a 2',3'-disubstituted ribonucleoside with a free 5' hydroxyl group. Some of these protecting groups are 2',3'-O-isopropylidene[113,114], -cyclopentylidene[115], -anisylidene [115] or -benzylidene[115,116]. These groups are removed slowly at room temperature by aqueous acids at pH 1, and proved too stable, resulting in isomerisation of the internucleotide bond. The benzylidene group can be modified by the introduction of para and ortho electron donating substituents and thus made more labile[31]. However, it became known that the effect of such a substituent (e.g. -OMe) is much

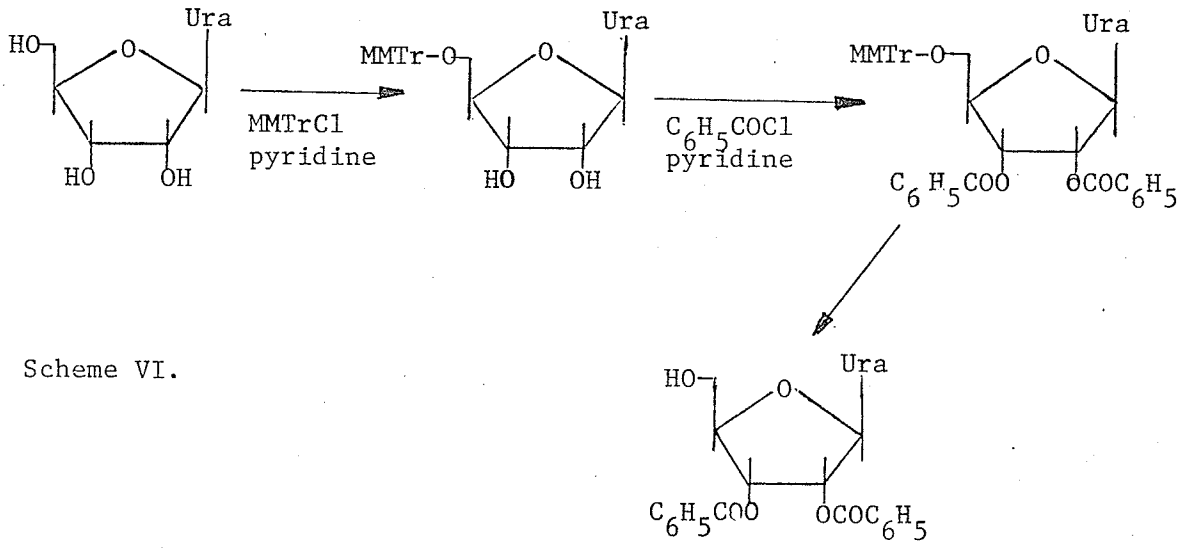
more pronounced if it is attached directly to the acetal carbon as in XXVIIa.



(XXVIIa)

This and 2',3'-O-ethoxymethylidene derivatives are obtained in high yields from exchange reactions between ribonucleosides and trimethyl-triethyl orthoesters[37] and are widely used in oligoribonucleotide synthesis[32-36]. All these derivatives are stable in alkaline medium.

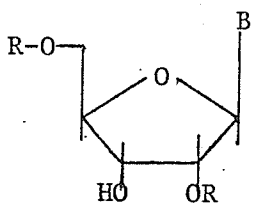
In an alternative strategy of oligonucleotide synthesis, Khorana and co-workers required 2',3'-O-diacylribonucleoside (acyl=acetyl or benzoyl) as the nucleoside component. They used a well known approach making use of the different reactivities of primary and secondary hydroxyl groups towards triphenylmethyl chloride (or its methoxy derivatives).



Scheme VI.

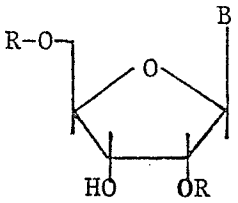
For example[41], uridine is directly monomethoxytritylated in pyridine to give 5'-O-substituted uridine which is then acylated (benzoylation is preferred) with acyl chloride in anhydrous pyridine. Detritylation is done by 80% acetic acid without isolating the intermediate product. The yields in these reactions are up to 80% (Scheme VI).

In a number of cases, 2',5'-di-O-protected nucleosides are the starting substances for oligoribonucleotide synthesis. Depending on whether the formation of the internucleotide bond is the final or an intermediate step, the protecting groups in the 2' and 5' positions can be identical or different. Reese and co-workers[16,42-47] have prepared the following three types:



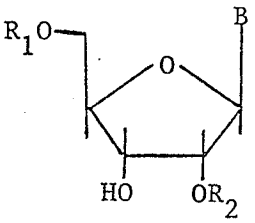
R = Alkali labile

Type I



R = Acid labile

Type II



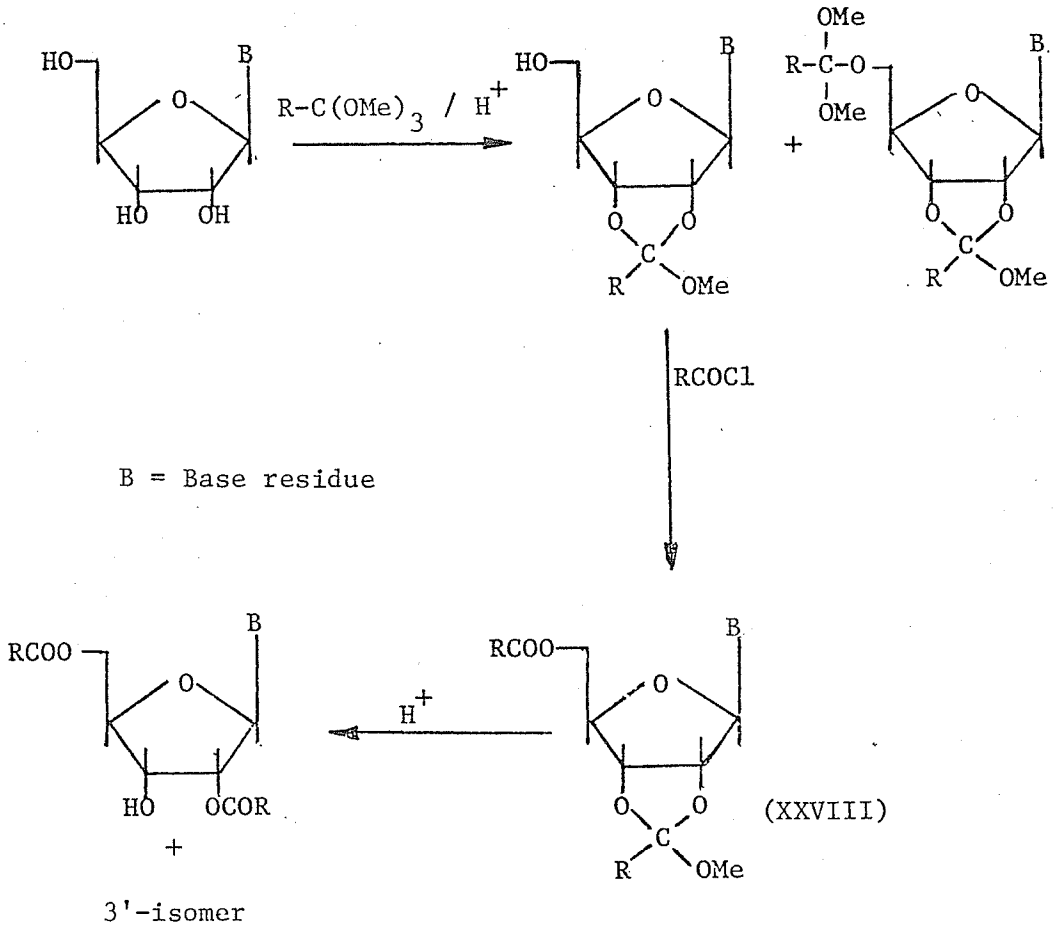
R_1 = Base labile

R_2 = Acid labile

Type III

B = Base residue

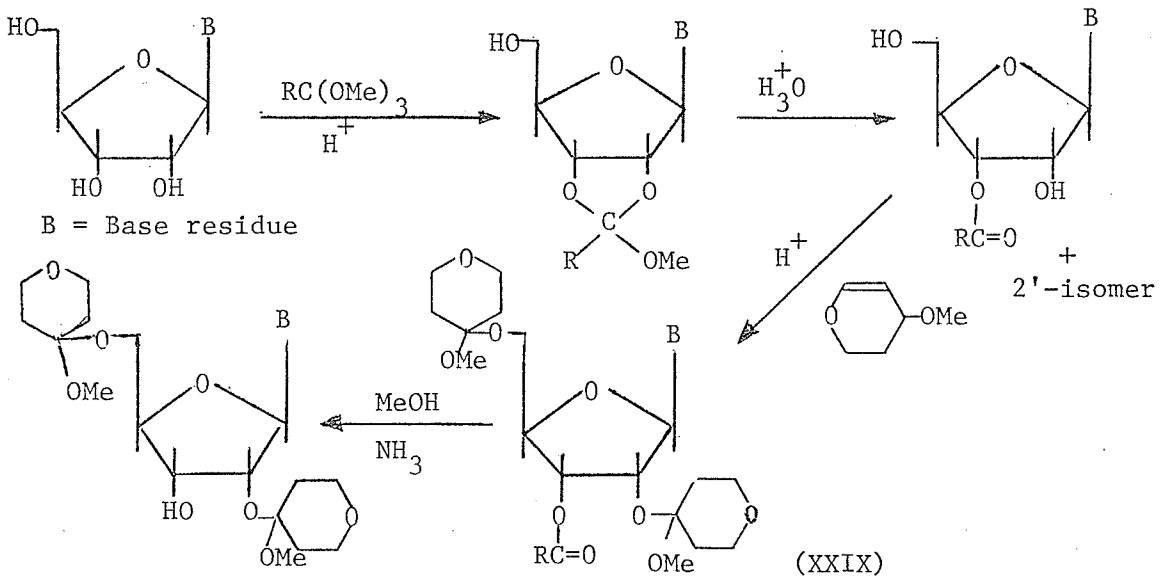
The following account summarises their procedures. Scheme VII describes the transformations required for making 2',5'-di-O-acylribonucleosides [42,44] (Type 1).



Scheme VII

Ribonucleosides or their 5'-derivatives undergo acid-catalysed exchange with trimethylorthoacetate or orthobenzoate to give the corresponding 2',3'-O-methoxyethylidene or benzylidene derivatives (R = Me or Ph) respectively. Treatment of the compound XXVIII with aqueous acids, under mild conditions, gives mixtures of the respective 2' (and 3') -acetates and -benzoates, from which it is often possible to obtain a pure crystalline isomer (usually the 3'-ester), in good yield.

Type II derivatives, where R is an acid labile group, as 2',5'-O-bisacetals of nucleosides are synthesised[45-47] by the following successive transformations (Scheme VIII).

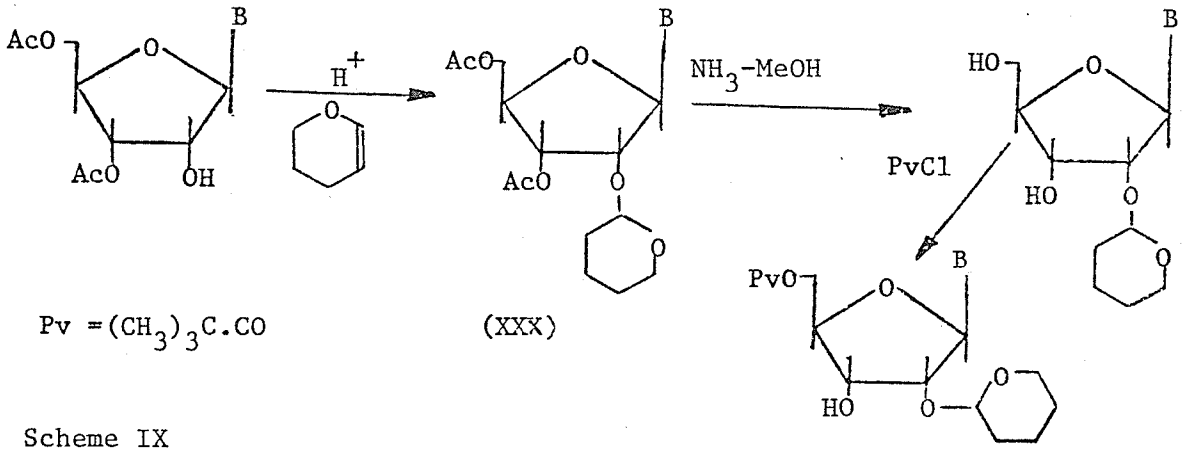


Scheme VIII

Acid catalysed exchange between a ribonucleoside (or its N-acyl derivative) and a trimethylorthoester gives the 2',3'-O-methoxy-alkylidene derivative which yields on mild hydrolysis and subsequent separation the 2' and 3'-O-acylribonucleosides. Reaction between the 3'-O-acyl derivative and excess of 4-methoxy-5,6-dihydropyran in the presence of mesitylene or toluenesulphonic acid gives a bisketal ester XXIX. Deacetylation by methanolic ammonia gives the desired product.

Type III compounds can be prepared in the following manner[16] (Scheme IX). The 3',5'-di-O-acetylribonucleoside (prepared by the orthoester exchange procedure[44] as described for type I derivatives) is treated with 2,3-dihydro-4-pyran in the presence of a small quantity of p-toluenesulphonic acid in dioxan to give XXIX which on treatment with methanolic ammonia gives a mixture of two diastereomers of 2'-O-tetra-

hydropyranylribonucleosides. The higher melting isomer of the latter compound is treated with a small excess of pivaloyl chloride in pyridine solution to give the 5'-O-pivaloyl-2'-O-tetrahydropyranyl derivative.



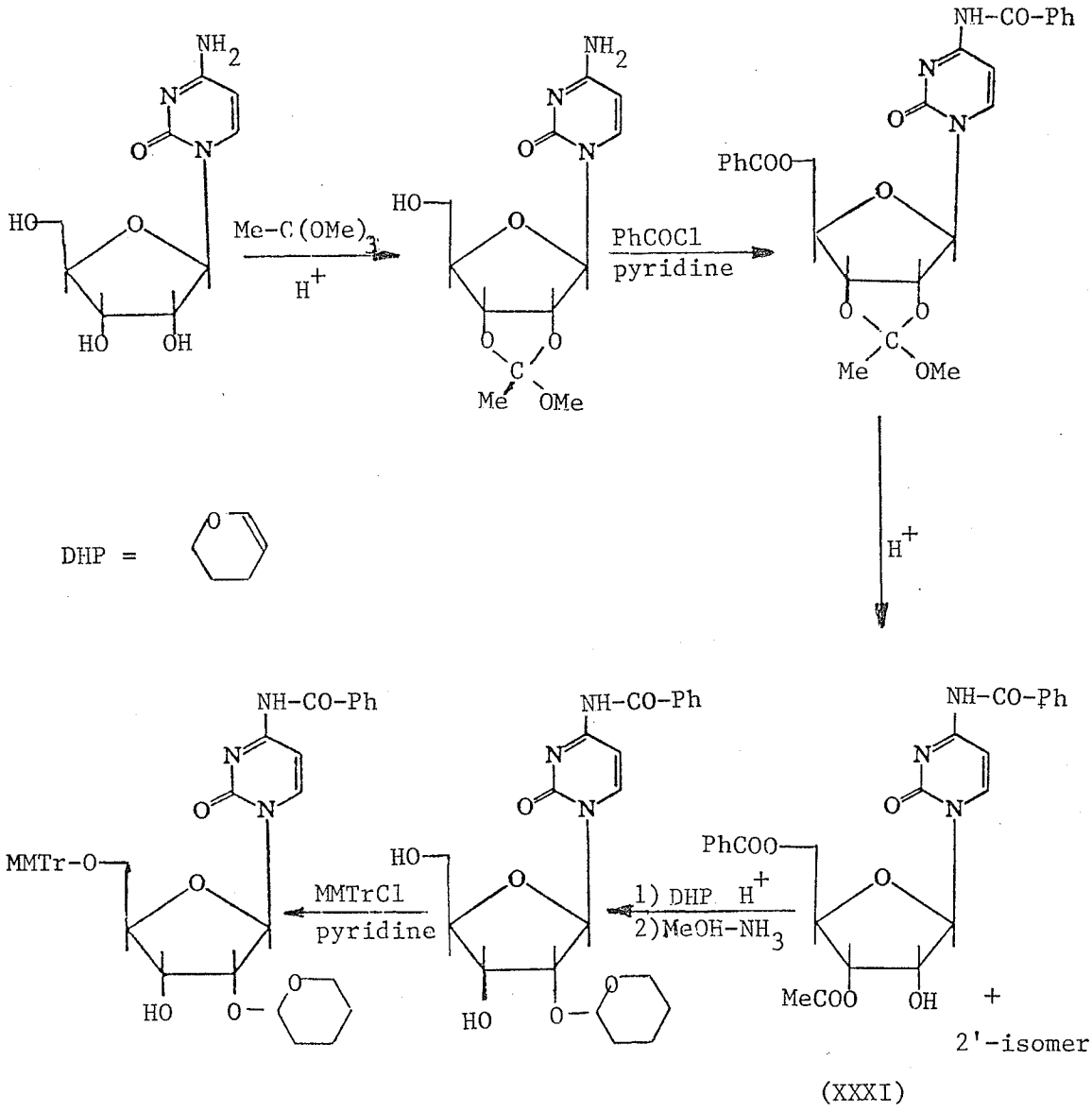
Pv = $(CH_3)_3C.CO$ (XXX)

Scheme IX

Type II compounds having two acid labile but different protecting groups have been synthesised by Neilson and Werstiuk[48]; Scheme X is an example. Cytidine is converted to 2',3'-O-methoxyethylidenecytidine by Reese's procedure[44] and the compound thus obtained is treated with benzoyl chloride in pyridine giving N^4,5'-O-dibenzoylcytidine in 95% yield over two steps from cytidine. Acetic acid (5%) opens the cyclic ortho-acetate ring system to give a mixture of the 3' and 2'-O-acetyl derivatives. Fractional crystallisation helps to get 3'-O-acetyl-N^4, 5'-O-dibenzoylcytidine in 35% yield. Reaction of XXX with dihydropyran and toluene-p-sulphonic acid in anhydrous dioxan gives the corresponding 2'-O-tetrahydropyranyl derivative which on deacetylation with methanolic ammonia results in a diasteromeric mixture of N^4-benzoyl-2'-O-tetrahydro-pyranylcytidine. Silica gel chromatography can separate this mixture into low Rf (high melting) and high Rf (low melting) components in 30 and 34%

yields respectively. Treatment of the low Rf compound with one equivalent of monomethoxytrityl chloride in anhydrous pyridine gives the expected N^4-benzoyl-5'-O-monomethoxytrityl-2'-O-tetrahydropyranyl-cytidine in 96% yield.

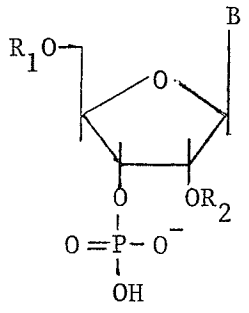
Scheme X. Synthesis of Type II protected nucleosides



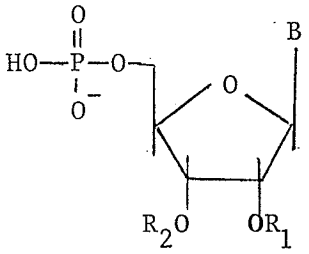
The 2'-hydroxy function in ribonucleosides has also been protected by the benzyl group[49,53] and such protected nucleosides have been used in synthesising uridylyl-(3'-5')-uridine by route B. Although this group is more stable than the acyl or α-alkoxyalkyl groups and hence appears to be more suitable for chain elongation, its removal has posed problems. Catalytic hydrogenation, the method used for debenzylation, is accompanied by hydrogenation of the 5,6-double bond of cytidine and uracil residues. Broom and Christensen[52] detected only 10-25% of the reduction of the double bond while complete 2'-O-debenzylation takes place in the case of cytidine. O-Benzylpurine ribonucleosides are quantitatively debenzylated at 3 atmospheres pressure at room temperature.

B. Nucleotide Components

In oligoribonucleotide synthesis, this can either be a ribonucleoside 3'-phosphate (M) as used in route A or a 5'-phosphate (N) as used in route B.



(M)



(N)

The choice of substituents for blocking the 2'- and 5' - in (M) or the 2'- and 3'-hydroxy groups in (N) as a rule is determined by whether the step in which the protected nucleotide is to be used is final

or intermediate in elongation of the oligonucleotide chain. In the first case R_1 and R_2 can be identical and should be similar in character to the blocking groups in the nucleoside component being used. In the second case R_1 and R_2 are chosen so that either can be selectively removed according to the requirement of the synthesis.

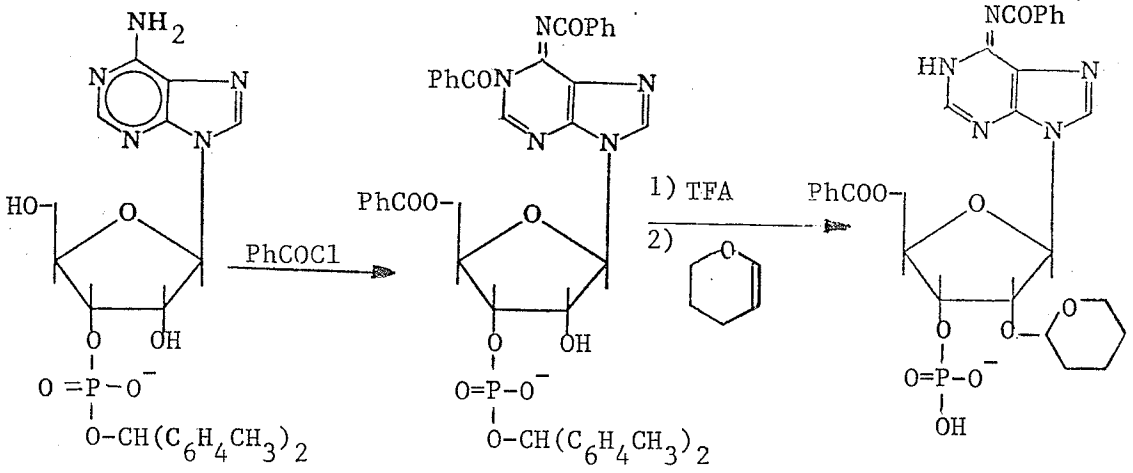
Khorana and co-workers preferred to use acetyl and benzoyl derivatives in cases where $R_1=R_2$ and in other cases where $R_1 \neq R_2$, R_1 was labile under acidic conditions such as mono- or dimethoxytrityl and R_2 was a group labile in alkaline conditions such as acetyl or benzoyl. One procedure[14] used for a pyrimidine-2'(3')-phosphate ($R_1 \neq R_2$) is to convert this compound to the corresponding 2',3'-cyclic phosphate which with monomethoxytrityl chloride gives the 5'-O-monomethoxytrityl-nucleoside-2',3'-cyclic phosphate in good yield. On incubation with pancreatic ribonuclease in aqueous DMF, this compound is quantitatively converted to the corresponding 3'-phosphate. This is followed by acylation using acetic or benzoic anhydride under anhydrous conditions in the presence of excess tetraethylammonium acetate or benzoate. Generally benzoyl protection is preferred.

Holy[56] and Smrt[55] also prepared type M derivatives in the pyrimidine and purine series, where $R_1 \neq R_2$ but R_1 and R_2 substituents have the opposite lability, i.e. R_1 is alkali-labile and R_2 is acid-labile. 2',3'-Cyclic phosphates are acetylated to 5'-O(N)-acetyl derivatives with acetic anhydride in pyridine and then the cyclic phosphate is selectively opened with enzymes to produce 3'-phosphates. Pancreatic ribonuclease is used for this purpose in the case of

pyrimidine derivatives and a mixture of T_1- and T_2-ribonucleases isolated from takadiastase is used for purine derivatives. As enzymatic hydrolysis is done at neutral pH, no deacetylation takes place. Finally the 2'-hydroxyl group is blocked with dihydropyran or vinylethyl ether. Yields at all steps are in the range of 80-90%.

Cramer and co-workers[57] have prepared N,$O^{5'}$-dibenzoyl-2'-O-tetrahydropyranyladenosine 3'-phosphate using the following method (Scheme XI). The 4:4-dimethylbenzhydryl ester of adenosine 3'-phosphate (XXXII) is acylated with benzoyl chloride in pyridine and the resulting tribenzoyl derivative is treated, without isolation, with trifluoroacetic acid (3 minutes,-15°), and then with dihydropyran in DMF to give N,$O^{5'}$-dibenzoyl-2'-O-tetrahydropyranyladenosine 3'-phosphate in 81% yield.

Scheme XI



XXXII

TFA = CF_3COOH

PURPOSE OF STUDY

It is apparent from the above discussion that lengthy procedures are required to suitably protect ribonucleosides which can then be used for oligoribonucleotide synthesis. Overall yields are low and extended syntheses difficult and inefficient. Many of the problems are the results of using unsatisfactory methods of protecting the 2'-hydroxyl group and of the necessity of using acidic or basic conditions for the removal of these protecting groups. Isomerisation of the protecting group between 2' and 3' hydroxyls is very common and separation of these isomers is difficult. Thus, there is a need to find reagents of greater selectivity with respect to protection and removal from reactive sites in ribonucleosides.

"Silylation" of organic compounds - substitution of a labile hydrogen atom with triorganosilyl groups - has found wide applications as an aid for chromatographic[65] and mass spectral studies. The value of silylation in organic synthesis has also become apparent in numerous investigations in the realm of organic chemistry during recent years[62-64].

This appreciation is based on a combination of factors: reactivity of a compound can be altered by blocking certain sensitive reaction centres in the molecule thus protecting the molecule from unwanted attack; several simple procedures are available for replacing reactive protons by trialkylsilyl groups; these protecting groups can be readily removed from the organic compound by hydrolysis; there is an improvement in the solubility of these compounds in non-polar solvents

and the volatility of the product increases as the result of the disappearance of hydrogen bonds, making it possible to analyse and separate these compounds by gas-liquid chromatography and mass spectrometry.

Among the triorganosilyl groups, the most studied has been the trimethylsilyl(TMS) moiety due to the ready availability of trimethylchlorosilane and by virtue of the relative compactness of the $Si(CH_3)_3$ group, which facilitates silicon elimination reactions. Though the TMS group has been used widely to protect the hydroxyl functions of carbohydrates, sterols and other alcohols, TMS ethers are too susceptible to solvolysis in protic media (either in the presence of an acid or a base) to be broadly useful in synthesis.

Sterically crowded alkylsilyl groups appeared more promising. The dimethylisopropylsilyloxy linkage is 10^2-10^3 times less readily solvolysed then the trimethylsilyloxy linkage[66,67], but is still too labile to survive such processes as the Grignard reaction, Wittig reaction or Jones(CrO_3) oxidation[58].

It appears that bulky substituents at the silicon atom retard the reaction as seen in the hydrolysis of disilazanes[68]. A similar steric effect of the substituent is observed at nitrogen in a study of the rate of methanolysis of silylamines: $C_6H_5(C_2H_5)N.Si(C_2H_5)_3$ silylates methanol more slowly than does $C_6H_5NH.Si(C_2H_5)_3$[69].

Akerman[70] has compared rates of acidic and basic hydrolysis of trimethyl, triethyl and t-butyldimethylphenoxysilanes. The substitution of a t-butyl group for a methyl group lowers the catalytic rate constant by a factor of 10^5. Compared with triethylphenoxysilane the catalytic

rate constant for t-butyldimethylphenoxysilane is lowered by a factor of 10^2. These low rate constants can, to a large extent, be ascribed to the steric influence of the t-butyl moiety. This group shields silicon from nucleophilic reagents that normally attack silicon. The steric effect is also evident in the relative ratios for reduction of ketones by t-butylsilanes e.g. in the reduction of alkyl substituted cyclohexanones di-tert-butylsilane is observed to be approximately 100 times less reactive than tri-sec -butylsilane[71].

For these reasons the tert-butyldimethylsilyl (TBDMS) protecting group has attracted a great deal of attention recently. Though TBDMS-chloride was synthesised by Sommer and Tyler[72] in 1954 and occasionally used in synthetic organic chemistry as, for example, in the isolation of ketone enolates[73], Corey and Venkateswarlu[58] first exploited the potential of the TBDMS group when they protected hydroxyl groups of a number of alcohols of interest in the synthesis of prostaglandins. They also discovered a very useful property that the TBDMS group could be cleaved by tetra-n-butylammonium fluoride in THF within 30 minutes at room temperature.

Immediately after this, Ogilvie[60,74] in an exhaustive study, showed the importance of the TBDMS group for protecting hydroxyl groups in deoxyribonucleosides.

Since these pioneering applications, the TBDMS protecting group has found a large number of uses, in a short period. Corey[75] for example employed this reagent for the preparation of a key intermediate in the synthesis of maytansine, a structurally complex anti-tumour agent. Beside its further applications by Corey[76] and others[77,78] in

prostaglandin synthesis, new uses have been investigated in the fields of steroids[79-84], carbohydrates [85,86] and cyclonucleosides[120]. Sundram[87] has used it for synthesising radiochemically-labeled reserpine.

Ogilvie et al[88] have recently shown the utility of TBDMS protecting group for synthesising the deoxyribonucleotides TpT and TpTpT in high yields by the triester approach. Besides, they have synthesised several other sterically crowded silylating reagents and studied the selectivity and acid lability of these silyl groups protecting the 3'- and 5'-hydroxyls of the thymidine molecule.

As pointed out earlier, blocking of ribonucleosides is much more difficult because of the presence of two secondary hydroxyl groups. TBDMS or other silyl protecting groups have never been used in a complex case where selective protection of cis hydroxyl groups was required.

Therefore it was planned to investigate the use of the TBDMS protecting group for blocking the hydroxyl functions of ribonucleosides in the following manner.

1. To make a complete study of the silylation reaction of uridine by TBDMS chloride in terms of preparation and separation of the mono, di and tri-substituted derivatives, and their characterisation. To determine the conditions for removing the TBDMS group(s) in acidic, basic and neutral media, and possible isomerisation. To show the usefulness of the protecting group in making hitherto inaccessible nucleoside derivatives.
2. To make a detailed examination of the problems and their solutions in using TBDMS protected ribonucleosides, particularly

2',5'-di-O-protected and 2'-O-protected derivatives, for dinucleotide synthesis by the triester and/or the diester methods, including desilylation of the protected dinucleotide.

3. To determine the compatibility of the TBDMS group with other protecting groups in ribonucleotide synthesis, as discussed above.
4. To make a brief study of the protection of adenosine, cytidine and guanosine employing the same approach as used to study the protection of uridine.
5. To make an exploratory study of the mass spectra of the TBDMS derivatives of ribonucleosides in important cases.

RESULTS AND DISCUSSION

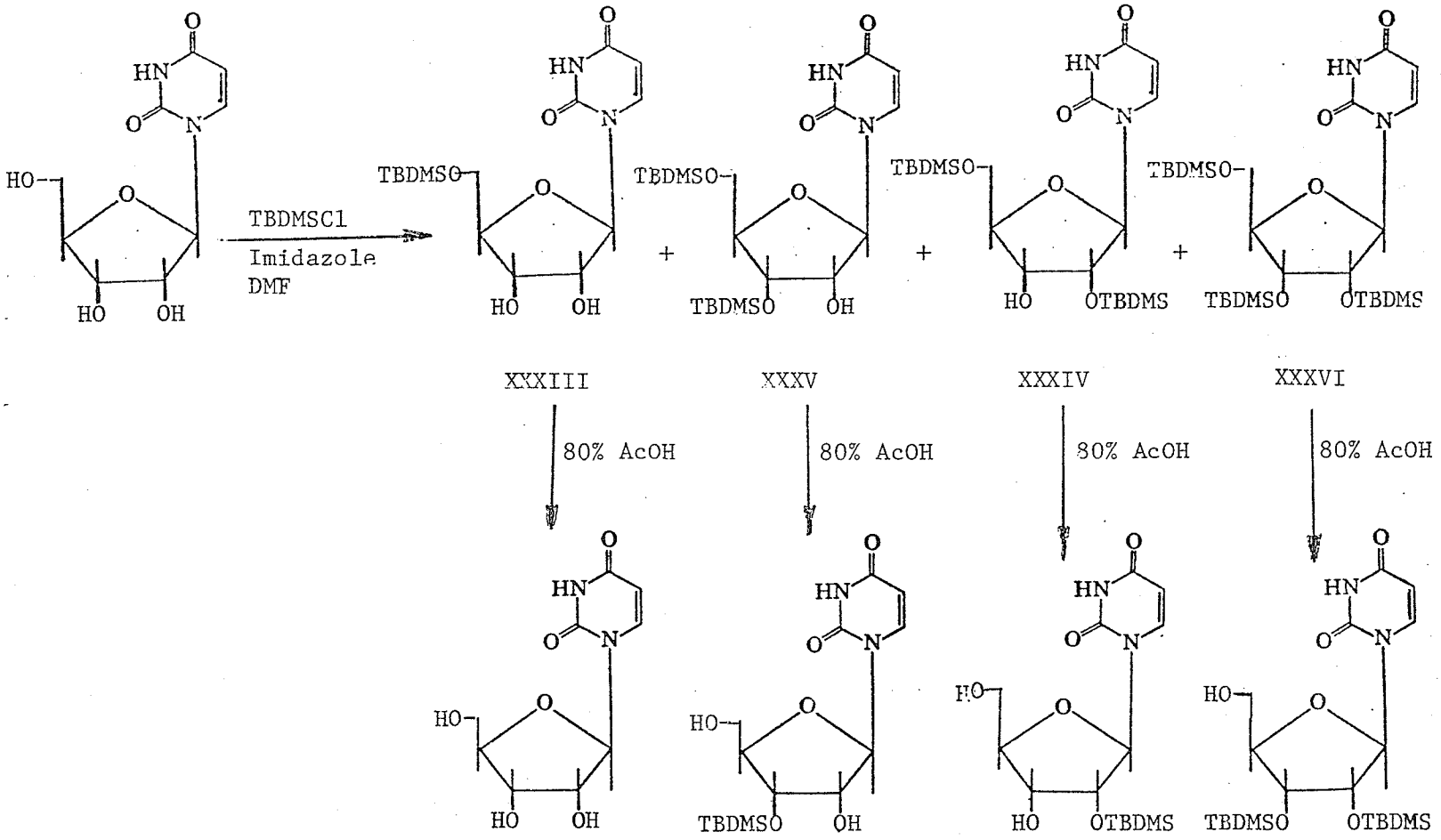
Silyation of Ribonucleosides

General. Uridine was chosen as the model ribonucleoside for evaluating the TBDMS protecting group, as this obviated the need for additional protection of nucleophilic sites on the base residue. Broadly speaking, the silyation reactions were carried out under the same conditions as described by Corey[58] for protecting prostaglandins and by Ogilvie[60,74] for deoxyribonucleosides.

The preparation of the TBDMS derivatives (Scheme XII) was performed , in general, by mixing uridine, TBDMSCl and imidazole in DMF at room temperature, when a homogenous solution was obtained immediately. Pyridine and DMSO replaced DMF in a few cases. Pyridine was found to be convenient while silylating 5'-O-monomethoxytrityluridine. The investigations on this reaction were carried out to find, beside other things, the optimum conditions for preparing 2',5'-di-O-TBDMS-uridine, 5'-O-monomethoxytrityl-2'-O-TBDMSuridine and 2',3',5'-tri-O-TBDMSuridine as these compounds were the starting materials for synthesising ribonucleotides. 2'-O-TBDMSuridine was also important for chain extension at the 3'-end, while 2',3'-di-O-TBDMSuridine could be used as a terminal nucleoside. Both of these compounds were prepared by the acidic hydrolysis of 2',5'-di-O-TBDMSuridine and 2',3',5'-tri-O-TBDMSuridine respectively (Scheme XII).

The direct silylation of uridine by TBDMSCl resulted in four main products, 5'-O-TBDMSuridine, 2',3',5'-tri-O-TBDMSuridine and the two isomers 2',5'-di-O-TBDMSuridine and 3',5'-di-O-TBDMSuridine. The effects of

Scheme XII. Synthesis of the TBDMS derivatives of uridine by direct silylation and their hydrolysis by 80% acetic acid.



different variables on total yields and product ratios are summarised in Table I.

The presence of imidazole was found to be essential by Corey while silylating prostaglandins with TBDMSCl in DMF. Ogilvie[60] has studied the influence of the amount of imidazole on product ratios and total yield in the silylation of deoxyribonucleosides. Similar observations were made in the case of uridine and the most desirable ratio of TBDMSCl:imidazole was found to be 1:2. The requirement of imidazole was not absolute if pyridine was used as a solvent in place of DMF as shown by Ogilvie[60]. Blackburne et al[86] were able to rapidly silylate D-glucal in pyridine at $-10^{o}C$. They observed that the presence of imidazole was unnecessary as they felt that though the reaction was much faster in DMF, the silylation reaction proceeded with considerably lower selectivity. Though no experiments were done for silylating uridine in the absence of imidazole, two important facts may be mentioned. In pyridine, there is no isomerisation (2'⇌3') of the TBDMS group, though in DMF, isomerisation leading to equilibration was noticed. This difference will also influence the final composition of the products.

Recent kinetic experiments have demonstrated the superiority of the silylating system used in these experiments. Kutschinski[89] has compared six reagents prepared from TBDMSCl--analogs of six trimethylsilyl silylation reagents- N-(TBDMS)acetamide, N,O-bis(TBDMS)acetamide, N-(TBDMS)-N-methyltrifluoroacetamide, N-(TBDMS)-imidazole and N-(TBDMS)-dimethylamine in DMF and acetonitrile. The most effective reagent for alcohols was found to be TBDMSCl:imidazole,1:2 mole ratio as used in the

Table I. Silylation of Uridine by tert -Butyldimethylsilyl Chloride

Reactants Ratio[1]	Solvent[2]	Time,Hrs.	Products as Percentages[3] XXXIII	XXXIV	XXXV	XXXVI
1:1.1:2.2	DMF	20	82	← 7 →		4
1:1.5:3.0	DMF	22	60	14	12	6
1:2.2:4.4	DMF	2	11	46	32	6
1:2.2:4.4	DMSO	2	27	35	36	1
1:2.2:4.4	Pyridine	3	20	41	31	2
1:2.5:5.0	DMF	16	15	37	24	18
1:2.5:5.0	DMF	42	15	30	30	18
1:3.5:7.0	DMF	8	← not determined →			63

1. Molar ratio of uridine, TBDMSCl and imidazole in order. In general, 0.125 mmol of uridine was used.
2. DMF and DMSO used were: 1 ml/mmol of uridine. Pyridine used was 4 ml/mmol of uridine.
3. Product ratios were determined by separating the reaction mixture on a silica gel plate, eluting them in methanol and determining their concentrations spectrophotometrically or using ether and ethyl acetate (the latter was particularly required for the compound XXXIII) for elution and actually isolating the products.

studies above.

