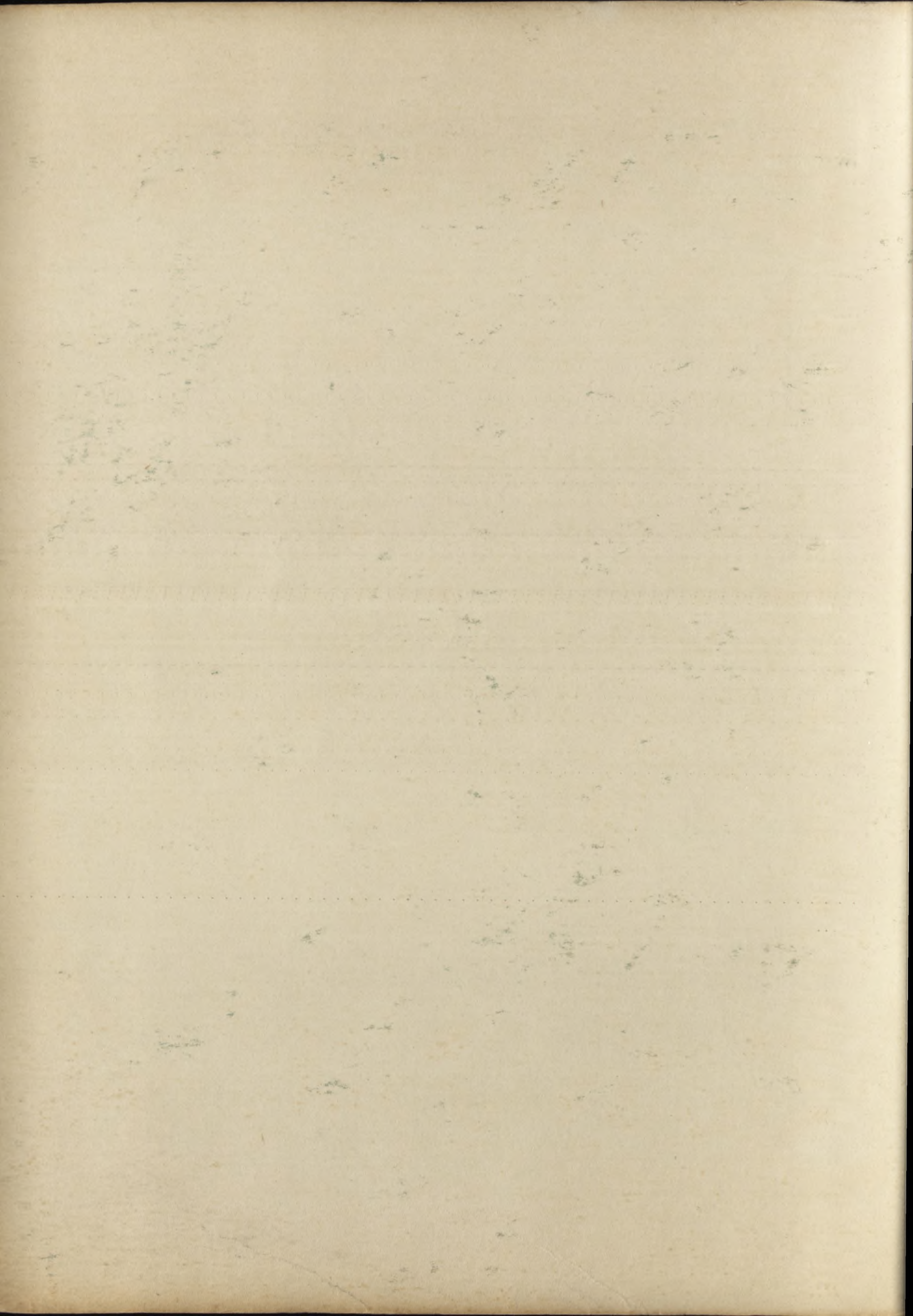

UNIWERSYTET IM. ADAMA MICKIEWICZA
INSTYTUT CHEMII

RECENT DEVELOPMENTS IN OLIGONUCLEOTIDE SYNTHESIS AND CHEMISTRY OF MINOR BASES OF tRNA

INTERNATIONAL CONFERENCE
September 13 and 14, 1974
POZNAŃ - KIEKRZ
POLAND

POZNAŃ 1974

CHAIRMAN OF THE CONFERENCE:

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EDITOR:

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Five years ago a group of several organic chemists from Poznań working in the field of natural products chemistry (alkaloids, peptides, terpenes) decided to try hard the new, dynamically developing research problems of nucleic acids chemistry. We began the new subject for two reasons:

- we felt that this research was needed in our country,
- we wanted to see, if we could manage this very fascinating but also very difficult problem, demanding the knowledge of all branches of chemistry research.

Our proposition was undertook without hesitation by undergraduate and postgraduate students, who were not afraid of the difficulties of this new subject as well as the strong competition, and a certain amount of ignorance helped them to take this decision. Our colleagues with greater experience showed reserve and scepticism, the more so as we had decided to deal with the problem from three different directions simultaneously:

- oligoribonucleotides synthesis of prescribed sequence,
- synthesis and chemistry of modified nucleosides,
- isolation and structural studies of tRNA.

After five years of very exhausting work in the new field we had behind us more failures than successes. We know that it would be much easier to obtain a partial success in one of the above mentioned fields, but we do not however regret the difficult and consistent way, that we have chosen, because it is extremely exciting thanks to its complexity, and our first successes give us great satisfaction.

Having mainly our younger colleagues and students in mind, we recently organized a conference "On the Recent Developments in Oligonucleotide Synthesis and in the Chemistry of Minor Bases of tRNA" held in Poznań - Kiekrz on September 13 and 14, 1974. We would like to thank the Institute of Organic Chemistry of the Polish Academy of Sciences and the Institute of Chemistry of Adam Mickiewicz University in Poznań for their financial sup-

port. We would like also to express our thanks to 14 outstanding scientists from nearly all over the world in vited to this conference, who gave the plenary lectures concerning their latest achievments within the areas discussed at the meeting. We are very glad that our guests kindly agreed to publish full texts of their lectures in this volume. The text of the lecture: "Some By-ways of Phosphotriester Approach to Oligonucleotide Synthesis" given by Dr. Colin B.Reese is not included here, because it will be published in the materials of the 5th International Conference of Organic Phosphorous Chemistry held in Gdańsk on September 16-22, 1974. The text of Dr. Girish B. Chheda's lecture: "Chemistry and Biochemistry of Anticodon Adjacent Hypermodified Nucleosides of tRNA" is not published either because Dr. G.B.Chheda was not unfortunately able to give us a copy in time.

During the conference 29 communications in the form of posters were also presented. The complete list of posters is inserted in Part III of this volume. Initially we wanted also to insert in this volume photographs of all the posters; it appeared however that for technical reasons this would be very difficult. (Thus, we desisted for this purpose). We want however to inform all interested, that we can deliver them the posters on microfilm.

We apologize for all editorial errors in this volume;we consider that the speedy publishing of it outweighed the need to improve its form.

124 scientists from Poland and abroad took part in the conference. We would like to express our thanks to all participants for their active and fruitful participation in our meeting. The full success of the conference encourages us to organize a similar meeting in 1976.

Maciej Wiewiórowski

Poznań in September 1974 Chairman of the conference

SYNTHESIS OF RIBOOLIGONUCLEOTIDES HAVING SEQUENCES OF TRANSFER RIBONUCLEIC ACIDS

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During the last decade the chemical synthesis of polynucleotides has been explored by many investigators. The most brilliant success in this field is perhaps the synthesis of a gene for yeast alanine tRNA by Khorana and coworkers.[1] This was accomplished by a combination of chemical synthesis of deoxyribooligonucleotides of upto icosanucleotide and enzymatic joining of them to each other employing DNA ligase.[2]

On the other hand, the chemical synthesis of riboligonucleotides, which have an additional 2'-hydroxyl group in each carbohydrate moiety, is rather difficult, mainly because of the following reasons. (1) Selective protection of the 2'-OH while leaving the 3'-OH unprotected usually requires lengthy pathways. (2) Migration of phosphate groups occurs rather easily under catalysis by acid or alkali. (3) Yields were relatively low in the condensation steps presumably due to steric hindrance. Nevertheless, the method for the synthesis of relatively short oligonucleotides was developed[3] and used for the synthesis of 64 trinucleotides which code for 20 amino acids in the protein biosynthesis.[4]

It is evident, however, that synthesis of longer polymers is necessary

to approach the synthesis of large molecule such as tRNA . This could not be achieved by the mere repititive joining of mononucleotides one by one and the block condensation method has to be developed. The minimum requirements for a ribooligonucleotide "block" are to have a free phosphate and/or OH groups on the chain terminus and suitable protecting groups in the middle nucleotides units on both the heterocyclic amino and 2'-OH groups.(see Fig. 1). Cyanoethyl phosphates[5], widely used in deoxyoligonucleotide synthesis are not suitable for the present purpose, because the alkali treatment used to eliminate the cyanoethyl group cause deprotection of 2'-OH groups and makes it impossible to elongate the chain by further condensations.

Synthesis of Ribooligonucleotides by the Use of Aromatic Phosphoramidates as Protected Phosphate Groups

Several years ago we studied the decomposition of nucleotide phosphoramidates by means of nitrite and found that thymidine 5'-phosphoramidate is converted quantitatively to the corresponding phosphate[6] under neutral conditions at room temperature. We tested,therefore, aromatic phosphoramidates, which are known to be stable in acidic and alkaline media.[7] (Fig. 2)

Fig. 1

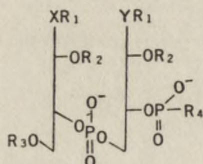
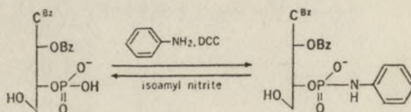


Fig. 2



N,2'-O-Dibenzoylcytidine 3'-phosphate was converted smoothly to the corresponding 3'-phosphoranisidate upon treatment with anisidine and DCC. The phosphoranisidate was then allowed to react with isoamyl nitrite in an aqueous acetic acid-pyridine buffer at pH 7.5 and room temperature. As expected, the 3'-phosphate was recovered in quantitative yield. By this experiment, it was proved that aromatic phosphoramidates could be used for the protection of the terminal phosphate and that other protecting groups, such as 2'-O-benzoyl or N-benzoyl, remain intact. A separate experiment showed that a 5'-monomethoxytrityl group, which is labile to acid, is also stable to this nitrite treatment.

Synthesis of Oligoribonucleotides Having the Sequences of Yeast Alanine tRNA.

Using this aromatic phosphoramidate we attempted to synthesize first a hexanucleotide CpCpApCpCpA having the sequence of yeast alanine tRNA[8] (Fig. 3) at the 3'- end.