Ogilive et al[88] have also compared reaction rates of TBDMS derivatives of imidazole and acetamide for silylating thymidine. They found N-(TBDMS)-imidazole and N-(TBDMS)-acetamide to be much slower reagents, though the slower reactions were found to be advantageous for monitoring a reaction by gas chromatography as this permitted greater control over the silylation process.

The silylation reaction could be easily followed by tlc: the development of the chromatogram in THF identified unreacted uridine and 5'-O-TBDMSuridine and the presence of the isomers 2',5' and 3',5'-di-O-TBDMSuridines and 2',3',5'-tri-O-TBDMSuridine was indicated by developing a tlc in low-polarity solvents like ether or ether-hexane(2:1). It may be useful at this stage to mention some observations regarding chromatographic mobility of the various derivatives of uridine. 5'-O-TBDMS-uridine moved more slowly than 2'(3')-O-TBDMSuridine in all of the solvents used and it followed the general pattern seen in TBDMS derivatives of deoxynucleosides[60]. 2',5'-di-O-TBDMSuridine moved faster than the two other isomers 3',5' and 2',3'-di-O-TBDMSuridines in low-polarity solvents. Out of the remaining three derivatives 2',3',5'-tri-O-TBDMSuridine was the most mobile of the possible silyl derivatives of uridine, but the mobilities of 2' and 3'-O-TBDMSuridines were the same in various solvents tried. However, these two derivatives can be distinguished either by GC (after derivatization) or by HPLC, as described later in this thesis.

There was an interesting correlation in the melting points of the seven silyl derivatives of uridine studied, which were highly influenced

by the positions of the substituents. The effect of the substituents on the melting points was in the order 3'TBDMS > 2'TBDMS > 5'TBDMS. The presence of a 5'TBDMS substituent depressed the melting point considerably. These points are well appreciated by noting their melting points which are 204-206°C(3'-O-TBDMSuridine), 191-194°C(2'-O-TBDMSuridine) and 136-139°C(5'-O-TBDMSuridine). These influences were also apparent in disubstituted derivatives, the melting points of which are 218-221°C (2',3'-di-O-TBDMSuridine), 136-137°C(3',5'-di-O-TBDMSuridine) and 121-122°C(2',5'-di-O-TBDMSuridine). The most dramatic impact of the presence of 5'TBDMS was seen in the case of 2',3',5'-tri-O-TBDMSuridine which melted at about 80°C.

Product Isolation. Though the silylation reaction was rapid, for example, 2 equivalents of TBDMSCl completely reacted with uridine in one hour, the longer reaction times as reported in Table I were used to ensure completion of the reaction for the purpose of finding the product ratios more accurately.

The preparative scale separation of the products was performed on thick layer silica gel plates. The separation of the isomers 2',5'-di-O-TBDMSuridine and 3',5'-di-O-TBDMSuridine required care in selecting the solvents for development because their R_f values on the plates were very close. Two or three developments in ether-hexane(2:1) were adequate to separate these two isomers satisfactorily. The separation of 5'-O-TBDMSuridine from unreacted uridine was achieved by developing the plate in ether or ethyl acetate. 2',3',5'-Tri-O-TBDMSuridine which moved faster than all other silyl derivatives could be separated when plates were developed in ether-hexane(2:1).

One very important point to be kept in view while separating 2',5'-di-O-TBDMSuridine and 3',5'-di-O-TBDMSuridine isomers, was the tendency of these two derivatives to interconvert to each other to a noticeable extent when in contact with silica gel for about 4 hours. Therefore it was necessary to develop the plates with no loss of time and then to elute them from silica gel rapidly, in ether. When this precaution was taken, the products were of single spot purity. In fact, this precaution was necessary in all cases where the 2'(3') TBDMS substituent had a free neighbouring hydroxyl group.

The 2',5'- and 3',5'-di-O-TBDMSuridines were obtained as white solids after the solvents were completely removed. In many cases, it was found helpful, after evaporating ether, to remove the trapped solvent and /or moisture by co-evaporating at $\sim 40^{o}C$ with hexane on a flat-bed evaporator, whereupon white solids were obtained. Both these compounds could be crystallised by adding hexane to their solutions in ether till a slight turbidity persisted. 5'-O-TBDMSuridine was eluted from silica gel by ethanol, the removal of which gave a white solid. Crystallisation was accomplished from ethanol-water. 2',3',5'-Tri-O-TBDMSuridine was readily eluted by ether, but it was difficult to obtain this compound as a solid. Only prolonged exposure to vacuum gave a solid. Attempts at crystallisation from different solvents were unsuccessful.

Product Ratio. When a lower ratio of TBDMSCl was used so as to mainly prepare the monosilylated derivative, the 5' isomer was the most predominant product as expected and the 2' and 3' isomers were never obtained in any isolable quantities. While using higher TBDMSCl ratios

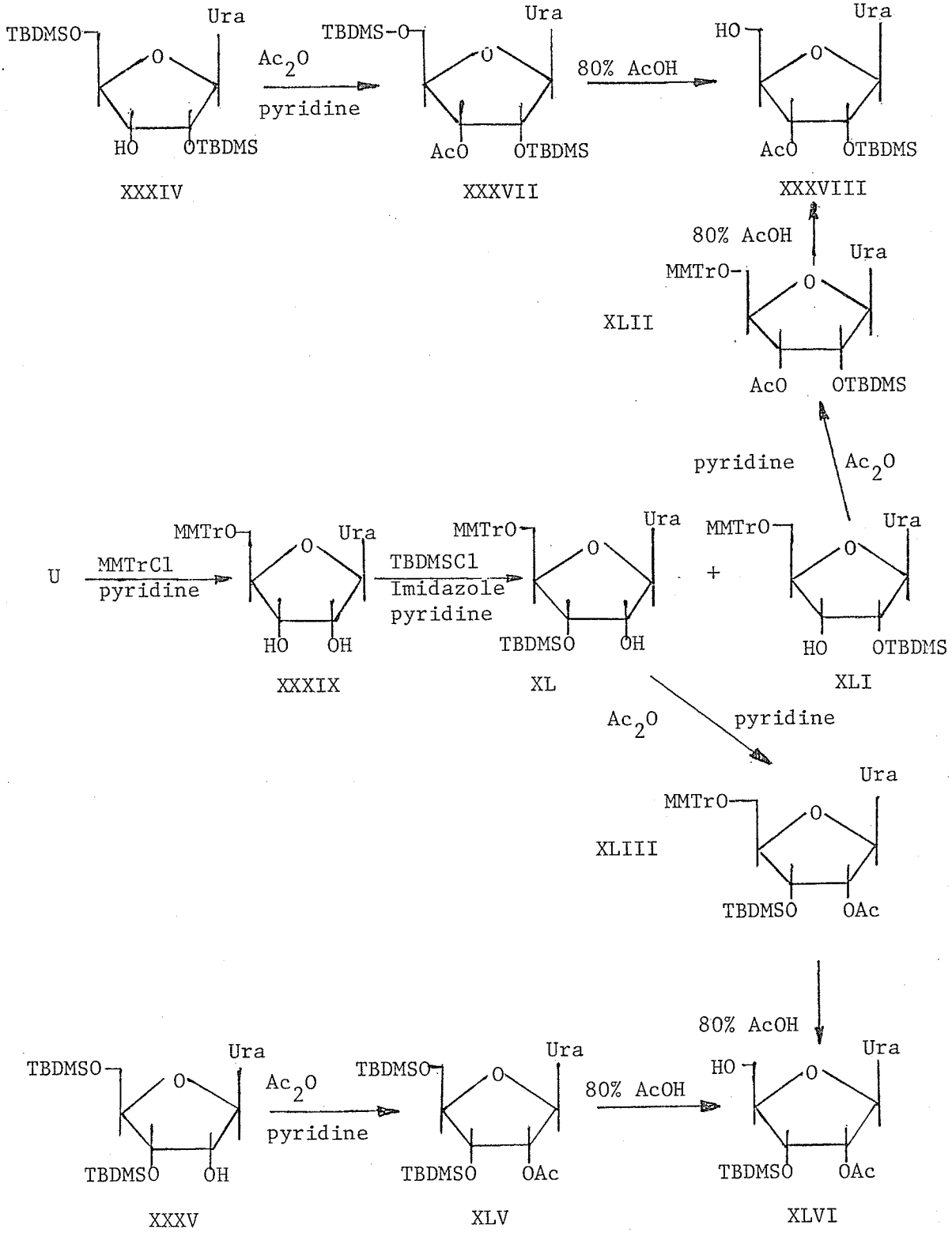
(2.2 equivalents) when di-O-TBDMS derivatives were the desired products, 5'-O-TBDMS(∿11%) and a small quantity of 2',3',5'-tri-O-derivative (∿2-6%) were also always produced.

A study of the effects of the reaction medium on the product composition was made, by replacing DMF with pyridine and DMSO. Under almost similar reaction conditions (2.2 equivalents of TBDMSCl + 4.4 equivalents of imidazole, reaction time, 2 hours) the amount of 5'-O-TBDMSuridine was minimum in the case of DMF(∿11%) and maximum in the case of DMSO(∿27%) while in pyridine it was formed to the extent of 20% (reaction time 3 hours). The corresponding total amounts of isomeric di-O-TBDMS derivatives varied in the same manner in these solvents. The relative proportions of 2',5' and 3',5' isomers were 2:1 in DMF, 4:3 in pyridine and 1:1 in DMSO. One reason for this variance could be different equilibration ratios of the isomers in these solvents (in the presence of imidazole). The predominance of 2',5'-di-O-TBDMSuridine over 3',5'-di-O-TBDMSuridine could be due to more acidic 2' protons, stemming from the electron-withdrawing force of the base moiety as well as steric influence from the 5'-position. The generally observed, selective or quasi-selective alkylation, acylation or tosylation on the 2'-position has been interpreted in this manner[90]. The base ring was not affected when uridine was silylated with 3.5 equivalents of TBDMSCl.

Silylation of 5'-O-Monomethoxytrityluridine. It was found desirable, for the purpose of ribonucleotide synthesis, to synthesise a 2',5'-di-O-protected uridine in which the 5'-hydroxyl group was protected by the acid labile monomethoxytrityl (MMTr) group and the 2'-hydroxyl function by the TBDMS group.

The simplest approach was to start with 5'-O-monomethoxytrityl-uridine as this compound can be easily obtained from the specific reaction of MMTrCl with the primary 5'-hydroxyl group in the presence of pyridine. Initially this compound was isolated and purified before being silylated in DMF, this solvent being found convenient for the silylation reaction with TBDMSCl. Later, it was found, as described earlier, that silylation in pyridine was not disadvantageous, except that it took a little longer to complete the reaction. This was not important, at least in this particular case, as the initial isolation and purification became unnecessary. The actual procedure was straightforward (Scheme XIII). Uridine was monomethoxytritylated using 1.15 equivalents of MMTrCl in pyridine (5 ml/mmol) at room temperature in 10 hours. Immediately afterwards, 1.2 equivalents of TBDMSCl and 2.4 equivalents of imidazole were added and the reaction was almost complete at room temperature in 14 hours. Two main products, 5'-O-monomethoxy-2'(3')-O-TBDMSuridines were formed, which were separated on silica gel plates using ether-hexane(3:2) for development. The faster product was the 2',5'-derivative which could be eluted in ether or ethyl acetate. Co-evaporation with hexane, to remove solvents, gave a white solid in 44% yield. This was used without further purification for the ribonucleotide synthesis. Crystallisation from benzene-hexane gave white crystals

Scheme XIII. Silylation of 5'-O-monomethoxytrityluridine and characterisation of the isomeric products.

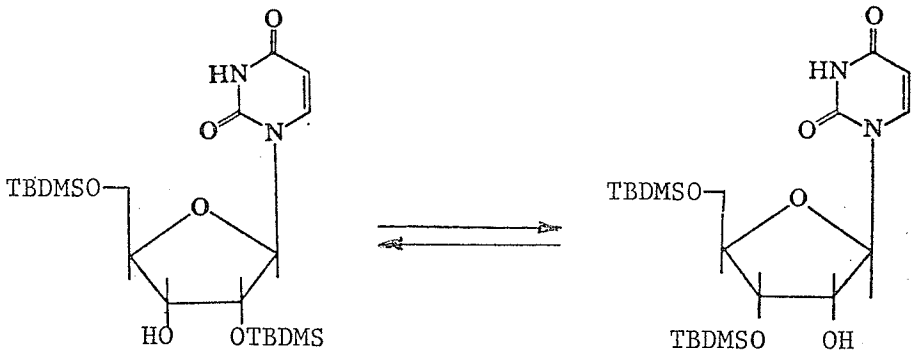


of melting point 141-143°C.

As explained later, to take advantage of the property of 2'⇌3' isomerisation of the TBDMS groups the 'unwanted' 3',5'-di-O-derivative was left on the plates for 3 days and then eluted and again separated on silica gel plates whereupon more of the 2',5' derivative was obtained, thereby increasing the 'effective' yield of the desired 2',5'-di-O-compound by about 20% in a typical case.

Stability Studies

Isomerisation on Silica Gel. This problem has been already referred to, while discussing the isolation of silyl derivatives. The finding that 2',5' and 3',5'-di-O-TBDMSuridines isomerised on silica gel plates was



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very important in this work, from the point of view of the preparation of pure 2',5'-di-O-protected compounds as starting materials for ribonucleotide synthesis and other synthetic applications. In this research Camag DSF-5 silica gel was used for making plates which were used without activation.

It was clear this type of isomerisation would occur in all TBDMS derivatives where a free hydroxyl group was available on the neighbouring

carbon atom. It was not possible to detect this isomerisation in the case of 2'-and 3'-O-TBDMSuridines as their R_f values were too close. In previous work on silylation of deoxyribonucleosides[60,74] (using the same silica gel) as well as in this work, no isomerisation of the kind 3'⇄5' was noticed.

In an interesting study of silica gel detritylation Lehrfeld[94] has referred to the work of Borgstrom[95] who observed acyl migration during the chromatographic purification of 2-O-acylglycerides on silica gel.

The isomerisation of 2',5' and 3',5'-di-O-TBDMSuridines was also observed in basic media such as wet pyridine or ammonia but there was no isomerisation in acidic medium like 80% acetic acid. Lehrfeld[94] examined four well-known commercial brands of silica gel for their detritylation activity and concluded that this activity was not directly related to the quantity of residual acid present in these silica gel samples. The detritylation activity was attributed to catalytic (adsorption) activity of the silica gel used. As silica gel is an acidic adsorbent, there should be no 2'⇄3' isomerisation due to its acidic character as shown independently. Therefore it is possible that isomerisation witnessed was the result of catalytic activity. The isomers 5'-O-monomethoxytrityl-2'-O-TBDMS- and 5'-O-monomethoxytrityl-3'-O-TBDMSuridines also exhibited the tendency to isomerise when in contact with silica gel.

Though it was necessary to take precautions so that products were not contaminated by other isomers, isomerisation proved advantageous in one aspect as it offered a convenient way of converting 3',5'-di-O-protected derivatives to more useful 2',5'-di-O-protected derivatives

(for ribonucleotide synthesis). In this way the "effective" yield of 2',5'-di-O-protected uridines was much higher than 44-45% reported earlier in this work. A contact time on the silica gel of 1-2 days was enough to equilibrate the 3',5'-di-O-protected isomer which was then eluted for further separation of the isomers on plates. This time could be shortened by heating the silica gel at 100°C for about 3 hours, but this was accompanied by some desilylation. The same purpose could be achieved by stirring the 3',5'-di-O-TBDMSuridine with pyridine and water. For a summary of the conditions under which isomerisation took place, see Table III.

Hydrolysis under Acidic Conditions. This study was important as it led to the methods for making 2'-and 2',3'-di-O-TBDMSuridines which can be used as nucleoside components in the synthesis of ribonucleotides. 3'-O-TBDMSuridine could prove useful, along with other derivatives, in chemical transformations. A knowledge of the stability of the TBDMS groups at different positions of the sugar moiety was important in its own right. A study of the stability of the 2'-O-TBDMS group in acetic acid was particularly important as it was planned to protect the 5'-hydroxyl with the acid-labile monomethoxytrityl group so that a dinucleotide chain could be extended from the 5' end, after its removal.

The results of the action of 80% acetic acid on the silyl derivatives of uridine at room temperature and 100°C are summarised in Table II. In none of these cases was any 2'⇄3' isomerisation noticed, unlike the behavior under mild basic conditons.

5'-O-TBDMSuridine was the most susceptible to acetic acid, as

∿95% of the silyl group was cleaved in 15 minutes at 100°C, and 98% was cleaved at room temperature in 18 hours. 2'- and 3'-O-TBDMS groups are much more stable towards 80% acetic acid and their presence retarded the rate of hydrolysis of the 5'-O-TBDMS group. For example, ∿10% of the starting material remained unreacted in the case of 2',5'- and 3',5'-di-O-TBDMSuridines even when the reaction time was twice as long (30 min.at 100°C). In the case of 2',3',5'-tri-O-TBDMSuridine, after 45 minutes at 100°C, about 2% of the starting material was present. A similar retarding influence was observed when acidic hydrolysis was conducted at room temperature. For example, 5% of the starting material remained unreacted after 28 hours in the case of 3',5'-di-O-TBDMSuridine and 32% of the starting material remained unreacted in the case of 2',3',5'-tri-O-TBDMSuridine.

These characteristics permitted the synthesis of 2'-, 3'-, 2',3'-silyl derivatives from 2',5'-, 3',5'- and 2',3',5'-silyl derivatives respectively. Thus it was possible to obtain 2'-and 3'-O-TBDMSuridines in 75% analytical yield from respective disilyl derivatives by the action of 80% acetic acid at room temperature for 21 hours or by heating at 100°C for 30 minutes. Similarly, 2',3'-di-O-TBDMSuridine could be easily obtained from 2',3',5'-tri-O-TBDMSuridine by heating with 80% acetic acid at 100°C for 45 minutes in 75% analytical yield (Scheme XII).

The difference in the stability of the TBDMS group protecting primary and secondary hydroxyls towards 80% acetic acid has been noted before. The TBDMS groups protecting primary and secondary hydroxyl functions of deoxyribonucleosides showed a difference of the same order[88].

The silyloxy linkage in certain prostaglandins was much more labile. For example, 10,11-epoxy-15-tri-p-xylylsilyl-PGA_2 and the corresponding benzylsilyl derivatives were smoothly desilylated by acetic acid-THF-water (3:1:1,v/v) at 26°C in 9 hours[91] (from secondary hydroxyl functions). On the other hand, the silyloxy linkage of some steroid derivatives showed a certain amount of resistance towards 80% acetic acid and hence more acidic conditions such as HCl or trifluoroacetic acid in acetone were required in some cases[80].

The monitoring of the acid hydrolysis reaction by tlc was not convenient using Eastman silica gel coated Chromatograms No. 13181, as these plates do not work well, in general, in the presence of acidic material. Drying by heating and prolonged contact with silica gel caused isomerisation thus making the identification of the products difficult. Glc analysis of the reaction mixture was attempted during a detailed study of the hydrolysis of trialkysilyl derivatives of thymidine by Westmore and co-workers[92]. They found that direct analysis of the reaction mixture was not feasible and it was necessary to remove, in vacuo, the acetic acid and water present. Of course, tlc can also be performed after removing acetic acid.

A study of the stability of TBDMS groups in the presence of p-toluenesulphonic acid monohydrate (in dioxane or acetone) was important as the introduction of other protecting groups like tetrahydropyranyl, 2',3'-isopropylidene and 2',3'-methoxyethylidene into a silylated uridine required the presence of this catalyst. The 5'-TBDMS group was most labile as seen previously in the case of 80% acetic acid. In 1.5 hours, 50% of

the 5'-TBDMS group was cleaved from 2',5'-di-O-TBDMSuridine. Prolonged action cleaved about 40% of the 2'-TBDMS group in 19 hours when 0.57 equivalent of the acid was used. Both of these reactions were carried out at room temperature.

2'-O-TBDMSuridine was converted to uridine by 0.1M methanolic-aqueous HCl, whereas 0.01M of the acid did not have any effect in 2 hours. Trifluoroacetic acid (33% in benzene, v/v) partially desilylated 3',5'-di-O-TBDMSuridine to uridine in 16 hours.

As expected, when 5'-O-monomethoxytrityl-2'-O-TBDMSuridine was detritylated by 80% acetic acid in 4 hours at room temperature, the 2'-TBDMS group remained unaffected.

The mechanism of acidic hydrolysis involved protonation of the oxygen atom, followed by nucleophilic attack by water on silicon as shown below in a general case[93].

$$H_3O^+ + R_3SiOR' \rightleftharpoons R_3SiOHR'^+ + H_2O$$

$$R_3SiOHR'^+ + H_2O \rightleftharpoons R_3SiOH_2^+ + R'OH$$

$$R_3SiOH_2^+ + H_2O \rightleftharpoons R_3SiOH + H_3O^+$$

Table II. Hydrolysis of the TBDMS, Isopropylidene and Monomethoxytrityl Derivatives of Uridine by 80% Acetic Acid.

Compound	Temp.	Time		Products as Percentages			
			Unreacted	2'-O-TBDMSU	3'-O-TBDMSU	2',3'-Di-O-TBDMSU	U
		hrs.	Material%				
U-OIP	RT	3.5	95	-	-	-	5
	RT	24	60	-	-	-	40
	85°C	1	2	-	-	-	98
5'-O-TBDMSU	RT	18	2	-	-	-	98
	100°C	0.25	5	-	-	-	95
	100°C	0.50	-	-	-	-	100
2',5'-Di-O-TBDMSU	RT	5	48	52	-	-	-
	RT	21	5	82	-	-	6
	100°C	0.50	11	75	-	-	14
3',5'-Di-O-TBDMSU	RT	4.50	58	-	42	-	-
	RT	28	5	-	69	-	26
	100°C	0.50	10	-	75	-	15
2',3',5'-Tri-O-TBDMSU	RT	5	75	-	-	25	-
	RT	28	32	← 5 →		62	-
	100°C	0.75	2	← 15 →		75	8
2',3'-Di-O-TBDMSU	100°C	0.50	95	← 5 →			
5'-O-MMTr-2'-O-TBDMSU	RT	4	-	98	-	-	2

Stability under Phosphorylating Conditions. One of the main objectives of preparing TBDMS derivatives was to be able to use them for the synthesis of ribonucleotides. 2',5'-Di-O-TBDMSuridine, 2'-O-TBDMSuridine and 2',3'-di-O-TBDMSuridine were of particular interest in this context. Therefore it was essential to study the stability (cleavage and isomerisation) of the TBDMS group at 2'(3') and 5' positions of uridine under the conditions used for ribonucleotide synthesis.

Pyridine, which is the most commonly used reaction medium for phosphorylation, did not cause isomerisation or hydrolysis when 2',5'-di-O-TBDMSuridine was stirred with this solvent for prolonged periods (up to 75 hours).

TPS, which is one of the most commonly used activating agents in the phosphorylating and condensation reactions, was next added to the above pyridine solution. Again, there was no isomerisation or cleavage of either of the two TBDMS groups in 24 hours. The addition of water, which is necessary after phosphorylation to break up pyrophosphates and to hydrolyse TPS, did not cause any isomerisation but it did result in a slight cleavage of the TBDMS group after 18 hours. In order to have correct data, TPS and water were added approximately in the same proportions as these are generally present in an actual phosphorylation reaction. It was observed that about 10% of the 5'-TBDMS group was cleaved when stirring with water was prolonged to 48 hours, although in actual practice it is not necessary to stir the phosphorylated reaction mixture for this length of time.

Treatment of 2',5'-di-O-TBDMSuridine with the pyridinium salt of β-cyanoethyl phosphate (in pyridine) for 12 hours caused cleavage

of 5% of the 5'-TBDMS groups. This type of cleavage increased to 40% after stirring for 66 hours. In an actual phosphorylation reaction, an excess of pyridine and TPS are present and, moreover, there is no need to stir for more than 6 hours. This experiment merely indicated the possible lability of 5'TBDMS towards the acid phosphate under extreme conditions.

DCC is another activating agent used in nucleotide synthesis. This was found to be unsuitable for phosphorylating a pyridine solution of 2',5'-di-O-TBDMSuridine as isomerisation to 3',5'-di-O-TBDMSuridine took place (∿40% in 23 hours).

These experiments indicated that under normal phosphorylating conditions, no cleavage or isomerisation of the TBDMS group occurred at the 2' or 5' position. Its stability was exceptional in pyridine as compared with acyl groups. For example, Reese[96] found that 2'-and 3'-O-acetylribonucleoside isomerised rapidly even in anhydrous pyridine. The 2'(3')-TBDMS group isomerised only in wet pyridine or in refluxing pyridine.

5'-O-Monomethoxytrityl-2'-O-TBDMSuridine was also tested under similar conditions. The monomethoxytrityl group was much less stable under acidic conditions. The 2'-TBDMS group isomerised to the 3'-TBDMS position, in the same manner as in 2',5'-di-O-TBDMSuridine discussed above.

Stability under Basic Conditions. In general, 2'(3')TBDMS isomerisation (2'⇌3') took place in the presence of a free neighbouring hydroxyl group under mildly basic conditions. There was a cleavage of the TBDMS group, preferentially from the 2'(3') positions as compared with the 5' position,

under stronger basic conditions.

While discussing the stability of the TBDMS group under phosphorylating conditions, it was mentioned that unlike acyl groups, the TBDMS group was very stable in dry pyridine. In the presence of moist pyridine and at refluxing temperature, it was not completely stable. However, when neighbouring hydroxyls were blocked, no isomerisation occurred.

As the silylation reaction was conducted primarily in DMF, it was interesting to study isomerisation in this solvent. In 5 hours, about 20% of 2',5'-di-O-TBDMSuridine had isomerised to 3',5'-di-O-TBDMSuridine. The presence of imidazole, which was the catalyst used invariably for silylation, hastened the isomerisation.

Ethanolic ammonia (7M) caused isomerisation and a small amount of cleavage at room temperature in the case of 2',5'- and 3',5'-di-O-TBDMS-uridine. There was complete desilylation in the case of 2',5'-di-O-TBDMSuridine by 0.5M NaOH (dioxan-water, 1:1) in about 36 hours at room temperature.

The action of ethanolic ammonia (7M-9M) at 60-70^{o}C was studied in greater detail, because of somewhat unexpected and interesting results. The results of this study are summarised in Table IV. The 2'-TBDMS group was found to be much more labile than the 5'-TBDMS group. For example, 2'-TBDMS was cleaved ($\sim$90%) in 4 hours, whereas there was hardly any desilylation in 3 hours in the case of 5'-O-TBDMSuridine.

Some steric influence of 5'-TBDMS group on the hydrolysis of 2'- and 3'-TBDMS groups was apparent. For example, only 25% cleavage of the 2'-TBDMS group was observed from 2',5'-di-O-TBDMSuridine in 2.5 hours.

Under these conditions, the 5'-O-TBDMS group was not completely stable. Thus 50% cleavage of the 5'-TBDMS group occurred in 10 hours from 2',5'-di-O-TBDMSuridine.

The behaviour of 2',3'-di-O-TBDMSuridine was particularly instructive. Even after 55 hours of heating, there was no desilylation. It showed that blockage of neighbouring hydroxyls completely prevented the hydrolysis of 2',3'-di-O-TBDMSuridine.

The reversal of the lability of 5'-and 2'(3')-TBDMS groups in acidic and basic media is not without a precedent. Hosoda et al[80] noticed a similar difference between alcoholic and phenolic ethers of steroids. The TBDMS ether of phenol is more stable to acid than that of alcohol. In contrast, the phenolic ether is susceptible to basic conditions at room temperature while the alcoholic ether is stable to base. These findings were supported by the data obtained in the present series of experiments (Table IV). It will be noticed that the 3'-TBDMS group was slightly more stable to the base attack as compared with the 2'-TBDMS group. The 2'-OH was more acidic (phenolic) as explained earlier, than the 3'-OH and thus would be more susceptible to base cleavage of its silyl ether.

The marked difference in the rate of hydrolysis of 2'(3')-O-TBDMS-uridine and 2',3'-di-O-TBDMSuridine in ammonia could be explained by the existence of a five-member transition state when a neighbouring hydroxyl group was free. The high reactivity of 2-trimethylsilyl-pyridine and similar systems (they cleaved under neutral conditions) has been explained on this basis[143].

Table III. Isomerisation of 2',5'/3',5'-Di-O-TBDMSuridines in Various Media.

Compound	Medium	Temp.	Time hrs.	∿ Ratio 2',5':3',5'	∿ Ratio 3',5':2',5'
2',5'-Di-O-TBDMSU	Silica gel plates*	RT	44	8:1	-
3',5'-Di-O-TBDMSU	Silica gel plates*	RT	44	-	8:1
3',5'-Di-O-TBDMSU	Silica gel plates*	100°C	2	-	8:1
3',5'-Di-O-TBDMSU	Pyridine-water	RT	6	-	4:1
3',5'-Di-O-TBDMSU	Pyridine-water	RT	22	-	3:2
2',5'-Di-O-TBDMSU	DMF	RT	3	2:1	-
2',5'-Di-O-TBDMSU	DMF	RT	24	1:1	-
2',5'-Di-O-TBDMSU	DMF-imidazole	RT	6	1:1	-
2',5'-Di-O-TBDMSU	Triethylamine	RT	1	No change	-
2',5'-Di-O-TBDMSU	Buffer pH 10.4 ($NaOH+NaHCO_3$)	RT	12	3:2	-
3',5'-Di-O-TBDMSU	Silica gel plates **		48	-	3:1

* Isomerisation appeared to be dependent on the age of the plates. These plates were prepared 10 weeks before.

** 24 hours old plate

Table IV. Action of 7M-9M Ethanolic Ammonia at 60-70°C on the TBDMS Derivatives of Uridine and Adenosine.

Compound	Time hrs.	Conc.	Isomerised product (2',5'/3',5')	5'-O-TBDMSU/A	U/A
2'-O-TBDMSU	4	9M	-	-	90%
5'-O-TBDMSU	3	9M	← No change →		
2',3'-Di-O-TBDMSU	55	7M	← No change →		
	5	9M	← No change →		
3',5'-Di-O-TBDMSU	3	7M	Present	Nil	Nil
	5	7M	Present	Present	Nil
2',5'-Di-O-TBDMSU*	2.5	9M	Present	25%	Nil
	5	7M	35%	35%	Nil
	10	7M	Nil	50%	50%
2',5'-Di-O-TBDMSA*	4	9M	30%	30%	5%
3',5'-Di-O-TBDMSA*	4	9M	23%	27%	2%

* Starting material present can be found by difference.

The Cleavage of Silyl Groups by Tetra-n-Butylammonium Fluoride(TBAF).

The importance of the silyl protecting groups in synthetic organic chemistry was greatly enhanced by Corey's[58] discovery that TBAF can cleave the silyl groups with great ease in a short reaction period (30 minutes). The cleavage of the silyl groups is therefore not dependent upon acidic or basic hydrolysis.

The TBDMS protecting groups of uridine, irrespective of their positions on the sugar moiety, were smoothly removed by this reagent in 30 minutes. It has been implied since the initial experiments by Corey[58] and other workers that the reaction proceeded well only in dry THF. It was shown in this work that this was not an essential condition. For example, 2'-O-TBDMSuridine could be desilylated by TBAF in a mixture of pyridine-THF-water (1:8:1, v/v) in 30 minutes.

Golding *et al*[97] have pointed out that acyl migration can take place when TBAF is used for removing the TBDMS protecting groups from a protected glycerol, possibly because the fluoride ion in THF was sufficiently basic to catalyse such 1,2 acyl migrations. A similar observation was made in this work while attempting to make 2'(3')-O-pivaloyl protected uridines (discussed later in this thesis).

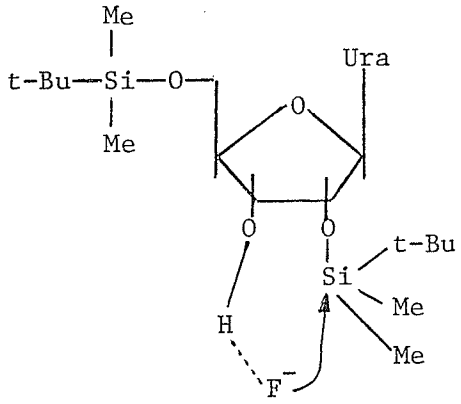
Ogilvie *et al*[98] have shown that the rate of nucleophilic attack by fluoride ions can be moderated in the presence of glacial acetic acid, probably because of strong solvation of fluoride ions by acetic acid. It was thought, therefore, that acyl migration may not occur if desilylation was done by TBAF in the presence of acetic acid. Therefore various combinations of glacial acetic acid, 80% acetic acid and other solvents with TBAF were studied for removing the TBDMS group.

The desilylation of 2'-O-TBDMSuridine by TBAF-H_2O-AcOH (1:16:20, molar ratio) went to the extent of 50% in 24 hours. When 2',5'-di-O-TBDMSuridine was treated with 10 equivalents of TBAF in the presence of 400 equivalents of glacial acetic acid (per silyl group), ∿50% desilylation had occurred from both the 2' and 5' positions after 12 hours and desilylation was complete after 26 hours. These reaction conditions allowed simultaneous cleavage of 2'- and 5'-TBDMS groups while previous work had shown the 2'-TBDMS group to be more base labile and the 5'-TBDMS group to be more acid labile. These studies showed that TBAF could be used, under appropriate conditions, for selective cleavage.

When the above experiment was repeated but using 5 equivalents of TBAF, the reaction mixture, on examination after 5 hours, showed the absence of 3',5'-di-O-TBDMSuridine though the starting substance was still present. This indicated that under these conditions, there was no migration (2'⇄3') of the TBDMS group. This experiment also showed there was a possibility of preventing acyl migration if desilylation was performed using glacial acetic acid and TBAF together. No further work was done to find whether acyl migration could be actually prevented in this way.

The hydrolysis by ethanolic ammonia at 60°C indicated that the 2'(3')-TBDMS group was much more base labile as compared with the 5'-TBDMS group. This finding could not be confirmed when TBAF was used for desilylation, due to the rapidity of this reaction. In one experiment where the activity of fluoride ion was moderated by desilylating with 20 equivalents of TBAF in methanol-THF (3:5 v/v), the 2'-TBDMS group was seen to cleave more readily than the 5'-TBDMS group. After 24 hours, about

one-half of 2',5'-di-O-TBDMSuridine was converted to uridine and the other half was present as 5'-O-TBDMSuridine. This preferential attack of the fluoride ion on the 2'(3') position, was similar to that seen in the case of ethanolic-ammonia at 70°C and could be due to the hydrogen bonding of fluoride ion to the adjacent free hydroxyl group. Miller et al[141,142] have noticed acceleration of reactions involving KF due to the formation of an hydrogen bond.



Compatibility of the TBDMS Groups with Other Protecting Groups

In earlier studies[60] on the use of the TBDMS group for the protection of hydroxyl groups of deoxyribonucleosides, the advantages of the TBDMS group with regard to its compatibility with other protecting groups have been described. It will be advantageous at this stage to know about the compatibility of the TBDMS protecting groups with other protecting groups in the ribo series. Unlike 2'-O-tetrahydropyranyl and methoxytetrahydropyranyl groups the 2'(3')-O-TBDMS group would be compatible with acid-labile protecting groups like mono-, di- or trimethoxytrityl for protecting 5'-hydroxyls because of the increased stability of

2'(3')-O-TBDMS groups towards 80% acetic acid (see Table II). This property has proved useful in the synthesis of oligoribonucleotides as described later in this research. The introduction of base-labile protecting groups like acetyl and benzoyl proved easy in the presence of absolute pyridine because there was no danger of 2'⇌3' isomerisation.

In selecting compatible protecting groups, the limiting factors, in general, are:

1. the tendency of the 2'(3')-TBDMS group to isomerise in basic media in the presence of a free neighbouring hydroxyl group,
2. the cleavage of the 5'-TBDMS group by 80% acetic acid at 100°C (15 minutes, ~95%) and at room temperature (5 hours, ca. 70%). The actual times may be longer depending on other substituents present. The susceptibility of the TBDMS group to p-toluenesulphonic acid, pyridine hydrochloride and other acidic conditions should also be kept in view,
3. the desilylation of the 2'(3')-TBDMS group in 7M ethanolic ammonium hydroxide at 60°C while the 5'TBDMS group remained largely unaffected under these conditions.

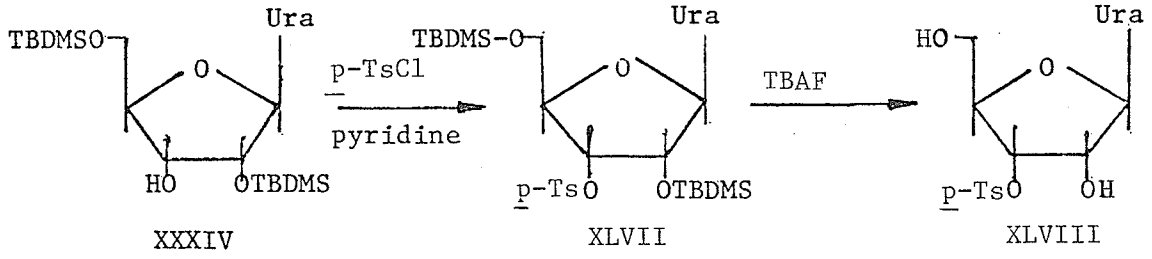
The Characterisation of the TBDMS Derivatives of Uridine

The positions of the TBDMS substituents in the uridine derivatives were determined by converting them into known compounds except in the case of 2',3',5'-tri-O-TBDMSuridine.

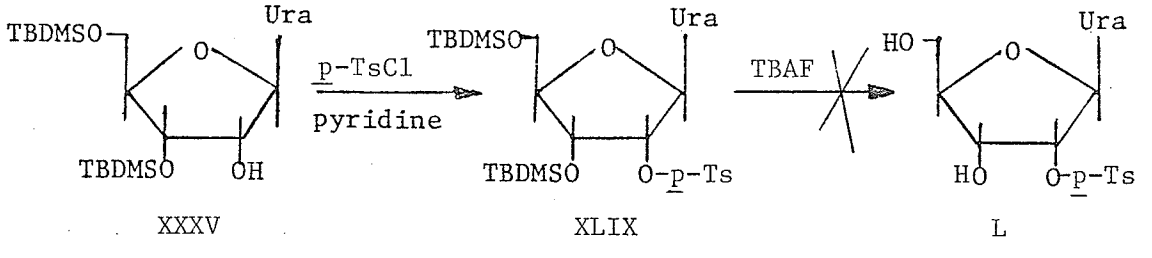
Tosyl Derivatives of Di-O-TBDMSuridines. The orientations of 2',5' and 3',5'-di-O-TBDMSuridines were determined by making the p-toluenesulphonyl (tosyl) derivatives since the TBDMS group did not isomerise under tosylation conditions.