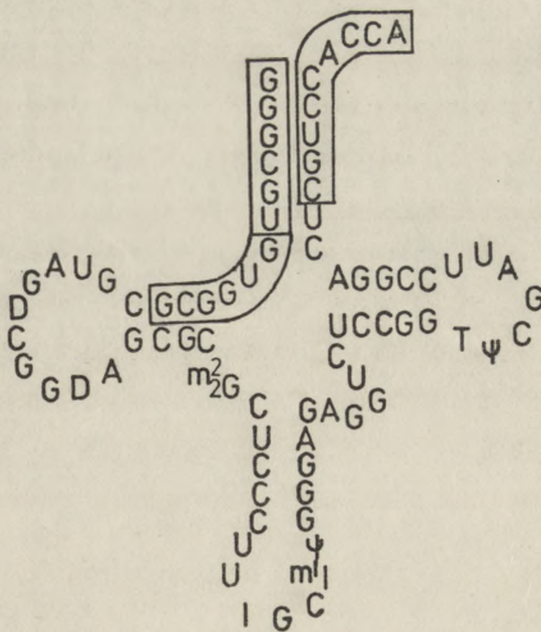
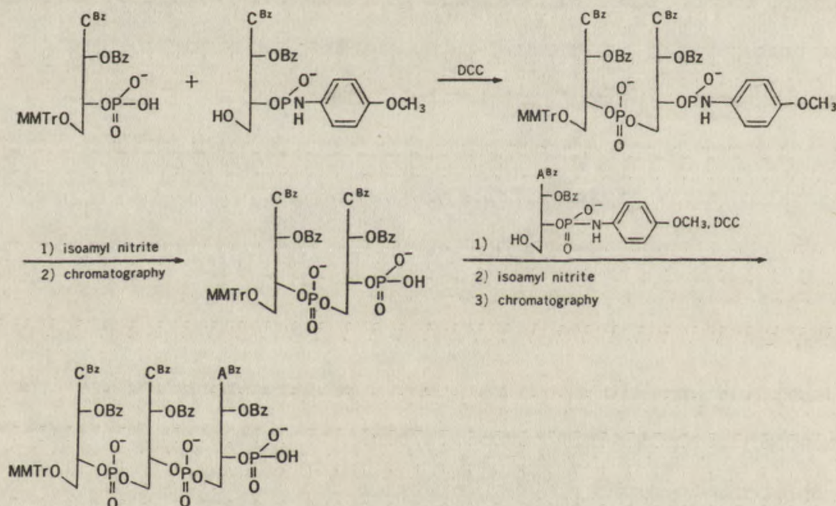


Fig. 3

This was achieved by condensing two trinucleotide units, MMTrC^{Bz}(OBz)p-C^{Bz}(OBz)pA^{Bz}(OBz)p* and HOC^{Bz}(OBz)pC^{Bz}(OBz)pA^{Bz}(OBz)$_2$.[9] The former trinucleotide was synthesized by reaction of C^{Bz}(OBz)p-NH-C_6H_4-OCH_3 with MMTrC^{Bz}-(OBz)p with DCC as condensing reagent.[10] (Fig. 4) After the appropriate

Fig. 4



work-up, the protected dinucleotide was treated with excess isoamyl nitrite to afford MMTrC^{Bz}(OBz)pC^{Bz}(OBz)p which was purified by DEAE-cellulose column chromatography. The trinucleotide was obtained by fur-

---

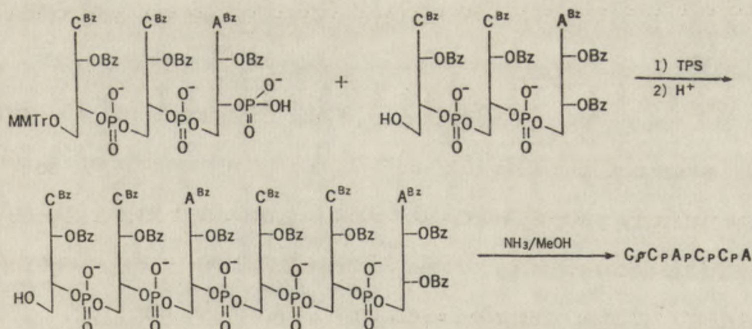
* The abbreviation used was as has been appeared in the J. Biol. Chem. 241, 531 (1966). For the protected ribonucleotide essentially the same system is used as described in references 9 and 10. MMTrC^{Bz}(OBz)p-C^{Bz}(OBz)pA^{Bz}(OBz)p refers to 5'-O-monomethoxytrityl-N,2'-O-dibenzoyl-cytidylyl-(3'-5')-N,2'-O-dibenzoylcytidylyl-(3'-5')-N,2'-O-dibenzoyl-adenosine 3'-phosphate.

ther condensation of a mononucleotide $A^{Bz}(OBz)p\text{-}NH\text{-}C_6H_4\text{-}OCH_3$ by the use of DCC. The yield in the final step was around 30%. Thus, properly protected trinucleotide became available in reasonable yield and in relatively large quantity (1 mmolar scale).

A trinucleotide CpGpUp corresponding to the 7th to 9th nucleotides of yeast alanine tRNA 3'-end was synthesized also by essentially the same procedure using phosphoramidate method.[11] The phosphoramidate method was also successfully applied to the synthesis of deoxyoligonucleotides.[12]

The condensation of the trinucleotide $MMTrC^{Bz}(OBz)pC^{Bz}(OBz)pA^{Bz}(OBz)p$ with a trinucleotide $HOC^{Bz}(OBz)pC^{Bz}(OBz)pA^{Bz}(OBz)_2$, which was synthesized by the method of Khorana[4], was attempted first by using DCC, but it was not possible to isolate any condensation products. As it was postulated[13]

Fig. 5

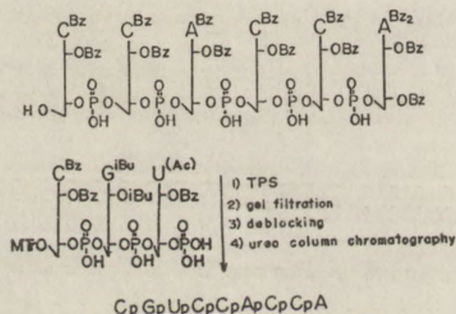


that cyclic trimetaphosphates might be intermediates in the activation of phosphomonoesters, the reactive intermediate in the condensation seems to be too bulky for access of 5'-OH end group. Employing triisopropylbenzenesulfonyl chloride (TPS)[14] as the condensing reagent in this reaction enabled a hexanucleotide $MMTrC^{Bz}(OBz)pC^{Bz}(OBz)pA^{Bz}(OBz)pC^{Bz}(OBz)p$-$C^{Bz}(OBz)pA^{Bz}(OBz)_2$ to be isolated by column chromatography successively on Sephadex LH-20 and TEAE-cellulose though in 15% yield. (Fig. 5.)

Thus it was first proved that the block condensation method is useful for synthesis of ribooligonucleotides of longer nucleotide units.

For further elongation of the hexanucleotide to nonanucleotide with the sequence authentic to alanine tRNA 3'-end, two routes were chosen[11] :

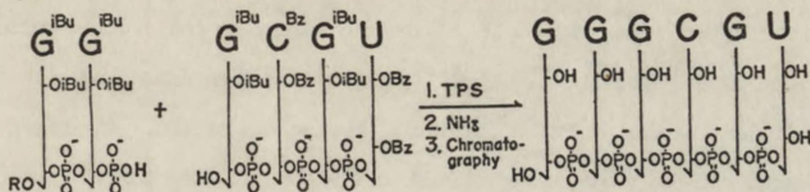
Fig. 6



(i) Condensation of a hexa with a trinucleotide and (ii) successive condensation of the hexa with a mono- and then a dinucleotide. (Fig. 6). The hexanucleotide was condensed with trinucleotide MMTrC^{Bz}(OBz)pG^{iBu}(OiBu)p-U(OBz)p by the use of TPS. Purification of the nonanucleotide was achieved after total deprotection using DEAE-cellulose in the presence of 7M urea[15] at 50°. Essentially pure CpGpUpCpCpApCpCpA was obtained in a yield of 8%. The successive condensation of mononucleotide MMTrU(OBz)p and dinucleotide MMTrC^{Bz}(OBz)pG^{iBu}(OiBu)p afforded yields of 8 and 5% in two steps. Characterization of these nonanucleotides by digestion with pancreatic RNase and RNase T1, as well as by spleen phosphodiesterase showed satisfactory results. Thus the nonanucleotide having the sequence of alanine tRNA 3'-end has been synthesized.

In order to obtain another end of the alanine tRNA molecule, a hexanucleotide GpGpGpCpGpU spanning 1st to 6th nucleotides of 5'-end, which was complementary to the nonanucleotide obtained as before, was synthesized. (Fig. 7). A dinucleotide iBuG^{iBu}(OiBu)pG^{iBu}(OiBu)p was isolated

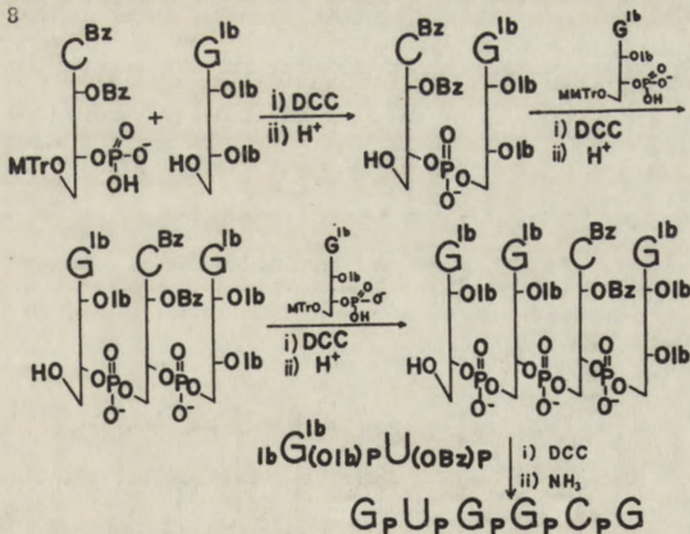
Fig. 7



from the polymerization mixture of Gp units and condensed with G^{iBu}(OiBu)p-C^{Bz}OBz)pG^{iBu}(OiBu)pU(OBz)$_2$, which was synthesized by the stepwise condensation, by the use of TPS. The isolated yield was 15%.

The second hexanucleotide, GpUpGpGpCpG corresponding to 7th to 12th nucleotides of alanine tRNA was synthesized by the method involving stepwise condensation of mononucleotides to yield tetranucleotide GpGpCpG and successive condensation with dinucleotide GpUp.[11] (Fig. 8) Pyridinium

Fig. 8

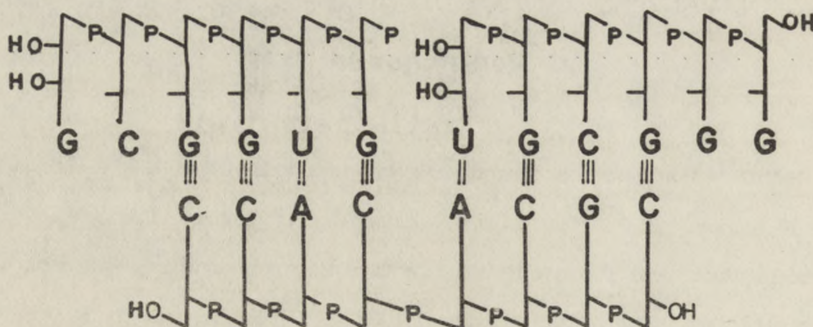


salt of MMTrC^{Bz}(OBz)p was condensed with two equivalents of G^{iBu}(OiBu)$_2$ by DCC (10 equiv.) at 23° for 3 days. After detritylation with acetic acid, the product was isolated by extraction with chloroform-n-butanol and precipitation. The dinucleotide C^{Bz}(OBz)pG^{iBu}(OiBu)$_2$ was then condensed with MMTrG^{iBu}(OiBu)p (4 equiv.) by the use of DCC at 23° for 5 days.

The product trinucleotide was purified on a column of TEAE-cellulose using triethylammonium acetate in a linear gradient as the eluting buffer. Trinucleotide $G^{iBu}(OiBu)pC^{Bz}(OBz)pG^{iBu}(OiBu)_2$ was then condensed with ten equivalent excess of $MMTrG^{iBu}(OiBu)p$ by the use of DCC. The yield of tetranucleotide, which was purified on a TEAE-cellulose column, was 23%. Relatively lower yields in these condensation steps may be due to the fact that this sequence contained three guanine residues. Another dinucleotide $iBuG^{iBu}(OiBu)pU(OBz)p$ was synthesized from pyridinium U(OBz)p-NH-naphthyl (β)[16] and slight excess of $iBuG^{iBu}(OiBu)p$ by DCC. The yield of the dinucleotide was 25%.

The condensation of tetranucleotide GpGpCpG and dinucleotide GpUp (3 equivalents) using DCC at 18° for 48 hrs gave a hexanucleotide GpUp-GpGpCpG after deprotection and purification through a column of DEAE-cellulose with 7M urea and subsequent Biogel filtration. For the phosphorylation of the 5'-terminus of the hexanucleotide, polynucleotide kinase[17] was employed. Incubation of the hexanucleotide with γ-^{32}P-ATP afforded ^{32}PGpUpGpGpCpG. This labeled hexanucleotide may be suitable for the joining with the former hexanucleotide on a deoxy-oligonucleotide template by the catalysis of DNA ligase.[2] (Fig. 9)

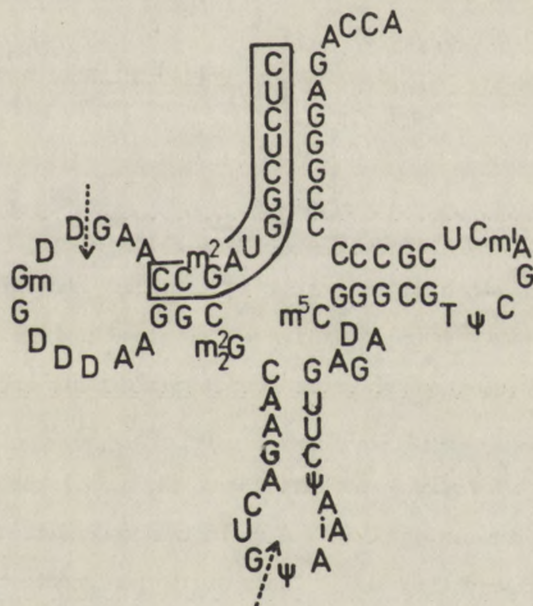
Fig. 9



Synthesis of Oligoribonucleotides Corresponding to the 5'-End of Yeast Tyrosine tRNA

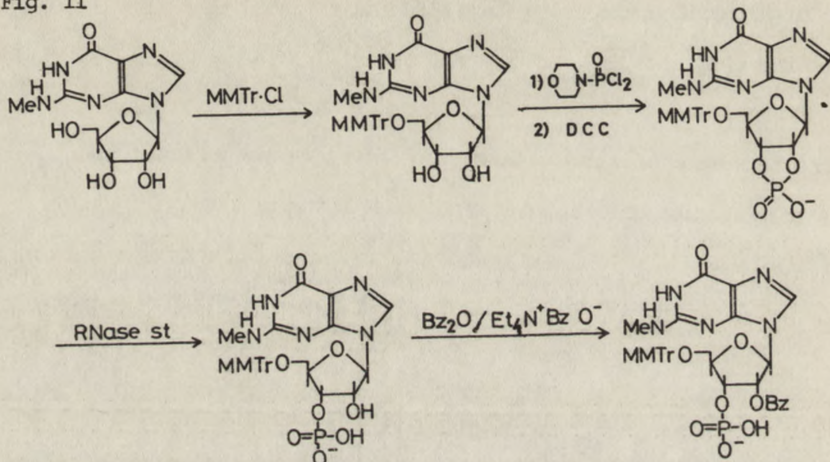
On the backgroud of results discussed above,[18] we now extended the synthesis to oligoribonucleotides having yeast tyrosine tRNA. (Fig. 10)

Fig. 10



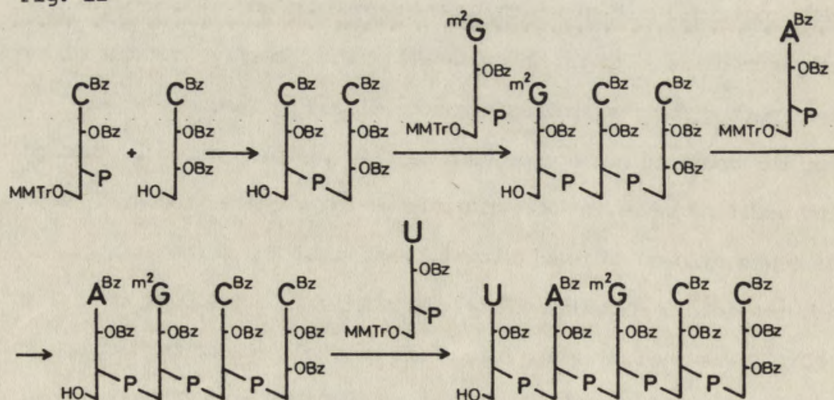
This tRNA contained a N^2-monomethylguanine residue and a nucleoside N^2-monomethylguanosine might be accessible rather easily, because of an excellent method for its synthesis was developed by Yamazaki et al.[19] By using the modified nucleotide such as m^2Gp we could directly incorporate the modified bases in tRNA molecule by the chemical means. N^2-methylguanosine was allowed to react with 1.2 equiv. of monomethoxytrityl chloride to yield 5'-O-methoxytrityl derivative in a yield of 66%. (Fig. 11) MMTrm^2G was then phosphorylated with morpholinophosphorodichloridate[20] and the resulting 2' (or 3')-phosphate was cyclized with DCC. 2',3'-Cyclic phosphate, thus obtained,was hydrolyzed by RNase St[21] to give m^2Gp in a quantitative yield. After the protection of 2'-OH group, the pro-

Fig. 11



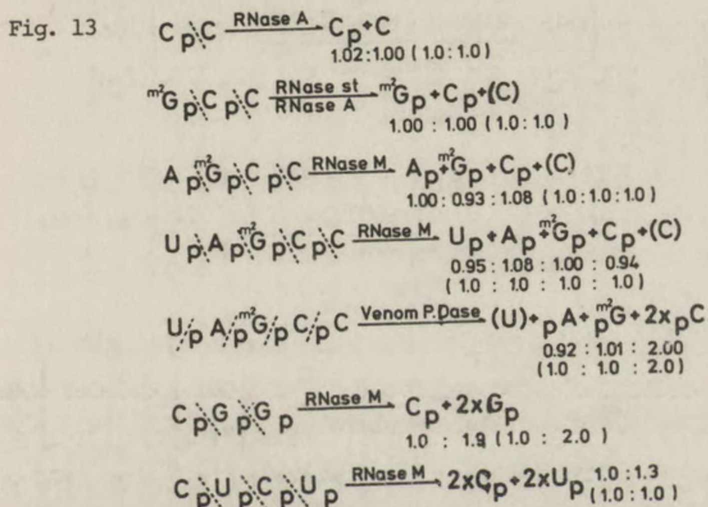
duct MMTr-m^{2}G(OBz)p was obtained in a yield of 47%. The terminal CpC unit was obtained by condensing MMTrC^{Bz}(OBz)p and slight excess of C^{Bz}-(OBz)$_2$. MMTrC^{Bz}(OBz)pC^{Bz}(OBz)$_2$ was obtained in a yield of 75%. The dinucleotide was then condensed with MMTr-m^{2}G(OBz)p obtained as above by DCC and the yield of trinucleotide was 38%. To the trinucleotide MMTr-A^{Bz}(OBz)p (5 equiv.) and MMTrU(OBz)p (4 equiv.) were successively condensed by the use of DCC. Yields in two condensation steps were 31 and 21 %, respectively (Fig. 12)[22]. These di- to pentanucleotides were character-

Fig. 12



ized by the digestion with RNase A, RNase St, RNase M and snake venom phosphodiesterase as shown in Fig. 13.

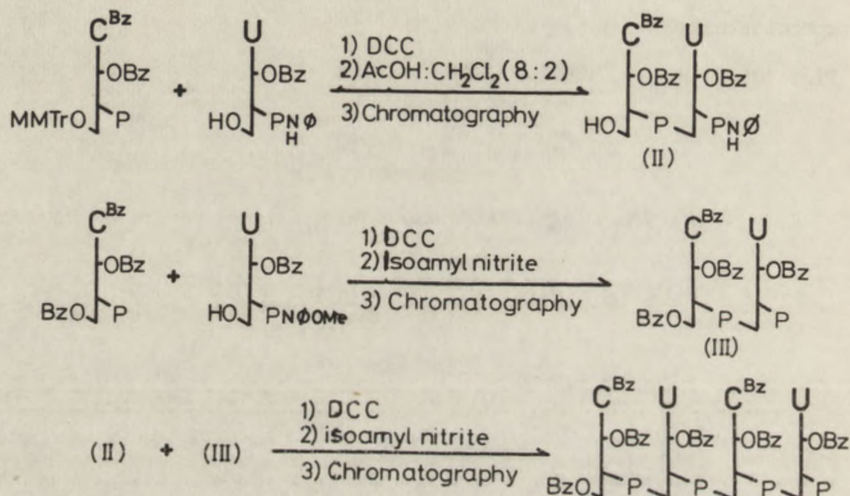
Fig. 13



This synthesis was the first example of the stepwise synthesis of ribopentanucleotide and yields in each condensation steps were as expected. By using much larger excess of mononucleotide units, it may be possible to increase the yield and to use for synthesizing longer oligonucleotides.

A tetranucleotide CpUpCpUp having the sequence corresponding to 1st to 4th nucleotides of tyrosine tRNA was synthesized by condensing two CpUp units. (Fig. 14) 2'-O-Benzoyluridine 3'-phosphoranisidate was allowed to react with 2 equivalents of 2',5',N-tribenzoylcytidine 3'-phosphate using DCC. By the purification through a column of TEAE-cellulose the protected dinucleotide BzOC^{Bz}(OBz)pU(OBz)p was obtained in a yield of 55%. For the synthesis of another dinucleotide, 5'-O-monomethoxytrityl-N,2'-O-dibenzoylcytidine 3'-phosphate was condensed with 2'-O-benzoyluridine 3'-phosphoranilidate by DCC. Yield of dinucleotide C^{Bz}(OBz)p-U(OBz)p-NH-C_6H_5 was 21%. Condensation of these two dinucleotides was

Fig. 14



conducted by the use of TPS (6 equiv.) at 28° for 8 hrs. After TEAE-cellulose column chromatography the protected tetranucleotide was obtained in a yield of 22%. For condensing dinucleotides having terminal phosphate residues TPS was the most effective reagent.

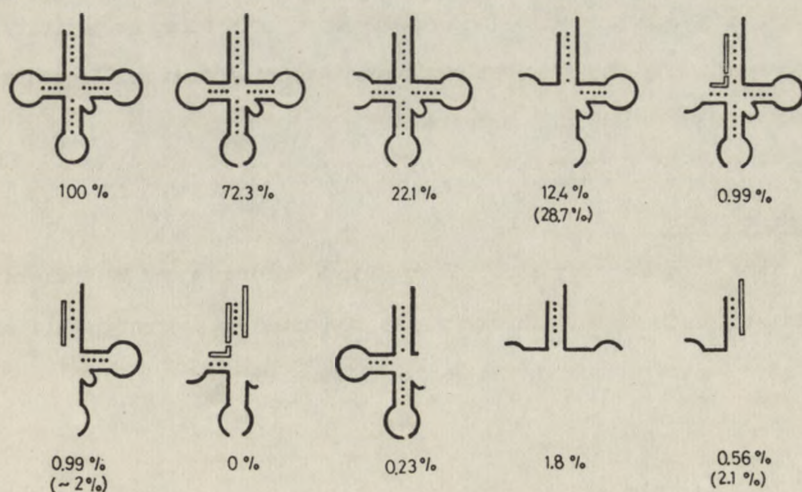
The third fragment CpGpGp corresponding to 5th to 7th nucleotides of tyrosine tRNA 5'-end was synthesized as follows. Several fold excess of 5'-O-monomethoxytrityl-N,2',5'-tribenzoylcytidine 3'-phosphate was condensed with N,2'-O-diisobutyrylguanosine 3'-phosphoranisidate by the use of DCC. Dinucleotide MMTrC^{Bz}(OBz)pG^{iBu}(OiBu)p was obtained in a yield of35%. The protected dinucleotide was then condensed with G^{iBu}-(OiBu)p-NH-C_6H_4-OCH_3 by DCC at room temperature for 50 hrs. Trinucleotide was obtained in a yield of 17%.

Condensation of these three blocks corresponding to 5'-end of the tyrosine tRNA is now in progress.

Reconstitution of Chemically Synthesized Oligoribonucleotides With the Fragment Obtained by RNase T1 Digestion of tRNA for Amino Acid Acceptor activity.

Since the first discovery of Holley et al.[23] that yeast alanine tRNA could be cleaved at the anti-codon region to two halves by a limited digestion with RNase T1, many investigators have shown[24] the recovery of amino acid acceptor activity by reconstituting these fragments. In the field of proteins the reconstitution of pancreatic RNase fragments, S-protein and S-peptide, showed full activity. Chemically synthesized S-peptide was shown to have its activity when it was reconstituted with natural S-protein.[25] We could assume, therefore, if we synthesize appropriate oligonucleotide having sequence of tRNA, these synthetic fragments could be reconstituted with fragments obtained by digestion of tRNA. Along this line we preliminary tested the reconstitution of 3'-end nonanucleotide, 5'-end hexanucleotide and 5'-end second hexanucleotide with RNase T1 digest of the Torula yeast tRNA.[26] Results were schematically shown in Fig. 15.