It is also known that sulphonyl esters, unlike their carboxylic counterparts, do not undergo "acyl" migration[99]. Another important consideration is that p-toluenesulphonates are reasonably stable to acid, neutral and mild basic conditions[100]. The methanesulphonyl (mesyl) derivatives were not preferred because of the possibility of facile removal of the group while cleaving the silyl groups by TBAF, although mesylation would be less impeded by steric hindrance as compared with tosylation[101].

The tosylation of the secondary hydroxyl groups in the case of silyl derivatives, was done by using 6 equivalents of p-toluenesulphonyl chloride at 0°C in the presence of pyridine.



XXXIV XLVII XLVIII



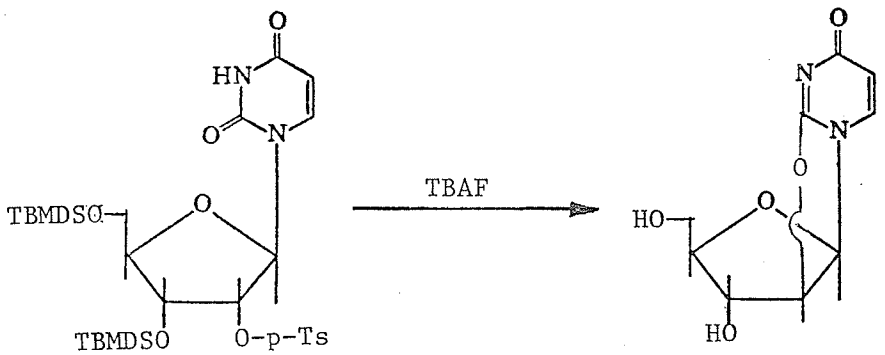
XXXV XLIX L

The products were separated from their respective starting materials and some side products by silica gel chromatography on plates using either hexane-ether (1:2) or benzene-ether (1:2 or 2:1). Ultraviolet absorption spectra ruled out any sulphonation of the uracil ring. The mass spectra, in both cases, showed a peak at m/e M-15(611) and a prominent peak at M-57(569), the latter peak is a characteristic of all nucleosides and many other classes of compounds protected by the TBDMS group. Partial mass spectra of these derivatives have been described later in a separate section.

Synthesis of 3'-O-p-Toluenesulphonyluridine. 2',5'-Di-O-TBDMS-3'-O-tosyluridine could be desilylated by TBAF in 45 minutes using 8 equivalents of the reagent. The product was separated by silica gel chromatography. After two crystallisations from absolute alcohol, a pure product of m.p. 204-205°C (literature value[102] 205-206°C) was obtained. UV absorption maxima in ethanol were at 261nm and 225nm.

The mass spectrum showed a parent peak at m/e 398. Some other fragments of interest were at m/e 112(B), 113(B+H), 155(p-tosyl), M-18, 298(M-uracil+H). The IR absorption bands due to sulphonic ester were at 1180 and 1355(KBr disc). This synthesis proved the structure of 2',5'-di-O-TBDMSuridine. According to the literature[103,139], the melting point of 2'-O-p-toluenesulphonyluridine is 172-174°C[139] and 175-176°C[103].

Characterisation of 3',5'-Di-O-TBDMSuridine. While there was no difficulty in tosylating 3',5'-di-O-TBDMSuridine as described above, the desilylation of the tosylated product did not proceed in the same way as seen in the case of 2',5'-di-O-TBDMSuridine derivative. A treatment with TBAF (∿4 equivalents) for 40 minutes followed by separation of the reaction products on a silica gel plate (developed in THF) indicated the presence of one major product (>80%). After elution with ethanol, this product exhibited UV absorption maxima at 249nm and 224nm. Such a dramatic hypsochromic shift in the UV absorption suggested the formation of an anhydro-linkage as shown below.



XLIX LI

It is known that mono-sulphonyloxylated derivatives of uridine such as 2'-O-tosyl-5'O-acetyluridine react readily with MeOH-NH_3[114,138] to form 2,2'-anhydrouridine,LI. The 2,2' oxygen linkage is readily formed under basic conditions as a result of a rearward nucleophilic attack upon C-2' by the 2-carbonyl oxygen, resulting in a displacement of the 2'-sulphonyloxyl group. 3'-O-Tosyluridine did not form a 2,3'-anhydrouridine by treatment with alkali or ammonia even under very vigorous conditions. The ready conversion of XLIX to an anhydro derivative LI appeared to be due to the presence of tosyl group at the 2' positon,

proving the structure of the starting substance to be 3',5'-di-TBDMS-uridine.

Characterisation of 5'-O-Monomethoxytrityl-2'-O-TBDMS and 5'-O-Monomethoxytrityl-3'-O-TBDMSuridines. (Scheme XIII)

The acetylation of the 3'(2')-hydroxyl group of the 2'(3')-O-TBDMS protected uridine was a simple reaction using excess of acetic anhydride in the presence of dry pyridine and there was no evidence of any 2' TBDMS $\rightleftharpoons$ 3'TBDMS isomerisation under these reaction conditions.

2'-O- and 3'-O-TBDMS derivatives of 5'-O-monomethoxytrityl-uridine were completely converted to their 3'-O and 2'-O-acetyl derivatives respectively in a 15 hour reaction period. The acid labile 5'-O-MMTr group was then removed by 80% acetic acid at room temperature in 4 hours. The 2'(3')-O-TBDMS group remained intact under these conditions, as discussed earlier. The resulting 2'(3')-O-acetyl-3'(2')-O-TBDMSuridines were purified by silica gel chromatography on plates. The ether eluants of both these compounds gave white solids on removal of ether which were finally purified by crystallisation from ether-hexane.

The two acetyl derivatives XLVI and XXXVIII were also prepared from 3', 5'-di-O-and 2', 5'-di-O-TBDMSuridines using identical conditions for the acetylation reaction. The structures of these disilyl derivatives have been proved as discussed above. The 5'-TBDMS group being more acid labile than the 2'(3')-TBDMS group was removed by treatment with 80% acetic acid at 100^{o}C for 75 minutes.

The melting points of the derivatives 2'-O-acetyl-3'-O-TBDMS-uridine (209-211^{o}C) and 3'-O-acetyl-2'-O-TBDMSuridine (201-203^{o}C)

prepared by two different routes, were identical. The mixed melting points did not show any depression, proving the identical nature of these substances and hence proving the structures of 2'-O and 3'-O-TBDMS-derivatives of 5'-O-monomethoxytrityluridine. As a check, the mixed melting point of 3'-O-acetyl-2'-O-TBDMSuridine and 2'-O-acetyl-3'-O-TBDMSuridine (∿1:1) was found to be 160-185°C.

In both cases, the mass spectra of the same compound prepared by different routes were identical in all respects. The mass spectra of the isomers have been discussed separately, later in this thesis.

Characterisation of 2'-O-, 3'-O- and 2',3'-Di-O-TBDMSuridines.

There was very little doubt about the structures of these derivatives, which were obtained by the removal of 5'-TBDMS groups from 2',5'-di-O-, 3',5'-di-O- and 2', 3', 5'-tri-O-TBDMSuridines by the action of 80% acetic acid, as there was no evidence of 2'TBDMS ⇄ 3'TBDMS isomerisation in acidic media. Further, all these derivatives have sharp melting points.

As the structures of 5'-O-monomethoxytrityl-2'(3')-O-TBDMS-uridines have already been proved, a method was available to determine the orientation of 2'-O- and 3'-O-TBDMSuridines by converting these two compounds to their respective 5'-O-monomethoxytrityl derivatives. The reaction of 2'-O- and 3'-O-TBDMSuridines with monomethoxytrityl chloride in the presence of pyridine gave mainly 5'-O-monomethoxytrityl compounds which were purified on silica gel plates. The melting point of 5'-O-monomethoxytrityl-2'-O-TBDMSuridine prepared by this method, after crystallisation, was the same as that of the compound obtained previously by silylation of 5'-O-monomethoxytrityluridine. The mixed melting point

was not depressed (141-143°C). Their R_f values were also identical and the mass spectra of both these compounds showed a parent m/e peak at 630 and a prominent peak at 573(M-57).

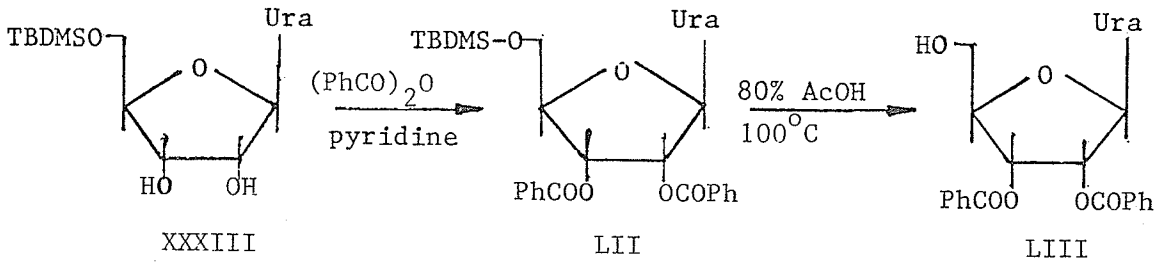
5'-O-Monomethoxytrityl-3'-O-TBDMSuridine was also identical to the compound previously obtained, with respect to R_f value, melting point and absence of any depression in their mixed melting points (106-109°C). The mass spectra showed a parent m/e peak at 630 and a prominent peak at 573(M-57) in both cases.

As 2'-O- and 3'-O-TBDMSuridines can not be distinguished by tlc, this method of converting them to 5'-monomethoxytrityl derivatives can be useful, as these derivatives have different R_f values for tlc in ether-hexane (2:1) and other solvent systems.

2',3'-Di-O-TBDMSuridine was characterised in a similar manner by first blocking 5'-hydroxyl with the monomethoxytrityl group and then removing the TBDMS groups by TBAF. The product thus obtained, after being crystallised from benzene, had the melting point 103-105°C, which was not depressed when a mixed melting point with authentic 5'-O-monomethyltrityluridine was measured.

Characterisation of 5'-O-TBDMSuridine.

The existence of free hydroxyl groups at 2' and 3' positions was determined by synthesising the known compound 2',3'-di-O-benzoyluridine LIII according to the following reaction sequence.



The dibenzoyl derivative LII was purified by chromatography on silica gel plates and was obtained in an excellent yield (∿98%). Its identity was proved from its mass spectrum which showed a prominent m/e peak at 509(M-57)-the loss of 57 amu being characteristic of all compounds having a TBDMS group. Its ultraviolet absorption maxima in ethanol at 257nm and 230nm also helped to confirm the structure of the compound LII.

The removal of the 5'-TBDMS group to obtain the desired product LIII was achieved to the extent of ∿50% by heating with 80% acetic acid at 100°C for 40 minutes, (no optimisation of the reaction was done). The compound LIII was easily separated from the unreacted material on silica gel plates using ether for development. It was crystallised, as described in literature[111], by dissolving in ethanol and adding hot cyclohexane. The melting point was 194-195°C (literature value[111] 195-197°C). It was also possible to crystallise this compound from ether-hexane; the melting point in this case was 194-197°C. The ultraviolet absorption maxima in ethanol were at 257nm and 235nm. The mass spectrum did not show a parent peak but prominent peaks at m/e 341(M-uracil) and 105 (C_6H_5CO) were significant.

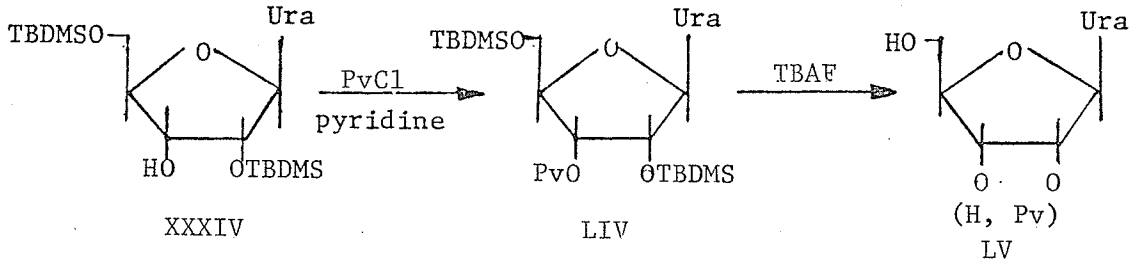
Characterisation of 2',3',5'-Tri-O-TBDMSuridine. A high resolution mass spectrum was run on this compound. The exact mass of the $(M-C_4H_9)^+$ peak was determined to be 529.258 (calculated for $C_{23}H_{45}Si_3N_2O_6$ = 529.259). Thus the empirical formula of the compound is $C_{27}H_{54}Si_3N_2O_6$, indicating the presence of three TBDMS groups in the uridine molecule. The UV spectrum showed that the uracil ring was not substituted. It was not possible to crystallise this compound.

Attempted Synthesis of 2'-O and 3'-O-Pivaloyluridines

A general synthesis of 2'-O- and 3'-O-acyluridines would require a pair of intermediates with selectively removable substituents on the primary and on the appropriate secondary position of the ribose system. Acid-labile or hydrogenolytically removable protecting groups were the obvious choice for the synthesis of these base-labile acyl derivatives. Recently, there has been interest in the synthesis of derivatives having large acyl substituents such as pivaloyl or a bulky naturally occurring substituent such as valyl, for the purpose of studying acyl migration and other phenomena.

For the purpose of the synthesis of pivaloyluridines, Jarman et al[107] considered 2',5'-and 3',5'-uridine derivatives protected by trityl and benzyl groups. As the trityl group was found to be unsatisfactory, presumably due to its bulkiness, the alternative benzyl function was used. Their procedure involved 7 steps and overall yield, based on uridine was about 2%. The main problem was to obtain 2',5'-and 3',5'-benzyluridines in a satisfactory yield larger than the normal 3%.

It was seen in this research that 2',5'-and 3',5'-di-O-TBDMS-derivatives could be obtained from their ribonucleosides in one step reactions in high yields (∿ 45% in the case of 2',5' derivatives of uridine and adenosine). Therefore, it was of interest to find whether 2'-and 3'-acyl derivatives of the type referred to above could be synthesised from their derivatives in a simpler way. The reaction sequence used for this purpose will be described below. The acylation of 2',5'-di-O-TBDMS-uridine with 4 equivalents of pivaloyl chloride in the presence of



XXXIV LIV LV

pyridine at room temperature was a slow reaction with the yield around 35% in 23 hours. The use of a greater excess of pivaloyl chloride did not help as the ring substituted compounds were formed. Considering the very low overall yields obtained in the existing procedures, the suggested route was still promising.

The product was purified on silica gel plates using ether-hexane (2:1) for the development and was eluted in ether. A white residue of melting point 118-122^{o}C was obtained on removal of the solvent. The mass spectrum of this trisubstituted product showed peaks at m/e 541 ($M-CH_3$) and 499(M-tBu).

The desilylation by TBAF presented problems. The product LV obtained after separation on silica gel plates appeared to be a mixture of 2'- and 3'-isomers, as evidenced by its melting point. The mass spectrum indicated that the product was 2'(3')-pivaloyluridine with the ions being of m/e 328(M), 310(M-18), 217(S) and 113(B+2).

The migration of the acetyl group has previously been reported in literature by Diana[109] in the fluoride displacement of mesylates. It was possible that an unprotected hydroxyl group will

participate in the migration of an acyl group under the influence of the basic properties of fluoride ions[110]. More recently, Golding et al[97] have pointed out that 1,2-and 1,3-diacyl glycerides were not obtainable as pure substances in a procedure employing a TBDMS protecting group which was removed by TBAF. Thus, there is a need of a method by which the TBDMS group can be removed without causing any acyl migration. In this connection, a preliminary study was made, as described earlier in this thesis, in which the reactivity of TBAF was moderated by the addition of glacial acetic acid. Though no further experiments were carried out to prove this, there is a strong possibility that a 'modified TBAF' can be found which may remove the silyl group without causing any acyl migration.

Protection of Adenosine, Cytidine and Guanosine by the TBDMS Group

Having completed the study of the protection of uridine by TBDMSCl, it was necessary, in order to establish the generality of this protecting group, to investigate the TBDMS protection of the other three ribonucleosides. Though these studies were not as complete as in the case of uridine, particularly in the case of cytidine and guanosine, there was no doubt left that the TBDMS protecting group was of general application to all ribonucleosides.

The main emphasis was to investigate i) the silylation reaction itself, ii) the separation of the reaction products obtained by direct silylation, iii) the acetic acid hydrolysis reaction so that 2'-O-, 3'-O- and 2'3'-di-O-TBDMS derivatives can be made. In the case of adenosine, all of the possible derivatives were prepared and 5'-O- and

3',5'-di-O-TBDMSadenosines were characterised by synthesising known compounds. The structures of the other derivatives were established by the help of mass spectrometry.

The Silylation of Adenosine. The reaction of TBDMSCl with adenosine proceeded in a similar way to that of uridine in all essential respects except that the reaction was slightly slower than uridine and cytidine reaction rates. There was no precipitation at any stage of the silylation reaction and the reaction mixture contained four main products, namely 5'-O-, 2',5'-di-O-, 3',5'-di-O- and 2',3',5'-tri-O-TBDMSadenosines, as found in the case of uridine.

The separation of the products on silica gel plates presented no problems and was somewhat easier than in the case of uridine. Ether-hexane 2:1 or 5:2 was found to be a suitable solvent system for separating the two disilyl isomers and the trisilyl derivative, but the separation of 5'-O-TBDMSadenosine from unreacted adenosine and salts required the development of plates in ethyl acetate or THF-ethyl acetate (1:1). The unreacted adenosine could be separated from salts, whenever required, by developing the plates in THF. All four products could be eluted from silica gel by ethyl acetate, but ether was also used for eluting 2',5'-di-O-, 3',5'-di-O- and 2',3',5'-tri-O-derivatives.

2',5'-Di-O- and 3',5'-di-O-TBDMSuridines were obtained as solids just by removing ethyl acetate in an aspirator. It will be no exaggeration to say that adenosine derivatives were the easiest to obtain as nice crystalline substances. Even 2',3',5'-tri-O-TBDMSadenosine could be easily crystallised (methanol-water), unlike the corresponding uridine

Scheme XIIIa. Silylation of adenosine by TBDMSCl and the action of 80% acetic acid on products of the silylation reaction.

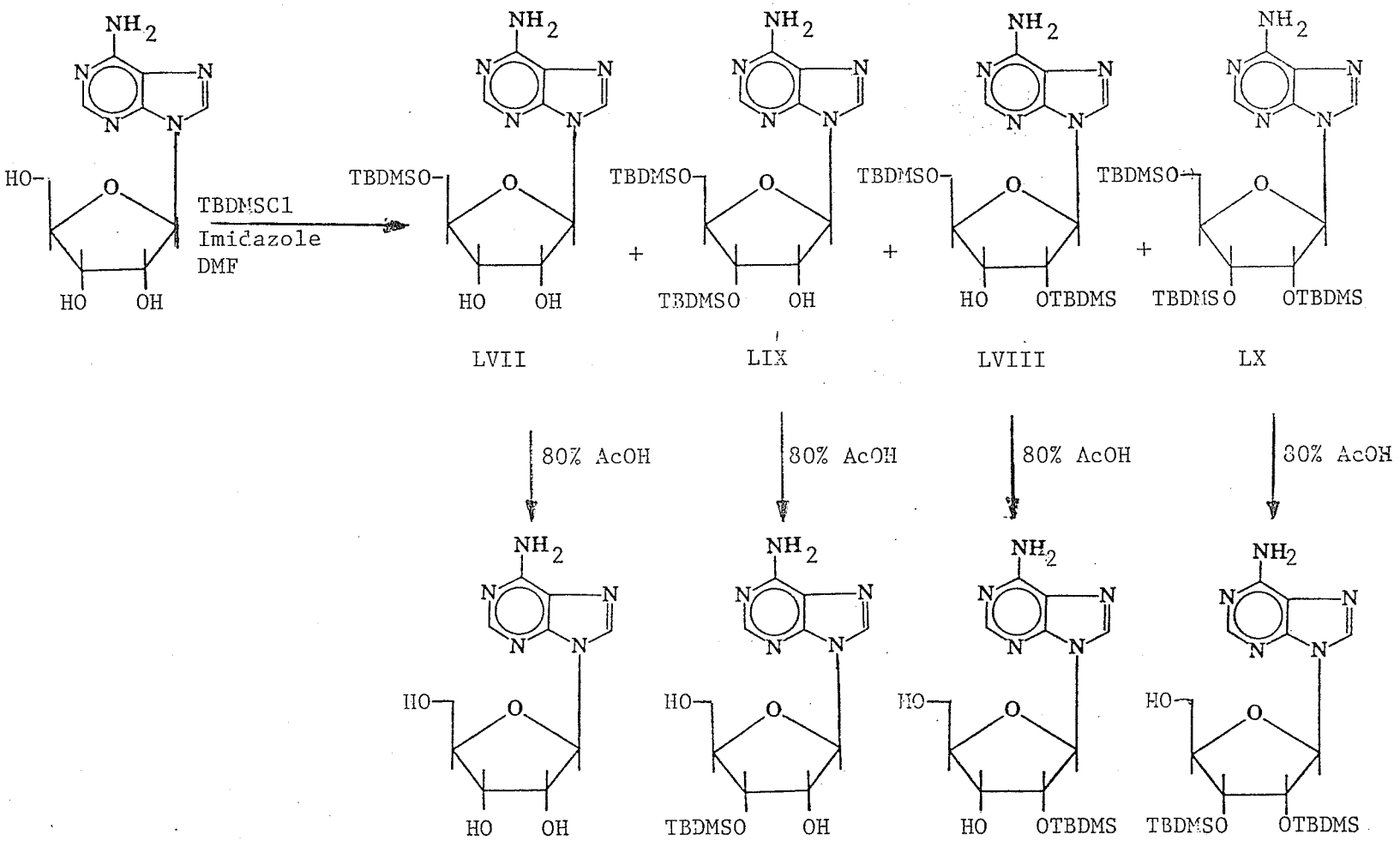


Table V. Silylation of Adenosine by tert -Butyldimethylsilyl Chloride

Reactants Ratio**	Solvent[2]	Time, Hrs.	← Products* as Percentages[3] →			
			LVII	LVIII	LIX	LX
1:1.1:2.2	DMF	20	39	9	8	-
1:1.5:3.0	DMF	7.5	30	18	21	1
1:2.2:4.4	DMF	3.5	20	37	37	5
1:2.2:4.4	DMF	12	17	45	25	8
1:3.3:6.6	DMF	2.5	22	45	20	3
1:4.4:8.8	DMF	2	1	21	5	71

For explanations of the superscripts, see Table I.

* LVII = 5'-O-TBDMSadenosine

LVIII = 2',5'-Di-O-TBDMSadenosine

LIX = 3',5'-Di-O-TBDMSadenosine

LX = 2',3',5'-Tri-O-TBDMSadenosine

** Reactants ratio is the molar ratio of adenosine : TBDMSCl : imidazole.

derivative which could not be crystallised from various solvents tried. 5'-O-TBDMSadenosine, like 5'-O-TBDMSuridine, was obtained as a white solid after removing the ethyl acetate solvent, without any difficulty.

The product ratios were determined for reaction in DMF only (see Table V). The maximum amount of 2',5'-di-O-TBDMSadenosine, which is the most useful derivative for ribonucleotide synthesis, was 45% which was comparable to that obtained in the case of the corresponding uridine derivative. This table may also prove useful for determining the optimum reactant ratio for synthesising the other three derivatives obtainable by direct silylation.

In an attempt to convert adenosine completely to its 2',3',5'-tri-O-TBDMS derivative, 6 equivalents of TBDMSCl were used (imidazole was twice the amount of TBDMSCl). This did not offer any particular advantage as 4.4 equivalents of TBDSCl gave a slightly higher yield (71%). It appeared that polysilylated compounds were formed when employing higher TBDMSCl ratios.

Stability Studies. With regard to 80% acetic acid lability, the TBDMS derivatives of adenosine were similar to uridine derivatives, except that the general order of stability was observed to be uridine < adenosine < cytidine, both at 100°C and room temperature. For example, in 21 hours 95% of 2',5'-di-O-TBDMSuridine was hydrolysed but the corresponding adenosine derivative was hydrolysed to the extent of 79% in the same time (room temperature). As found in the case of uridine, the 5'-TBDMS group was more labile than 2'(3')-TBDMS group (see Table VI). This made it

Table VI. Hydrolysis of the TBDMS derivatives of Adenosine and Cytidine by 80% Acetic Acid.

Compound	Temp.	Time	Products as Percentages			
		hrs.	Unreacted Material %	2'-O-TBDMSA	3'-O-TBDMSA	2',3'-Di-O-TBDMSA	A
2',5'-Di-O-TBDMSA	RT	10	42	53	-	-	N.D.
		20	21	70	-	-	N.D.
		24	16	78	-	-	N.D.
	100°C	0.60	33	58	-	-	N.D.
3',5'-Di-O-TBDMSA	RT	28	22	-	67	-	6
	100°C	0.60	40	-	55	-	2
2',3',5'-Tri-O-TBDMSA	100°C	0.33	74	-	-	22	-
		0.66	39	-	-	58	-
		1	13	-	-	82	-

N.D. = Not Determined

				← Cytidine Derivatives →			C
2',5'-Di-O-TBDMSC	RT	24	51	49	49	-	-
2',3',5'-Tri-O-TBDMSC	100°C	0.33	72	-	-	25	-
		1	43	-	-	52	-

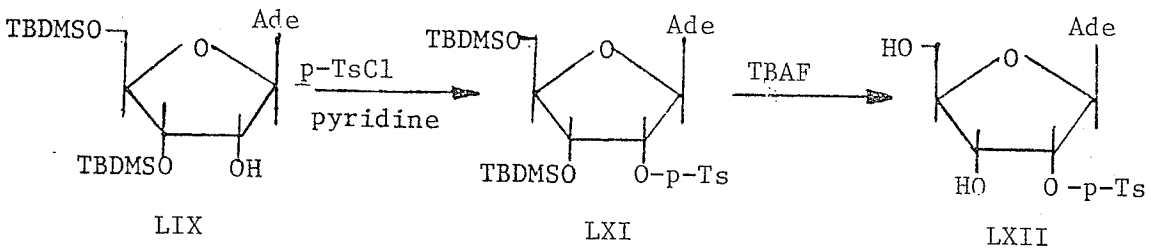
possible to easily make those derivatives which could not be obtained by direct silylation of adenosine. While Table VI may serve as a guide for selecting the correct hydrolysis conditions, the criterion was to prevent hydrolysis of 2'-and 3'-TBDMS groups by avoiding prolonged contact time.

There was no evidence of 2'-TBDMS $\rightleftarrows$ 3'-TBDMS isomerisation in the acidic medium. Isomerisation did take place in basic media The rate of isomerisation on silica gel was less than that observed in the case of uridine derivatives, although no quantitative data were obtained.

The action of 7M ethanolic ammonia on adenosine derivatives was similar to that in the case of uridine derivatives. As found before, the 2'(3')-TBDMS group was much more base-labile than the 5'-TBDMS group. Although based on limited data, it can be said that adenosine derivatives were slightly more base-labile than uridine derivatives. Further,the 2'-TBDMS group was marginally more base-labile than the 3'-TBDMS group, probably due to smaller steric hindrance caused by the 5'-TBDMS group or greater acidity of the 2'-oxygen function (see Table IV).

Characterisation of the TBDMS Derivatives of Adenosine

3',5'-Di-O-TBDMSadenosine. This compound was characterised by synthesising the known compound 2'-O-p-tosyladenosine.



LIX LXI LXII

The advantages of utilising tosyl derivatives for orientation purposes have been mentioned before while discussing the structures of 2',5'- and 3',5'-di-O-TBDMSuridines.

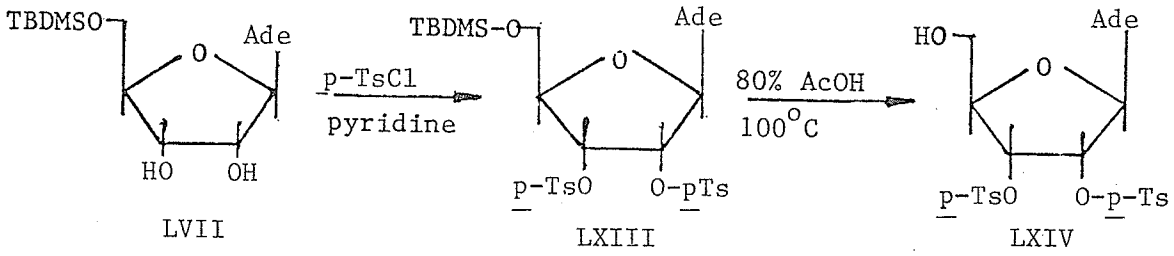
When 5 equivalents of p-TsCl were used, about 85% of the disilyl derivative was tosylated in 64 hours at 0°C. The product LXI was separated on silica gel plates using benzene-ether(1:1) for development. The product was eluted in ether, the removal of which gave a solid of melting point 173-176°C (without crystallisation). The mass spectrum showed fragments of m/e 634 ($M-CH_3$) and 592 (M-t-Bu). The partial mass spectrum of the compound has been described in the section on Mass Spectrometry.

This compound was desilylated with TBAF and the products separated on silica gel plates using EtOAc-THF(1:1) for development. The main product was obtained in 92% yield and had a melting point of 212-220°C (dec.), which on crystallisation from methanol-water improved to 227-229°C (literature values[103,104] 229-230°C).

The overall yield of 2'-O-p-tosyladenosine was 71% on the basis of 3',5'-di-O-TBDMSadenosine used. This method provided a convenient route to this derivative, which is a precursor to 8,2'-anhydroadenosine, which has been previously difficult to prepare in pure form and in high yield. This yield is comparable to that obtained recently by Moffatt et al[103] using organotin derivatives.

5'-O-TBDMSadenosine. The position of the TBDMS group in this compound was located by blocking the 2'- and 3'-hydroxyl by p-tosyl groups and then removing the 5'-TBDMS group to obtain the known compound 2',3'-di-

-O-tosyladenosine.



When 5'-O-TBDMSadenosine was treated with 8 equivalents of p-TsCl in the presence of pyridine at 0°C, for 24 hours, two products and no starting material was seen by tlc in ether. Possibly these were the desired compound LXIII and 5'-O-TBDMS-2'(3')-O-tosyladenosine in the ratio 1:1. Further addition of p-TsCl and an increase in the reaction time resulted in the formation of poly-substituted derivatives, due to the tosylation of the adenine base. Sasaki et al [90] previously noted some problems during ditosylation of 5'-O-acetyladenosine. These difficulties were presumably due to steric reasons.

No optimisation of the reaction was done, as the main purpose was to characterise the 5'-derivative. The product was purified by precipitation with hexane from ether solution to form a solid of melting point 157-159°C. The mass spectrum indicated the presence of fragments of m/e 674(M-15) and 632(M-57).

This compound LXIII was desilylated by heating with 80% acetic acid at 100°C for one hour. The required product LXIV was separated on a 10 x 20 cm of silica gel plate and on crystallisation from ethanol had a melting point 205-207°C (literature value[90] 207-209°C). UV absorption

maxima in ethanol were at 261nm and 226nm.

Other supporting evidence for the characterisation of this compound was obtained by preparing its 2',3'-O-isopropylidene derivative based on the procedure used by Hampton[105]. The reaction proceeded at a much slower rate than found in the case of adenosine by Hampton[105]. After the work up of the reaction mixture, the predominant compound was 2',3'-O-isopropylideneadenosine, possibly due to cleavage of the 5'-TBDMS group. This did not really matter as far as the characterisation of 5'-O-TBDMSadenosine was concerned since 2'(3')-TBDMS groups were too stable to come off under these reaction conditions. The melting point of the isolated compound was 215-218°C (literature value[105] 217.5-218°C). The mass spectrum was identical to that obtained by Dolhun and Wiebers[106] for this compound.

This kind of derivative could be useful in characterising 5'-O-TBDMScytidine and 5'-O-TBDMSguanosine, as the mass spectra of their 2',3'-O-isopropylidene derivatives have been studied in detail by the above authors[106].

2'-and 3'-O-TBDMSadenosines. These derivatives were obtained from 2',5'- and 3',5'-di-O-derivatives respectively by removing the acid-labile 5'-TBDMS group with the help of 80% acetic acid. As no 2' $\rightleftarrows$ 3' isomerisation was seen under these conditions, the structures of these compounds followed from their parent compounds. The structure of 3',5'-di-O-TBDMS-adenosine was proved by making a known compound as described above. This clearly established the structure of 3'-O-TBDMSadenosine. The structure of 5'-O-TBDMSadenosine was also known, thus leaving only 2'-O-TBDMSadenosine whose structure was not proved directly by synthetic

methods. Additional evidence for characterising mono-TBDMSadenosines was obtained from a study of their mass spectra. This has been discussed in detail in the section on Mass Spectrometry and Chromatography of the TBDMS derivatives of ribonucleosides.

The Silylation of Cytidine. This reaction was studied in a similar manner to those already described in detail for uridine and adenosine. The rate of silylation reaction lay somewhere between that of uridine and adenosine. Four main products were obtained as in previous silylation reactions (see Table VII A).

The completion of the reaction was monitored by tlc in THF. The proportions of various products obtained using different quantities of reagents are described in Table VII A. Ether-hexane (5:1 or 4:1) was found to be the most suitable developing solvent for separating the disilyl and the trisilyl derivatives on silica gel plates. Ether could be used alone when 5'-O-TBDMScytidine was the main product. The development in ethyl acetate gave a beautiful separation of the four main products at Rf 0.12, 0.30, 0.52 and 0.62 when plates were lightly to moderately loaded.

The approximate ratio of faster to slower di-O-silyl isomers was 3:2 when 2.2 equivalents of TBDMSCl were used. A slight effect on selectivity was noted when pyridine replaced DMF as the reaction medium, in that the proportion of the slower disilyl isomer increased. The 2',5'-di-O-TBDMS derivative (faster isomer) was obtained as a solid when ether or ethyl acetate eluants were removed and the residue coevaporated with hexane at 40°C. Alternatively, after the removal of solvents, refluxing with hexane gave a white solid on cooling.

Table VII A. Silylation of Cytidine by tert -Butyldimethylsilyl Chloride

Reactants Ratio[1]	Solvent[2]	Time,Hrs.	Products as Percentages[3,4,5], 5'-	2',5'-	3',5'-	2',3',5'-
1:1.1:2.2	DMF	1	40	4	4	-
1:1.8:4.0	DMF	12	42	27	18	5
1:2.2:4.4	DMF	2	26	37	25	6
1:2.2:4.4	Pyridine	4.5	14	38	32	5
1:3.3:6.6	DMF	2	16	24	24	25
1:4.4:8.8	DMF	2	1	10	5	82

1. Molar ratio of cytidine, TBDMSCl and imidazole in order. In general, 0.125 mmol of cytidine was used.
2. DMF used: 1 ml/mmol of cytidine. Pyridine used: 4 ml/mmol of cytidine.
3. Products were eluted in methanol and concentrations determined spectrophotometrically.
4. Product names have been indicated by mentioning the position(s) of TBDMS substituent(s).
5. Position of the TBDMS substituent(s) determined by tlc and/or mass spectral analysis.

Stability Studies. The rate of isomerisation of the disilyl derivatives (2',5' and 3',5') on silica gel was lower than that of the corresponding uridine derivatives. The effect of heat on this isomerisation on silica gel was also studied. For example, the 2',5'-di-O-TBDMScytidine at 100°C for 30 minutes isomerised to the extent of 15%. A similar experiment at room temperature for 14 hours did not show any isomerisation due to silica gel. When the silica gel plates were heated at 100°C for 2.5 hours, the ratio of the faster to slower isomer was ∿ 1:1, but this was accompanied by ∿ 10-20% of desilylation.

Some other isomerisation studies on the 2',5'-di-O-TBDMScytidine were made in the following media.

Pyridine: There was no isomerisation after stirring with dry pyridine for 65 hours at room temperature; but there was a small amount of isomerisation (∿4%) when it was heated with pyridine at 50°C for 5 hours.

Pyridine-water: There was isomerisation and after 7 hours of stirring at room temperature, two isomers were present in the ratio of the faster to the slower of 3:4.

Ethanol: There was no isomerisation when stirred for 24 hours at room temperature.

Ethanolic ammonia(7M): After 65 hours at room temperature the ratio of the faster to the slower isomer was ∿ 1:1. There was partial hydrolysis to 5'-O-TBDMScytidine.

Characterisation. 2',3'-Di-O- and 2',5'-di-O-TBDMScytidines were characterised on the basis of their mass spectra as discussed later in a separate section. Their R_f values were in the same order as found in the case of uridine and adenosine derivatives.

The Silylation of Guanosine. It has been observed that the silylation of deoxyguanosine needed a relatively large excess of TBDMSCl reagent[60]. A similar trend was observed in the case of guanosine. An additional factor was the low solubility of guanosine in DMF, so that even 3.3 equivalents of TBDMSCl could silylate only 80% of guanosine in 23 hours at room temperature.

Upon increasing the amount of DMF (2ml/mmol) and TBDMSCl (4.4 equivalents), guanosine was completely silylated in 2.5 hours. Even when using this much excess of the silylating reagent, the disilyl products were formed to the extent of 40% (both isomers) and there was no trisilyl derivative formed (see Table VII B).

The silylation reaction was also investigated in DMSO, as guanosine was easily soluble in this solvent. The use of this solvent favoured the formation of the trisilyl derivative but there was no increase in the amount of the disilyl derivatives. For example, 4.4 equivalents of TBDMSCl gave 36% of two disilyl derivatives and 3.3 equivalents gave only 10% of the two disilyl derivatives.

The separation of the reaction products on silica gel plates required much more care, particularly when DMSO was the reaction medium. The separation was satisfactory when plates were not heavily loaded and these were initially developed in chloroform-methanol(8:1). Two or three developments were required for a clean separation of the two disilyl and the trisilyl derivatives.