Fig. 15



If we assume the amount of [^{14}C]-alanine incorporation to the native tRNA as 100%, reconstituted molecule from 3'- and 5'-half showed 72% activity. If this molecule lacked a part of dihydro-U loop, the amount incorporated dropped to 22%. Removal of the second 5'-quarter further decreased to 12.4%. However, if we used 4 equivalent amoutn of 5'-quarter, the incorporation increased to 29%. Replacement of this 5'-quarter by two synthetic hexanucleotides showed about 1% incorporation. Use of only one terminal hexanucleotide showed the same range of activity. Increasing the amount of hexanucleotide to 4 fold, the incorporation was doubled. Reconstitution of 5'-half and two 3'fragments as shown in the Figure gave only 0.2% activity. The use of 3 synthetic oligonucleotides with natural two lower fragments showed no activity. However, if we used 3'-end nonanucleotide and 5'-quarter, the incorporation around 0.6% was observed. This activity increased to 2% by the use of 4 fold excess of 5'-quarter fragment. Reconstitution of two upper quarters as shown gave also 2% activity.

Although the amount of the incorporation was very small and provide any fruitful information, this is the first case in that chemically synthesized ribooligonucleotides showed the amino acid acceptor activity.

A possibility for elucidation of structure-function relationship of tRNAs was proved by this experiment.

Acknowledgements

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BLOCK COUPLING SYNTHESIS OF OLIGORIBONUCLEOTIDES

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Introduction. Transfer RNA, the protein biosynthesis adaptor, is a superb example of a polyfunctional molecule. Continual conformation change in its tertiary structure allows participation in many different interactions, some of which, anticodon recognition and cognate aminoacid attachment, have been established, while others, aminoacyl-tRNA synthetase recognition, ribosome recognition, and "punctuation" machinery, are as yet unknown. These particular structure-function relationships may never be solved using complete native molecules, however, specific interactions may be determined using oligoribonucleotide "fragments" corresponding to portions of the primary sequence. Indeed, this approach has already been adopted. Partial RNase T-1 digestions on $tRNA_f^{Met}$ (E. coli) provided an intact anticodon region which was shown to have similar, but weaker, binding to its parent (1). A stem region showing partial alanyl-tRNA synthetase activity was isolated from careful RNase T-1 digest of $tRNA_{II}^{Ala}$ (yeast) (2). Fragments from enzymic digestions are, however, limited as their sequences and lengths are controlled by (a) the availability of single molecular species of tRNA and (b) the specificity of the enzymes used. Chemical syntheses on the other hand can theoretically provide all possible oligoribonucleotides. Unfortunately, five years ago, the status of oligoribonucleotide synthesis was not sufficiently developed to provide say, decamers of predetermined sequence. Synthesis of all 64 triribonucleotides had been accomplished but the preparative scale was quite small (3). A recent extension of these studies has allowed an all-chemical block synthesis of the stem region of $tRNA^{Ala}$ (yeast) (4).

BLOCK COUPLING SYNTHESIS OF OLIGORIBONUCLEOTIDES

Function	Protecting group	Deblocking conditions
Phosphate	2,2,2-trichloroethyl	Cu/Zn in DMF
NH_2 on Bases	benzoyl	7N NH_4OH
2'-OH of riboside	tetrahydropyranyl	pH2
5'-OH of riboside	triphenylmethoxyacetyl	0.15N NH_3-CH_3OH

Phosphate Protection. Introduction of phosphotriester protection into the synthesis of oligodeoxyribonucleotides allowed convenient larger scale isolation and purification using silica gel chromatography in organic solvents (5). Full protection of phosphate groupings is preferred as charged phosphodiesters are susceptible to undesirable side reactions during subsequent coupling reactions. Applied to synthesis of oligoribonucleotides, yields increased substantially even when using approximately equimolar quantities of reactants. A number of "third" groups has been employed: 2-cyanoethyl (5), substituted phenyls (6), both removable by mild base, and 2,2,2-trichloroethyl removable by reductive cleavage. First introduced for carboxyl protection, the potential of the latter for phosphate protection was soon realized (7). This last group has been our choice throughout our endeavours.

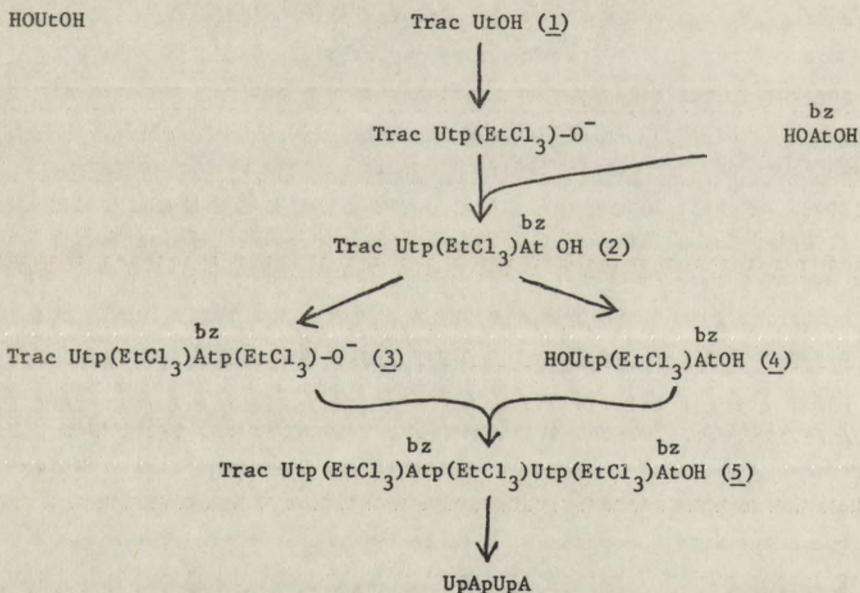
Base Protection. Benzamide protection of the heterocyclic bases, adenine, cytosine and guanine, has been used throughout. During the synthesis of protected nucleosides, fractional crystallisation is necessary to separate 2'- and 3'-acetates after acidic opening of cyclic orthoacetates (8). In both the cytidine and the guanosine series, N-benzoyl derivatives were superior. No trouble has been experienced in benzamide removal during final deblocking.

BLOCK COUPLING SYNTHESIS OF OLIGORIBONUCLEOTIDES

2'-Hydroxyl Protection. Protection of the 2'-hydroxyl group of ribose is of major importance. Should this group be free in the presence of base, phosphate migration results, leading to undesirable 2', 5'-phosphodiester linkages. Preference for acid-labile protection over base-labile carboxylic esters (3) led to the selection of tetrahydropyranyl ethers for their convenient preparation and, under conditions for their removal (pH 2), negligible phosphate isomerisation (9). Reaction of dihydropyran with alcohols results in the formation of a chiral acetal carbon giving pairs of diastereoisomers (9). Each diasteromer of 2'-O-tetrahydropyranylribofuranoside has quite different physical properties from its partner because of the existence of an additional hydrogen bond between the 3'-hydroxyl and the tetrahydropyranyl ring oxygen in one of the isomers (10). To overcome this problem a symmetrical ketal group has been introduced (11). However, we have taken advantage of these differing solubilities: the lower R_F diastereomers, being less soluble in pyridine, are converted to 5'-substituted derivatives and used as the first member of a sequence assembled by a stepwise synthesis, while the higher R_F diastereomers are used in the other positions of the sequence.

5'-Hydroxyl Protection. In our early work 5'-p-methoxyltrityl protection was employed and a successful stepwise synthesis was developed (12). Pentamers can be made in good yield by this method. However, when couplings involving guanosine were encountered, efficiency dropped making the preparation of longer material rather difficult. A fragment coupling method had to be developed. Since a free 5'-hydroxyl group is required to couple preformed protected blocks, a means of special protection and specific removal had to be developed. Obviously, the trityl group is unsatisfactory since the acidic conditions needed for its removal will also cleave tetrahydropyranyl ethers. For specific removal, only non-reductive neutral or mild basic conditions are available. 5'-Alkoxy- or aryloxyacetates appeared promising and a 5'-phenoxyacetyl group was used in a synthesis of UpU, but certain shortcomings became obvious (13). Trityl groups are detected as bright yellow-orange coloration on t.l.c. plates after treatment with an acidic ceric sulphate spray and heating. Compounds containing trityl groups can

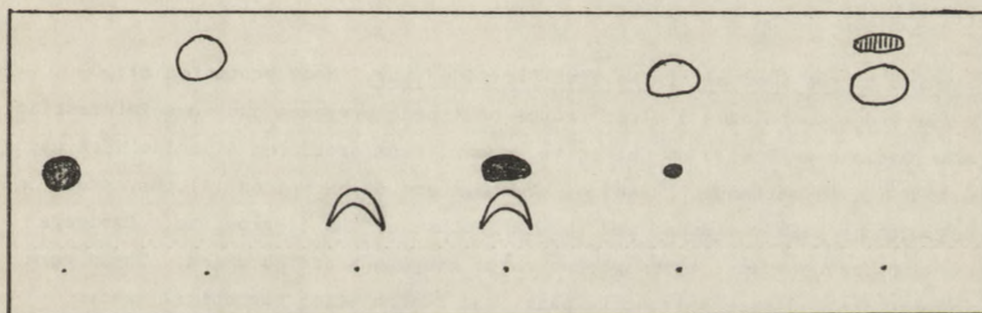
be conveniently followed during reactions and their subsequent work-up and chromatography. Phenoxyacetate gave brown colorations and were indistinguishable from other nucleoside derivatives and TPS break-down products. The combination of the trityl detection and alkoxyacetyl base lability led to the development of the triphenylmethoxyacetyl (trityloxyacetyl or Trac) derivatives (13).

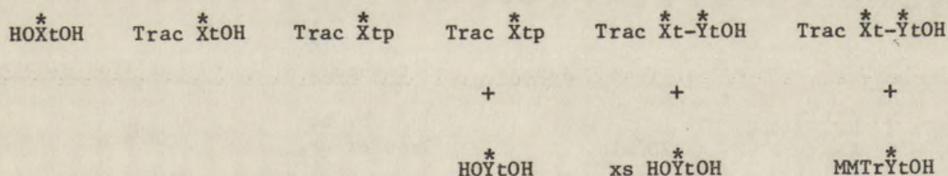


Synthesis of UpApUpA (14). Selective 5'-esterification of 2'-O-tetrahydropyranyluridine using trityloxyacetate and TPS gave 5'-trityloxyacetate (1) which on phosphorylation with mono-2,2,2-trichloroethylphosphate and TPS, and subsequent coupling with N^6-benzoyl-2'-O-tetrahydropyranyladenosine, gave protected dinucleoside monophosphate (2). This material was divided into two equal portions, one of which was phosphorylated to give dinucleotide (3) and the other treated with 0.15 N NH_3 in methanol to remove the Trac group selectively giving dinucleoside monophosphate (4). Subsequent coupling of 3 and 4 gave protected tetranucleotide (5) which on complete deblocking (13) gave UpApUpA as shown by standard enzymic digestion analysis. Yields of all

steps were high (80%).

Monitor of Coupling using thin layer chromatography. All couplings were followed by t.l.c. on silica gel plates using 10% CH_3OH-CH_2Cl_2 as solvent.





where ○ is yellow
◍ is orange
● is brown

and $\overset{*}{X}$ and $\overset{*}{Y}$ = A^{bz}, C^{bz}, G^{bz} or U.

Phosphorylation gives a charged product whose R_F is low, thus the end point of reaction can be easily determined. Coupling with the next nucleoside derivative gives a neutral dinucleotide of high R_F so the second part of the two-step process can be followed. Normally, column chromatography separates this product from excess nucleoside, HO$\overset{*}{Y}$tOH, but not always. Careful treatment of the mixture with monomethoxytrityl chloride forms monomethoxytrityl ethers of primary alcohols. Successful column chromatography separation is then possible. Tritylation also provides a check for existence of any di-

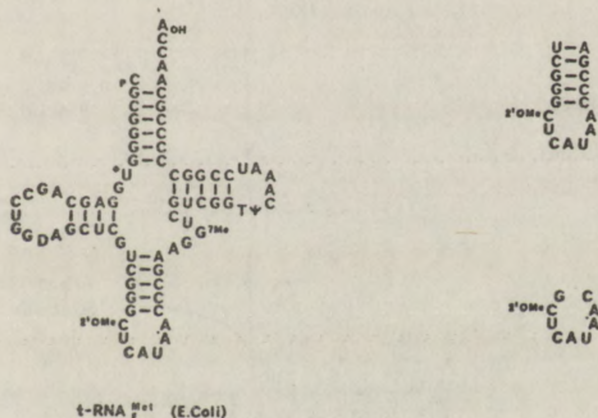
nucleotides containing 3'-3' linkages (these have a free primary hydroxyl). None of this material has ever been detected. The neighbouring bulky 2'-O-tetrahydropyranyl grouping apparently forbids coupling at the secondary 3'-hydroxyl group of $HO\overset{*}{Y}tOH$ and allows selective reaction at the primary 5'-position.

Studies on the Removal of the Trac Grouping (15). Many protected oligonucleotides containing 5'-Trac groups have been prepared and some interesting observations were made on selective removal from protected dinucleotides using 0.15 N NH_3 in methanol. Rates of cleavage are dependent on (a) the nature and sequence of base residues, and (b) the nature of the 3'-grouping. Cleavage products are complex, although two major compounds are obtained. These were found to be a diastereoisomeric pair, the P atom being the chiral centre. Separation is possible in some of the cases.

Compound	$t_{\frac{1}{2}}$ of Trac Cleavage	Compound	$t_{\frac{1}{2}}$ of Trac cleavage
Trac Ut-$\overset{bz}{A}$tOH	20 min.	Trac $\overset{bz}{A}$t-$\overset{bz}{C}t_2$	2 h.
Trac Ut-$\overset{bz}{C}$tOH	20 min.	Trac $\overset{bz}{C}$t-Ut_2	2 h.
Trac $\overset{bz}{G}$t-$\overset{bz}{G}$tOH	1 h.	Trac $\overset{bz}{G}$t-$\overset{bz}{G}t_2$	5 h.
Trac $\overset{bz}{G}$t-$\overset{bz}{C}$tOH	1 h.	Trac $\overset{bz}{A}$t-Utp	< 5 min.
Trac $\overset{bz}{A}$t-UtOH	1 h.	Trac Ut-$\overset{bz}{A}$tp	< 5 min.
Trac $\overset{bz}{A}$t-$\overset{bz}{C}$tOH	30 min.		
Trac $\overset{bz}{C}$t-$\overset{bz}{A}$tOH	20 min.		
Trac $\overset{bz}{C}$t-UtOH	30 min.		

BLOCK COUPLING SYNTHESIS OF OLIGORIBONUCLEOTIDES

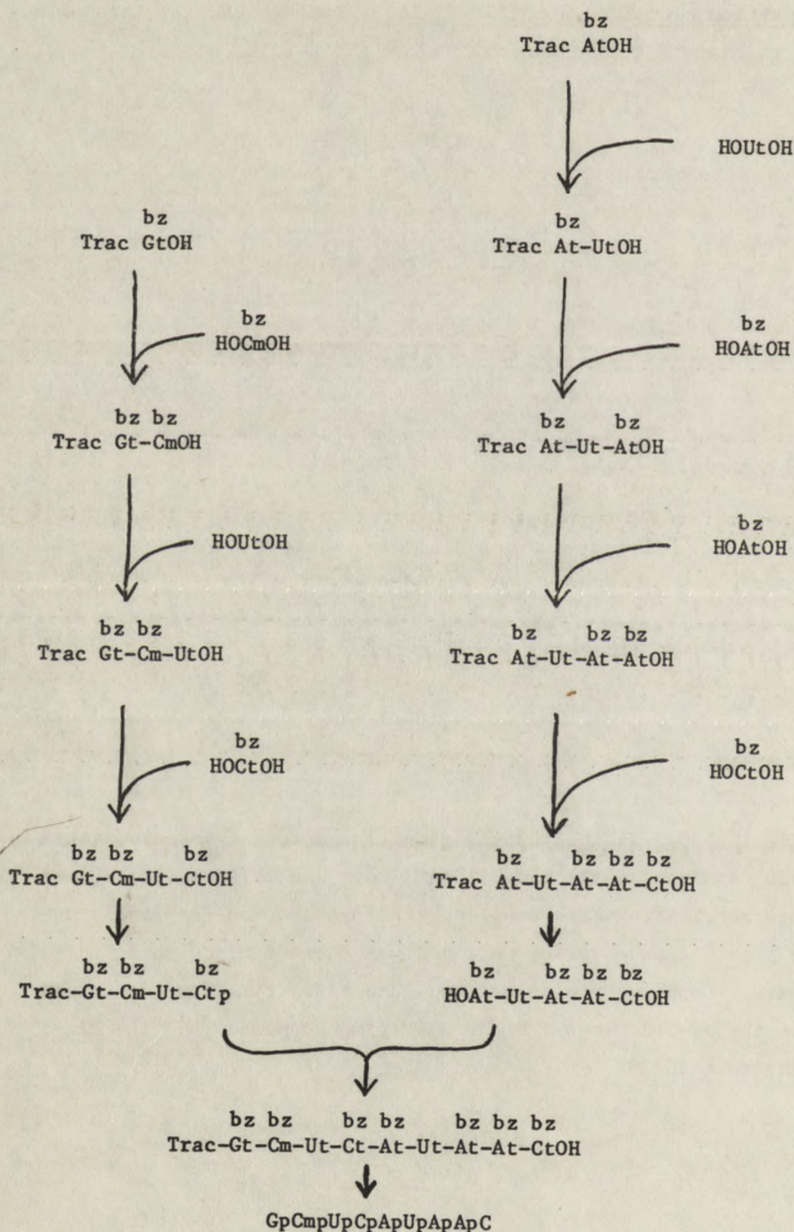
Synthesis of Nonaribonucleotide, GCmUCAUAAC (16). The sequence of this oligomer corresponds to that of the anticodon loop of $tRNA_f^{Met}$ (*E. coli*).



t-RNA $_f^{Met}$ (E.Coli)

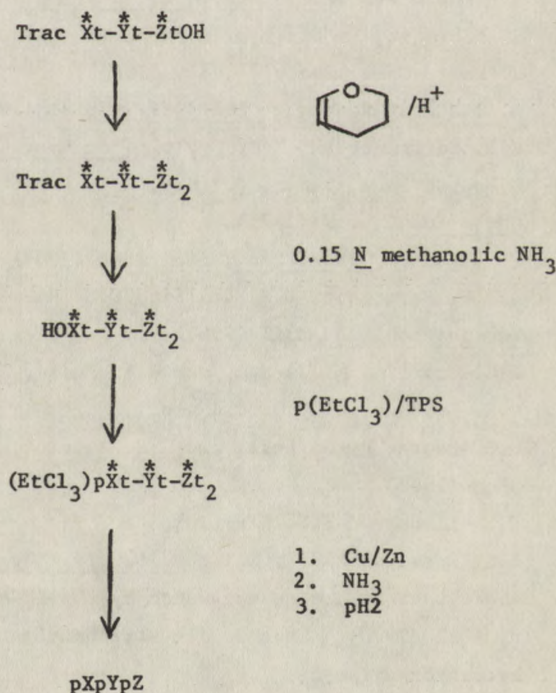
Synthesis outline is shown in the accompanying flow sheet. Protected tetranucleotide, GCmUC, and pentanucleotide, AUAAC, were assembled stepwise, and then coupled together. After each coupling, the product was completely deblocked and the free oligonucleotide obtained was characterized using enzymic digestion analysis. Coupling yields between adenosine, cytidine and uridine residues were in the 50-70% range. Those involving guanosine and the final fragment coupling were 20-30%.

BLOCK COUPLING SYNTHESIS OF OLIGORIBONUCLEOTIDES



BLOCK COUPLING SYNTHESIS OF OLIGORIBONUCLEOTIDES

5'-Phosphorylation. Selective removal of the triphenylmethoxyacetyl group from blocked oligoribonucleotides allows 5'-phosphorylation at the protected level. An outline is summarized for the synthesis of pXpYpZ. The 3'-terminal hydroxyl group is protected as a tetrahydropyranyl ether. The Trac group can then be removed and the regenerated 5'-hydroxyl phosphorylated with TPS-activated mono 2,2,2-trichloroethylphosphate. Complete deblocking gives oligomers with a 5'-phosphate in good yield (17).



Conclusion. A synthetic method has been developed for oligomers of any sequence and length up to approximately ten major residues of the major bases. Oligomers can have 5'-, 3'- or 3', 5'-terminal phosphate groups. Preparative scale is large and milligram amounts of material can be conveniently obtained.

BLOCK COUPLING SYNTHESIS OF OLIGORIBONUCLEOTIDES

Acknowledgment: The expertise of my colleague, Dr. Eva S. Werstiuk, is gratefully acknowledged. Research was supported by the Medical Research Council of Canada.

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14. Abbreviations are as suggested by the IUPAC-IUB commission, Biochem. 9, 4022 (1970). Trac is 5'-O-triphenylmethoxyacetyl, t is 2'-O-tetrahydropyranyl.
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NUCLEOTIDE SYNTHESIS BY OXIDATION-REDUCTION CONDENSATION.

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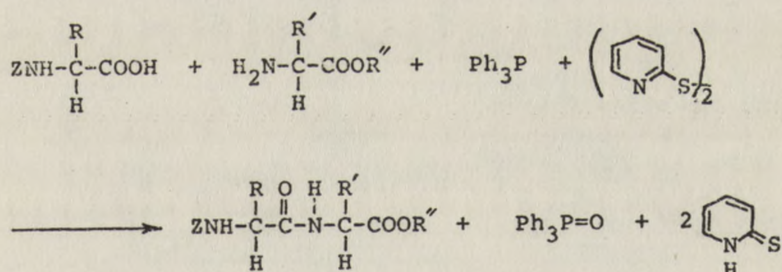
I. INTRODUCTION

A useful and practical method of peptide synthesis[1] via the oxidation-reduction condensation by use of triphenylphosphine [Ph_3P] and 2,2'-dipyridyl disulfide [$(PyS)_2$] is shown in the following equation.



In this condensation reaction, dehydration from free carboxylic components and free amino components proceeds through the elimination of one oxygen atom (reduction) and two hydrogen atoms (oxidation) with [Ph_3P] and [$(PyS)_2$], respectively, to afford peptides, triphenylphosphine oxide and 2 mol of pyrid-2-thione. This new condensation reaction was successfully applied to the syntheses of oligopeptides [oxytocin[2], LH-RH[3] and ACTH(1-24)[4]] in solution or on solid support as illustrated in Scheme I.

Scheme I.

Oxytocin(in solution)

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

LH-RH (in solid phase)

Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

ACTH(1-24) (in solid phase)

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-

Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-NH_2

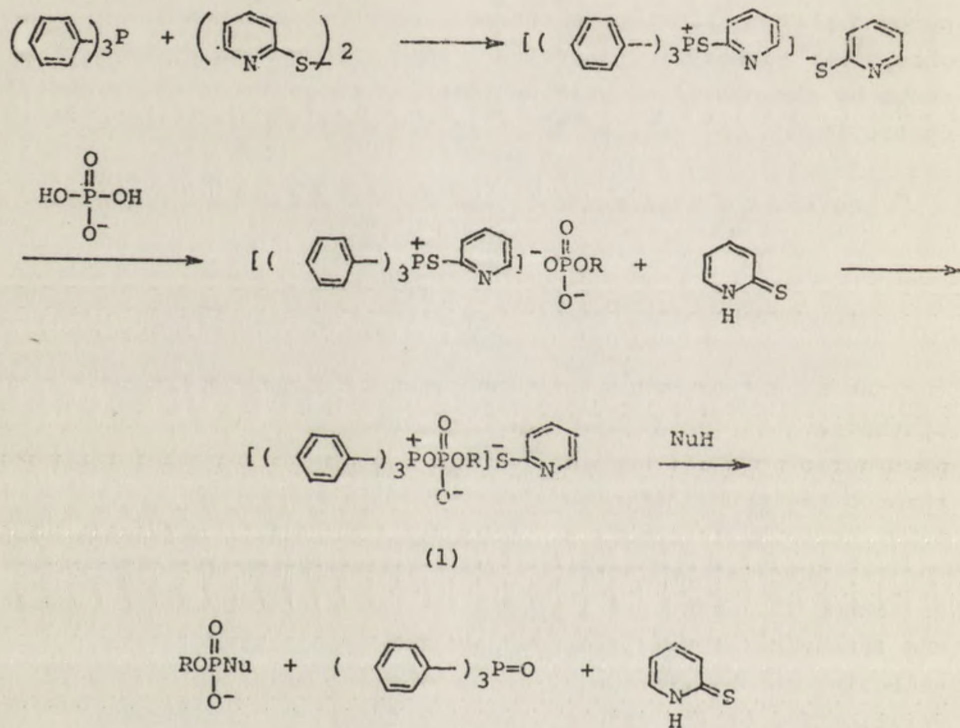
We have further examined the application of this condensation reaction to the intermolecular dehydrations between various phosphates and nucleophilic components as phosphates, alcohols, amines and carboxylic acids, with the formations of pyrophosphates, phosphoric esters, phosphoroamidates and phosphoric carboxylic anhydrides, which are generally brought about by the use of organic dehydrating reagents as dicyclohexylcarbodiimide[5], 2,4,6,-triisopropylbenzenesulfonyl chloride[6] or mesitylenesulfonyl chloride[7].