Monitoring of the reaction using Eastman Chromotograms No.13181 (coated TLC sheets) for identifying the disilyl and the trisilyl guanosines was best done by developing the tlc in chloroform-ethanol (12:1). The

Table VII B. Silylation of Guanosine by tert-Butyldimethylsilyl Chloride

Reactants Ratio[1]	Solvent[2] (ml/mmol)	Time, Hrs.	Products as Percentages[3,4,5,]			
			5'-	2'.5'-	3',5'-	2'.3',5'-
1:2.2:4.4	DMF(1.0)	19	35	3		-
1:3.3:6.6	DMSO(1.2)	12	60	6	4	-
1:4.4:8.8	DMF(2.0)	2	60	40		-
1:4.4:8.8	DMSO(2.4)	4	11	18	18	34
1:6:12	DMSO(2.0)	2	-	24	16	55

1. Molar ratio of guanosine, TBDMSCl and imidazole in order. In general, 0.125 ml of DMF/mmol of guanosine was used.
2. The amount mentioned is in ml per mmol of guanosine.
3. Products were eluted in methanol and concentrations determined spectrophotometrically.
4. Product names have been indicated by mentioning the position(s) of the TBDMS substituent(s).
5. Positions of the TBDMS substituent(s) determined by tlc method only.

completion of the reaction was seen by tlc when more polar solvents like chloroform-ethanol(2:1) were used.

Two products were eluted from the silica gel using methanol, without any 2' $\rightleftarrows$ 3' isomerisation if longer contact time was not allowed ($\sim$ 2 hours). There was no isomerisation on silica gel when ethyl acetate was used for elution, but it was needed in much larger amounts.

It was easy to obtain the disilyl derivatives of single spot purity when methanol-chloroform(4:1) was used for elution. This solvent was found to be particularly useful because solids were easily obtained on removing these solvents.

Characterisation. No attempt was made to characterise guanosine derivatives by synthesising known compounds. A preliminary study showed that mass spectrometry of partially silylated guanosine derivatives may not be very useful and hence it was not possible to characterise the TBDMS derivatives of guanosine using this simpler technique. On the other hand chromatographic behaviour of TBDMS derivatives could be more useful on the assumption that R_f values of guanosine derivatives will follow the same order as seen in the case of characterised derivatives of uridine, adenosine and cytidine.

Mass Spectrometry and Chromatography of the TBDMS Derivatives of Ribonucleosides.

The TBDMS group, while having exceptionally useful properties from a synthetic viewpoint also imparted valuable characteristic properties to nucleosides for the purpose of mass spectrometry and chromatography. A brief account of the techniques found useful in this study is given in this section.

Mass Spectrometry. A study of the mass spectra of the TBDMS and other sterically crowded silyl derivatives of deoxynucleosides and ribonucleosides (particularly uridine) forms the subject matter of a doctoral thesis by M.A.Quilliam[144] and a part of the data included in this discussion was taken from this source. For the purpose of this research, a study of the mass spectra of the TBDMS derivatives was of great interest not only for identifying the compounds themselves but also as an aid to distinguish isomeric monosilyl and disilyl derivatives of ribonucleosides. Previous studies[145] showed that it was possible to differentiate the 3'- and 5'-O-TBDMS derivatives of thymidine and deoxyadenosine on the basis of their mass spectra. The present study proved that even 2'-and 3'-O-TBDMS derivatives of ribonucleosides could be distinguished. Whenever possible, structures of the intermediate products obtained during syntheses were determined taking advantage of the facility with which the TBDMS derivatives could be characterised by mass spectrometry. The mass spectrometry of some of these compounds is discussed in the last part of this section.

Comments on the Mass Spectra of the TBDMS Derivatives of Ribonucleosides

These comments are based on the data given in Tables VIII to XII and Figures 1 to 8. It may also be noted that the mass spectra were normalised by correcting for mass discrimination in the quadrupole mass filter by multiplying the intensity of an ion by its m/e value before normalisation, since the transmission of the mass filter is inversely proportional to the mass of the ion.

Some general comments are as follows:

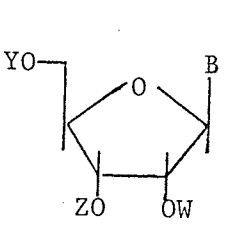
1. Molecular ion is very weak or not detectable at all.
2. The distinguishing feature in the mass spectra of a TBDMS derivative is the presence of a relatively prominent ion at $(M-57)^+$ which confirms the molecular weight of a compound. The ready loss of a tert -butyl radical from the molecular ion is characteristic of many classes of compounds studied, such as deoxynucleosides[60,144,145], carbohydrates[85,147,148], prostaglandins[149], steroids[150,151], and lipids[152].
3. Other prominent ions occur at m/e 75, X_2SiOH^+ and m/e 73, $X_2SiCH_3^+$ ($X=CH_3$).
4. Characteristic ions are observed for various positions of silyloxy groups on the sugar moiety as shown below. These assignments are supported by studies on uridine derivatives of established structures (discussed earlier in this thesis). These generalisations have been used to determine the orientation of adenosine and cytidine derivatives.

Some structurally significant ion types are given in Scheme XIIIb.

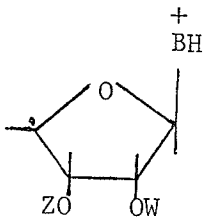
Specific comments are as follows:

$(M-30)^+$. This ion indicated the presence of a 5'-OH group. It is usually prominent in the case of purine nucleosides since its formation is promoted by location of the positive charge on the purine base, and transfer of H to the base.

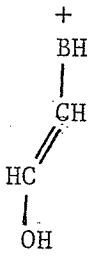
Scheme XIIIb. Possible structures of some of the ion-fragments of the TBDMS derivatives of ribonucleosides.



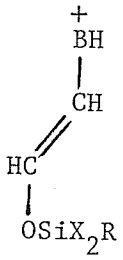
M



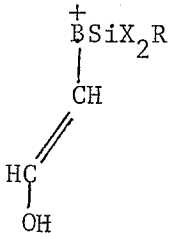
$(M-30)^+$

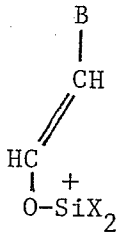


$(B+44)^+$



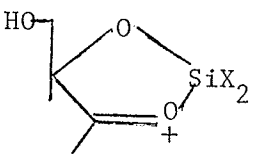
and/or



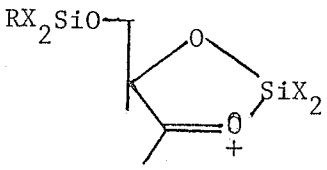


$(B+SiX_2R+43)^+$

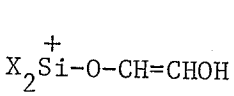
$(B+SiX_2+42)^+$



$(SiX_2+71)^+$



$(SiX_2+RX_2Si+70)^+$



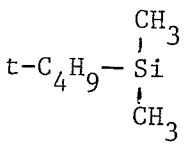
$(SiX_2+59)^+$

Y, Z, W = H or RX_2Si

R = $t\text{-}C_4H_9$

X = CH_3

RX_2Si represents



$(B+44)^+$. The formation of this ion is favoured by the presence of 2'-OH group. H migration to the base from elsewhere in the molecule is also required.

$(B+SiX_2R+43)^+$. This ion is a structural analogue of $(B+44)^+$ in which a 2'- or 5'-O-silyl group replaces one H. Since RX_2Si migrates less readily than H, this ion type should be favoured for 2'-O-silyl derivatives.

$(B+SiX_2+42)^+$. This fragment ion is formed most easily when a 2'-O-silyl group is present. It is more abundant for pyrimidine than for purine nucleosides, presumably because in the latter case there is greater probability of location of the positive charge on the nucleobase than on the silyl residue.

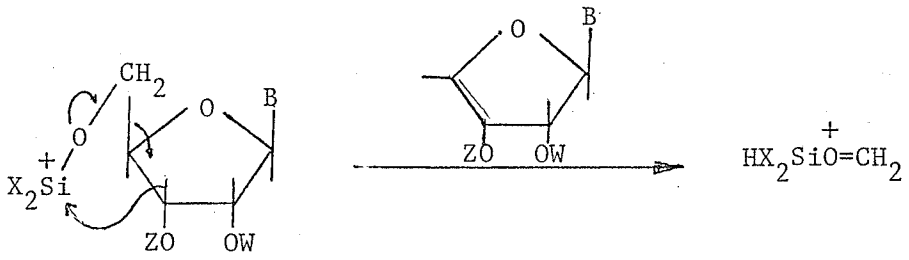
$(B+SiX_2)^+$. This ion is formed by transfer of the positively charged SiX_2 residue to the nucleobase. Since the 3'-O-silyl residue is not as sterically accessible to the base, this ion type is of low abundance in the case of 3'-O-silyl derivatives unless 2'- and/or 5'-O-silyl groups are also present.

$(SiX_2+71)^+$. This ion type is most readily formed when a 3'-O- silyl group is present, and results from interaction of a 3'-O-$\overset{+}{S}iX_2$ with 4'O.

$(SiX_2+SiX_2R+70)^+$. This is the structural analogue of $(SiX_2+71)^+$ when a 5'-O-silyl group is present.

$(SiX_2+59)^+$. Empirically, this ion seems to be formed most readily when 3' and/or 5'-O-silyl groups are present.

$RX_2SiOCH_2^+$ and $HX_2SiOCH_2^+$. The major sources of these ions should be the 5'-O-silyl group. The latter appears to be a strong indicator of the presence of such a group and may be formed by the reaction:



Characterisation of Mono-silyladenosines.

A study of the fragmentation pattern of uridine derivatives, whose structures have been proved by synthetic methods, was useful in establishing the identities of adenosine derivatives. Table VIII lists diagnostically important ions in the mass spectra of the mono-silyl ribonucleosides.

2'-O-TBDMSadenosine. The strongest indicators of the presence of the 2'-O-silyl group are the relatively strong peak at $(B+SiX_2R+43)^+$ and the relatively weak peak at $(B+44)^+$. Also consistent with this assignment are the peaks at $(B+SiX_2+42)^+$ and $(B+SiX_2)^+$ which, while not very strong, are the most abundant of the three isomers. An ion at $(M-30)^+$ indicates the presence of the 5'-OH group.

3'-O-TBDMSadenosine. An abundant peak at $(B+44)^+$ indicates the presence of the 2'-OH group, while the ion at $(M-30)^+$ indicates a 5'-OH group. A relatively weak ion at $(B+SiX_2R+43)^+$ indicates the absence of a 2'-O-silyl group. The ion at $(SiX_2+71)^+$ has the highest abundance of the three isomers, consistent with the presence of a 3'-O-silyl group.

5'-O-TBDMSadenosine. The absence of $(M-30)^+$ indicates the presence of a

Table VIII. Some Diagnostically Important Ions in the Mass Spectra of Mono-silyl Ribonucleosides

Uridine				Adenosine				
	Position of RX_2Si				Position of RX_2Si			
	2'	3'	5'		2'	3'	5'	
m/e	RI%	RI%	RI%	m/e	RI%	RI%	RI%	Ion type[+]
358	-	-	-	381	0.14	0.23	0.13	M
343	-	-	-	366	5.7	3.9	2.2	M-Me
328	-	-	-	351	5.6	3.6	-	M-30
301	39	37	46	324	100	58	71	M-57
269	-	1.8	0.5	292	31	6.6	0.15	$B+SiX_2R+43$
211*	8.4	9.4	3.0	234	5.2	2.0	0.78	$B+SiX_2+42$
179	7.2	9.2	1.1	202	3.6	3.1	0.71	B+68
169	100	46	28	192	6.7	2.3	1.7	$B+SiX_2$
155	5.9	45	100	178	7.3	39	100	B+44
147	4.7	25	18.3	147	0.82	1.1	1.9	
145	4.9	12.5	12.6	145	2.3	2.4	2.1	RX_2SiOCH_2
141	7.2	6.0	10.2	164	11.0	32	36	B+30
129	24	42	15	129	7.3	11.8	3.5	SiX_2+71
117	16.3	72	58	117	7.7	14.4	17.5	SiX_2+59
113	34	100	19.8	136	86	100	49	B+2H
112	9.1	13.5	17.0	135	14.1	24	13.0	B+H
89	2.4	5.8	15.6	89	1.8	3.6	5.3	HX_2SiOCH_2
75	50	70	55	75	36	33	24	X_2SiOH
73	39	41	37	73	27	20	16.7	X_2SiCH_3

* Same m/e as $RX_2SiC_5H_5O_2$.

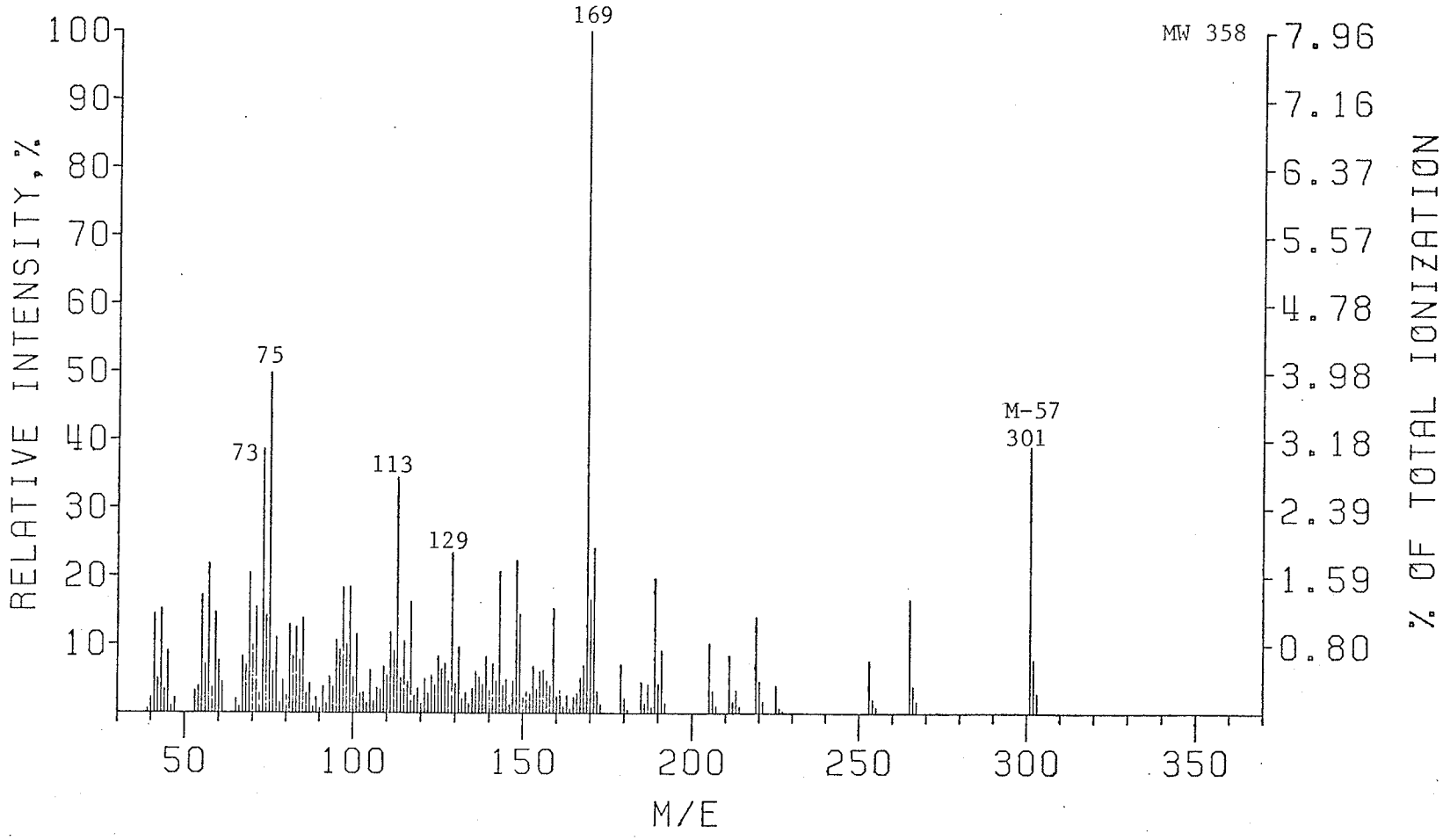


Figure 1. Mass spectrum of 2'-O-TBDMSuridine

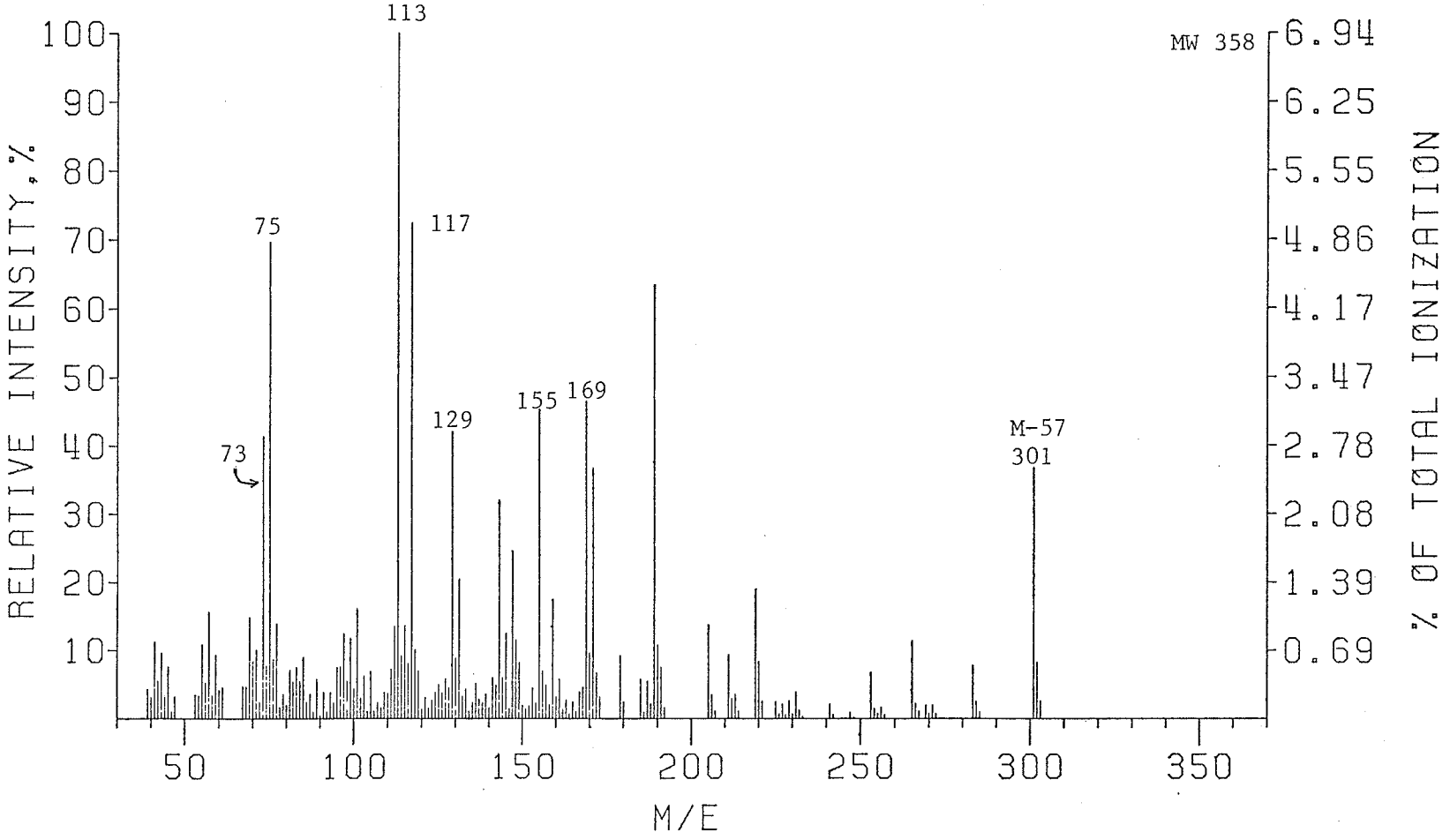


Figure 2. Mass spectrum of 3'-O-TBDMSuridine

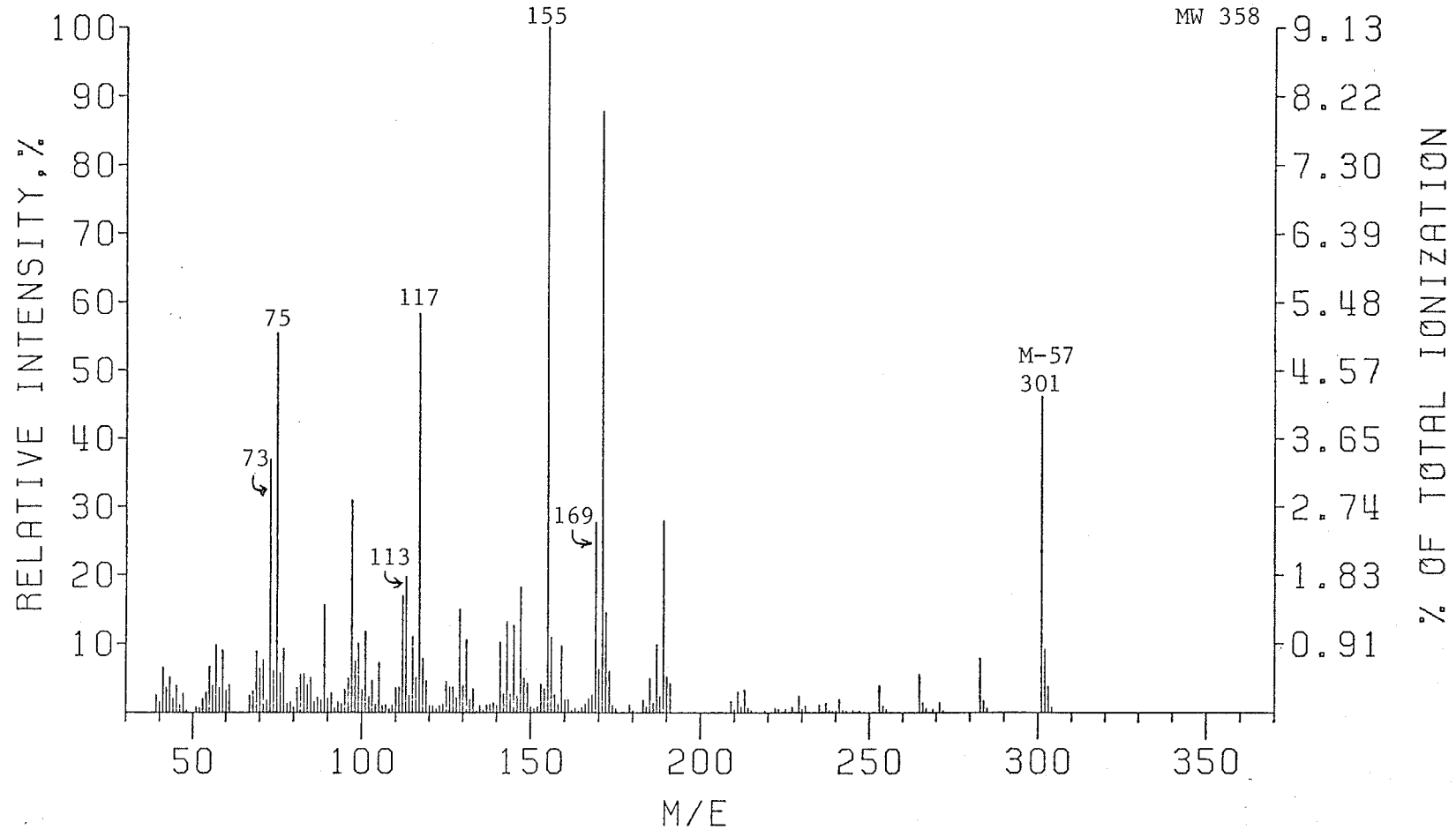


Figure 3. Mass spectrum of 5'-O-TBDMSuridine (XXXIII)

5'-O-silyl group. The weak peaks at $(B+SiX_2R+43)^+$ and $(B+SiX_2+42)^+$ and the strong peak at $(B+44)^+$ indicate a 2'-OH group. The ion at HX_2SiOCH_2 has the highest relative abundance for this isomer, consistent with the presence of a 5'-O-silyl group.

Characterisation of Di-O-TBDMS Derivatives of Adenosine and Cytidine.

The following discussion is based on the data given in Table IX.

2',3'-Di-O-TBDMSadenosine. The presence of $(M-30)^+$ indicates the presence of a 5'-OH group. The weakness of the $(SiX_2+SiX_2R+70)^+$ ion indicates the presence of a 3'- or 5'-OH group. The weakness of $(B+44)^+$ indicates a 2'-O-silyl group rather than 2'-OH group.

2',5'-Di-O-TBDMSadenosine. The absence of $(M-30)^+$ indicates the presence of a 5'-O-silyl group rather than 5'-OH group. Abundant ions at $(B+SiX_2R+43)^+$ and $(B+SiX_2+42)^+$ indicate a 2'-O-silyl group while weakness of $(SiX_2+SiX_2R+70)^+$ indicates a 3'- or 5'-OH group.

3',5'-Di-O-TBDMSadenosine. The absence of $(M-30)^+$ shows the presence of a 5'-O-silyl group rather than 5'-OH group. The relative weaknesses of the $(B+SiX_2R+70)^+$ and $(B+SiX_2+42)^+$ peaks and strength of the $(B+44)^+$ peak indicate a 2'-OH group. The abundant $(SiX_2+SiX_2R+70)^+$ ion indicates a 3',5'-O-silyl group and prominent ions at $RX_2SiOCH_2^+$ and $HX_2SiOCH_2^+$ confirm the presence of the 5'-O-silyl group.

2',3'-Di-O-TBDMScytidine. The presence of the 5'-OH group is indicated by a small peak at $(M-30)^+$. The weakness of $(SiX_2+SiX_2R+70)^+$ indicates a 3'- or 5'-OH group.

2',5'-Di-O-TBDMScytidine. The presence of the 5'-O-silyl group is

Table IX. Some Diagnostically Important Ions in the Mass Spectra of Di-silyl Ribonucleosides

Uridine				Adenosine				Cytidine			
	Positions of RX_2Si on				Positions of RX_2Si on				RX_2Si on		
	2',3'	2',5'	3',5'		2',3'	2',5'	3',5'		2',3'	2',5'	Ion type[+]
m/e	RI%	RI%	RI%	m/e	RI%	RI%	RI%	m/e	RI%	RI%	
472	-	-	-	495	0.11	-	-	471	-	-	M
457	2.4	1.8	1.6	480	4.6	4.7	3.1	456	1.4	0.15	$M-CH_3$
442	-	-	-	465	2.7	-	-	441	0.46	-	M-30
415	60	53	51	438	100	92	53	414	47	8.5	M-57
269	10.0	39	7.7	292	4.6	78	7.1	268	9.4	2.5	$B+SiX_2R+43$
261	-	0.8	20	261	1.8	2.9	26	261	0.54	0.38	SiX_2+SiX_2R+70
211*	10	34	10.7	234	5.9	12.4	3.4	210	9.7	6.5	$B+SiX_2+42$
187	3.0	26	39	187	-	15.2	10.5	187	5.3	10.1	
179	17.3	2.7	2.7	202	8.8	0.56	0.49	178	15.7	0.82	B+68
169	100	94	19.4	192	8.5	6.7	4.2	168	100	100	$B+SiX_2$
155	2.6	4.4	4.5	178	4.1	5.0	19.4	154	3.2	3.1	B+44

(continued)

Table IX. (continued)

Positions of RX_2Si on				Positions of RX_2Si on				RX_2Si on			
	2',3'	2',5'	3',5'		2',3'	2',5'	3',5'		2',3'	2',5'	Ion type[+]
m/e	RI%	RI%	RI%	m/e	RI%	RI%	RI%	m/e	RI%	RI%	
147	41	12.9	16.2	147	22	8.6	11.8	147	21	5.8	
145	3.1	16.8	34	145	4.6	13.0	19.9	145	5.3	7.5	RX_2SiOCH_2
129	9.3	18.2	30	129	11.5	17.1	17.7	129	15.7	9.4	SiX_2+71
117	12.6	41	48	117	10.7	33	27	117	14.5	16.3	SiX_2+59
113	6.9	6.3	6.2	136	31	40	44	112	27	30	B+2H
112	4.7	3.6	4.6	135	21	18.9	12.0	111	4.6	7.3	B+H
89	3.6	40	71	89	5.3	16.2	68	89	4.6	14.7	HX_2SiOCH_2
75	28	51	40	75	18.2	45	30	75	41	30	X_2SiOH
73	58	100	100	73	52	100	100	73	76	62	X_2SiCH_3

* Same m/e as $RX_2SiC_5H_5O_2$

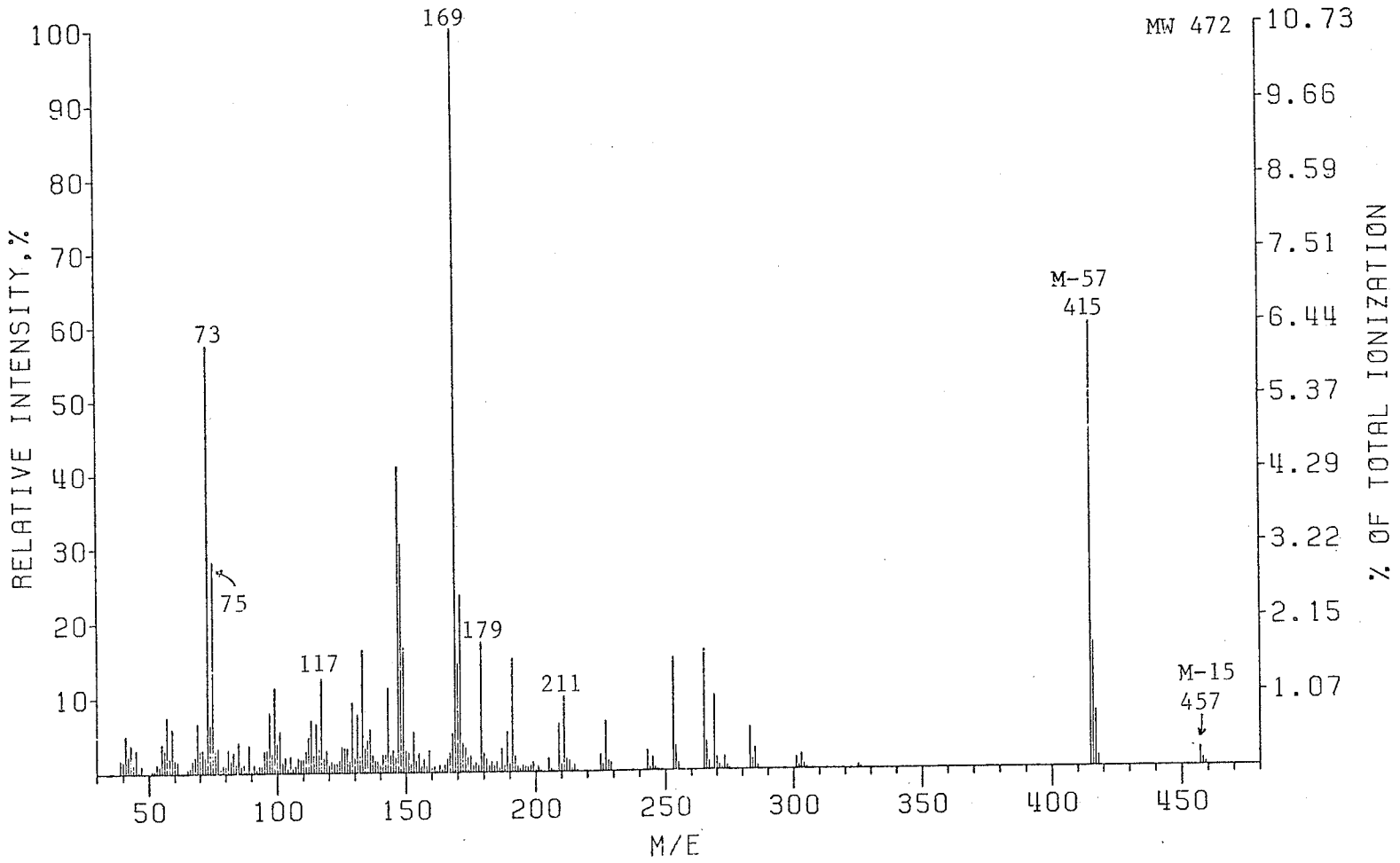


Figure 4. Mass spectrum of 2',3'-di-O-TBDMSuridine

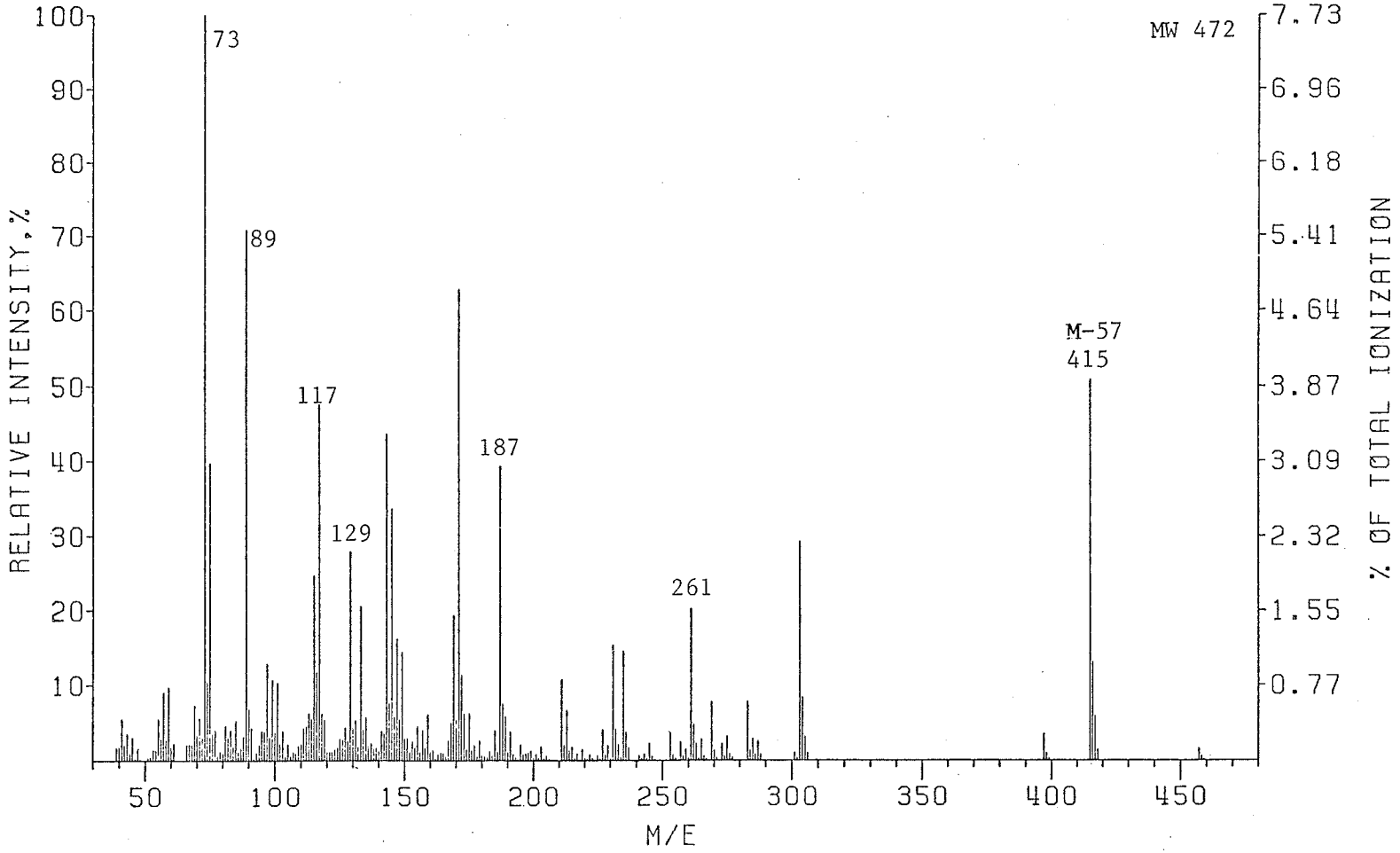


Figure 5. Mass spectrum of 3',5'-di-O-TBDMSuridine (XXXV).

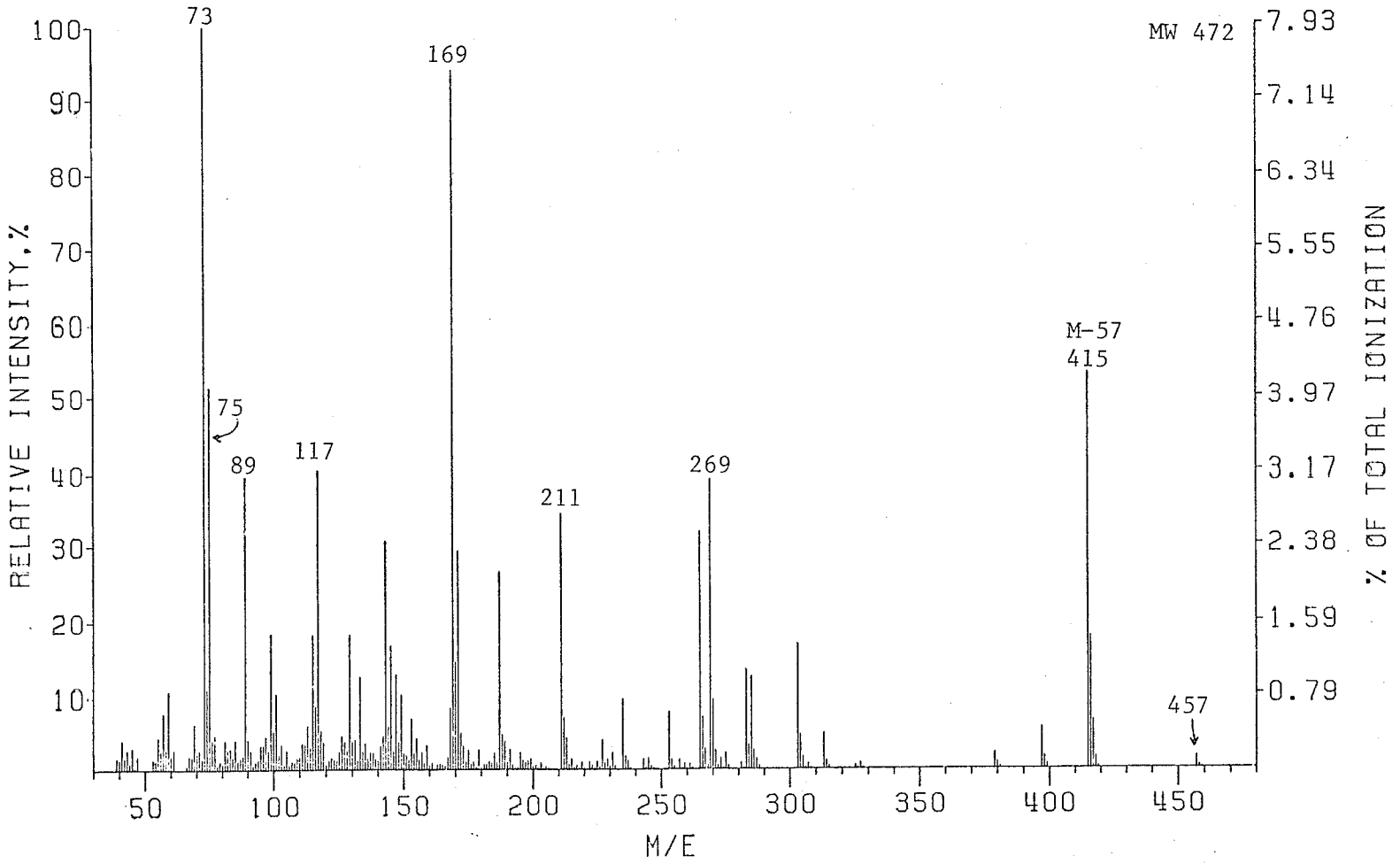


Figure 6. Mass spectrum of 2',5'-di-O-TBDMSuridine (XXXIV).