The results will be described in the following chapters.

II. PHOSPHORYLATION BY OXIDATION-REDUCTION CONDENSATION

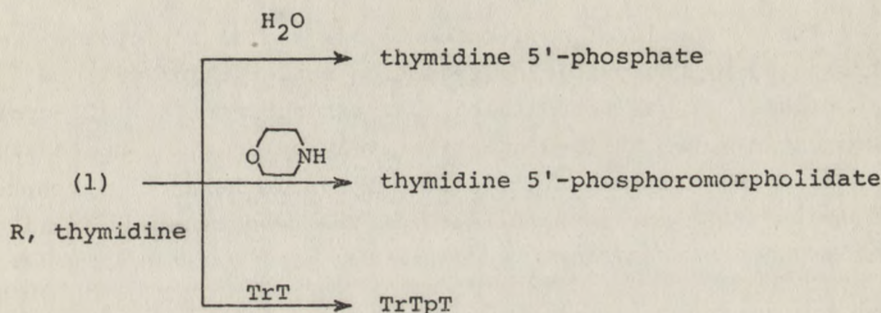
As suggested by the previous studies on the peptide synthesis, the dehydration reaction between monoesters of phosphoric acid and nucleophilic components is expected to proceed through the paths, if occurs, as shown in Scheme II. As shown in other successful methods of phophorylation, it is necessary that the key intermediate, the phosphoroxyphosphonium salt (1) shown in Scheme II, exists as a comparatively so stable one to exclude the formation of sym-pyrophosphate for application to the selective phophorylation of alcohol, amine or phosphoric acid. Verification of its stable existence under appropriate condition has been obtained as follows; when 3'-O-acetylthymidine 5'-phosphate was allowed to react with excess Ph_3P-$(PyS)_2$ in a small amount of pyridine, (1) was formed quite rapidly and could be kept as a stable salt in a highly concentrated solution as indicated by the following findings, though it was not isolated. First, symmetrical pyrophosphate was no longer produced even though the solution of (1) was further diluted with pyridine. Second, thymidine 5'-phosphate, a starting material, was recovered quantitatively by hydrolysis of the intermidiate (1) at room temperature.

Scheme II.



Third, thymidine 5'-phosphoromorpholidate or 5'-O-trityl-thymidylyl-(3'⟶5')-3'-O-acetylthymidine (TrTpTOAc) was produced by the addition of morpholine or 5'-O-tritylthymidine (TrT), respectively, to the solution of (1) (Scheme III).

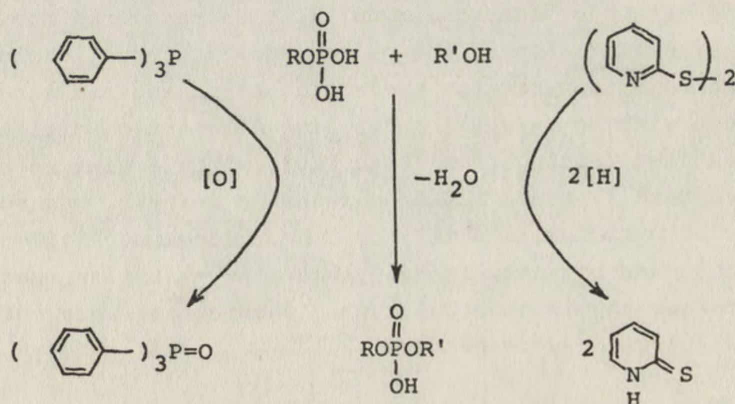
Scheme III



Examination about its stability by tlc and paper electrophoresis showed no detectable decomposition after 1 day at room temperature. Further, paper chromatography showed that 90% of thymidine 5'-phosphate was obtained along with 10% of symmetrical pyrophosphate after the solution of (1) had stood for 60 hr at room temperature, followed by mild alkaline hydrolysis.

Thus, the dehydration between monoesters of phosphoric acid and nucleophilic components is also shown to proceed by eliminating one oxygen atom with triphenylphosphine (reduction) and two hydrogen atoms with 2,2'-dipyridyl disulfide (oxidation) to afford mixed esters of phosphoric acid, triphenylphosphine oxide, and 2 mol of pyrid-2-thione as shown in Scheme IV.

Scheme IV



III PREPARATION OF (2',3'- AND 3',5'-) CYCLIC NUCLEOTIDES

The synthesis of nucleoside 2',3'- and 3',5'- cyclic phosphates by the oxidation-reduction condensation will be described. It was established that ribonucleoside 2',3'-cyclic phosphates were obtained quantitatively by treating nucleoside 2'-(or 3'-) phosphates with Ph_3P—$(PyS)_2$ in hexamethylphosphorotriamide (HMPA) or methanol-water. The results are summarized in Table I.

Table I

SYNTHESIS OF (2',3'- and 3',5'-) CYCLIC NUCLEOTIDES

Nucleotide	Yield (%)	Nucleotide	Yield (%)
2',3'-cyclic AMP	quant.	3',5'-cyclic AMP	85
2',3'-cyclic CMP	quant.	3',5'-cyclic CMP	56
2',3'-cyclic GMP	quant.	3',5'-cyclic GMP	85
2',3'-cyclic UMP	quant.	3',5'-cyclic UMP	80
4',5'-cyclic FMN*	quant.	3',5'-cyclic TMP	70

* FMN refers to Riboflavine 5'-phosphate.

In the case of nucleoside 3',5'-cyclic phosphates, undesirable pyrophosphates were formed when the reactions were carried out under the same conditions as mentioned above. However, cyclization to the nucleoside 3',5'-cyclic phosphates was the major course when nucleoside 5'-phosphates were allowed to react with Ph_3P-$(PyS)_2$ under high dilution conditions at temperatures ranging from 70 to 120°. When a mixture of adenosine 5'-phosphate (1 mmol) and 5 equiv of Ph_3P-$(PyS)_2$ was refluxed in anhydrous pyridine (150 ml) for 3 hr, adenosine 3',5'-cyclic phosphate was obtained in 86% yield. In a similar manner, uridine and thymidine 3',5'-cyclic phosphates were obtained in 80 and 70% yields, respectively.

Since the solubility of the N,N'-dicyclohexyl-4-morpholinocarboxamidinium salts of cytidine and guanosine 5'-phosphates was increased by the addition of triphenylphosphine, cytidine 3',5'-cyclic phosphate was successfully obtained in 56% yield from cytidine 5'-phosphate without the protection of the amino group of cytidine. Guanosine 3',5'-cyclic phosphate was also obtained in 85% yield by the use of lyophillized powder of the N,N'-dicyclohexyl-4-morpholinocarboxamidinium salt of guanosine 5'-phosphate. Nearly the same result was obtained when N^2-benzoylguanosine 5'-phosphate, easily soluble in pyridine, was used in place of the salt of guanosine 5'-phosphate in the above experiment.

IV SYNTHESIS OF OLIGONUCLEOTIDES[8)]

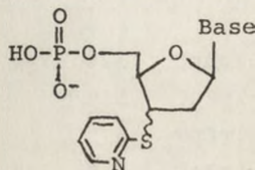
Next, it was established that $C_{3'-5'}$-linked deoxyribooligonucleotides were successfully synthesized by the present method. The reaction of 5'-O-tritylthymidine (0.6 mmol) with 3'-O-acetylthymidine 5'-phosphate (0.5 mmol) and 5 equiv of $Ph_3P-(PyS)_2$ in 5 ml of anhydrous pyridine at room temperature for 8 hr afforded thymidylyl- (3'→ 5')-thymidine in 90% yield after removal of the protecting groups in the usual manner. Thymidylyl-(3'→ 5')-thymidylyl-(3'→ 5')-thymidine was obtained in 65% yield when 5'-O-tritylthymidylyl-(3'→ 5')-thymidine and 2 equiv of 3'-O-acetylthymidine 5'-phosphate were allowed to react with 10 equiv of $Ph_3P-(PyS)_2$ under the same condition. Similarly various trinucleoside diphosphates can be prepared in high yields as shown in Table II.

Table II Synthesis of Trinucleoside Diphosphates

d-TpTpT	65%	d-TpApC	50%	d-ApApA	60%
d-TpTpA	55%	d-TpCpC	53%	d-ApApC	55%
d-TpTpC	58%	d-TpCpT	63%	d-CpApT	55%
d-TpApT	60%	d-ApTpT	61%	d-TpGpT	63%
d-TpApA	58%	d-ApTpA	55%	d-TpGpG	50%

Further, TpTpTpT was obtained in 50% yield from TrTpTpT and 3 equiv of 3'-O-acetylthymidine 5'-phosphate. The oligothymidylate, on degradation with spleen phosphodiesterase, gave thymidine 3'-phosphate and thymidine in the expected ratio. A dinucleotide, 5'-O-phosphorylthymidylyl-(3'→5')-thymidine, was also obtained in 65% yield when a mixture of β-cyanoethyl thymidine 5'-phosphate and 3'-O-acetylthymidine 5'-phosphate was treated with 8 equiv of Ph_3P-$(PyS)_2$ at room temparature for 5 hr. On the other hand, in the case of β-cyanoethyl thymidine 5'-phosphate synthesized by the reaction with excess of β-cyanoethanol and thymidine 5'-phosphate, it was found that sym-pyrophosphate (P^1,P^2-dithymidine 5'-pyrophosphate) was always produced as the by-product.
For example, when thymidine 5'-phosphate (1 mmol) and β-cyanoethanol (30 mmol) were treated with 5 equiv of Ph_3P-$(PyS)_2$ in 2 ml of pyridine at room temperature, β-cyanoethyl thymidine 5'-phosphate was obtained in 68% yield along with 32% yield of the sym-pyrophosphate (the ratio of β-cyanoethanol/pyridine=1 : 1 v/v). But, the yield of the sym-pyrophosphate was reduced to 20% when 15 equiv of β-cyanoethanol and 3 ml of pyridine were used in the above experiment (β-cyanoethanol/pyridine=1 : 3 v/v). The result shows that the dilution of the reaction mixture with an excess amount of β-cyanoethanol causes the velocity of the formation of phosphoroxyphosphonium salt (1) to slow down, which led to the production of the undesirable sym-pyrophosphate.

Concerning a side reaction in this oxidation-reduction condensation applied to nucleotide synthesis it was recently found that a small amount of S-pyridylthio nucleotide is formed during the internucleotide bond formation.



V SYNTHESIS OF OLIGONUCLEOTIDES BY THE USE OF PHOSHORODIANILIDATES[9)]

It is well known that protected dinucleotides having 5'-phosphomonoester are important intermediates in the synt. is of deoxyribooligonucleotides of defined sequences. The preparation of such dinucleotides is usually performed by the coupling of 3'-protected nucleotides with 5'-protected nucleotides, followed by the selective removal of the 5'-phosphate protecting group. The β-cyanoethyl group has been used most commonly for protection of 5'-phosphate of mononucleotides and, in addition, several othe: useful protecting groups have been proposed recently for this purpose.

In connection with this problem , a convenient and general method for the synthesis of such dinucleotides is established by the use of nucleoside 5'-phosphorodianilidates (2) on the basis of the following facts; first, phosphorodianilidates (2) are found to be prepared easily by treating nucleoside 5'-phosphate and aniline with $Ph_3P-(PyS)_2$. Second , the dianilidates (2) thus prepared are more stable in comparison with the corresponding phosphoromonoanilidates (3) under basic conditions, which makes it possible to remove other protecting groups selectively. Concerning the removal of the anilino groups of 5'-phosphate, it was reported by Ikehara et al.[10)] that amidates derived from the aromatic primary amines can be readily cleaved to give the parent 5'-phosphate group by treatment with isoamyl nitrite under mild conditions.

Phosphorodianilidates (2) were prepared as follows; for example, when N^6-benzoyldeoxyadenosine 5'-phosphate (1 mmol) was treated with aniline (10 mmol) in the presence of $Ph_3P-(PyS)_2$ (10 mmol) in anhydrous pyridine (10 ml) at room temperature for 2 days, the dianilidate was formed and was purified with silica gel tlc developed by tetrahydrofuran.

Thymidine, deoxycytidine and deoxyguanosine phosphorodianilidates (2) can also be prepared in 60-65% yields along with the corresponding phosphoroanilidates (3) in 35-40% yields. The dianilidates (2) can easily be separated from the corresponding monoanilidates (3) by tlc as described above. These results are shown in Table III.

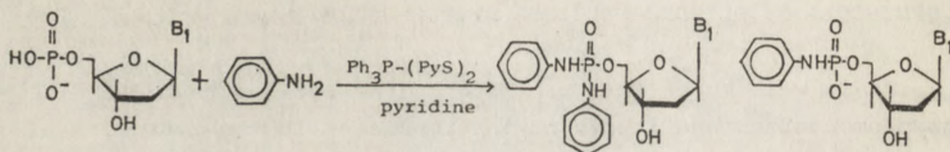


Table III Synthesis of Nucleotide 5'-Phosphorodiamidate[a)]

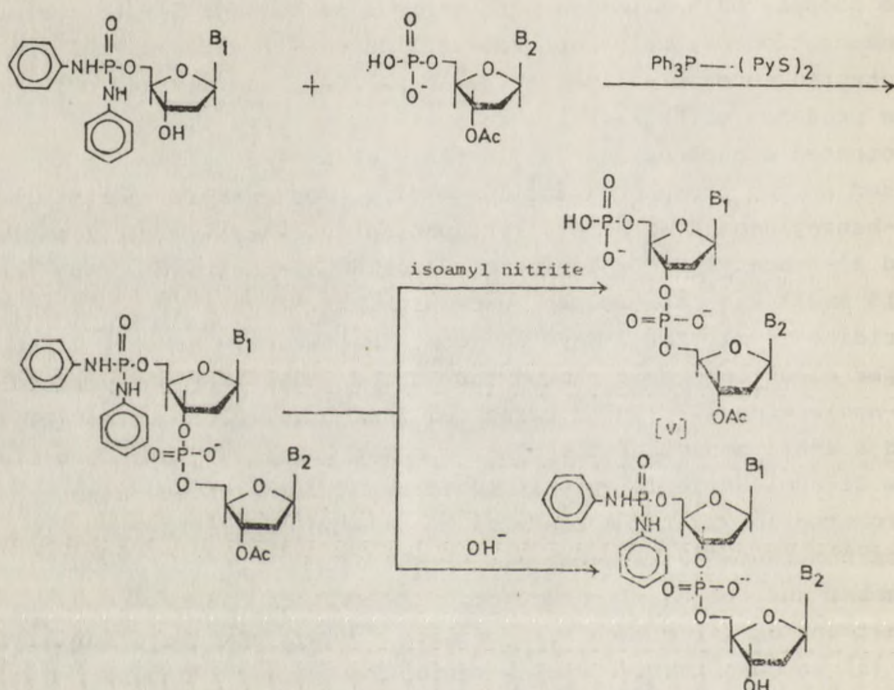
B_1	Amine	Reaction time (day)	Yields (%) Phosphoro-monoamidate (3)	Phosphoro-diamidate (2)
thymine	aniline	2	35	65
thymine	morpholine	5 (hr)	82.5	17.5
		1	57	43
		3	17	64[b)]
N^6-benzoyl adenine	aniline	2	35	65
N^4-anisoyl cytosine	aniline	2	41	59
N^2-benzoyl guanine	aniline	2	40	60

a) Ten equiv each of Ph_3P-$(PyS)_2$ and 10 equiv of amine were used on parent nucleoside 5'-phosphate.

b) TppT was also produced in 17% yield.

Then, the preparation of dinucleotides was tried by treating the phosphorodianilidates with various nucleoside 5'-phosphates. Condensation of the phosphorodianilidates (2) with suitable protected mononucleotides (e.q., d-pA^{Bz}OAc) was carried out in the presence of Ph_3P-$(PyS)_2$ in pyridine to give the protected dinucleotides (4) in the yields ranging from 50 to 75% based on the phosphorodianilidate (2). For example, a mixture of N^6-benzoyldeoxyadenosine 5'-phosphorodianilidate (2) (0.1 mmol) and 3'-O-acetyl N^6-benzoyldeoxyadenosine 5'-phosphate (d-pA^{Bz}OAc, 0.15 mmol) was allowed to react with Ph_3P-$(PyS)_2$ (1 mmol) in pyridine (1 ml) for 3 days at room temperature. At this stage, paper electrophoresis showed four spots; that is , the desired di-nucleotide (4) (70%), unreacted d-pA^{Bz}OAc (0.06 mmol), (2)(20%) and a small amount of the other product which was not identified. The di-nucleotide (4) was isolated by DEAE-cellulose column chromatography using a liner salt gradient of triethylammonium bicarbonate in 50% ethanol (1ℓ of 50% ethanol in the mixing chamber and the equal volume of 0.1 M salt in the reservoir). Treatment of (4) with alkali or with isoamyl nitrite afforded (6) or (5) in quantitative yield, respectively, according to the following Scheme.

Scheme V



Similary various other dinucleotides d-pA^{Bz}pT, d-pA^{Bz}pC^{An}, d-pTpT, d-pTpA^{Bz}, d-pTpC^{An} were also prepared in good yields (Table IV).

Table IV Synthesis of Protected Dinucleotides Bearing 5'-Phosphate Groups.

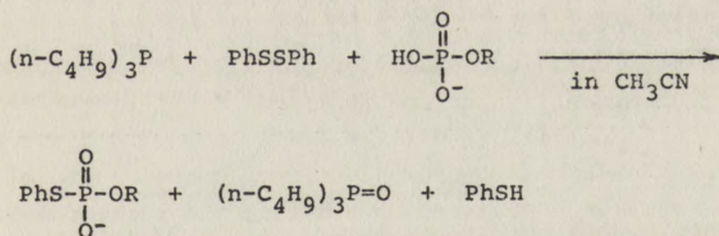
Dinucleotide	3'-OH$^{b)}$ containing component	5'-Phosphate containing component	Amount (mmol)	Ph_3-P(PyS)$_2$ (mmol)	Yield (%)
d-pTpTOAc	d-pT	d-pTOAc	0.1	0.5	70
d-pTpC^{An}OAc	d-pT	d-pC^{An}OAc	0.07	0.5	50
d-pTpA^{Bz}OAc	d-pT	d-pA^{Bz}OAc	0.07	0.5	51
d-pA^{Bz}pTOAc	d-pA^{Bz}	d-pTOAc	0.1	1	75
d-pA^{Bz}pA^{Bz}OAc	d-pA^{Bz}	d-pA^{Bz}OAc	0.1	1	70
d-pA^{Bz}pC^{An}OAc	d-pA^{Bz}	d-pC^{An}OAc	0.1	1	70

a) Yields were determined spectrophotometrically in water.

b) In each case, 0.05 mmol amount of component was used.

VI PREPARATION OF S-PHENYL NUCLEOSIDE PHOSPHOROTHIOATES[11)]

A convenient method for the synthesis of S-phenyl nucleoside phosphorothioates by the reaction of nucleotides with diphenyl disulfide in the presence of tri-n-butylphosphine will be described in this section. Ethylthio group reported by Nussbaum in 1965 has been proved to be particularly valuable for the protection of phosphate esters in oligonucleotide synthesis. This type of nucleotides can be prepared by the reaction of protected nucleosides with S-ethyl phosphorothioate by the use of dicyclohexylcarbodiimide as a condensing agent, but this method appears to be a lack of generality because of the instability of S-ethyl phosphorothioate under these reaction conditions. It is established that S-phenyl nucleoside phosphorothioates are easily prepared from the corresponding nucleotides by the use of diphenyl disulfide and tri-n-butylphosphine. An important feature of the present method is that it doesn't involve the use of S-alkyl phosphorothioate and protected nucleosides, which are sometimes not easily available.



R=nucleoside residue

Thus, when a mixture of one equiv. of pyridinium salt of 3'-O-acetylthymidine 5'-phosphate and 10 equiv of diphenyl disulfide in dry acetonitrile was treated with 10 equiv of tri-n-butylphosphine at room temperature for 30 min, S-phenyl 3'-O-acetylthymidine 5'-phosphorothioate [$\lambda_{max}^{H_2O}$ 267 (ε=10,100), 243 nm; $\lambda_{min}^{H_2O}$ 250, 236 nm] was obtained in 96% yield. When thymidine 5'-phosphate was employed in place of 3'-O-acetylthymidine 5'-phosphate, S-phenyl thymidine 5'-phosphorothioate was obtained in 91% yield. In this case, little coupling reaction between phosphate and 3'-hydroxyl group on sugar moiety was observed.

In a similar manner, S-phenyl N^6-benzoyladenosine 5'-phosphorothioate [$\lambda_{max}^{H_2O}$ 283 (ε=18,500), 238 nm; $\lambda_{min}^{H_2O}$ 262, 230 nm], and S-phenyl N^4-anisoylcytidine 5'-phosphorothioate [$\lambda_{max}^{H_2O}$ 303 (ε=22,450), 244 nm; $\lambda_{min}^{H_2O}$ 254, 237 nm] were obtained in 80% and 71% yields, respectively.

In the above reactions, it is noted that tri-n-butylphosphine is remarkably effective for the synthesis of S-phenyl nucleoside phosphorothioates. When triphenylphosphine was used in place of tri-n-butylphosphine, the symmetrical dinucleoside pyrophosphates were formed as main products.

VII NEW METHOD FOR THE SYNTHESIS OF DI- AND TRI-NUCLEOTIDES[12].

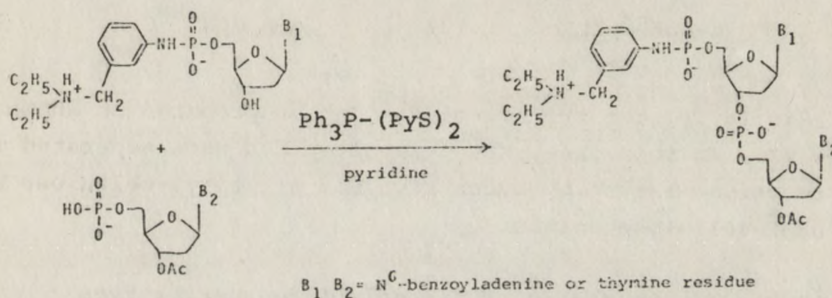
An attempt to prepare oligonucleotides by the use of a protecting group which acts as a purification handle for separation has been tried.

We have found that it is much easier to purify the oligonucleotides produced when the nucleotides having 3-(N,N-diethylaminomethyl)anilino group for protection of phosphoric site have been employed.

In this section, a convenient preparative method of such nucleoside phosphroanilidates and their use for the synthesis of the di- and tri- nucleotides will be described. The 5'-phosphoroanilidates of N^6-benzoyl 2'-deoxyadenosine (d-NpA^{Bz}) and of thymidine (d-NpT) were prepared by treating the corresponding nucleoside 5'-phosphate with 3-(N,N-diethylaminomethyl)aniline in the presence of Ph_3P-$(PyS)_2$ in 80% and 86%, respectively. Because of the character to form the inner salts, they are neutral and stable substances. Therefore, isolation and purification from the reaction mixture becomes very easy by washing with water followed by trityl-cellulose and DEAE-cellulose column chromatography.