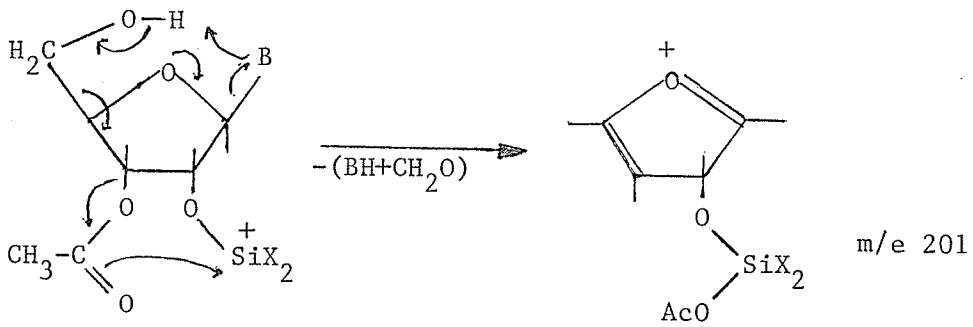
indicated by the absence of $(M-30)^+$ and a relatively strong peak at $HX_2SiOCH_2^+$. The weakness of $(SiX_2+SiX_2R+70)^+$ would then indicate a 3'-OH group meaning that the remaining silyl group occupies the 2' position. However, $(B+SiX_2R+43)^+$ is weaker than expected for this assignment, when compared with uridine derivatives. On the other hand, as for 2',5'-di-O-silyluridine, $(B+SiX_2)^+$ is very intense.

Mass Spectra of Some Other TBDMS Derivatives

Tri-O-TBDMSribonucleosides. The mass spectra confirm the identities of these derivatives. See Table X.

Acetyl-Silyluridines. This discussion is based on the data given in Table XI. The identities of the mono-acetyl-mono-silyluridines were established by synthetic methods. Relative intensities of certain ions are consistent with previous ideas. Thus $(B+SiX_2)^+$ is more intense for the 2'-O-silyl isomer and $(SiX_2+71)^+$ is more intense for the 3'-O-silyl isomer. An interesting intensity difference between the two isomers occurs at m/e 201, which is much more abundant for the 2'-O-silyl isomer. Possibly, hydrogen transfer to the base occurs more readily as H^+ (mechanism A) than as H^- (mechanism B).

Mechanism A



m/e 201

Table X. Some Diagnostically Important Ions in the Mass Spectra of Tri-silyl Ribonucleosides.

Uridine		Adenosine		Cytidine		
m/e	RI%	m/e	RI%	m/e	RI%	Ion type[+]
586	-	609	0.1	585	-	M
529	97	552	100	528	33	M-R
269	4.0	292	17.8	268	3.2	$B+SiX_2R+43$
261	26	261	15.3	261	8.0	SiX_2+SiX_2R+70
211*	17	234	7.8	210	5.3	$B+SiX_2+42$
169	46	192	3.5	168	45	$B+SiX_2$
113	1.4	136	10.1	112	3.6	B+2H
89	52	89	46	89	8.0	HX_2SiOCH_2
75	14.7	75	10.0	75	100	X_2SiOH
73	100	73	98	73	61	X_2SiCH_3

* Same m/e as $RX_2SiC_5H_5O_2$.

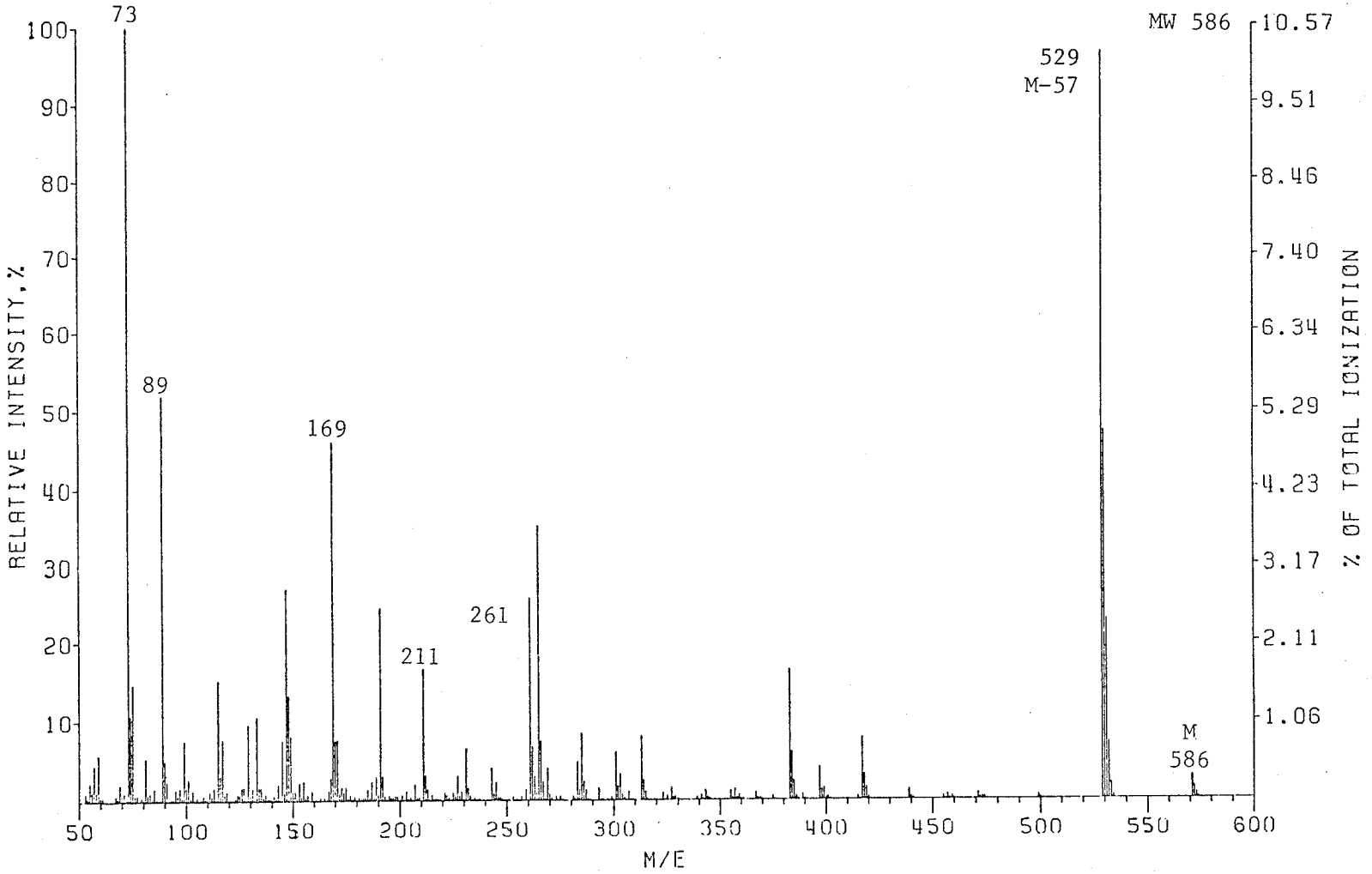


Figure 7. Mass spectrum of 2',3',5'-tri-O-TBDMSuridine (XXXVI).

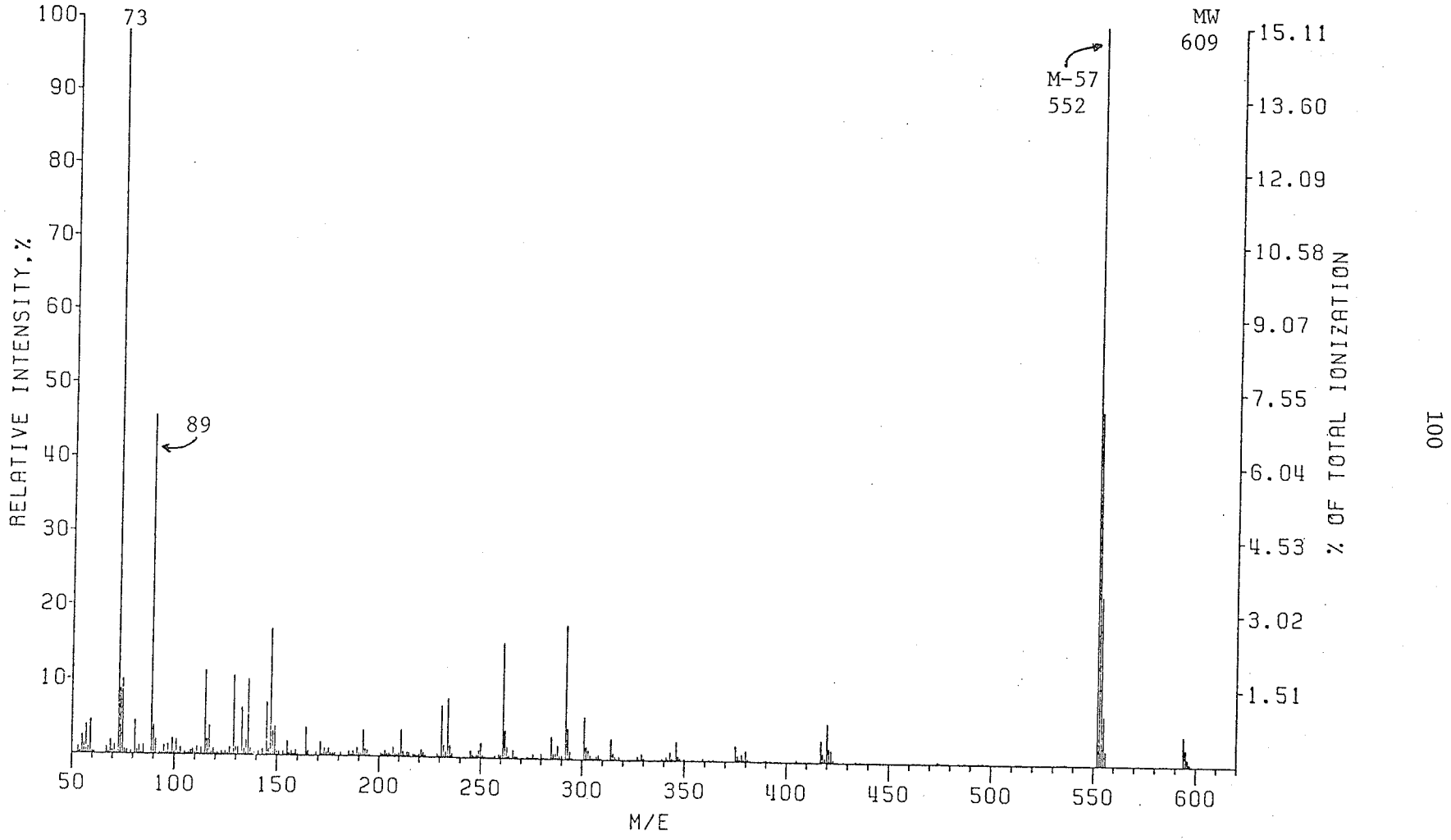
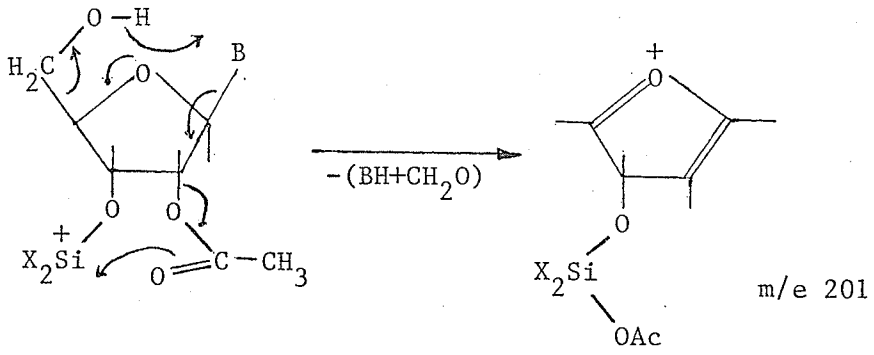


Figure 8. Mass spectrum of 2',3',5'-tri-O-TBDMSadenosine (LX).

Table XI. Some Diagnostically Important Ions in the Mass Spectra of Acetyl-silyl Uridines.

OH ⟶	5'	5'	
RX_2Si ⟶	2'	3'	
Ac ⟶	3'	2'	
m/e	RI%	RI%	Ion type$^+$
400	-	-	M
343	11.5	100	M-57
283	1.8	36	M-57-HOAc
253	4.9	1.9	M-57-HOAc-CH_2O
231	4.9	17.8	
201	72	2.0	SiX_2+OAc+$C_4H_4O_2$
179	2.4	21	B+68
171	15.8	44	
169	43	10.6	B+SiX_2
129	7.4	16.2	SiX_2+71
117	100	49	X_2SiOAc
113	12.9	23	B+2H
75	34	39	X_2SiOH
73	26	21	X_2SiCH_3

Mechanism B



Mono-O-tosyl-di-O-TBDMSribonucleosides. The data for uridines and adenosine derivatives are given in Table XII.

Case of Uridine. The 2'-O-silyl isomer is recognised by the more abundant ions at $(B+SiX_2+42)^+$ and $(B+SiX_2)^+$ and the 3'-O-silyl isomer by the more intense ion at $(SiX_2+SiX_2R+70)^+$.

Case of Adenosine. The 2'-O-silyl isomer is recognised by a small peak at m/e 331 ($BCH=CHOtosyl^+$) and the 3'-O-silyl isomer by the large peak at $(SiX_2+SiX_2R+70)^+$.

Liquid Chromatography. All TBDMS derivatives of ribonucleosides have good liquid chromatographic properties due to low polarity of silyl ethers and the stability of TBDMS ethers towards hydrolysis. Whereas tlc was very useful in this project for monitoring the reactions, thick layer chromatography on silica gel plates was the most valuable method for the preparative isolation of crystalline samples of most silyl derivatives. The mobility of silyl ethers in non-polar solvents is dependent on the number of silyl groups, the position of substituents in partial derivatives and on the nature of the nucleobase. Thus, the fully protected compounds have the greatest mobility while for the

Table XII. Some Diagnostically Important Ions in the Mass Spectra of Mono-tosyl-di-silyl-ribonucleosides.

	Uridine			Adenosine		
Tosyl →	2'	3'		2'	3'	
RX_2Si →	3',5'	2',5'		3',5'	2',5'	
m/e	RI%	RI%	m/e	RI%	Ri%	Ion type$^+$
626	0.08	-	649	-	-	M
569	11.3	27	592	19.1	29	M-R
308	-	-	331	2.3	-	B+tosyl+42
261	9.1	2.2	261	9.5	2.2	SiX_2+SiX_2R+70
229	25	44	229	7.8	7.6	
211*	5.8	52	234	4.7	7.6	B+SiX_2+42
191	4.0	29	191	2.8	2.4	
171	9.8	19.4	171	15.8	6.0	O-tosyl
169	13.9	67	192	2.9	3.7	B+SiX_2
155	11.6	28	155	20	9.5	tosyl
149	16.3	28	149	26	12.4	
145	13.7	8.2	145	14.0	4.4	RX_2SiOCH_2
137	35	7.8	137	6.9	5.5	
129	17.7	28	129	22	12.9	SiX_2+71
117	14.6	22	117	18.4	6.4	SiX_2+59
115	19.5	22	115	16.7	6.7	RX_2Si
113	4.8	11.0	136	22	32	B+2H
91	25	64	91	37	13.7	$C_7H_7^+$
89	75	42	89	63	13.4	HX_2SiOCH_2
75	43	78	75	27	8.1	X_2SiOH
73	100	100	73	100	30	X_2SiCH_3
57	18.5	47	57	96	100	$C_4H_9^+$

* Same m/e as $RX_2SiC_5H_5O_2$

partially protected derivatives, 2'(3')-O-TBDMS compounds moved faster than 5'-O-TBDMS isomers. The order of mobilities in a relatively non-polar solvent was found to be the same in all cases examined. For example, 2',5'-di-O-TBDMS derivatives of uridine, adenosine and cytidine are more mobile than the 3',5'-di-O-derivatives. The only limitation is that 2'-and 3'-O-TBDMS derivatives can not be separated by any of the solvents used so far.

Gas Chromatography. This subject has been investigated by M.A.Quilliam[144] in the case of the TBDMS and other sterically crowded silyl derivatives of nucleosides. Although this study is not as extensive for ribonucleosides as in the case of deoxyribonucleosides, a number of interesting observations have been made. It was found, for example, that direct analysis of partially silylated derivatives of ribonucleosides by gas chromatography was complicated by intramolecular rearrangements. This meant, in effect, that further derivatization of the compounds to be chromatographed was necessary.

High Performance Liquid Chromatography (HPLC) was investigated only briefly because the instrument became available towards the end of this project. Fig.9 illustrates the successful HPLC analysis of a mixture of di-O- and tri-O-TBDMSuridines. HPLC appears very promising for separating the TBDMS derivatives of ribonucleosides and ribonucleotides. With its higher resolution, it may be possible to separate all the partial derivatives of ribonucleosides. In one case attempted, long retention times and severe tailing were encountered for 5'-O-TBDMSuridine. The solvent system and conditions still need to be optimised for three mono-

silyluridines. The retention data are given below (Table XIII), under the conditions as described in Figure 1.

Table XIII: Relative retention times of TBDMS derivatives of uridine by High Performance Liquid Chromatography

Compound	Relative Retention Time[a]
2',3',5'-Tri-O-TBDMSuridine	1.000
2',5'-Di-O-TBDMSuridine	1.081
2',3'-Di-O-TBDMSurindine	1.494
3',5'-Di-O-TBDMSuridine	1.722
2'-O-TBDMSuridine	4.23[b]
3'-O-TBDMSuridine	4.71[b]
5'-O-TBDMSuridine	26.94[c]

a = uncorrected for dead volume
b = tailing
c = severe tailing.

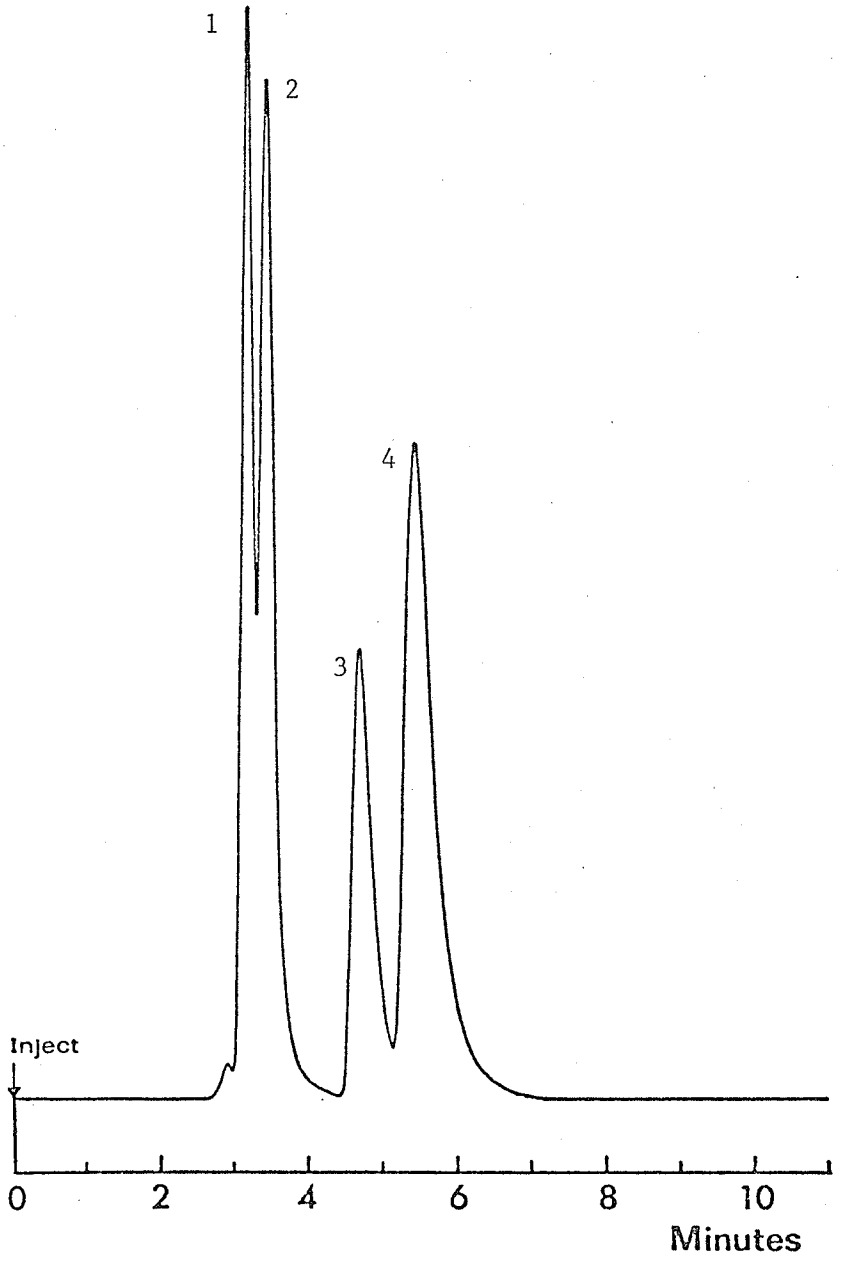


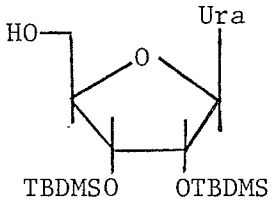
Figure 9. High performance liquid chromatograph of a mixture of 2',3',5'-tri-O-TBDMSuridine (1, 2.075 µg), 2'5'-di-O-TBDMSuridine (2, 1.83 µg), 2',3'-di-O-TBDMSuridine (3, 1.83 µg), 3',5'-di-O-TBDMSuridine (4, 2.95 µg).

Conditions: 3mm-ID X 0.25m column packed with 5µ Sperisorb Silica; 0.4 ml/min ethyl acetate mobile phase; UV detection at 280nm.

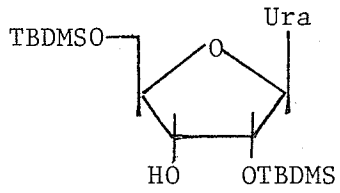
Building Blocks for Oligoribonucleotide Synthesis

A general approach to oligoribonucleotide synthesis requires four building blocks derived from each ribonucleoside: a terminal 2',3'-, a terminal 2',5'-, a non-terminal 2'-, and a non-terminal 2',5'-protected derivative (I to IV, respectively). In this research, methods have been developed, as explained earlier, by which all the four types of 2'-TBDMS protected uridine derivatives can be easily available.

Terminal nucleoside units

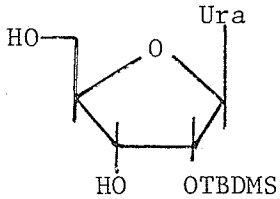


I

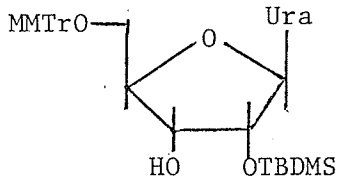


II

Non-terminal nucleoside units



III



IV

In the following pages, the use of these building blocks for the synthesis of ribonucleotides has been illustrated.

It is now appropriate to summarise the advantages offered by the TBDMS protected derivatives:

1. Ready accessibility of 2',5'-protected derivatives as building blocks mentioned above by simple synthetic methods is a a great advantage over many known protecting groups for ribonucleotide synthesis.

2. These derivatives can be obtained very easily as crystalline substances and it is then possible to avoid contamination with positional isomers and other impurities which

could lead to oligomers with incorrectly orientated internucleotidic linkages.

3. From studies on isomerisation under phosphorylating conditions, it is clear that no special precautions are needed to minimise isomerisation of the TBDMS group as is necessary, for example, when using acyl groups.

4. Their adapatability to various analytical techniques such as GC, MS, GC/MS and HPLC.

5. Their compatibility with a variety of conditions in synthetic transformations in organic chemistry.

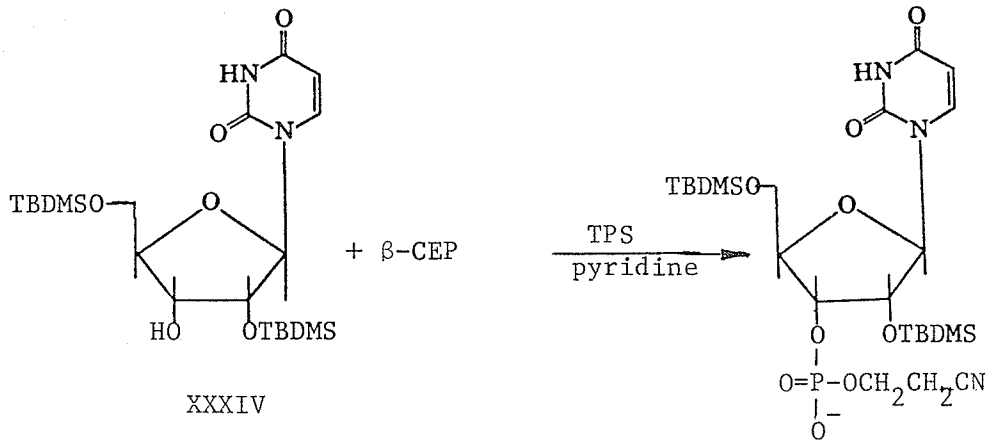
6. Since the TBDMS group is devoid of chirality, its use introduces no stereochemical complications which is an important factor in the case of complex molecules.

Use of the TBDMS Protecting Group in the Synthesis of Diribonucleoside Monophosphates (Triester Method)

β-Cyanoethyl Ester of 2',5'-Di-O-TBDMSuridine-3'-phosphate(LXXIII): Synthesis and isolation on silica gel plates

The basic feature of the triester approach for nucleotide synthesis as reported by various workers[18,22,19,24] is a two-step reaction, i.e. phosphorylation followed by subsequent coupling at each synthetic step. It is well known that initial phosphorylation does not give a pure 3'-phosphodiester component[27,121]. It was, therefore, thought that this problem could be overcome by first isolating the 3'-phosphodiester on silica gel plates, followed by the use of this pure material for condensation with a nucleoside component.

Several experiments were performed to find the optimum conditions for phosphorylating 2',5'-di-O-TBDMSuridine and isolating the product on plates.



XXXIV

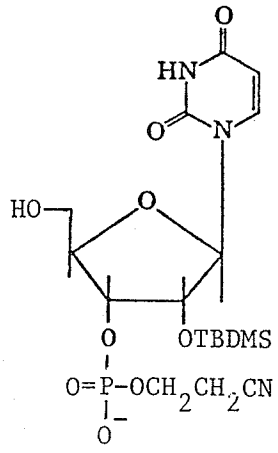
LXXIII

Initially 2 equivalents of the pyridinium salt of β-cyanoethyl phosphate and 4 equivalents of the activating agent TPS were used but the rate of phosphorylation was slower (12 hours instead of 6 hours for 95% completion) than for deoxyribonucleosides[18]. This was not unexpected for protected ribonucleosides. Letsinger and Grams[21] phosphorylated 2',5'-di-O-(1-ethoxyethyl)uridine in 16 hours using 2.3 equivalents of β-cyanoethyl phosphate and 4.6 equivalents of TPS. In the present research, most of the initial phosphorylation reactions were performed using 3 equivalents of β-cyanoethyl phosphate and 4 equivalents of TPS when reaction was complete in 6 hours. The absence of starting material could be easily seen by tlc in ether or ethyl acetate. The reaction was quenched by the addition of cold water and the reaction mixture was stirred for a minimum of 16 hours to decompose the pyrophosphates. An examination of the reaction mixture this stage by tlc indicated the presence of three substances. Their R_f values are given below and they have been referred to as Fast, Middle and Slow.

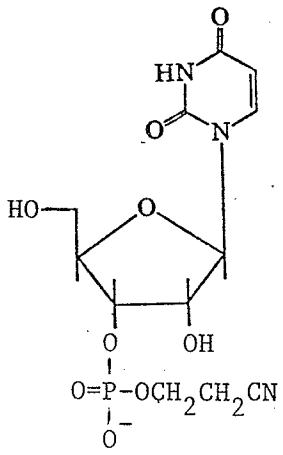
	$CHCl_3$-EtOH (1:1)	$CHCl_3$-EtOH (2:1)	$CHCl_3$-EtOH (3:1)
Fast	0.54	0.56	0.57
Middle	0.48	0.33	0.29
Slow	0.06	0.04	0.03

The fast component was predominant in all cases (∿ 80%). Though no examination of the minor products was made, one explanation for their presence was that a small amount of TBDMS groups was being cleaved during

the long stirring with TPS acid, thus giving the two compounds,



and

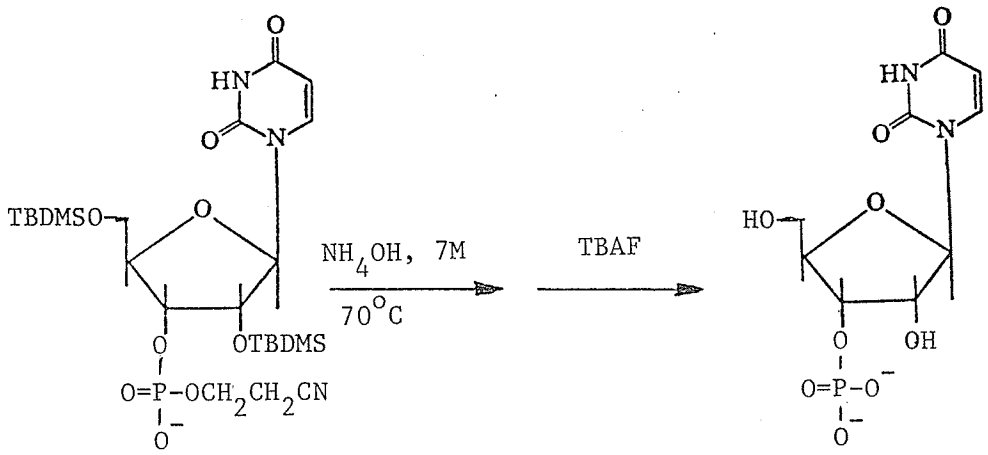


This may not be the only reason as desilylation should be caused to a much smaller extent as learned from studies on the effect of TPS, pyridine and water on 2',5'-di-O-TBDMSuridine (discussed earlier). Further, there is evidence[121] that reaction of β-cyanoethyl phosphate and 5'-tritylthymidine gave a mixture of two trityl-containing phosphate derivatives indicating that the presence of side-products was not unknown during phosphorylation with β-cyanoethyl phosphate.

Pyridine was removed before applying the reaction mixture to silica gel plates so that it would not interfere in the separation of various products. The plates were first developed in ether when a fast-moving non-nucleotidic product separated, but no further investigation was done on this side-product. These plates were then developed 2 or 3 times in chloroform-ethanol(1:1) when a major band and a minor slower band separated. The major band material was eluted in ethanol giving LXXIII in a yield of 50%. The ultraviolet absorption spectrum showed a maximum at 260nm in ethanol.

The nucleotidic character of the material was determined by electrophoresis in a phosphate buffer of pH 7.9. The major component

had an electrophoretic mobility of 0.58. When this was decyanoethylated by heating with 7M NH_4OH at 70°C and then desilylated by treating with TBAF, its electrophoretic mobility changed to 1.05 (Tp=1.00).



LXXIII LXXVIII

In many cases, the faster (major) band material, when examined after being eluted from silica gel, did not prove to be a single substance as seen by tlc in $CHCl_3$-EtOH(1:1) and electrophoresis. Two substances of R_f 0.48 (major) and 0.19 with respective electrophoretic mobilities of 0.58 and 0.83 were present. The proportion of the major (desired) compound varied from 95% to 50%, probably depending on work up procedures used. It is most likely that the impurity resulted from decyanoethylation during work up as indicated by higher values of electrophoretic mobility of the minor component.

Rechromatography on silica gel plates helped to ensure the purity of the phosphodiester (LXXIII) but it resulted in much lower overall

yields (30%). This compound did not remain pure for long at room temperature. In one particular case, a product having Em 0.59 originally was examined after three weeks when two products with Em 1.2(30%) and 0.66(70%) were identified.

Synthesis of a Dinucleoside Monophosphate Using Isolated β-Cyanoethyl Ester of 2',5'-Di-O-TBDMSuridine-3'-phosphate(LXXIII) (Scheme XIV) (Small Scale Preparation)

Having isolated and confirmed the nucleotidic nature of the phosphodiester(LXXIII), the next step was to use it for synthesising a dinucleotide. A small scale preparation was done starting with 0.021 mmol of pure LXXIII and condensing with 2 equivalents of 2',3'-O-isopropylideneuridine in the presence of 2 equivalents of TPS for 23 hours. In the end, the reaction mixture was stirred with water for 9 hours.

Deprotection was achieved, after removing the solvents, by treating the reaction mixture directly with 3 equivalents of TBAF (assuming 100% conversion) for 30 minutes. An attempt was made to find the nature of various substances present in the reaction mixture by the assistance of paper chromatography in solvent A and electrophoresis. Em and R_f^A values and relative amounts of constituents in a particular band are tabulated below. The faster bands were not investigated.

Bands	R_f^A	Em (Relative Percentages)
I	0.37	0.88 (50), 1.05 (40)
II	0.48	0.60 (80), 1.00 (20)
III	0.64	0.61 (20), 0.71 (70)
IV	0.77	0.41 (80), 0.68 (20)

Electrophoresis indicated that each band contained impurities. On rechromatography, bands II and III gave different components, as shown below:

Bands	R_f^A (Relative Percentages)
II	0.20 (20), 0.40 (80)
III	0.63 (70), 0.22 (20), 0.37 (10)

These data indicated the presence of five substances in the desilylated reaction mixture, with Em values 1.00, 0.41, 0.71, 0.88 and 0.61. The first two were easily identified as Up and U-OIP (authentic U-OIP also moved like this) respectively. Out of the remaining three, the substance with Em 0.6 (R_f^A 0.40) was found to be the dinucleotide UpU-OIP (LXXVI, Scheme XIV), the yield being 57%. The procedure for its characterisation is described below.

But first it was necessary to know whether TBAF influenced the electrophoretic mobility and the paper chromatographic properties of a deprotected dinucleotide.

Enzymatic Degradation and Chromatographic Behaviour of Authentic UpU Treated with Tetra-n-butylammonium fluoride(TBAF)

For this purpose, authentic UpU(ammonium salt, Sigma Chemical Co.) was treated with excess of TBAF in THF solution for 15 hours. Electrophoresis on samples taken after 40 minutes and 15 hours indicated no difference in the mobility of these two samples and authentic UpU. On the other hand,paper chromatography in solvent A revealed the existence of two bands, one at R_f 0.14, as UpU, and a wide band at R_f 0.65. The electrophoretic mobility of the R_f0.65 substance, after being eluted from papers and lyophilised, was the same as that of the R_f0.14 substance

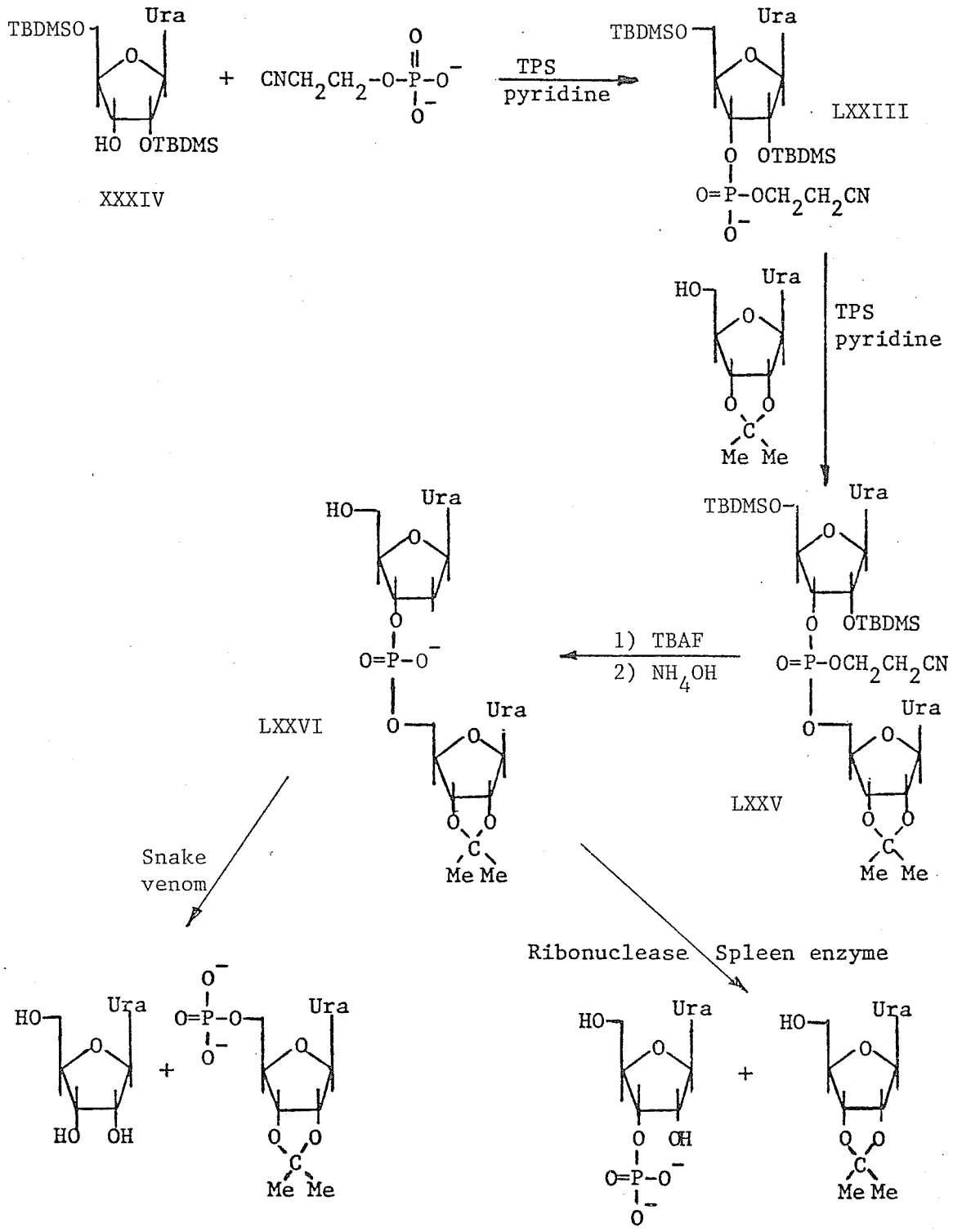
and authentic UpU. Rechromatography in solvent A of the R_f 0.65 substance showed it to be no longer moving fast, its R_f being the same as that of pure UpU. In another similar experiment where the quantity of TBAF was thrice as much, more than 70% of the faster moving product (R_f 0.72) did not move normally like UpU after one rechromatography on paper in solvent A unlike the result obtained when the quantity of TBAF was only one-third. The mobility changed though, from R_f^A 0.72 to 0.54 (UpU =0.39). The faster moving product (R_f0.72) was degraded by ribonuclease to uridine and Up. These experiments indicated that the mobility of a part of the nucleotides changed after treatment with TBAF. As well the enzyme ribonuclease was not inhibited by the 'impurity' which caused UpU to move much faster on papers in solvent A. The same results were obtained when UpU was treated with TBAF for one hour and 18 hours.

Characterisation of the β-Cyanoethyl Ester of 2',5'-Di-O-TBDMSuridylyl-(3'-5')-2',3'-O-isopropylideneuridine (LXXV, Scheme XIV)

The deprotection of LXXV resulting in a product of R_f 0.4 (UpU-OIP) has been discussed above. A part of the R_f 0.4 substance, when incubated with snake venom enzyme, was completely degraded to the expected nucleotide pU-OIP (LXXVII) and uridine nucleoside as shown by paper chromatography in solvent A, the ratio of U and pU-OIP being equal to 1.00.

Two characteristics of snake enzyme should be stated. This enzyme requires the nucleotide to have a free 3'hydroxyl. Though presence of a 3'-phosphoryl terminal group confers resistance on the substrate, small groups like methyl at the 2' position[122] or 2',3'-isopropylidene[123] do not affect the ability of the enzyme to recognise a non-phosphorylated 3'-

Scheme XIV. Synthesis and characterisation of 2',5'-di-O-TBDMS-uridylyl-(3'-5')-2',3'-O-isopropylideneuridine (LXXV).



hydroxyl.

Laskowski et al[153] have found that dinucleoside moncphosphates having 2'-5', 3'-5' and 5'-5' linkages are hydrolysed by the action of snake venom enzyme. This meant that degradation studies by 3'-5' specific enzymes as spleen phosphodiestrase and/or pancreatic ribonuclease were essential to distinguish 3'-5' linked compounds from 2'-5' linked compounds.

To determine the exact mode of internucleotide linkage present in the dinucleotides LXXV and LXXVI, one part of the R_f0.4 substance was incubated with spleen enzyme for 8 hours, when approximately 80% of the dinucleotide LXXVI degraded to Up and U-OIP. It was thought that the presence of the undegradable material could arise for two reasons. The spleen enzyme might have been inactivated by fluoride ions as TBAF was used to deprotect the dinucleotide LXXV. As rechromatography had been performed to obtain the R_f^A 0.4 product, only a trace amount of fluoride ions could be present in UpU-OIP. The effect of the presence of fluoride ions on the activity of spleen phosphodiestrase has been investigated by Hilmoe[124] using different quantities of NaF and the enzyme is not very sensitive to fluoride (and arsenite)ions.

Another reason could be the use of insufficient quantity of TBAF (3 equivalents) for a limited time (30 minutes). This factor has a bearing on the degradability of the dinucleotide as shown in the following account.

Desilylation of a Dinucleotide using 'Limited' and 'Excess' amounts of TBAF

To study the effect of limited and excess quantities of TBAF, the

dinucleotide LXXXIV was treated with i) 2 equivalents of TBAF for 35 min. and ii) 44 equivalents for 4 hours. The products were separated in both cases by paper chromatography in solvent A and in both the cases two products at R_f^A 0.50 and 0.85 were obtained. The R_f 0.50 material obtained in this first case was rechromatographed in solvent A to remove any fluoride ions which may 'inhibit' the enzyme to be used in the next step. This compound degraded to the extent of ∿60% when incubated with ribonuclease for 4 hours. The ratio of Up to U-OIP was 1:1.35. Beside the undegraded dinucleotide at R_f^A 0.35, minor products of R_f 0.44 and 0.52 were also present. The R_f 0.85 material was again treated with TBAF for a limited period but there was no UpU-OIP present when the reaction mixture was submitted to paper chromatography in solvent A.

In the second case, the results were entirely different. Though two materials at R_f 0.51 and 0.89 were present in a ratio of 3:1, the R_f 0.51 band was completly degradable by incubation with ribonuclease for 2.5 hours. The ratio of Up to U-OIP was 1:1.18. This indicated that use of a limited amount of TBAF was not desirable as the R_f 0.4 product was partially degradable by ribonuclease. The problems and the correct procedure for converting the R_f 0.85 material to UpU-OIP has been discussed separately later in this thesis.

Some Considerations on Possible Isomerisation of the Internucleotide Linkage. The next possibility to be considered was the isomerisation of the internucleotide linkage 3'-5' $\rightleftarrows$ 2'-5' in an acidic medium. The dinucleotide could have isomerised after the reaction was quenched, during the 9 hours in which the reaction mixture was stirred in the presence of TPS acid or when it was deprotected. This view could be supported by

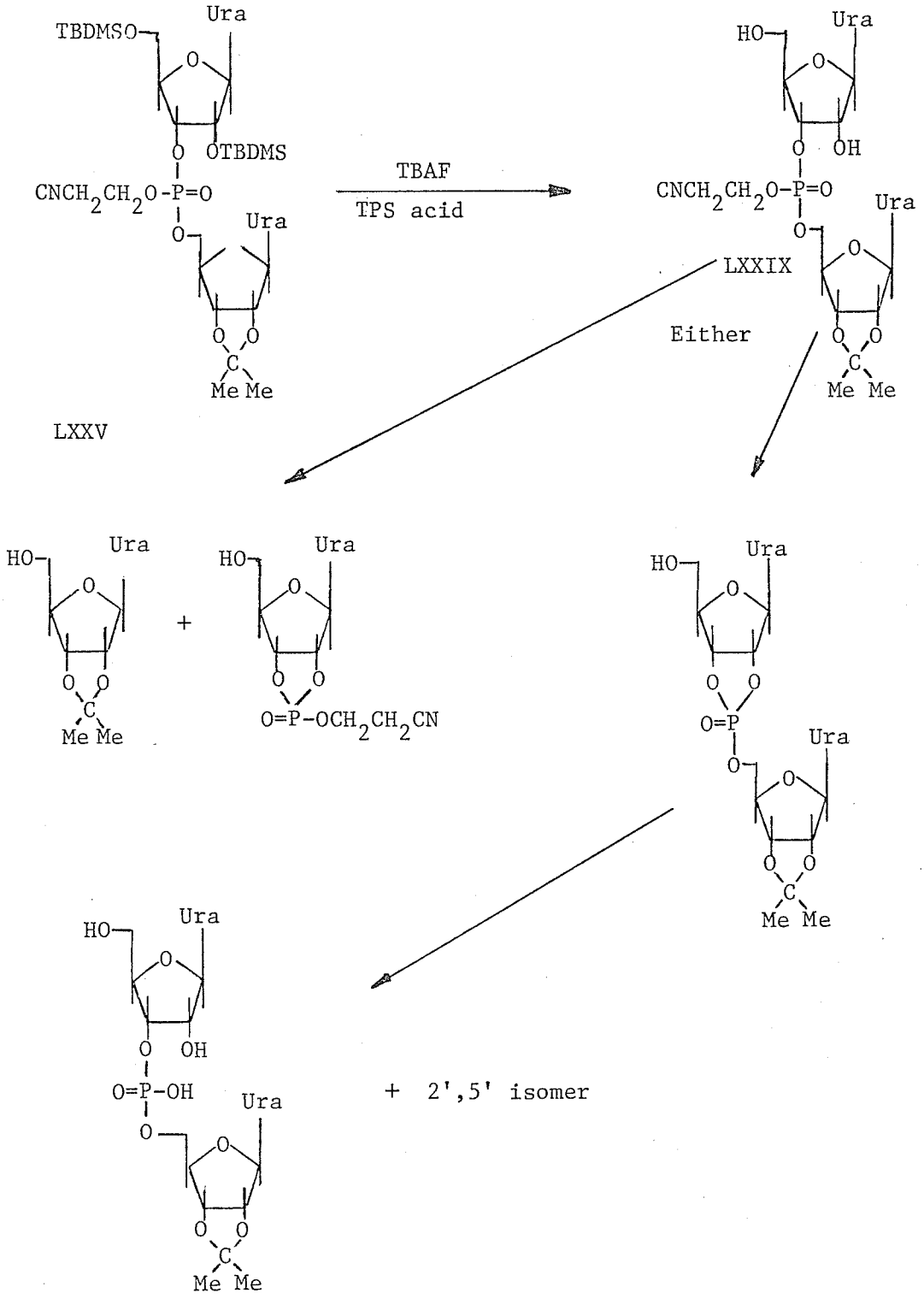
the observations made in the following experiment.

3',5'-Di-O-TBDMSuridine (0.2 mmol) was phosphorylated by β-cyanoethyl phosphate (0.6 mmol) in the presence of TPS (0.8 mmol) and pyridine in 6 hours. The reaction mixture, after being stirred with water, was extracted with chloroform. The phosphorylated product was then condensed with 2'.3'-O-isopropylideneuridine (0.4 mmol) in the presence of TPS (0.6 mmol). The reaction mixture was then stirred with water overnight. On removal of solvents, tlc in EtOAc-THF(2:1) indicated the presence of two new products at R_f 0.32 and 0.82. Whereas the latter was the TPS derivative of 2',3'-isopropylideneuridine (its formation has been discussed separately) the R_f 0.32 substance appeared to be the dinucleotide.

For rapid identification of the dinucleotide formed, approximately 40% of the reaction mixture was directly deprotected by stirring with TBAF (0.5 mmol) for 2.5 hours. The product which separated on papers at R_f 0.39 was found to be partially degradable (∿40%) by ribonuclease. This unexpected degradation could only be explained on the basis of isomerisation of the internucleotide bond as it is well known that ribonuclease does not degrade 2'-5' linked nucleotides. The isomerisation of the internucleotide bond could have happened when the reaction mixture was stirred with water in the presence of TPS acid or when the dinucleotide was being deprotected by TBAF (in the presence of TPS acid) as per the following line of argument.

Smrt[125] has recently pointed out how isomerisation of the fully-protected internucleotide bond can take place when only a trace amount

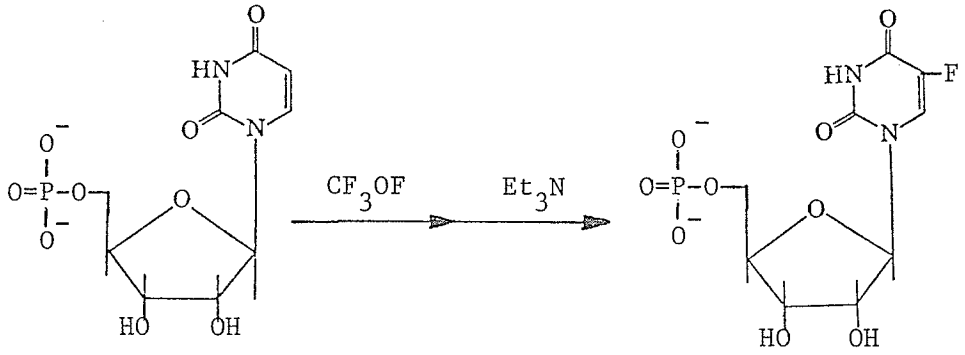
Scheme XV. Possible mechanism of the isomerisation of the internucleotide linkage during deprotection of the dinucleotide LXXV, as based on Smrt's suggestion[125].



of 2'-hydroxyl group is present in the neighbourhood of a protected phosphate group. He studied the action of 90% acetic acid on a dinucleotide in which the 2'-hydroxyl was protected by the tetrahydropyranyl group. After 20 hours, the reaction mixture contained Up, its β-cyanoethyl ester, the nucleoside component and 2'-5' and 3'-5' linked dinucleotides. The decomposition reaction was initiated when a trace amount of 2'-O-tetrahydropyranyl group was split off and the resulting derivative with a free 2'-hydroxyl underwent intramolecular cyclisation accompanied by removal of either the β-cyanoethyl group or a nucleoside. The cyclic phosphotriester thus obtained was opened with the formation of the phosphodiester (2'-5' and 3'-5'). The acidity of the phosphodiester autocatalytically accelerated the removal of the tetrahydropyranyl group thus starting a chain reaction(Scheme XV).

Partial Enzymatic Degradation and Tightly-Held Fluoride Ions

Recently Robins[1,126] has made significant observations about the effect of fluoride ions on nucleotides. While preparing 5-fluorouridine-5'-monophosphate (LXXXI) by direct fluorination of uridine-5'-monophosphate



LXXX LXXXI

5'-FUMP

sodium salt using trifluoromethyl hypofluorite in trichlorofluoromethane, they found the presence of tightly associated fluoride with initially precipitated samples of LXXXI (disodium salt). They found that analytical values for fluorine were essentially twice the theoretical amount. The ^{19}F NMR spectrum contained a peak with the appropriate splitting for the C_5-F substituents plus broad absorption in the range expected for fluoride anion. A ^{31}P NMR spectrum showed no evidence of P-F bonding. Reprecipitation from aqueous alcoholic solutions failed to reduce the analytical value for fluorine content. Robins and co-workers[1,126] prepared pure LXXXI by adsorbing the impure substance on a carbon column followed by elution with various solvents and a crystallisation.

When they attempted the direct fluorination of UpU (tetra-n-butyl ammonium salt), the product was hydrolysed by snake enzyme and alkaline phosphatase to 5-fluorouridine as the only UV absorbing substance. However, more careful examination of the reaction mixture indicated that along with identifiable fluorinated side products, there were some other products which were not hydrolysed to 5-fluorouridine by pancreatic ribonuclease. The desired 5-fluorouridylyl(3'-5')-5-fluorouridine was present in less than 20% yield (enzymatically degradable). Three separate enzymatic determinations gave 2'(3')-FUMP/ 5-fluorouridine = 0.85/1; 1.15/1; 1.2/1. The paper chromatographic behaviour of the fluoride associated substances was not reported. These authors also pointed out another example of a tightly associated ion (Hg^{2+} presumably) in nucleoside synthesis.

Smith et al[128] have also mentioned the necessity for careful chromatography to remove contaminating fluoride in the synthesis of a

nucleoside phosphofluoridate; for example when preparing thymidine-5'-phosphofluoridate from thymidine-5',(2,4-dinitrophenyl)phosphate by the action of sodium fluoride.

Synthesis of β-Cyanoethyl Ester of 2',5'-Di-O-TBDMSuridylyl-(3'-5')-2',3'-O-isopropylideneuridine(LXXV) and Isolation on Silica Gel Plates (Modified Procedure).

To exclude the possibility of isomerisation of the internucleotide linkage, it was necessary to modify the procedure for the phosphorylation and condensation reactions so that contact with TPS acid might be minimal. Another important object was to be able to isolate the pure dinucleotide as a fully-protected molecule on silica gel plates.

Although a yield of 57% was achieved in the small scale experiment for synthesising the title compound(LXXV) from isolated β-cyanoethyl ester of 2',5'-di-O-TBDMSuridine-3'-phosphate, this approach was discontinued. Rechromatography was almost always necessary and the overall yield was of the order of about 30%. Therefore an extraction procedure was adopted to isolate the monophosphorylated product.

As breaking up of the pyrophosphate involved stirring with water, more pyridine was added to protect from cleavage the more labile group. In this and most other condensation reactions, 2 equivalents each of TPS and nucleoside component were used. The effect of variation of this ratio was not studied. Unlike the previous procedure, instead of stirring with water after the condensation reaction, the reaction mixture was extracted with chloroform immediately after addition of water. The chloroform extract had at least three products with R_f values of 0.30

(U-OIP), 0.47 (the desired product) and 0.80 (5'-TPS derivative of U-OIP) after tlc in ethyl acetate. The last named product was the most predominant. A good reproducible separation on plates required complete removal of pyridine and this was achieved by co-evaporating with toluene. Care was necessary in choosing solvents for developing, particularly when different solvent systems were being tried in an attempt to get a perfect separation. For example, the relative positions of U-OIP and the product bands changed when EtOAc was replaced by ether-EtOAc(1:1). The product band moved faster than U-OIP in EtOAc but it was slower in ether-EtOAc(1:1). Elution of the product was very slow in EtOAc. THF was not used because a syrup was obtained on removing THF which never became a solid, even though distilled THF was used. Methanol was used for rapid elution to minimise decyanoethylation on silica gel. A yield of 20% was achieved, but no optimisation was attempted.

Characterisation by Enzymatic Hydrolysis. The fully protected dinucleotide LXXV was deprotected by reaction with excess (~40 equivalents) of TBAF in THF for 7 hours. Paper chromatography in solvent A showed two products of R_f 0.49 and 0.85 in the approximate ratio 1:3 respectively.

The R_f 0.49 substance was completely degraded by bovine pancreatic ribonuclease to give Up (R_f^A 0.09) and U-OIP (R_f^A 0.74) in a ratio of 1:1.05. Spleen enzyme also degraded this substance completely to give two substances of R_f^A 0.10 (Up) and 0.76 (U-OIP). This clearly established that only a 3'-5' link was present in the dinucleotide synthesised. It was also obvious that no isomerisation of the internucleotide bond took place when employing the modified method.

Desilylation Studies. Complete deprotection of a nucleotide is of primary importance as in many applications it is the unprotected nucleotide which is required. In the desilylation reaction described above about 75% of the product had a higher R_f^A value of 0.85 and apparently it was not the 'normal' product.

In an attempt to obtain to obtain a single product of R_f^A 0.40 the same dinucleotide as used above was first treated with TBAF (50 equivalents) for 11 hours and then with 15N NH_4OH for 24 hours. Again two products were obtained. In this case when the faster product was examined, it was degraded to the extent of ∿80% by pancreatic ribonuclease. Obviously, this was not due to the presence of any 2'-5' linkage as the same compound later degraded completely by first converting the R_f^A 0.85 product to 0.4 product by treatment with conc.NH_4OH. The reason(s) for its higher mobility and its partial resistance to ribonuclease degradation must have something to do with the presence of the fluoride ion which was either tightly associated in the manner referred to by Robins et al[126] and described above in detail, or in some other hitherto unknown way.

It became necessary at this stage to find some way to have a fully degradable dinucleotide which had a'normal' R_f value. First it was ensured that TBDMS groups were completely cleaved by treating the R_f 0.8 substance with a further amount (40 equivalents) of TBAF for 10 hours. Paper chromatography in solvent A separated two main products of R_f 0.41 and 0.80 in the approximate ratio of 3:7. It was ensured that a simple re-chromatography of the R_f^A 0.8 material did not give a 'normal' nucleotide of R_f^A ∿0.4 as seen in one of the cases when UpU was treated with TBAF.

The suggestion that a simple rechromatography could convert R_f 0.85 material to R_f 0.4 material was further considered. In three cases out of the four examined for desilylation, there was no change at all in the mobility of R_f 0.85 material after rechromatography in solvent A. In one case, about 30% of the material appeared at R_f 0.41 and 15% each of two substances had R_f values of 0.73 and 0.6. The remaining portion still had the higher mobility (R_f 0.86). Beside these examples, there were other situations where R_f 0.85 material was rechromatographed but without showing any change in R_f value. For example, in several cases the R_f 0.85 material was treated with spleen enzyme and the reaction mixture was separated in solvent A. Neither was there any degradation by the enzyme, nor was there any product at R_f 0.4. Therefore it appeared that a simple rechromatography was not a satisfactory procedure for obtaining a product of the 'normal' mobility from the R_f 0.85 material.

Further, there was no doubt that 'excess' treatment with TBAF did not prevent the R_f 0.85 material from completely changing to R_f 0.4 material. On the contrary, in several cases when only a limited quantity of TBAF was used for short periods (30-60 minutes), the product consisted of only one fast band of R_f 0.85.

Another point was that when the R_f 0.85 substance was treated with 7M NH_4OH for 11 hours, and then chromatographed in solvent A, about 80% of the product was at R_f 0.4 and was totally degradable by ribonuclease and spleen enzyme. In all previous separations on papers in solvent A after treatment with TBAF the approximate ratio between the slow and the fast product was 1:3 or 1:4 respectively (20-25% of R_f 0.4 product). It appeared that use of more concentrated ammonia for longer periods helped

to convert much larger amounts of the R_f 0.8 substance to the 'normal' dinucleotide of R_f^A 0.4. There seemed little doubt that the remaining 20% could also be converted to the desired product by repeating the ammonia treatment. Even repeated treatment with TBAF followed by chromatography in solvent A, which is probably not as suitable as the ammonia treatment procedure, gave 100% conversion.

The effectiveness of ammonia treatment could be due to two reasons. It is conceivable that fluoride ion did not remove a silyl group adjacent to a 3'-phosphate anion. In this case the R_f 0.85 material is 2'-O-TBDMS-uridylyl-(3'-5')-2',3'-O-isopropylideneuridine and ammonia treatment cleaved the 2'-TBDMS group. It has been shown before that the 2'-silyl is quite susceptible to base cleavage.

Alternatively, the abnormal mobility is due to the presence of tightly associated fluoride ions, which can be removed by ammonia treatment. In this case it might be possible to remove the fluoride ions by adsorbing the 'impure' material on a carbon column as used by Robins et al[126] for purifying a mononucleotide contaminated with tightly associated fluoride ions.

Synthesis of β-Cyanoethyl Derivative of 5'-O-Monomethoxytrityl-2'-O-TBDMS-uridylyl-(3'-5')-2',3'-isopropylideneuridine(LXXXIII) (Scheme XVI)

The monomethoxytrityl (as well as dimethoxytrityl and trityl) group has been widely used for protecting the 5'-hydroxyl group in deoxy- and ribonucleoside synthesis. It has the advantage of being selectively cleaved by 80% acetic acid at room temperature in 4 hours, so that it is possible to synthesise tri and higher nucleotides by extending the chain from the 5'-end of the dinucleotides. It was of great interest, therefore,

to study the compatibility of the MMTr group with the TBDMS group in general and to use 5'-O-MMTr-2'-O-TBDMSribonucleosides, in particular, for the synthesis of oligoribonucleotides.

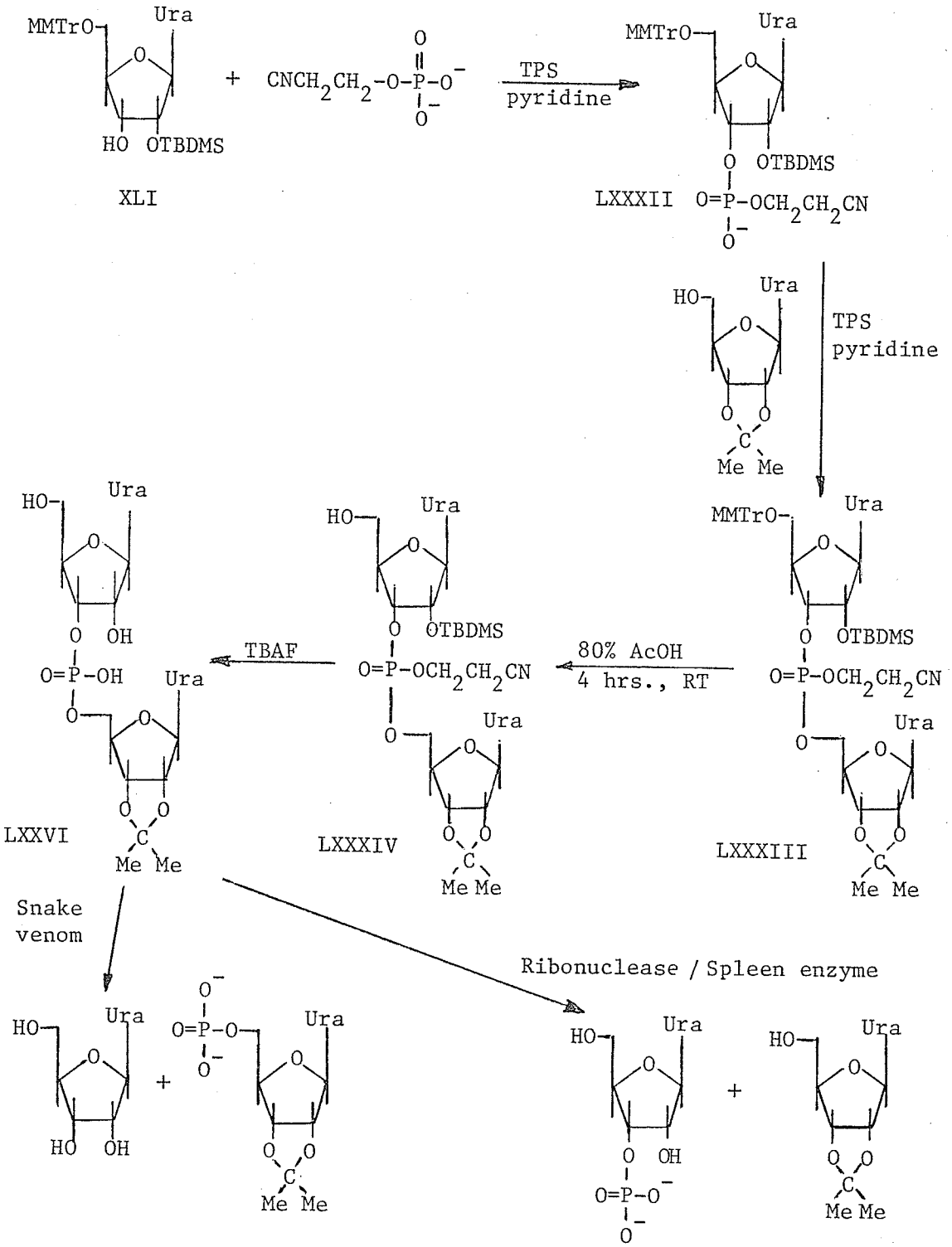
A very convenient one-flask procedure was developed for preparing the derivative 5'-O-MMTr-2'-O-TBDMSuridine directly from uridine in 44% yield, as described earlier in this thesis.

The presence of the MMTr group is very helpful in the identification of various components of a reaction mixture by tlc analysis, as it gives a distinctive yellow colour after being sprayed with $HClO_4$(10%) and warmed by hot air.

5'-O-MMTr-2'-O-TBDMSuridine(XLI) was phosphorylated in 6 hours at room temperature using 3 equivalents of the pyridinium salt of β-cyanoethyl phosphate and 4 equivalents of TPS. A reduction in the amount of TPS to 3 equivalents increased the reaction time to 11 hours. Similarly a reduction in the amount of β-cyanoethyl phosphate required a longer reaction time. Side products were present in this case also as seen before in the phosphorylation of 2',5'-di-O-TBDMSuridine. At least two trityl containing substances were indicated by tlc in MeOH-ether(1:3) when the reaction mixture was tested before and after pyrophosphate breaking. No further investigation was made to find the nature of these two substances.

For the isolation of the monophosphorylated product, the conventional extraction procedure was followed. The chloroform extract on removing the solvent gave a reddish brown flaky solid which was then dried,and condensed with 2 equivalents of U-OIP in the presence of 2 equivalents of TPS for 22 hours. The presence of newly formed dinucleo-

Scheme XVI. Synthesis and characterisation of 5'-O-MMTr-2'-TBDMS-uridylyl-(3'-5')-2',3'-O-isopropylideneuridine (LXXXIII).



tide was indicated by tlc in EtOAc-THF(1:1) and MeOH-ether(1:8). The latter solvent system was preferred because whenever THF was used as a developer, it was not possible to see a good yellow colour spot due to the MMTr group after spraying with $HClO_4$. The fully protected dinucleotide LXXXIII and other water-insoluble substances were isolated by pouring the reaction mixture into ice and water. The precipitated substances were then separated on silica gel plates by two developments in ethyl acetate. A substantial amount of sulphonated U-OIP was present which moved very fast on the plates. Two other main products were U-OIP (R_f 0.41) and the desired product (R_f 0.59), which was eluted in a large excess of ethyl acetate. This was a single spot material as seen by tlc in MeOH-ether(1:8). The product was obtained as a solid in a yield of 21%.

Two other methods were also tried for isolating the fully protected dinucleotide. In one case, after quenching the reaction with water, the reaction mixture was extracted in chloroform. After removing solvents, including residual pyridine, the residue was dissolved in ethanol and separated on silica gel plates using ether as a developer. The three main products beside tritanol were sulphonated U-OIP (R_f 0.68), U-OIP (R_f 0.22) and the desired product (R_f 0.13) which was eluted in THF. This was obtained as an oil after removing THF, unlike the previous case, though dry THF was used. Because of this difficulty, the use of this solvent was avoided for eluting purposes whenever possible. The yield as determined spectrophotometrically was 26.5%.

These compounds, isolated by different methods, were characterised by enzymatic methods after deprotection as described below.

Deblocking of 5'-O-Monomethoxytrityl Group. The removal of the 5'-O-MMTr group is an important and sensitive step as its satisfactory cleavage, without affecting other parts of the molecule, will make possible the extension of the oligoribonucleotide chain at the 5'-end. Moreover, all protecting groups must be eventually removed after nucleotides have been synthesised. The usual procedure for the removal of the 5'-MMTr group requires treatment with 80% acetic acid at room temperature for 4 hours. This procedure was applicable to the synthesised dinucleotide LXXXIII as other protecting groups like 2'-O-TBDMS and 2',3'-O-iso pylidene were not affected.

The purification of the detritylated dinucleotide LXXXIV was done on silica gel plates by developing the plates in EtOAc-THF(4:1) when a major product separated at R_f 0.41 as well as two minor products (one of them was the starting material) and tritanol. The desired product was eluted from silica gel plates in methanol and after removing solvents was obtained as a solid in 93% yield. It was precipitated as a white solid (m.p. 137-147^{o}C) by adding hexane to its solution in ethyl acetate. Tlc in MeOH-ether(1:4) showed it was a single spot material, of R_f 0.12.

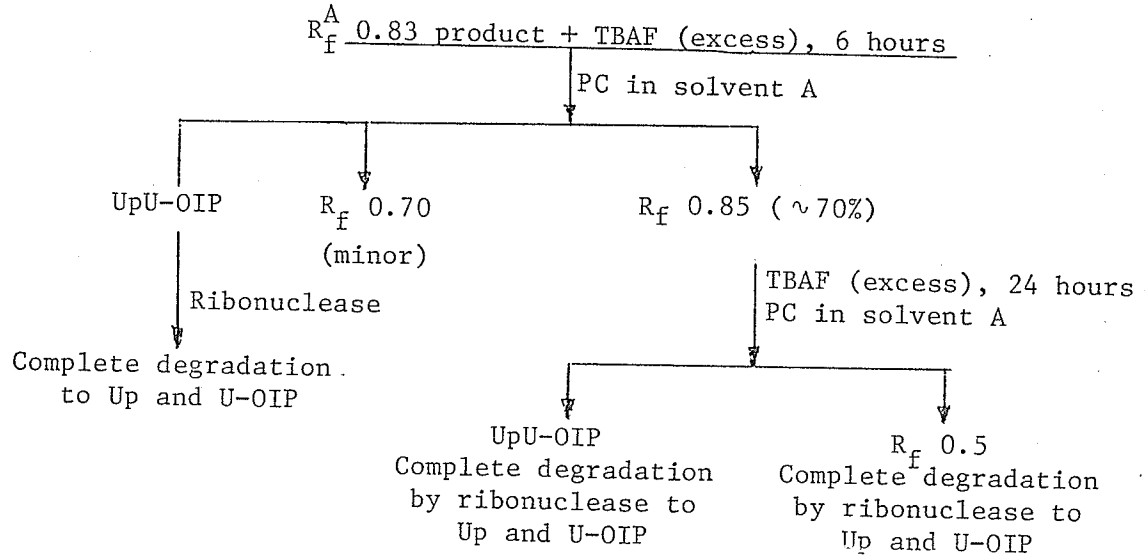
Characterisation by Enzymatic Hydrolysis. The detritylated product was treated with TBAF (26 equivalents) at room temperature for 5 hours and the products were separated by paper chromatography. At least 25% of the product appeared at R_f^A 0.43 and the rest was at R_f^A 0.83.

The R_f^A 0.43 product was completely degraded by ribonuclease giving

Up and U-OIP in the ratio 1:1.1. This clearly demonstrated that the dinucleotide had only a 3'-5' linkage. There remained the problem of complete conversion of the detritylated nucleotide(LXXXIV) to a product of R_f^A ∿0.4, which is discussed below.

Conversion of the Faster Product (R_f^A ∿0.8) to UpU-OIP. The presence of a faster moving product was noticed before while purifying the desilylatd dinucleotide LXXVI obtained from 2',5'-di-O-TBDMSuridine. The various possible reasons for the presence of this unexpected product and methods to convert it to degradable UpU-OIP have been discussed earlier.

A slightly different procedure was used in this case as shown in the following flow sheet. It was possible to convert nearly 100% of the detritylated dinucleotide LXXXIV to 'normal' degradable UpU-OIP. This procedure employing repeated treatment with TBAF was used on the assumption that it might be the uncleaved 2'-TBDMS group which was responsible for the higher mobility.



PC = Paper Chromatography.

It has been mentioned before that ammonia treatment was very effective in converting the fast moving product to the normal R_f^A 0.4 product UpU-OIP. It is quite likely that purification at each step to UpU-OIP was assisted in the present procedure by the repeated action of ammonia (from solvent A) on the 'impure' product followed by rechromatography on papers.

To restate, the following two precautions were observed while obtaining a completely degradable dinucleotide,

i) Used excess of TBAF (∿50 equivalents)

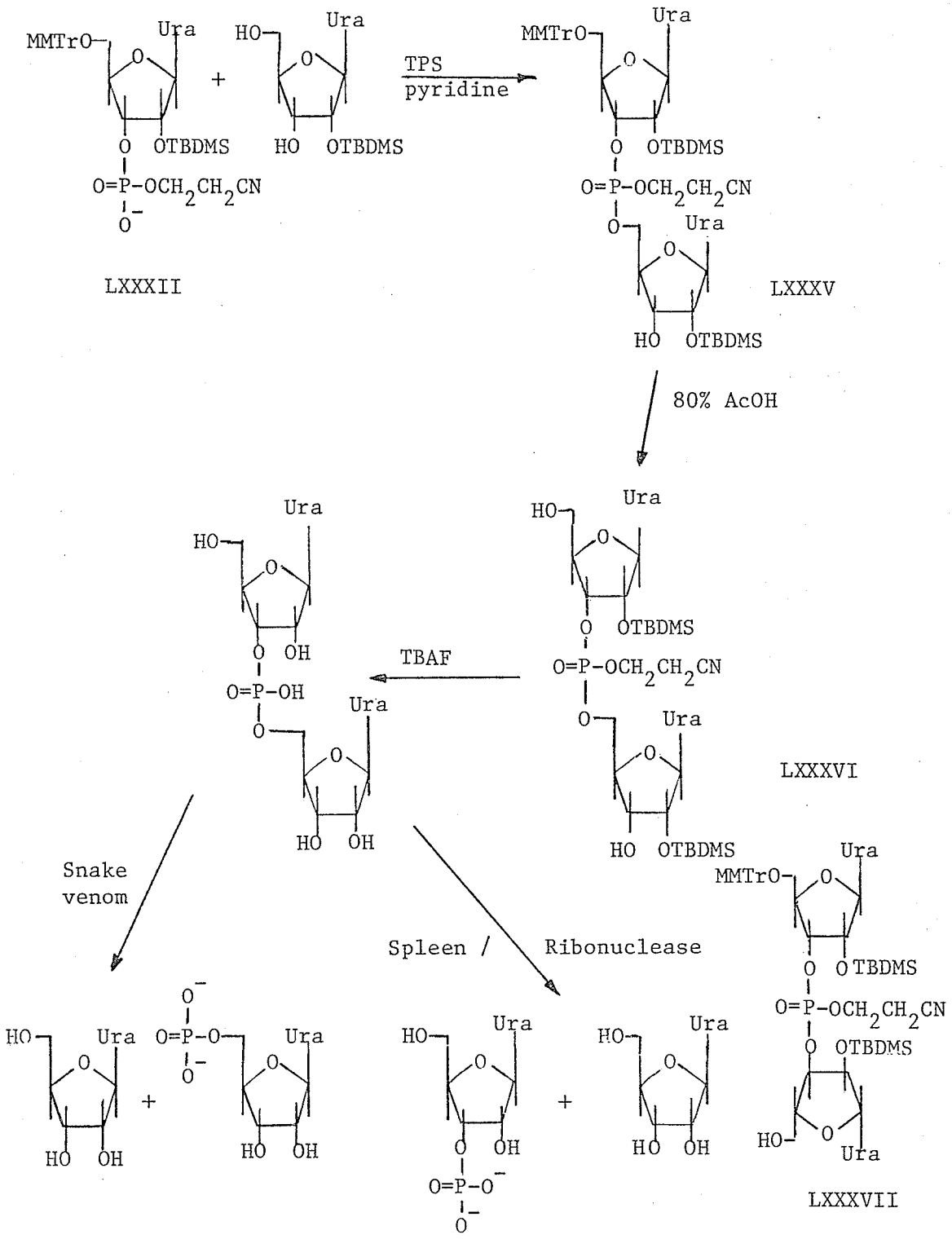
ii) The faster band was treated with concentrated ammonia and the products then purified by paper chromatography in solvent A.

Synthesis of β-Cyanoethyl Derivative of 5'-O-Monomethoxytrityl-2'-O-TBDMSuridylyl-(3'-5')-2'-O-TBDMSuridine (LXXXV) (Scheme XVII)

2'-O-Protected ribonucleosides have sometimes been used as the nucleoside component in the ribonucleotide synthesis. For example, Letsinger and Grams[21] have used 2'-O-(1-ethoxyethyl)uridine for the synthesis of UpU. The use of 2'-protected ribonucleosides has the advantage that no deblocking is necessary when the nucleotide chain has to be extended from the 3'-end. It is essential that the protecting group at the 2'-position should inhibit the formation of a 3'-3' linkage. To examine the suitability of the TBDMS group for this purpose, the dinucleotide LXXXV was synthesised.

2'-O-TBDMSuridine was prepared by the hydrolysis of 2',5'-di-O-TBDMSuridine as described before. The fully protected dinucleotide LXXXV was isolated on silica gel plates using ether as a developer and

Scheme XVII. Synthesis and characterisation of 5'-O-MMTr-2'-O-TBDMS-uridylyl-(3'-5')-2'-O-TBDMSuridine (LXXXV).



two closely moving products of R_f 0.10 and 0.15 separated after five developments. They moved more slowly than 2'-O-TBDMSuridine. Both of these products had a trityl group and both compounds were enzymatically degraded by ribonuclease, spleen and snake venom enzymes to the extent of ∿97%. It is very likely that these two substances were diastereomers due to the presence of a chiral phosphotriester group. Reese et al[129], Neilson et al[131], and Narang et al[130] have reported the existence of diastereomers in ribo- and deoxyribonucleotide triesters. A small amount of 3'-3' linked dinucleotide LXXXVII was present(∿3%).

Comments on the Yields of the Dinucleotides.

Though no optimisation of the reactions leading to the dinucleotide synthesis was attempted, a few comments on this subject are appropriate. There was evidence of a considerable amount of sulphonation of 2',3'-O-isopropylideneuridine at the condensation stage when the β-cyanoethyl ester of 2',5'-di-O-protected-uridine-3'-phosphate was stirred with 2 equivalents each of TPS and 2',3'-O-isopropylideneuridine for 24 hours in pyridine. This fact was not recognised till late in this work because previous investigations[133] had shown that a small amount (9%) of 5'-hydroxyl (in 2',3'-di-O-benzoyluridine) reacted with TPS in 24 hours at room temperature. The presence of a considerable amount of sulphonated product was proved by isolating and identifying this compound.

This was also confirmed by reacting TPS and 2',3'-O-isopropylideneuridine under exactly similar conditions as used for the condensation reaction. In 25 hours, about 47% of the TPS derivative was obtained.

The product obtained had UV absorption maxima in ethanol at 259nm and and 230nm and the mass spectrum showed a parent peak at m/e 550. Other fragments obtained were of m/e 535(M-15) and 439(M-Base). It was apparent that the extent of sulphonation of the 5'-hydroxyl group by TPS was dependent on the nature of the protecting groups present on the 2'and 3'-positions of the uridine molecule. Further evidence supporting this viewpoint was available by studying the reaction of 2',3'-di-O-TBDMSuridine and TPS in pyridine. There was practically no reaction (∿5%) in 24 hours.

It is doubtful that TPS was an ideal activating agent in the present synthesis. Perhaps due to similar difficulties, modifications[133] of Khorana's procedure have been suggested and these are based on ^{31}P-NMR studies on the mechanism of internucleotide bond formation. The requirements are preactivation of the nucleotide component during several hours for the formation of the metaphosphates and the use of as little excess of arenesulphonyl chloride as possible.

Beside the problem of sulphonation noted in this work, it is known that the use of arenesulphonyl chloride gives rise to the formation of symmetric pyrophosphates as by-products. Further, the separation of arenesulphonic acid sometimes tends to be quite difficult. Narang et al[122,130,137] and Neilson et al[135] have found arylsulphonyl 1,2,4-triazoles and tetrazoles to be much more promising activating agents.

EXPERIMENTAL

General Methods

All phosphorylation and condensation reactions were carried out in glass joint flasks. The reactants were dried by the evaporation of pyridine at reduced pressure and air was admitted at the end through a column (30 cm x 2 cm) of anhydrous magnesium perchlorate. All reactions were run at room temperature in daylight. Silylation reactions were carried out in screw-capped vials or glass-stoppered small flasks at room temperature or at 37°C.

Paper chromatography was performed by the descending technique using Whatman 3MM papers, the paper sizes used were either 22 inches x 9 inches or 12 inches x 4 inches (for Shandon's 12 inches x 7 inches diameter tank). The solvent systems used were: Solvent A, isopropyl alcohol-concentrated ammonium hydroxide-water (7:1:2) and solvent B', n-butanol-ethanol-water (4:1:5, organic phase). The solvents were prepared on a volume basis. Whenever required a particular band was cut out and eluted in water and lyophilised to get a solid or an oil.

Thin layer chromatography was run on Eastman 13181 silica gel Chromatogram sheets with fluorescent indicator. In earlier experiments, Eastman 6060, silica gel sheets were used. Dimensions of the strips were 6.6 cm x 2 cm. When acetic acid was present, it was removed under suitable conditions before the samples were spotted on chromatogam strips. Thick layer chromatography was done on glass plates (20 cm x 20 cm) coated with a 1 mm thick layer of silica gel DSF-5 (Camag).

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 one hour. The buffers used were:

i) Triethylammonium bicarbonate buffer, 0.05M of pH 7.5, prepared by adding 15.15 gm of triethylamine into 3 litres of water, followed by slow addition of 20 gm of dry ice and adjusting the pH to 7.5 by adding very dilute triethylamine or dry ice.

ii) Phosphate buffer pH 8.0, prepared by dissolving 0.0033M potassium dihydrogen phosphate and 0.063M disodium hydrogen phosphate in 3 litres of water.

Electrophoretic mobilities (Em) were calculated using a standard thymidine-3'-phosphate as 1.00. For visualising nucleosides and nucleotides on tlcs and papers, an ultraviolet light source (Mineralite, output 254nm) was used.

Equipment

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Ultraviolet spectra were obtained on a Cary Model 14 or 15 recording spectrophotometers. Infrared spectra were obtained on a Perkin-Elmer 700 recording spectrophotometer and samples were prepared in KBr discs unless otherwise mentioned.

Mass spectra were obtained in most cases on a Finnigan 1015 RF-quadrupole mass spectrometer. In a few cases, mass spectra were obtained on a Hitachi-Perkin Elmer RMU-6D single focussing 8" radius magnetic sector mass spectrometer and in one case for determining exact mass values, a CEC 110-B double-focussing mass spectrometer of the UpJohn company (Kalamazoo, Michigan) was used.

High performance liquid chromatography was performed on a Spectra-Physics Model 3500 instrument with a 10 µl volume valve injector and a UV absorption monitor (λ = 280nm) with 5µ Spherisorb Silica. Ethyl acetate was used as mobile phase with flow rates from 0.4 to 3.2 ml/min.

Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, Tennessee. Samples were prepared by crystallisation or precipitation, followed by heating in an oil bath at 60°C under reduced press pressure.

Reagents and Chemicals

Molecular Sieves used were "LINDE" type 3A, diameter 1/16", as supplied by Matheson Coleman and Bell.

Dimethylformamide (DMF). Reagent grade DMF was refluxed over purified calcium hydride (Fisher) and then distilled at atmospheric pressure directly into a reagent bottle containing molecular sieves.

Pyridine. Technical grade pyridine was kept overnight on p-toluenesulphonyl chloride and then refluxed for a minimum 2 hours. It was distilled using a Vigreux column, excluding moisture, into a flask containing calcium hydride and refluxed again. Pyridine was then distilled using a Vigreux column into a reagent bottle containing molecular sieves.

Tetrahydrofuran (THF) dry. Reagent grade THF was kept overnight on sodium hydroxide pellets and then decanted and distilled into a flask. Sufficient $LiAlH_4$ was added to react with the moisture followed by refluxing and distillation into a bottle containing molecular sieves, well protected from light.

Pyridinium β-cyanoethyl phoshate was prepared by first obtaining the pyridinium form of the resin Dowex 50W-X8 by washing this resin in a column with the following solutions in order: 0.1M HCl until clear, water till neutral, pyridine-water (1:9 v/v) until basic and finally with water so that the eluant is neutral. The barium salt of β-cyanoethyl phosphate dihydrate (7 gm, 21.7 mmol,Sigma Chemical Co.) was dissolved by prolonged stirring in about 500 ml of water and the small quantity of an insoluble matter was removed by filtration. The clear solution was passed very slowly over 50 ml of the pyridinium form of the Dowex resin in a column, which was then washed slowly with an equal volume of water-pyridine (1:9). All of the washings were concentrated to a small volume on a flat bed evaporator, lyophilised and dissolved in 50 ml of dry pyridine. One ml of the solution contained about 0.4 mmol of the reagent.

Tetra-n-butylammonium fluoride (TBAF) was prepared by neutralising tetra-n-butylammonium hydroxide (30 ml of 25% solution in methanol, J.T.Baker Chemical Co.) in a glass beaker to pH 7.0, by a weak aqueous solution of HF (diluted and contained in a teflon beaker). Solvents were removed by azeotropic distillation after adding benzene-acetonitrile (1:1, v/v), on a flat bed evaporator. The residue was left overnight on a vacuum pump and then lyophilised. A light dry powder thus obtained was dissolved in 25 ml of dry THF and stored over molecular sieves, protected from light. Ocassionally a syrup was obtained after lyophilisation, which was dried over P_2O_5 in a vacuum desiccator for a day. One ml of this solution contained 1 mmol of the reagent. A similar procedure for obtaining crystals of TBAF has been described by Clark and Emsley[140].

tert -Butyldimethylsilyl chloride was purchased from Willowbrook Laboratories, Waukesha, Wisconsin, and kept in a desiccator stored in a refrigerator. Before using, it was brought to room temperature to avoid any condensation of moisture. This can be prepared according to the procedure given by Sommer and Tyler[72].

Triisopropylbenzenesulphonyl chloride (TPS) was prepared by Peter Ng of this department.

Nucleosides were purchased from Sigma Chemical Co.

2',3'-O-Isopropylideneuridine was synthesised according to the procedure described by Tipson[132].

Solvents for Chromatography: Reagent grade solvents, purchased in bulk, were used directly without any further purification except in the case of THF, which was kept overnight on NaOH pellets, decanted and distilled.

Enzyme Assays

Spleen Phosphodiesterase(Bovine, Type I, Sigma Chemical Co.)

One ml of sodium pyrophosphate buffer (0.01M, adjusted to pH 6.5 with H_3PO_4) was injected into a vial containing about 23 units of the enzyme. About 0.1 ml of this solution and 0.2 ml of 0.5M ammonium acetate (adjusted to pH 6.5 with acetic acid) were added to the nucleotide to be tested (0.1 to 1 mg) in a small vial. This solution was incubated at 37°C for 5 hours, unless otherwise stated, and applied to a 4 cm length of a Whatman paper. The paper was developed in solvent A and the nucleoside and nucleotide bands were cut out, eluted with water and diluted to 10ml, 5ml or 2ml depending on the amount of nucleotide used for the test.

Snake Venom phosphodiesterase (Russel's Viper Venom, Calbiochem, 200 units per vial)

One ml of tris-(hydroxymethyl)-aminoethane(TRIS) buffer (adjusted to pH 9.2 with 0.1M HCl) was injected into a vial as supplied by the manufacturer. About 0.1ml of this enzyme solution was added to the nucleotide (0.1 to 1mg) and incubated at 37°C for 7 hours or as stated. The solution was then worked up as described above for the spleen enzyme.

Ribonuclease (from Bovine Pancrease, Protease Free, Type XIIA, Activity 100 Kunitz units per mg, Sigma Chemical Co.)

5mg of the enzyme was dissolved in 1ml of TRIS buffer (pH 7.8, made by mixing about 34ml of 0.1M HCl with 50ml of 0.1M TRIS). About 30µl of the enzyme solution was added to 0.1-0.5mg of the nucleotide along with 0.2ml of TRIS buffer(pH 7.5) and incubated at 37°C for 3 hours or as mentioned. The solution was worked up as described for the spleen enzyme.

Synthetic Methods

General Procedure for Synthesising TBDMS Derivatives of Ribonucleosides

The ribonucleoside or its derivative, imidazole and TBDMSCl were added to a small flask or a screw capped vial and dissolved immediately in dry DMF (1 ml per mmol of ribonucleoside except in the case of guanosine). The reaction mixture was stirred at room temperature until a tlc, developed in a suitable solvent (see silylation of individual nucleosides) indicated completion of the reaction. Methanol was added to quench the reaction and the solution thus obtained was applied to silica gel plates. Three or four plates per mmol of ribonucleoside were required. In general, plates were not loaded too heavily so that the isomeric 2',5'- and 3',5'-di-O-TBDMS derivatives could separate cleanly without necessitating rechromatography. Plates were developed in suitable solvents as described in the silylation of specific nucleosides. The slower moving 5'-O-TBDMS derivatives were separated from unreacted nucleosides by redeveloping the plates in more polar solvents than those used for separating di- and tri-silyl derivatives. All silica gel bands containing ribonucleosides having a silyl group on either the 2' or 3' position were eluted immediately in appropriate solvents to prevent any isomerisation on silica gel. Single spot materials could then be obtained in all cases.

2',5'-Di-O-TBDMS and 3',5'-Di-O-TBDMSuridines

Uridine (976 mg, 4 mmol), imidazole (1196 mg, 17.6 mmol) and TBDMSCl (1325 mg, 8.8 mmol) were dissolved together in 4 ml of DMF and stirred at room temperature for 2 hours when no uridine remained as indicated by tlc in THF. The viscous reaction mixture was diluted in methanol and applied on 16 silica gel plates, which were developed three times in ether-hexane (2:1) in quick succession. Three fast bands separated, the fastest being 2',3',5'-tri-O-TBDMSuridine followed by the bands of 2',5'-di-O-TBDMSuridine and 3',5'-di-O-TBDMSuridine. The slowest product was 5'-O-TBDMSuridine which stayed at the base line. This was moved by THF by developing the plate to a short height. All of the three fast bands were eluted in ether, about 600-700 ml was used in each case to ensure complete and rapid recovery. The solvents were removed on a flat bed evaporator, and completly removed by co-evaporation with hexane to leave 2',5'-di-O-TBDMSuridine. Yield: 850 mg (1.8 mmol, 45%), crystallised from ether-hexane, m.p. 121-122°C. Chromatographic data are given in Table XV, spectral properties (UV and IR) in Table XIV while mass spectral data are described in Table IX (also see Figure 6).

Anal. Cald. for $C_{21}H_{40}O_6N_2Si_2$: C, 53.25; H, 8.53; Si 11.88

Found: C, 53.47; H, 8.58; Si 11.96

3',5'-Di-O-TBDMSuridine was also obtained as a white solid as described above. Crystallised from ether-hexane. Yield: 600 mg (1.27 mmol, 32%). M.p. 136-137°C. Physical data are given in Tables XV, XIV, IX and Figure 5.

Anal. Cald. for $C_{21}H_{40}O_6N_2Si_2$: C, 53.25; H, 8.53; Si 11.88

Found: C, 53.62; H, 8.70; Si 11.61

In addition 140 mg (10%) of 5'-O-TBDMSuridine was obtained as well as 140 mg (6%) of 2',3',5'-tri-O-TBDMSuridine.

5'-O-TBDMSuridine (XXXIII)

Uridine (488 mg, 2mmol), imidazole (300 mg, 4.4 mmol) and TBDMSCl (331 mg, 2.2 mmol) were dissolved in DMF (2 ml) and stirred at room temperature for 20 hours. After diluting with methanol the solution was applied to five silica gel plates. One development in ether-hexane (2:1) moved di-O- and tri-O-TBDMS derivatives higher up on the plates. Two subsequent developments in ether separated the required compound from unreacted uridine on the base line and the product was eluted in ethanol. A white solid (580 mg, 82%) was obtained on removing ethanol. On crystallisation from ethanol-water, its m.p. was 136-139°C. Chromatographic data is listed in Table XV, spectral properties (UV and IR) in Table XIV while mass spectral data are described in Table VIII and Figure 3.

Anal. Cald. for $C_{15}H_{26}N_2O_6Si$: C, 50.26; H, 7.31; Si, 7.83

Found: C, 50.20; H, 7.38; Si, 7.87

2',3',5'-Tri-O-TBDMSuridine (XXXVI)

Uridine (244 mg, 1mmol), imidazole (952 mg, 14 mmol) and TBDMSCl (1050 mg, 7mmol) were dissolved in 2 ml of DMF and stirred at room temperature for 8 hours. Methanol was added and the solution was applied to four plates which were developed in ether. The main band was at R_f 0.80 which was eluted in 250 ml of ether. On removing solvent, a viscous solid was obtained which was hard to solidify and crystallise. Yield: 500 mg (85%). Chromatographic, mass spectral, UV and IR data are given in Tables XV, X (also see Figure 7) and XIV respectively.

2'-O-TBDMSuridine

2',5'-Di-O-TBDMSuridine (472 mg, 1 mmol) was treated with 10 ml of 80% acetic acid on a steam bath for 40 minutes. The reaction mixture was diluted with ethanol and the solvents were removed under reduced pressure at room temperature. The residue was dissolved in methanol and applied on four silica gel plates which were developed in ether. Three bands appeared at R_f 0.9 (starting material~10%), 0.6 (the required product) and one at the origin (uridine~12%). The main band was eluted in THF and a white solid (260 mg, 73%), m.p. 189-192°C, was obtained on removing the solvent. After crystallisation from ether-hexane (9:1) the melting point was 191-194°C. Mass spectral, chromatographic and spectral (UV and IR) data are given in tables VIII (also see Figure 1), XV and XIV respectively.

Anal. Cald. for $C_{15}H_{26}N_2O_6Si$: C, 50.26; H, 7.31; Si, 7.83

Found: C, 50.48; H, 7.47; Si, 7.77

3'-O-TBDMSuridine

3',5'-Di-O-TBDMSuridine (188 mg, 0.4 mmol) was stirred with 4 ml of 80% acetic acid at room temperature for 24 hours. The reaction mixture was diluted with ethanol and the solvents were removed under reduced pressure. The residue was dissolved in methanol and applied to two silica gel plates which were developed in ether. Two main bands, beside the uridine band at the origin, at R_f 0.78 and 0.57 were obtained. The R_f 0.57 band was eluted in 400 ml of ether. On removing the solvent a white solid was obtained. Yield: 90 mg (63%). On crystallisation from ether-hexane it had a melting point of 204-206°C.

The band at Rf 0.78 was the starting matrial (64 mg). Chromatographic data are recorded in Table XV. The spectral properties are listed in Table XIV. The mass spectrum is given in Table VIII and Figure 1.

Anal. Cald. for $C_{15}H_{26}N_2O_6Si$: C, 50.26; H, 7.31; Si, 7.83

Found: C, 50.35; H, 7.41; Si, 7.90

2',3'-Di-O-TBDMSuridine

2',3',5'-Tri-O-TBDMSuridine (XXXVI) (500 mg, 0.85 mmol) and 10 ml of 80% acetic acid were heated on a steam bath for 45 minutes. The reaction mixture was diluted with ethanol and the solvents were removed on a flat bed evaporator. The residue was dissolved in methanol and applied to four plates which were developed in ether. Three bands were seen at R_f 0.35 (2' or 3'-O-TBDMSuridine), 0.65 (the desired product) and 0.92 (starting material). The R_f 0.65 material was eluted in ethanol and after evaporating the solvent, 290 mg (72%) of a white solid was obtained. On crystallisation from ether-hexane the m.p. was 224-226^{o}C.

Chromatographic data are given in Table XV. The spectral data are listed in Table XIV and the mass spectrum is given in Table IX and figure 4.

Anal. Cald. for $C_{21}H_{40}O_6N_2Si_2$: C, 53.25; H, 8.53; Si, 11.88

Found: C, 53.49; H, 8.46; Si, 11.64

5'-O-Monomethoxytrityl-2'-O-TBDMSuridine (XLI) and 5'-O-Monomethoxytrityl-2'-O-TBDMSuridine (XL)

Method I. Uridine (244 mg, 1mmol) and monomethoxytrityl chloride (243 mg, 1.15) were dissolved in 5 ml of dry pyridine and stirred at room temperature for 10 hours. A tlc in methanol-ether (1:8) showed the presence of very little uridine.

Silylation was carried out by adding TBDMSCl (180 mg, 1.2 mmol) and imidazole (163 mg, 2.4 mmol) in the same flask and stirring for a further 14 hours. Reaction was monitored by tlc in ether followed by spraying with $HClO_4$. Two main spots at R_f 0.47 and 0.76 indicated the presence of two isomeric di-O-TBDMS derivatives. Methanol was added to destroy excess of the reagents and solvents were removed under reduced pressure. Residual pyridine was removed by co-evaporating with toluene. The residue was dissolved in chloroform and some methanol and applied to four silica gel plates. Two developments in ether-hexane (3:2) separated the reaction products. The two main bands were eluted in ethyl acetate (ether was equally good). On removing most of the solvent and co-evaporating with hexane, a white solid was obtained in both cases. The faster band material was the compound XLI and the slower band product was the compound XL. Yield of the latter compound was 190 mg (30%).

The yield of the compound XLI was 280 mg (0.442 mmol, 44%). This was used for dinucleotide synthesis without any further purification. M.p. 136-142°C. On crystallisation from benzene-hexane, the melting point improved to 141-143°C. Chromatographic and spectral data are given in Tables XV and XIV respectively. The mass spectrum showed a parent peak

at m/e 630 and a prominent peak at 573 (M-57).

Elemental analysis of the compound XLI is given below.

Anal. Cald. for $C_{35}H_{42}O_7N_2Si$: C, 66.64; H, 6.71; Si, 4.45

Found: C, 66.88; H, 6.79; Si, 4.62

Method II. Recrystallised 2'-O-TBDMSuridine (40 mg, 0.11 mmol) was dried by co-evaporation of pyridine and dissolved in 0.5 ml of pyridine and monomethoxytrityl chloride (28 mg, 0.089 mmol) was added. The reaction was partially complete after 16 hours of stirring, as seen by the appearance of a new spot on tlc in ether at R_f 0.72. No attempt was made to complete the reaction so that it was not necessary to quench the reaction as this step could lead to isomerisation of the 2'- and 3'-TBDMS groups. Pyridine was removed, the residue dissolved in chloroform and applied to a silica gel plate (10 X 20 cm). On development in ether-hexane (1:1) the major band (the desired product) appeared at R_f 0.19, starting material remained at R_f 0.05 and minor products were seen at R_f at 0.69, 0.85 and 0.97. The product band was eluted in ether and crystallised from benzene-hexane, m.p. 141-143°C. R_f values and UV spectra were the same as for the compound prepared by Method I. The mixed melting point was 141-144°C. The mass spectrum was identical to that obtained in the first case.

5'-O-Monomethoxytrityl-2',3'-Di-O-TBDMSuridine

2',3'-Di-O-TBDMSuridine (47 mg, 0.1 mmol) was dried by co-evaporation of pyridine and dissolved in 0.5 ml of pyridine. After adding monomethoxytrityl chloride (34 mg, 0.11 mmol) the reaction mixture was stirred at room temperature for 20 hours. Tlc indicated formation of a

new product. In an attempt to complete the reaction, more monomethoxytrityl chloride (11 mg, 0.36 mmol) was added but about 20% of the starting material was still present. Imidazole (10 mg, 0.15 mmol) was added and the reaction was stopped by adding methanol. Solvents were removed at reduced pressure and the residue was co-evaporated with toluene to remove residual pyridine. The residue was dissolved in methanol and applied to one silica gel plate which was developed in ether-hexane (2:1). The major band of the product appeared at R_f 0.69 and starting material was at R_f0.38. Two minor products at R_f 0.48 and 0.86 were not identified. The desired product was eluted in ether which was removed and the residue was dissolved in ethanol. On addition of water the product precipitated to yield 53 mg (74%). M.p. 101-105°C. Ultraviolet absorption spectrum in methanol: λmax 264nm and 232nm, λmin 247nm. R_f values are given in Table XV.

A part of the product was treated with TBAF for 30 minutes at room temperature, when only one product was obtained which had same R_f values and melting point as an authentic sample of 5'-O-monomethoxytrityluridine.

3'-O-Acetyl-2'-O-TBDMSuridine (XXXVIII)

Method I. 5'-O-Monomethoxytrityl-2'-O-TBDMSuridine (70 mg, 0.11 mmol) was dried by evaporation of pyridine (1x2ml). It was dissolved in pyridine (1 ml) and stirred with acetic anhydride (0.1 ml, 1 mmol) for 16 hours at room temperature. Reaction was complete as seen by tlc in ether-hexane (2:1). Solvents were removed by co-evaporation with 80% acetic acid and the residue was stirred with 3 ml of the latter for 5 hours. The solvents were removed under reduced pressure after adding

ethanol. The residue was dissolved in methanol and applied to a silica gel plate which was developed in ether-hexane (2:1). The main band was eluted in ethyl acetate. On removing the solvent 24 mg of a white solid was left (60%) and on crystallisation from ether-hexane, m.p. 201-203°C. Chromatographic properties are given in Table XV and mass spectral data are given in Table XI.

Method II. 2',5'-Di-O-TBDMSuridine (60 mg, 0.127 mmol) was dried by co-evaporation of moisture with pyridine (2 x 2 ml). The residue was dissolved in pyridine (1 ml) and stirred with acetic anhydride (0.1 ml, ∿ 1 mmol) at room temperature for 20 hours. A tlc in ether-hexane (2:1) indicated absence of the starting material. The reaction mixture was diluted with ethanol and solvents were removed under reduced pressure. The residual pyridine was removed by co-evaporating with toluene and the residue was heated on a steam bath with 80% acetic acid for 2 hours to remove the 5'-TBDMS group. The reaction mixture was diluted with ethanol and solvents were removed under reduced pressure. A tlc in ethyl acetate showed the formation of a slower moving product. The residue was dissolved in methanol and applied to a silica gel plate which was developed in ether when three bands appeared at R_f 0.62 (the desired product), 0.74 (a small amount of unidentified substance) and 0.86 (∿ 20% of 2',5'-di-O-TBDMS-3'-O-acetyluridine). The R_f 0.62 band was eluted in ether, the removal of which gave the required product (36 mg, 70%). This was crystallised from ether-hexane, m.p. 200-202°C. A mixed melting point of the products obtained by the two methods described did not show any depression (m.p. 199-202°C). The mass spectra of the compounds obtained by the Methods I and II were identical.

2'-O-Acetyl-3'-O-TBDMSuridine (XLVI)

Method I. The procedure was exactly as used in method I for compound XXXVIII. Tlc in ethyl acetate indicated the presence of a new product at R_f 0.72. Yield: 68%. On crystallisation from ether-hexane, its m.p. was 207-209°C. Chromatographic properties are given in Table XV and mass spectral data are listed in Table XI.

Method II. 3',5'-Di-O-TBDMSuridine (60 mg, 0.127 mmol) was dried by co-evaporation of moisture with pyridine (2 x 2 ml), dissolved in pyridine (1 ml) and stirred with acetic anhydride (0.1 ml, ∿1 mmol) for 21 hours at room temperature, when the reaction was complete and a new product at R_f 0.47 (ether-hexane, 2:1) appeared. Two minor products at R_f 0.00 and 0.71 were also present. The solvents were removed after diluting with 80% acetic acid. The residue was treated with 80% acetic acid on a steam bath for 1.25 hours. The solvents were removed after diluting with ethanol and the residue dissolved in methanol and applied to a silica gel plate which was developed in ether. Three products at R_f 0.07 (possibly 2'(3')-O-acetyluridine), 0.35 (the desired product) and 0.75 (the acetylated starting material) were separated.

The R_f 0.35 band was eluted in ether on removal of which a residue (24 mg, 60%) was left. On crystallisation from ether-hexane, a white solid, m.p. 209-211°C was obtained. The mixed melting point of the compounds obtained by each method was 208-210°C. Mass spectral and chromatographic properties are given in Table XI and XV respectively.

3',5'-Di-O-TBDMS-2'-O-p-toluenesulphonyluridine (XLIX)

3',5'-Di-O-TBDMSuridine (80 mg, 0.17 mmol) was dissolved in dry pyridine (1 ml) and the solution was cooled to 0°C. p-Toluenesulphonyl chloride (190 mg, 1mmol) was added and kept in a refrigerator for 17 hours. Tlc in ether-hexane (2:1) indicated the complete conversion to a new product at R_f 0.47. Methanol was added and solvents were removed at reduced pressure. The residue was dissolved in methanol and the solution applied to a silica gel plate which was developed in hexane-ether (1:2). The main band was eluted in ether and after removing a part of the solvent, hexane was added to precipitate when a white solid of melting point 121-125°C was obtained. Yield: 70 mg (0.112 mmol, 66%). Its ultraviolet absorption spectrum in ethanol showed maxima at 260nm and 222nm and a minimum at 243nm.

O^2:2'-Cyclouridine (LI)

3',5'-Di-O-TBDMS-2'-O-p-toluenesulphonyluridine (86 mg, 0.13 mmol) was dissolved in THF (1 ml) and stirred with TBAF (4 equivalents) for 50 minutes. Tlc indicated the presence of a new product which moved more slowly than uridine. The reaction mixture was applied to a silica gel plate and developed in THF. The main band (R_f 0.25) was eluted first in ethanol and then in water. Ultraviolet absorption spectra in water and ethanol showed maxima at 249nm and 224nm.

2',5'-Di-O-TBDMS-3'-O-p-toluenesulphonyluridine (XLVII)

2',5'-Di-O-TBDMSuridine (80 mg, 0.17 mmol) was dried by evaporation of pyridine. Pyridine (1 ml) was added and the solution was cooled. p-Toluenesulphonyl chloride (190 mg, 1mmol) was added and the reaction mixture was kept in a refrigerator for 36 hours. A tlc in ether-benzene (1:2) indicated completion of the reaction. The R_f changed from 0.48 to 0.58. Methanol was added to quench the reaction.

The solvents were removed under reduced pressure, and the residue after being dissolved in methanol was applied to a silica gel plate which was developed in benzene-ether (2:1). The main band was eluted in ether. On removing ether and co-evaporating with hexane, a white solid was obtained. Yield: 71 mg (65%). Ultraviolet absorption spectrum in ethanol showed maxima at 262nm and 224nm and a minimum at 241nm. Chromatographic properties are given in Table XIV and mass spectral data is listed in Table XII.

3'-O-p-Toluenesulphonyluridine (XLVIII)

Compound XLVII (62 mg, 0.1 mmol) was dissolved in THF (1ml) and treated with TBAF (0.84 mmol) for 45 minutes. A tlc in ether indicated completion of the reaction. The solution was applied to a silica gel plate which was developed in THF. The main band was eluted in THF. On removing the solvent a white solid was obtained, m.p. 190-194^{o}C (dec.). On crystallisation from ethanol the melting point was 194-198^{o}C. Another crystallisation from absolute ethanol improved it to 204-206^{o}C. (literature value[102] 205-206^{o}C). The ultraviolet absorption spectrum in ethanol showed maxima at 261nm and 225nm and a minimum at 241nm.

Chromatographic properties are recorded in Table XIV. The mass spectrum showed a parent peak at m/e 398.

5'-O-TBDMS-2',3'-Di-O-benzoyluridine (LII) and 2',3'-Di-O-benzoyluridine (LIII)

5'-O-TBDMSuridine (60 mg, 0.17 mmol) was dried by co-evaporation of pyridine (1 x 2 ml), dissolved in pyridine (1 ml) and the solution was stirred with benzoic anhydride (300 mg, 1.33 mmol) at room temperature for 24 hours. The reaction mixture was poured into ice-water and extracted with chloroform (3 x 4 ml). The solvents were removed and the reaction mixture was applied to a silica gel plate which was developed in ether-hexane (2:1) when two bands separated. The fast band was most likely the unhydrolysed benzoic anhydride. The lower band was eluted in ether on removal of which a white solid (95 mg, 98%) was obtained. The dibenzoyl derivative of 5'-O- TBDMSuridine was identified by UV absorption maxima in ethanol (257nm and 230nm) and the mass spectrum which showed a prominent peak at m/e 509 (M-57). On crystallisation from ether-hexane, it had a melting point of 184-186°C.

This product was heated with 80% acetic acid for 40 minutes on a steam bath and the solvents were removed after diluting with ethanol. The residue was dissolved in methanol,applied to a silica gel plate and developed twice in ether, when two bands at R_f 0.87 (unreacted material) and R_f 0.57 were obtained. The R_f 0.57 band was eluted in ether and the residue obtained after removing the solvent was crystallised by dissolving in absolute ethanol and adding cyclohexane (m.p. 193-195°C). This

Table XIV. Ultraviolet (UV) and Infrared (IR) Spectral Properties of the TBDMS Derivatives of Uridine.

Compound	UV absorption, nm		IR absorption*, cm^{-1}				
	λmax	λmin	C=O	C-Me_3	Si-Me_2	Si-O-C and C-O-C	Si-C
2'-O-TBDMSU	262	231	1670	1390	1260	1065	780
3'-O-TBDMSU	262	231	1675	1395	1260	1045	785
5'-O-TBDMSU	262	231	1710	1380	1250	1040	765
2',3'-Di-O-TBDMSU	262	231	1685	1395	1270	1075	780
2',5'-Di-O-TBDMSU	262	231	1690	1380	1250	1060	775
3',5'-Di-O-TBDMSU	262	231	1700	1390	1260	1065	775
2',3',5'-Tri-O-TBDMSU	263	231	1670	1370	1255	1075	775
5'-O-MMTr-2'-O-TBDMSU	262,231	249	1680	1390	1250	1040	780
5'-O-MMTr-3'-O-TBDMSU	262,231	249					

* Assignments based on literature values[84,85,146].

Table XV. Thin-layer Chromatographic Properties of Uridine Derivatives.

Compound	Ether-Hexane (2:1)	Ether	EtOAc	THF
U	0.0	0.0	0.0	0.12
5'-O-MMTrU	0.01	0.02	0.04	0.27
2'-O-TBDMSU	0.04	0.17	0.27	-
3'-O-TBDMSU	0.04	0.17	0.26	0.82
5'-O-TBDMSU	0.02	0.02	0.05	0.71
2',3'-Di-O-TBDMSU	0.21	0.57	-	-
2',5'-Di-O-TBDMSU	0.25	0 47	0.77	-
3',5'-Di-O-TBDMSU	0.12	0.59	0.68	0.90
2',3',5'-Tri-O-TBDMSU	0.54	0.80	-	-
5'-O-MMTr-2'-O-TBDMSU	0.28	0.68	-	0.85
5'-O-MMTr-3'-O-TBDMSU	0.07	0.50	-	0.77
5'-O-MMTr-2',3'-Di-O-TBDMSU	-	0.80	-	-
5'-O-MMTr-3'-O-Acetyl-2'-O-TBDMSU	0.34	0.71	0.76	-
5'-O-MMTr-2'-O-Acetyl-3'-O-TBDMSU	0.31	0.53	0.72	-
3'-O-Acetyl-2'-O-TBDMSU	0. 27	0.40	0.42	-
2'-O-Acetyl-3'-O-TBDMSU	0.17	0.36	0.38	-

was also crystallised from ether-hexane (m.p. 194-195°C). The literature value[111] is 195-197°C. The ultraviolet absorption spectrum showed maxima at 257nm and 231nm (in ethanol). The mass spectrum showed a peak at m/e 341 (M-Base). The chromatographic data are given in Table XIV.

Stability of TBDMS Group Under Acidic Conditions

Experiment 1. 5 mg of TBDMS derivatives of the ribonucleosides were treated with 0.5 ml of 80% acetic acid either at room temperature or heated on a steam bath under reflux for the desired length of time. To quench the reaction excess of ethanol was added and the solvents were removed on a flat bed evaporator, repeating the dilution and evaporation to ensure complete removal of acetic acid. Tlc was done at this stage to identify the products formed. The reaction mixture was applied to one-third or one-fourth of a silica gel plate which was developed in an appropriate solvent. Various bands were eluted using methanol or ethanol directly into 50 ml or 100 ml volumetric flasks. Concentrations were determined spectrophotometrically. Results are recorded in Tables II and VI.

Experiment 2. 2'-O-TBDMSuridine was stirred with 0.01M methanolic hydrochloric acid for two hours when no hydrolysis was noticed. In another experiment when 0.1M hydrochloric acid was used, there was complete hydrolysis to uridine when tested after 2 hours. HCl was removed before doing tlc.

Experiment 3. 2',5'-Di-O-TBDMSuridine (4.7 mg, 0.01 mmol) was dissolved in dioxan and stirred with p-toluenesulphonic acid monohydrate (3 mg, 0.015 mmol). In 1.5 hours, about 50% was converted to

2'-O-TBDMSuridine and after 19 hours no starting material was left. About 40% of the product was uridine and the rest was 2'-O-TBDMSuridine. Similar results were obtained when the experiment was repeated with 5'-O-TBDMSuridine.

Effect of Solvents on the Desilylation of Protected Nucleosides by Tetra-n-butylammonium fluoride (TBAF)

Experiment 1. 2'-O-TBDMSuridine (4 mg, 0.01 mmol) was dissolved in 0.2 ml of a mixture of pyridine-THF-water (1:8:1, v/v) and treated with TBAF (0.03 mmol, 0.05 ml). Complete desilylation to uridine had taken place when tested after 30 minutes, indicating that the presence of pyridine and water presented no problems in desilylation reactions and the rate of desilylation was not appreciably retarded.

Experiment 2. 2'-O-TBDMSuridine was stirred with acetic acid-water-TBAF (∿ molar ratio 20:16:1). After 30 minutes, 30% conversion to uridine was seen and after 24 hours desilylation had increased to approximately 50%.

Experiment 3. 2',5'-Di-O-TBDMSuridine (4.7 mg, 0.01 mmol) was treated with glacial acetic acid (0.5 ml, 8 mmol) and TBAF (0.2 mmol, 0.3 ml). After 5 hours, there was present a lot of uridine and a small quantity of 2' and 5'-TBDMSuridine but the starting material was absent. After 26 hours, reactants were almost all converted to uridine.

The experiment was repeated using a molar ratio of 1:5:400 (TBDMS group:TBAF:glacial acetic acid) instead of 1:10:400 ratio used above. After 5 hours, acetic acid was removed after diluting with ethanol and the reaction mixture was examined by tlc. There was present

some starting material but no 3',5' isomer was present. Other products were 2'-O-TBDMSuridine and uridine.

Experiment 4. 2',5'-Di-O-TBDMSuridine (4.7 mg, 0.01 mmol) was dissolved in THF (0.5 ml) and stirred with TBAF (0.2 mmol, 0.15 ml). After 1.5 hours, tlc showed the presence of 5'-O-TBDMSuridine. After 5 hours uridine and the latter were present in 1:2 ratio. After 24 hours, the ratio was 1:1.

Stability of TBDMS Protecting Group Under General Reaction Work Up Conditions

Experiment 1. 2',5'-Di-O-TBDMSuridine was dissolved in methanol and some pyridine hydrochloride was added. Cleavage of the silyl group was seen as described below :

After 30 minutes	15% of 2'-O-TBDMSuridine was present
60 minutes	40% of 2'-O-TBDMSuridine was present
5 hours	No starting material was present. Only 2'-O-TBDMSuridine was the product
36 hours	Uridine was the only product

When the same treatment was given to 5'-O-monomethoxytrityl-2'-O-TBDMS-uridine no starting material was present after 3 hours, mostly due to loss of the MMTr group. After 15 hours, most of the product was uridine.

Experiment 2. 2',5'-Di-O-TBDMSuridine was dissolved in pyridine and a small quantity of water was added. After 30 minutes of stirring isomerisation was detected by tlc in ether.

When 5'-O-monomethoxytrityl-2'-O-TBDMSuridine was stirred with moist pyridine, a test made after 4 hours showed considerable

isomerisation. There was no cleavage of either of the protecting groups after 72 hours.

Stability of TBDMS Group Under Phosphorylating Conditions

Experiment 1. 2',5'-Di-O-TBDMSuridine and dry pyridine (5 ml / 0.01 mmol) were stirred together. No isomerisation or hydrolysis was noticed after 75 hours as seen by tlc in ether and ether-hexane(2:1).

Next, TPS was added (3.6 mmol/mmol of the nucleoside) and stirred for 2.5 hours when no isomerisation or hydrolysis was observed.

Water (8 ml/mmol of TPS) was then added to the above solution and stirred for 18 hours. No isomerisation or cleavage of the TBDMS group was seen. When excess of water was added and stirring continued for 48 hours, approximately 10% of the product was 2'-O-TBDMSuridine indicating partial cleavage of the 5' TBDMS group.

Experiment 2. 2',5'-Di-O-TBDMSuridine and β-cyanoethyl phosphate (0.4 mmol/ml in pyridine) were stirred together. After 12 hours, about 5% desilylation of the 5' TBDMS group had occurred. In 39 hours, the product contained about 20% of 2'-O-TBDMSuridine and after 66 hours, the product had 40% of 2'-O-TBDMSuridine.

Experiment 3. 2',5'-Di-O-TBDMSuridine (24 mg, 0.05 mmol) and β-cyanoethyl phosphate (0.1 mmol, 0.25 ml) were dried by co-evaporating pyridine. The residue was dissolved in pyridine (0.5 ml) and DCC (120 mg, 0.6 mmol) was added. The phosphorylation reaction was not complete in 47 hours and besides the starting material, its 3',5' isomer was also formed in appreciable amount in 19 hours.

2',5'-Di-O-TBDMSadenosine and 3',5'-Di-O-TBDMSadenosine

Adenosine (535 mg, 2 mmol), imidazole (599 mg, 8.8 mmol) and TBDMSCl (661 mg, 4.4 mmol) were dissolved in DMF (2 ml) and stirred at room temperature for 3.5 hours. Reaction was almost complete in 1.5 hours as seen by tlcs in ether and ethyl acetate. The clear but viscous solution of reaction products was diluted in methanol and chloroform and applied on 8 silica gel plates. Three developments in ether-hexane (2:1) clearly separated three products, the fastest being the 2',3',5'-tri-O-TBDMSadenosine and the next two were 2',5'-di-O-TBDMS and 3',5'-di-O-TBDMS derivatives respectively. On short development in ethyl acetate 5'-O-TBDMSadenosine separated from the base line, taking care there was no mixing of bands which had been separated in earlier developments. Traces of unreacted adenosine moved from the base line when a short development in THF was done. Both of the disilyl derivatives were eluted with ethyl acetate. Yield of 2',5' derivative was 370 mg (0.75 mmol, 37%), m.p, 169-172^{o}C. On crystallisation from ether-hexane, m.p. 171-174^{o}C. Chromatographic and mass spectral data are given in Tables XVI and IX respectively.

Yield of the 3',5' derivative on removing solvent was 378 mg (0.75 mmol, 38%) having m.p. 193-197^{o}C. On crystallisation from ether-hexane the m.p. improved to 196-197^{o}C. The above mentioned Tables give physical data for this isomer also.

The 5'-O-TBDMSadenosine band was eluted in ethanol and a white residue (118 mg, 20%) was obtained on removing the solvent.

5'-O-TBDMSadenosine (LVII)

Adenosine (267 mg, 1mmol) was reacted with imidazole (150 mg, 2.2 mmol) and TBDMSCl (165 mg, 1.1 mmol) in DMF (1 ml) at room temperature for 20 hours. The reaction mixture was diluted with methanol and the solution was applied to three silica gel plates. The plates were developed in ether-hexane (4:1) till a prominent band separated from the base line. This band was eluted in ethanol and the solvent was removed, obtaining the desired product (152 mg, 40%). Crystallisation from ether-hexane yielded a white solid, m.p. 174-177°C. Chromatographic and UV spectral data are given in Table XVI. The mass spectrum showed a parent ion peak at m/e 381. Other important fragments observed in the mass spectrum are listed in Table VIII.

2',3',5'-Tri-O-TBDMSadenosine (LX)

Adenosine (267 mg, 1 mmol) was stirred with imidazole (600 mg, 8.8 mmol), TBDMSCl (662 mg, 4.4 mmol) and DMF (1.5 ml) for two hours. The reaction mixture after being diluted with methanol was applied to three silica gel plates, which were then developed in ether-hexane (5:2). The main band was eluted in ether, on removal of which a white residue was obtained. Co-evaporation with hexane converted it into a nice crystalline substance which weighed 432 mg (71%). On crystallisation from methanol-water (3:1), it had m.p. 142-144°C. Chromatographic data are given in Table XVI. Important fragments observed in the mass spectrum are listed in Table X.

2'-O-TBDMSadenosine

2',5'-Di-O-TBDMSadenosine (100 mg, 2 mmol) was heated with 3 ml of 80% acetic acid for 55 minutes on a steam bath. The reaction mixture was diluted with ethanol and solvents were removed at reduced pressure. A tlc in ether indicated a change in R_f value of the main product from 0.63 to 0.26. The solution was applied to one silica gel plate and developed in ether. Two main bands appeared at R_f 0.62 and 0.19. The slower band was immediately eluted in ethyl acetate. On removing the solvent and co-evaporating with hexane a white solid, m.p. 202-212°C was obtained. On crystallisation from ether-hexane, needles of m.p. 214-217°C were obtained. Yield: 56 mg (74%). Chromatographic data are given in Table XVI. The mass spectrum showed a parent ion peak at 381. Other important fragment ions are listed in Table VIII.

3'-O-TBDMSadenosine

3',5'-Di-O-TBDMSadenosine (117 mg, 0.24 mmol) was heated with 80% acetic acid (3 ml) for one hour on a steam bath. The reaction mixture was diluted with ethanol and the solvents were removed at reduced pressure. The residue was dissolved in ethyl acetate and applied to one silica gel plate which was developed twice in ether. Two main bands separated at R_f 0.43 (the starting material) and 0.18 (the desired product), beside a minor unidentified band at R_f 0.64 and a small amount of adenosine at the base line. The R_f 0.18 band was eluted in ethyl acetate, the removal of which gave a waxy solid. Ether was added and the mixture heated to partly dissolve the waxy solid. A white solid (65 mg, 72%) was obtained after filtering the above cooled mixture.

On crystallisation from ether-EtOAc (1:1) it had m.p. 216-218°C. Chromatographic data are given in Table XVI. The mass spectrum showed a parent ion peak at 381. Other diagnostically important fragment ions are listed in Table VIII.

2',3'-Di-O-TBDMSadenosine

2',3',5'-Tri-O-TBDMSadenosine (280 mg, 0.46 mmol) was heated with 5 ml of 80% acetic acid on a steam bath for one hour. The reaction mixture was diluted with ethanol and the solvents were removed at reduced pressure. The residue, after being dissolved in methanol, was applied to three silica gel plates which were developed in ether-hexane (3:1). Two main bands appeared at R_f 0.8 and 0.47. The R_f 0.47 band was eluted in ethanol and a white solid was obtained after removing the solvent, which weighed 187 mg (82%). On crystallisation from ether-hexane it had m.p. 252-257°C. Chromatographic data are given in Table XVI. Important fragment ions obtained in the mass spectrum are listed in Table IX.

2',3'-Di-O-p-Toluenesulphonyl-5'-O-TBDMSadenosine (LXIII)

5'-O-TBDMSadenosine (15 mg, 0,043) was dried with pyridine (2 ml) and then dissolved in pyridine (0.5 ml) and cooled to 0°C. p-Toluene-sulphonyl chloride (66 mg, 0.34 mmol) was added and the solution was stirred at room temperature for 14 hours. Tlc in ether showed the absence of the starting material and the presence of two new spots with close mobilities. Water was added to the cooled reaction mixture which was then extracted with chloroform. The latter extract was applied to a part of a silica gel plate which was developed in ether-benzene (2:1)

when two major bands, approximately in the ratio 1:1, separated at R_f 0.30 and 0.17. There were small faster and slower bands also. The R_f 0.30 band material was eluted in ether,on removal of which a solid was obtained. On crystallisation from ether-hexane it had m.p. 157-159°C. The ultraviolet absorption spectrum in methanol showed maxima at 260nm, 272nm(shoulder) and 236nm. The mass spectrum indicated fragment ions at m/e 674 (M-15) and 632 (M-57).

2',3'-Di-O-p-Toluenesulphonyladenosine (LXIV)

2',3'-Di-O-p-toluenesulphonyl-5'-O-TBDMSuridine as obtained above was treated with 80% acetic acid on a steam bath for one hour. The reaction mixture was diluted with ethanol and the solvents were removed at reduced pressure. The residue was dissolved in methanol and the solution was applied to a part of a silica gel plate which was developed in ether. The main product was eluted in ether and the solvent was removed. The residue was crystallised from ethanol giving a solid of melting point 205-207°C (literature value[90] 207-209°C). The ultraviolet absorption spectrum in ethanol showed maxima at 261nm and 226nm.

3',5'-Di-O-TBDMS-2'-O-p-toluenesulphonyladenosine (LXI)

3',5'-Di-O-TBDMSadenosine (150 mg, 0.30 mmol) was dried by co-evaporation of 3 ml of pyridine, and then dissolved in pyridine (2.5 ml) and cooled to 0°C for 15 minutes. p-Toluenesulphonyl chloride (300 mg, 1.56 mmol) was added and the solution was stirred at 0°C for 64 hours. A tlc after 40 hours, in ether-benzene (2:1), indicated about 25% of the starting material was still present. R_f of the main product changed from 0.19 to 0.30. Methanol was added and the reaction mixture was kept

in ice for one hour. Solvents were removed at reduced pressure and the residual pyridine was removed by co-evaporating with toluene. The residue was dissolved in methanol, the solution was applied to two silica gel plates and developed twice in benzene-ether (1:1). The major band at R_f 0.50 was eluted in ether. On removing the solvents and co-evaporating with hexane a white solid was obtained which weighed 152 mg (78%), m.p. 173-176°C. Mass spectral and chromatographic data are given in Tables XII and XVI respectively. The ultraviolet absorption spectra in ethanol showed maxima at 261nm and 228nm.

2'-O-p-Toluenesulphonyladenosine (LXII)

The compound obtained above (139 mg, 0.214 mmol) was dissolved in 1 ml of dry THF and stirred with TBAF (1.28 mmol, 1.8 ml) for 35 minutes. A tlc in ether showed a change in R_f value from 0.56 to 0.15. The reaction mixture was applied to two silica gel plates and developed in EtOAc-THF (1:1). Two bands appeared at R_f 0.46 and 0.08. The main band at R_f 0.46 was eluted in methanol. Solvents were removed and the residue was co-evaporated with hexane when a white solid was obtained, 79 mg (92%), m.p. 212-220°C (dec.). On crystallisation from methanol-water the melting point improved to 227-229°C (literature value [103,104] 229-230°C). The ultraviolet absorption spectrum in ethanol showed maxima at 261nm and 228nm.

2',5'-Di-O-TBDMS-3'-O-p-toluenesulphonyladenosine

2',5'-Di-O-TBDMSadenosine (150 mg, 0.30 mmol) was dried by co-evaporating with pyridine. The residue was dissolved in pyridine (2.5 ml) and after cooling in ice, p-toluenesulphonyl chloride

Table XVI. Thin-layer Chromatographic Properties of Adenosine Derivatives

Compound	Ether-Hexane (2:1)	Ether	EtOAc
A	0.00	0.00	0.00
5'-O-TBDMSA	-	0.06	0.14
2'-O-TBDMSA	_	0.26	-
3'-O-TBDMSA	-	0.25	-
2',3'-Di-O-TBDMSA	0.19	0.55	-
3',5'-Di-O-TBDMSA	0.14	0.40	0.51
2',5'-Di-O-TBDMSA	0.24	0.62	0.62
2',3',5'-Tri-O-TBDMSA	0.42	0.74	0.77

(300 mg, 1.56 mmol) was added and the reaction mixture was kept in a refrigerator for 106 hours. A tlc in ether-benzene (2:1) indicated the presence of a new product. Methanol was added to quench the reaction and the solvents were removed at reduced pressure. The residual pyridine was removed by co-evaporating with toluene. The residue was dissolved in methanol and the solution was applied to two plates which were developed twice in ether-benzene (1:1). The major band was at R_f 0.43 beside bands at R_f 0.30, 0.69 and 0.88. The R_f 0.43 band material was eluted in methanol. On removing the solvent and co-evaporating with hexane a white solid was obtained, 126 mg (65%). Mass spectral and chromatographic data are given in Tables XII and XVI respectively.

β-Cyanoethyl Ester of 2',5'-Di-O-TBDMSuridine-3'-phosphate (LXXIII)

2',5'-Di-O-TBDMSuridine (141 mg, 0.3 mmol) and the pyridinium salt of β-cyanoethyl phosphate (0.9 mmol, 2.25 ml) were dried by co-evaporation of moisture with pyridine (5 x 1 ml) at room temperature. Pyridine (1.2 ml) and TPS (366 mg, 1.2 mmol) were added and the reaction mixture was stirred for 15 hours. A tlc in ether and EtOAc indicated the absence of 2',5'-di-O-TBDMSuridine. Water (1.2 ml) at 0°C was added to the ice-cooled reaction mixture and stirred for 22 hours. A tlc in $CHCl_3$-EtOH (1:1) showed the presence of at least three substances (also see Discussion).

Solvents were removed and an ethanol solution of the residue was applied immediately to two silica gel plates, which were first developed in ether when a very fast band appeared. Plates were than developed thrice in $CHCl_3$-EtOH (1:1), when two bands separated. The major (faster)

band was eluted in ethanol, which on removal of the solvent weighed 84 mg (50%, compound LXXIII). Em values were 0.6 (95%) and 0.83 (5%). R_f values in $CHCl_3$-EtOH (1:1) were 0.45 (major) and 0.19.

The slower (minor) component was obtained as a solid and weighed 25 mg. Em was 0.73 and R_f in $CHCl_3$-EtOH (1:1) was 0.18. This compound probably lacked the 5' TBDMS group from the desired product.

Both faster and slower moving substances were heated with 7M ethanolic-ammonia at 70°C for 3 hours. Solvents were then removed and the residues were treated with TBAF for 30 minutes. Electrophoretic mobilities of both products were 1.05 (Tp=1.00). Further characterisation of the faster substance LXXIII was done by condensing it with 2',3'-O-isopropylideneuridine (details given separately) and the product was degraded completely by snake venom enzyme and partially by spleen enzyme (see Discussion for an explanation of the last result).

β-Cyanoethyl Ester of 2',5'-Di-O-TBDMSuridylyl-(3'-5')-2',3'-O-isopropylideneuridine (LXXV) Using Isolated Monophosphate LXXIII (Small Scale Preparation)

Compound LXXIII (16 mg, 0.0208 mmol), Em 0.6, was dried by co-evaporation of moisture with pyridine (2x1ml). 2',3'-O-Isopropylideneuridine (16 mg, 0.056 mmol) was added and the reaction mixture was dried by evaporating pyridine (3x1ml). Pyridine (0.5 ml) and TPS (33 mg, 0.112 mmol) were added and the solution was stirred for 23 hours.

Water (0.4 ml) was added and the solution was stirred for 9 hours. Solvents were removed and the residue dried in vacuo. TBAF (0.18 mmol, 0.2 ml) was added and the reaction mixture was stirred for 30 minutes.

The reaction mixture was separated by paper chromatography in solvent A, using three papers. Four bands separated at R_f 0.77, 0.64, 0.48 and 0.37. Electrophoresis showed these bands to be mixtures. The relative proportions of various constituents and Em values are given in Discussion. Electrophoresis of the R_f 0.37 material indicated that there was no dinucleotide present, Em values being 0.88 (50%) and 1.05 (40%). The material of R_f 0.48, after being eluted in water and lyophilised, was purified by rechromatography on papers in solvent A, when two products of R_f 0.20 (15%) and 0.41 (85%) were obtained. The major band material, after being eluted and lyophilised, weighed 7 mg (57% of LXXVI), Em 0.6 (pH 8.1 buffer) and $R_f^{B'}$ 0.12.

1 mg of this material was incubated with snake venom enzyme for 8 hours resulting in complete degradation to pU-OIP and U in the ratio 1:1. Another part (1mg) was treated with spleen enzyme for 8 hours, when 80% degraded to Up and U-OIP in the ratio 0.86:1.

Paper Chromatographic and Electrophoretic Properties of Tetra-n-butylammonium fluoride (TBAF) Treated Nucleosides and Nucleotides.

Nucleosides. 2',5'-Di-O-TBDMSuridine was dissolved in THF and stirred with TBAF (4 equivalents). Samples were taken after 30 minutes and 4 hours and spotted on a paper along with uridine. The paper was developed in solvent A. Both samples gave the same results: only a single spot corresponding to free nucleoside was seen. The same result was obtained using 12 equivalents of TBAF. A tlc in THF indicated no difference.

Nucleotides.

Experiment 1. UpU (ammonium salt, 1mg, 0.002 mmol, Sigma Chemical

Co.) was stirred with THF (0.1 ml) and TBAF (0.05 mmol) for 15 hours. Samples were taken after 40 minutes and 15 hours for electrophoresis and paper chromatography. There was no difference in the electrophoretic mobility of the two samples and authentic UpU.

Paper chromatography in solvent A revealed the presence of two substances at R_f 0.14 (UpU) and a wide band at R_f 0.65. The faster band after elution and lyophilisation was rechromatographed in solvent A. Now, its R_f was the same as that of R_f 0.14 substance and pure UpU.

Experiment 2. UpU (1 mg, 0.002 mmol) was treated with TBAF (0.14 mmol, 0.14 ml) for 18 hours and the reaction mixture was applied to a paper for chromatography in solvent A. Two components at R_f 0.26 (UpU) and 0.77 separated in the ratio 1:3 respectively. Rechromatography on the R_f 0.77 material in solvent A gave two products at R_f 0.36 (UpU = 0.39) and 0.54 in $\sim$3:7 ratio respectively. The R_f 0.77 material was incubated with ribonuclease for 3 hours and the products were separated by paper chromatography in solvent A when two products at R_f 0.24 and 0.56 (U=0.54) were obtained. There was present a small amount of UV absorbing substance at R_f 0.72. The R_f 0.56 material was rechromatographed in solvent A when it moved like U, though it contained a small amount of R_f 0.20 material (possibly Up). Similar results were obtained when UpU was stirred with TBAF for one hour only.

β-Cyanoethyl Ester of 2',5'-Di-O-TBDMSuridylyl-(3'-5')-2',3'-O-isopropylideneuridine (LXXV) (Isolation on Silica Gel Plates)

2',5'-Di-O-TBDMSuridine (95 mg, 0.2 mmol) and the pyridinium salt of β-cyanoethyl phosphate (0.6 mmol, 1.5 ml) were dried by co-evaporating moisture with pyridine (5 x 1 ml). Pyridine (0.7 ml) and TPS (189 mg, 0.61 mmol) were added and the reaction mixture was stirred for 15 hours. Samples were taken for tlc in ether, at intervals. There was no isomerisation to 3',5'-di-O-TBDMSuridine, though a trace of 2'-O-TBDMS-uridine was indicated. Cold water (1 ml) and pyridine (1 ml) were added and the reaction mixture was stirred for 20 hours. A sample taken after 8 hours of stirring, for paper chromatography in solvent B', showed the presence of two products at R_f 0.40 and 0.75 in the ratio 1:1 approximately.

More water (∿2 ml) was added and the reaction mixture was extracted with chloroform (3 x 4 ml). The chloroform extract was dried over sodium sulphate. Solvents were removed and the residue was dried by co-evaporation of pyridine (3 x 1 ml). Pyridine (0.8 ml) and TPS (121 mg, 0.4 mmol) were added and the reaction mixture was stirred for 24 hours.

Cold water (1 ml) and pyridine (2 ml) were added and the reaction mixture was extracted with chloroform (3 x 4 ml). The chloroform extract was dried over sodium sulphate for 12 hours. Solvents were removed and a methanol solution of the residue was applied to two silica gel plates which were first developed in ether and then in EtOAc-ether (1:1). The slowest band at R_f 0.43 was eluted in ethyl acetate and methanol. Solvents were removed and a residue weighing 35 mg (20% of LXXV) was

obtained. Chromatographic and spectral data are given in Tables XVII and XVIII.

Compound LXXV (13 mg, 0.125 mmol) was stirred with TBAF (1.5 mmol, 1.5 ml) in THF for 7 hours and the reaction mixture was chromatographed on two papers in solvent A. Two products at R_f 0.49 and 0.85 separated, their approximate proportions being 1:3 respectively.

The R_f 0.49 material (LXXVI) was eluted and lyophilised. 0.5 mg of this substance was incubated with pancreatic ribonuclease when it degraded completely to Up and U-OIP. The same amount was also treated with spleen enzyme which completely degraded it to Up and U-OIP. See Discussion for a procedure to convert the R_f 0.85 substance to UpU-OIP.

β-Cyanoethyl Ester of 5'-O-Monomethoxytrityl-2'-O-TBDMSuridylyl-(3'-5')-2'-3'-O-isopropylideneuridine (LXXXIII)

Method I. 5'-O-Monomethoxytrityl-2'-O-TBDMSuridine (540 mg, 0.86 mmol) and the pyridinium salt of β-cyanoethyl phosphate in pyridine (2.54 mmol, 6.4 ml) were dried by co-evaporation of moisture with pyridine (5 x 1 ml). The residue was dissolved in pyridine (2.5 ml), TPS (1040 mg, 3.44 mmol) was added and the solution was stirred for 7 hours. Tlc in ether and ethyl acetate showed the absence of the starting material. The reaction mixture was cooled in ice and ice-cooled water (2 ml) was added and the solution was stirred again for 18 hours. Tlc in methanol-ether (1:3) showed the presence of two trityl containing compounds.

More water (2 ml) was added and the reaction mixture was extracted with chloroform (3 x 4 ml). The chloroform extract was washed with

water (5 ml) and then dried over sodium sulphate. Tlc in methanol-ether (1:3) showed the presence of two trityl containing compounds. Tritanol was also present.

Solvents were removed when a flaky reddish-brown solid was obtained. The residue was dried by evaporating pyridine (3 x 1 ml). 2',3'-O-Isopropylideneuridine (496 mg, 1.72 mmol) was added and the reaction mixture was dried again by co-evaporating moisture with pyridine (3 x 1 ml). Pyridine (2 ml) and TPS (520 mg, 1.72 mmol) were added and the reaction mixture was stirred for 22 hours. A sample taken after 12 hours for tlc in EtOAc-THF (1:1) showed the presence of a new product which was moving somewhat faster than U-OIP.

The reaction mixture was poured into 600 ml of ice and water. The precipitated material was filtered and left over filter paper to dry overnight. The solids were dissolved in THF and applied to nine silica gel plates which were developed twice in ethyl acetate. Two bands separated at R_f 0.41 (U-OIP) and 0.59 (the desired product) as well as a prominent fast band of sulphonated derivative of U-OIP.

The R_f 0.59 material was eluted in excess of ethyl acetate. On removing the solvent 200 mg of residue was left (21%). Tlc in methanol-ether (1:8) showed it to be a single spot substance of R_f 0.66. Spectral and chromatographic data is given in Tables XVII and XVIII.

Compound LXXXIII (15 mg, 0.0146 mmol) was treated with 1 ml of 80% acetic acid at room temperature for 5 hours. Ethanol was added and the solvents were removed at reduced pressure. Tlc in methanol-ether (1:5) showed the absence of the starting material. The reaction mixture in methanol was applied to a part of a silica gel plate which was

developed in ethyl-acetate (4:1). The main band was eluted in ethanol (100 ml). Solvent was removed leaving a residue weighing 8 mg.(LXXXIV)

TBAF (0.525 mmol, 0.75 ml) was added to the residue and the mixture was applied to two papers for chromatography in solvent A. Two products separated at R_f 0.43 and 0.83 in approximate ratio of 3:7 respectively. The R_f 0.43 material was eluted, then lyophilised and about one-third was incubated with an appropriate quantity (25 µl) of ribonuclease for 3 hours. There was complete degradation to two new products having R_f^A 0.11 (Up) and 0.77 (U-OIP) in the ratio 1:1.1 respectively. See Discussion for a procedure by which the R_f 0.83 substance was also converted to degradable UpU-OIP.

Method II. 5'-O-Monomethoxytrityl-2'-O-TBDMSuridine (130 mg, 0.2 mmol) and pyridinium salt of β-cyanoethyl phosphate (0.6 mmol) were dried by co-evaporation of moisture with pyridine (5 x 1 ml). TPS (242 mg, 0.8 mmol) and pyridine (0.6 ml) were added and the reaction mixture was stirred for 23 hours. Cold water (1 ml) was added to the ice-cooled reaction mixture and stirred again for 16 hours. More water (1 ml) was added and the reaction mixture was extracted with chloroform (3 x 4 ml). The chloroform extract was then washed with water (2 ml) and dried over sodium sulphate for several hours.

Solvents were removed and the residue dried by co-evaporation of moisture with pyridine (5 x 1 ml). Pyridine (0.8 ml) and TPS (122 mg, 0.4 mmol) were added. 2',3'-O-Isopropylideneuridine (115 mg, 0.4 mmol) was separately dried by evaporation of pyridine (2 x 2 ml), dissolved in pyridine (1 ml) and mixed with the phosphorylated product dried before.

The reaction mixture was stirred for 24 hours. Tlc in THF-EtOAc (1:1) and methanol-ether (1:8) indicated the presence of a new product which had the same R_f value as found for the compound LXXXIII prepared by Method I.

The reaction mixture was cooled in ice and cold water (3 ml) was added and extracted with chloroform (3 x 4 ml). Solvents were removed and the residual pyridine was removed by co-evaporating with toluene (2 x 3 ml). The solid thus obtained was dissolved in methanol or THF and applied to two silica gel plates which were developed in ether. The main bands were at R_f 0.94 (tritanol), 0.68 (sulphonated U-OIP), 0.22 (U-OIP) and 0.13 (the desired product). The R_f 0.13 band material was eluted in THF, on removal of which an oil was obtained. Yield as determined spectrophotometrically was 26.5%. Chromatographic and spectral data are given in Tables XVII and XVIII.

The compound was deprotected and characterised by enzymatic hydrolysis as described in Method I.

β-Cyanoethyl Ester of 5'-O-Monomethoxytrityl-2'-O-TBDMSuridylyl-(3'-5')-2'-O-TBDMSuridine (LXXXV)

5'-O-Monomethoxytrityl-2'-O-TBDMSuridine (315 mg, 0.5 mmol) and the pyridinium salt of β-cyanoethyl phosphate in pyridine (1.25 mmol, 3.1 ml) were dried by evaporation of pyridine (4 x 2 ml). TPS (605 mg, 2 mmol) and pyridine (2 ml) were added and the reaction mixture was stirred for 8 hours. Cold water (1.5 ml) was added to the ice-cooled solution which was stirred for a further 36 hour period. More water (1.5 ml) was added and the solution was extracted with chloroform

(3 x 4 ml). The chloroform extract was washed with water (2 ml) which was then dried over sodium sulphate for one hour.

The solvents were removed and the residue was dried with pyridine (2 x 2 ml) by its evaporation. 2'-O-TBDMSuridine was added and the mixture was dried with further evaporation of pyridine (3 x 2 ml). Pyridine (4 ml) and TPS (302 mg, 1 mmol) were added and the solution was stirred for 24 hours. More TPS (150 mg, 0.5 mmol) was added and the reaction mixture was stirred for 17 hours. Tlc in ether indicated the presence of a considerable amount of a slower moving substance. β-Cyanoethyl alcohol (1 ml) was added and the reaction mixture was stirred for 23 hours. Tlc indicated no difference in the amount of the unreacted slower moving compound.

The reaction mixture was applied directly on five plates which were developed first in ethyl acetate and then in THF. The various bands were not well separated, therefore these were all eluted in THF. Most of the solvent was removed and the solution was applied on four plates, which were developed four times in ether. The two bands at R_f 0.10 and 0.15 (possibly diastereomers of LXXXV), which moved slower than 2'-O-TBDMSuridine, were eluted in ethyl acetate. The solvent was removed giving 70 mg and 30 mg of the residues from R_f 0.15 and 0.10 bands respectively. Total yield of LXXXV was 18%.

The R_f 0.15 product (9 mg, 0.008 mmol) was treated with 80% acetic acid at room temperature for 2.5 hours. Solvents were removed after the reaction mixture had been diluted with ethanol. The residue was dried by leaving it on a vacuum pump for 12 hours. TBAF (0.084 mmol, 0.12 ml)

was added and the solution, after being stirred for 35 minutes, was applied to two papers. Chromatography in solvent A separated four products at R_f 0.18 (UpU), 0.35, 0.62 (minor) and 0.85. The R_f 0.18 band after being eluted and lyophilised was divided into three parts, which were treated with the following enzymes. There was degradation in all cases to the extent of 97%. The undegraded substance was most likely the 3'-3' linked dinucleotide LXXXVII.

Ribonuclease incubation for 5 hours gave two products at R_f^A 0.10 (Up) and 0.44 (U) in the ratio 1:0.90 respectively.

Snake venom enzyme incubation for 7 hours gave two products at R_f^A 0.06 (pU) and 0.45 (U) in the ratio 0.95:1.0 respectively

Spleen enzyme degraded in 7 hours to two products at R_f^A 0.12 (Up) and 0.50 (U) in the ratio 1:1.1 respectively

The R_f 0.10 product was deprotected and enzymatically characterised as described above. Chromatographic properties are given in Table XVII.

Stability of Phosphodiester Linkage Adjacent to 2'-TBDMS Group on Silica Gel

A mixture of 5'-O-monomethoxytrityl-2'-O-TBDMSuridylyl-(3'-5')-2',3'-O-isopropylideneuridine and its β-cyanoethyl ester was left on silica gel plates for more than two weeks. After elution in methanol, it was applied on a plate and developed three times in ethyl acetate. Two closely moving bands (possibly diastereomers) separated and some material was left on the origin. The latter was eluted in methanol and the presence of trityl group was checked.

Following the removal of methanol, this was treated with 80%

acetic acid for 4 hours at room temperature. The reaction mixture was diluted with ethanol and solvents were removed at reduced pressure. The residue was treated with TBAF to remove the silyl groups. The reaction mixture was applied to two papers for chromatography in solvent A. Two bands at R_f 0.44 (UpU-OIP) and 0.87 separated.

The R_f 0.44 band substance after being eluted and lyophilised was incubated with spleen enzyme for 6 hours. Two products were separated after paper chromatography in solvent A, at R_f 0.125 (Up) and 0.71 (U-OIP) in the ratio 1:1.15 respectively. There was no undegraded material left indicating the absence of any isomerised 2'-5' linked starting material.

5'-O-Triisopropylbenzenesulphonyl-2',3'-O-isopropylideneuridine

2',3'-O-Isopropylideneuridine (64 mg, 0.22 mmol) was dissolved in pyridine (1 ml) and stirred with TPS (130 mg, 0.43 mmol) for 22 hours. A tlc in ether showed the presence of a considerable amount of a new product at R_f 0.5. The reaction mixture was cooled in ice and methanol (1 ml) was added to quench the reaction. The solvents were removed under reduced pressure and the residual pyridine was removed by co-evaporating with toluene. The residue was dissolved in methanol and applied to a plate which was developed in ether. Four bands appeared at R_f 0.25 (U-OIP), 0.69 (minor), 0.81 (the desired product) and 0.90 (minor, unidentified). The R_f 0.81 band was eluted in ether. On removing the solvent, the residue weighed 60 mg (46%). The R_f 0.25 band was eluted in acetone, and on removing this solvent 24 mg of 2',3'-O-isopropylideneuridine (38%) was obtained. The ultraviolet absorption spectrum

in ethanol showed maxima at 259nm and 230nm. The mass spectrum of the product showed a parent peak at m/e 550 and prominent peaks at 535(M-15) and 439(M-Base).

Table XVII. Thin Layer Chromatographic Properties of Dinucleotides

Compound	MeOH-Ether 1:8	EtOAc-THF 2:1	THF	EtOAc	EtOH	Solvents A	Solvents B'
U-OIP	0.62	0.38	-	0.30	-	-	-
5'-O-MMTr-2'-O-TBDMSUp(CE)U-OIP (LXXXIII)	0.77	0.60	0.66	-	-	-	-
5'-OH-2'-O-TBDMSUp(CE)U-OIP (LXXXIV)	-	-	0.39	-	-	-	-
5'-O-MMTr-2'-O-TBDMSUp(CE)U-2'-O-TBDMS (LXXXV)	0.66	0.48	0.77	-	-	-	-
2',5'-Di-O-TBDMSUp(CE)U-OIP (LXXV)	-	-	-	0.47	-	-	-
5'-OH-2'-O-TBDMSUp(CE)U-2'-O-TBDMS (LXXXVI)	0.10	-	0.20	-	-	-	-
UpU-OIP (LXXVI)	-	-	-	-	0.58	0.57	0.3

Table XVIII. Paper Chromatographic and Electrophoretic Properties of Dinucleotides

Compound	Solvent A	Solvent B'	Em
5'-O-MMTr-2'-O-TBDMSUp(CE)U-OIP (LXXVIII)	-	0.95	-
5'-O-MMTr-2'-O-TBDMSUpU-OIP	0.88	0.70	-
5'-OH-2'-O-TBDMSUp(CE)U-OIP (LXXIV)	-	0.87	-
5'-OH-2'-O-TBDMDUpU-OIP	0.82	-	-
2',5'-Di-O-TBDMSUpU-OIP	0.91	-	-
2',5'-Di-O-TBDMSUp(CE) (LXXIII)	0.85	0.45	0.58
UpU-OIP (LXXVI)	0.40	0.12	0.67
UpU	0.19	-	0.62
Up	0.11	-	-
pU-OIP	0.05	-	-
5'-O-MMTr-2'-O-TBDMSUp(CE) (LXXXII)	0.91	-	-

TBDMS Derivatives of Cytidine. Direct silylation of cytidine gave four TBDMS-O-derivatives of cytidine as observed before in the case of uridine and adenosine. A general procedure was described in the beginning of the section on Synthetic Methods. The specific details are described below.

Cytidine (243 mg, 1 mmol), imidazole (300 mg, 4.4 mmol), TBDMSCl (332 mg, 2.2 mmol) and DMF (1 ml) were stirred together at room temperature for 2 hours. A tlc in THF indicated the absence of cytidine. The reaction mixture was diluted in methanol and the solution was applied to four silica gel plates which were developed twice in ether-hexane (5:1). There were seen three bands, beside the UV absorbing material on the origin. A fourth band of 5'-O-TBDMScytidine separated from the base line when the plates were developed to a short length in THF. The slowest band was eluted in ethanol and the three faster bands were eluted in ethyl acetate. The four silyl derivatives obtained on removing the solvents were as described below.

2',3',5'-Tri-O-TBDMScytidine (35 mg, 6%) was obtained from the fastest band. Its R_f (THF) was 0.8 and the mass spectrum showed a prominent peak at m/e 528 (M-57).

2',5'-Di-O-TBDMScytidine (174 mg, 37%) was obtained from the band immediately behind the fastest band. It R_f in THF was 0.63 and the mass spectrum showed a prominent peak at m/e 414 (M-57). It was crystallised from ether-hexane and its m.p. was 205-206°C.

Anal. Cald. for $C_{21}H_{41}N_3O_5Si_2$; C, 53.47; H, 8.76; Si 11.90

Found: C, 53.68; H, 9.18; Si 12.49

3',5'-Di-O-TBDMScytidine (117 mg, 25%) was obtained from the band immediately behind that of the 2',5' isomer. It R_f in THF was 0.30. 5'-O-TBDMScytidine (92 mg, 26%) was obtained from the slowest band. Its R_f in THF was 0.03.

2',3'-Di-O-TBDMScytidine. 2',3',5'-Tri-O-TBDMScytidine (60 mg, 0.103 mmol) was heated with 80% acetic acid on a steam bath for one hour. The reaction mixture was diluted with ethanol and the solvents were removed at reduced pressure. The residue was dissolved in ethanol and applied to a silica gel plate which was developed in ether (twice). The slower of the two bands obtained was eluted in ethanol. The removal of the solvent gave a white solid (30 mg, 60%). This could be crystallised from ether-hexane and had m.p. 234-238 (dec.). The mass spectrum showed a prominent peak at m/e 414 (M-57). Its R_f in THF was 0.45.

TBDMS Derivatives of Guanosine. The direct silylation of guanosine was performed according to the general procedure in the beginning of the section on Synthetic Methods.

Guanosine (283 mg, 1mmol), imidazole (600 mg, 8.8 mmol), TBDMSCl (664 mg, 4.4 mmol) and DMF (2 ml) were stirred together at room temperature for 2 hours. A tlc in chloroform-ethanol (2:1) indicated the absence of guanosine. The reaction mixture was diluted with methanol and applied to five silica gel plates which were developed three times in chloform-ethanol (8:1). Three bands separated which were eluted in chloform-methanol (8:1). The solvents were removed giving the following compounds. 2',5'-Di-O-TBDMSguanosine (100 mg, 20%) was obtained from the fastest band. Its R_f in chloform-ethanol (4:1) was 0.62.

3',5'-Di-O-TBDMSguanosine (98 mg, 20%) was obtained from the middle band and its R_f value in chloroform-ethanol (4:1) was 0.30. 5'-O-TBDMSguanosine (240 mg, 60%) was obtained from the slowest band. Its R_f in chloroform-ethanol (4:1) was 0.05.

2',3',5'-Tri-O-TBDMSguanosine. Guanosine (71 mg, 0.25 mmol) was stirred with imidazole (204 mg, 3 mmol), TBDMSCl (225 mg, 1.5 mmol) and DMSO (0.5 ml) for two hours at room temperature. A tlc in chloroform-ethanol (2:1) indicated the absence of guanosine. The reaction mixture was diluted with methanol and applied to two silica gel plates which were thoroughly dried before developing them in chloroform-methanol (8:1).

The three separated bands were eluted in chloroform-methanol (4:1) and the solvents were removed. The fastest band material was the desired product which weighed 86 mg (55%) and had R_f value of 0.71 in chloroform-ethanol (4:1).

The middle band was 2'5'-di-O-TBDMSguanosine and weighed 32 mg (24%). The slowest band was 3',5'-di-O-TBDMSguanosine (21 mg, 16%).

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