When a solution of d-NpA^{Bz} (0.1 mmol) and 3'-O-acetyl thymidine 5'-phosphate (pTOAc) (0.15 mmol) in dry pyridine (0.8 ml) was treated with Ph_3P (0.5 mmol) and $(PyS)_2$ (0.5 mmol) at room temperature for two days, the corresponding dinucleotide derivative, d-NpA^{Bz}pTOAc, was formed in 73% yield detected spectrophotometrically along with

P^1,P^2-di-3'-O-acetylthymidine 5'-pyrophosphate, pyrid-2-thione and triphenylphosphine oxide.



Purification of the dinucleotide was carried out as follow; the reaction mixture was dissolved in pyridine (2 ml) and was added dropwise to dry ether under vigorous stirring. The precipitate was collected by centrifuge and washed three times with dry ether. It was dissolved in water and chromatographed on DEAE cellulose. First d-NpA^{Bz} was washed out with water and then a linear gradient of triethylammonium bicarbonate solution from 0 to 0.05 M was used as an eluent. The desired dinucleotide, d-NpA^{Bz}pTOAc, was the first to be eluted at about 0.01 M. After the eluate was treated with isoamyl nitrite and then with methanolic ammonia for removal of the protecting groups, thymidylyl (5'→ 3') deoxyadenosine 5'-phosphate (d-pApT) was isolated in 70% yield based on d-NpA^{Bz}.

In a similar manner, the dinucleotide derivatives were obtained in good yields. The results are summarized in Table V.

According to this method, it takes only five days for their preparation because the dinucleotide derivatives are eluted first on column chromatography.

Table V Synthesis of Dinucleotides Derivatives

Compound	Yield	Compound	Yield
d-NpTpTOAc	65%	d-NpA^{Bz}pA^{Bz}OAc	67%
d-NpTpA^{Bz}OAc	62%	d-NpA^{Bz}pUOAc	59%
d-NpA^{Bz}pTOAc	70%	d-NpUpG^{iBu}OiBu	70%

Similarly, the trinucleotides can be prepared as shown in Table VI. In this case, the trinucleotides were separated from the reaction mixture by successive use of trityl-cellulose column and DEAE-cellulose column.

Table VI Synthesis of Trinucleotide Derivatives

Compound	Yield	Compound	Yield
d-NpA^{Bz}pTpTOAc	68%	d-NpA^{Bz}pTpG^{iBu}OiBu	21%
d-NpTpTpTOAc	40%	d-NpTpTpC^{An}OAc	46%
d-NpA^{Bz}pTpC^{An}OAc	61%	d-NpTpC^{An}pA^{Bz}OAc	51%

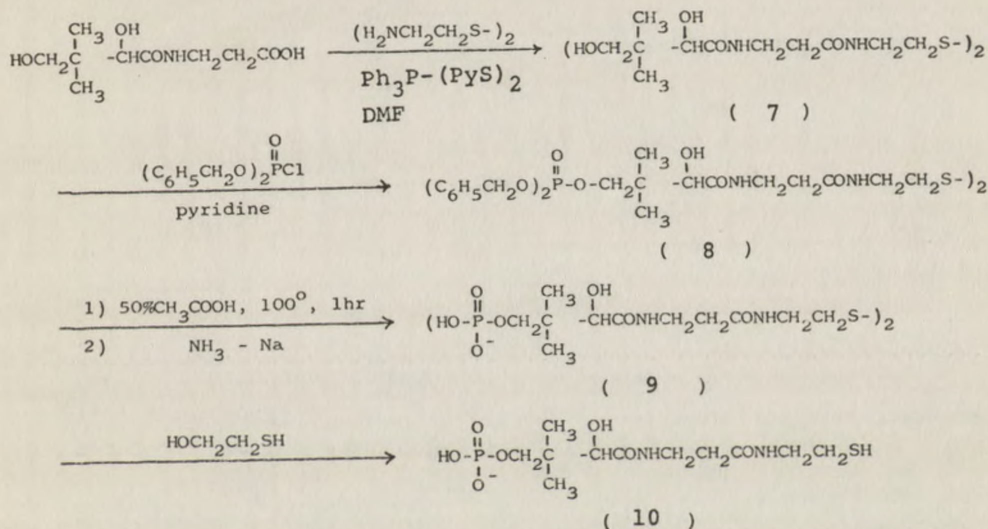
VIII TOTAL SYNTHESIS OF COENZYME A[13)]

In this section, the total synthesis of coenzyme A (16) by utilization of oxidation-reduction condensation in each important step will be described in detail.

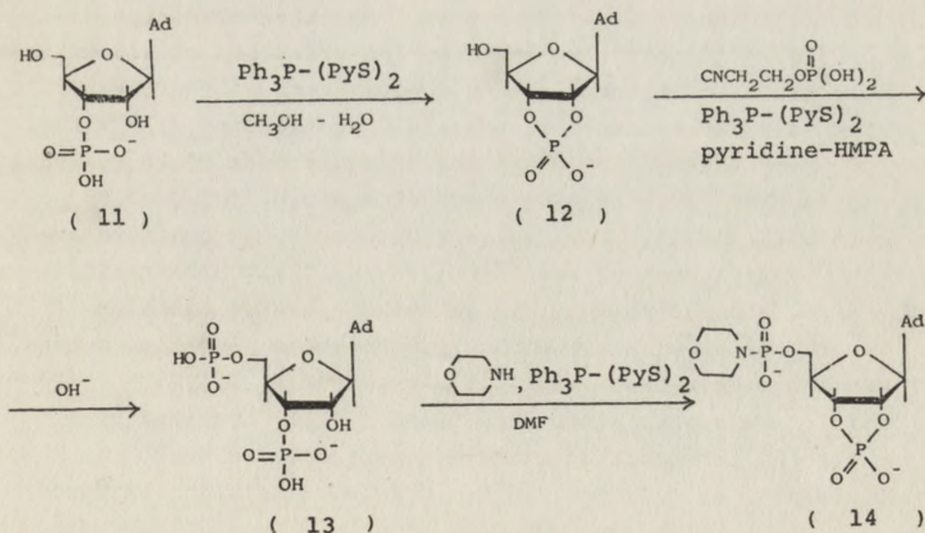
Four independent papers in the total synthesis of coemzyme A (16) have been published, each of which employed in principle the condensation of D-pantetheine derivatives with adenine nucleotides, differing in the procedures of the formations of pyrophosphate and cysteamine bonds. The present method is characterized in building up coemzyme A structure using the oxidation-reduction condensation in each important step as shown in Scheme VI.

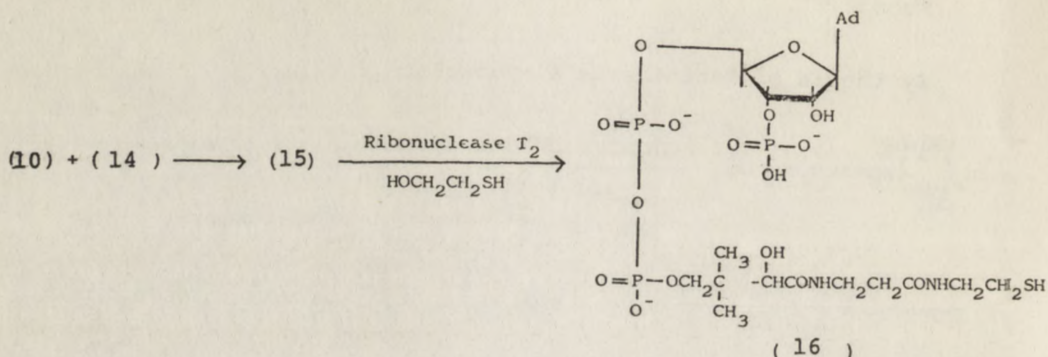
Scheme VI.

Synthesis of Pantetheine 4'-Phosphate.



Synthesis of Adenosine 2',3'-Cyclic Phosphate 5'-Phosphoromorpholidate





Two components, adenosine 2',3'-cyclic phosphate 5'-phosphoromorpholidate (14) and D-pantethine 4'-phosphate (16), were prepared by the following procedure; the (9) was prepared and isolated in 60% yield as its barium salt by treating D-pantethine (7) with dibenzyl phosphorochloridate in anhydrous pyridine according to the ordinary method. Trearment of D-pantothenic acid with 0.7 equiv of cysteamine and one equiv each of $Ph_3P-(PyS)_2$ in DMF gave a colorless glass substance (7) in 80% yield. The (10) was prepared by reduction of pantethine 4'-phosphate (7) with 2-mercaptoethanol. The other component, (14), was prepared in quantitative yield by the treatment of (13) with an excess amount of morpholine and 5 equiv each of $Ph_3P-(PyS)_2$ in DMF. The (13) was obtained in 60% yield by treating (12) with 2 equiv of β-cyanoethyl phosphate and 10 equiv each of $Ph_3P-(PyS)_2$ in pyridine-HMPA for 2 days at room temperature, followed by treatment with alkali. The (12) was obtained in a quantitative yield by the treatment of adenosine 2'-(or 3'-)phosphate (11) with 3 equiv each of $Ph_3P-(PyS)_2$ in methanol-water solution.

In accordance with Moffatt's pyrophosphate formation method,[14] (14) was allowed to react with (10) in anhydrous pyridine at room temperature overnight. The crude product (15), obtained by evaporation of the reaction mixture, was incubated with ribonuclease T_2 at 37°, for 4.5 hr in aqueous solution adjusted

to pH 4.6 to give crude coenzyme A (16). After the reaction mixture was adjusted to pH 6.0 with ammonium hydroxide and treated with 2-mercaptoethanol, purification was effected by chromatography on DEAE-cellulose column using a liner salt gradient to yield analytically pure coenzyme A (16). (adenosine phosphorus=1 : 2.96). Coenzyme A thus obtained was chromatographically and electrophoretically identical with the commercial sample. The total yield was 60% based on (14). The phosphotransacetylase assay showed its purity to be 98%. Satisfactory analytical data were obtained for all the compounds described above, which showed only one spot on paper chromatogram.

It is noted that the oxidation-reduction condensation is useful for the preparation of two important intermediates of coenzyme A, namely adenosine 2',3'-cyclic phosphate 5'-phosphoromorpholidate and pantethine, especially in the preparation of D-pantethine directly from D-pantothenoic acid and cysteamine in high yield.

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PHOSPHORYLATING REAGENTS FOR OLIGONUCLEOTIDE SYNTHESES

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Abstract. A general approach to the synthesis of oligonucleotides is proposed. The development of phosphorylating reagents to implement this approach is discussed. The reagents are applied to the synthesis of oligonucleotide models to arrive at optimum conditions to be used with the actual nucleosides.

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1. Introduction

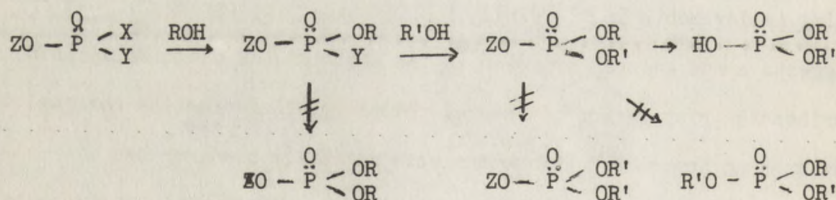
The contributions of several groups of investigators[1-8] have resulted in outstanding achievements in the field of oligonucleotide synthesis. This paper suggests a new general approach to the synthesis of oligonucleotides, and describes the preparation of several phosphorylating reagents designed to implement such an approach. The mechanistic rationale used in the development of the reagents is the oxyphosphorane concept[9-10]. This concept assumes the formation of intermediates with 5-coordinate phosphorus having one or more P-O bonds (oxyphosphoranes) in the nucleophilic displacements of compounds with 4-coordinate phosphorus. The concept gives rules for the formation, the decomposition, and the stereochemistry of the oxyphosphoranes with trigonal bipyramidal skeleton. The development of these rules has been discussed in detail[10-15].

The new phosphorylating reagents have been applied to the synthesis of unsymmetrical phosphodiesters, in general[16-21], and of oligonucleotide models, in particular. It is hoped that these studies will lead to the selection of the most practical reagents, and of the optimum experimental conditions, to be applied to the naturally occurring nucleosides in subsequent researches.

2. Hypothetical Scheme for Oligonucleotide Synthesis by Means of CEP-X[22] and CAP-X[22] Reagents

To reduce to a minimum the use of protective groups for the hydroxyl groups of the nucleosides, a reagent for oligonucleotide synthesis should have the ability to phosphorylate selectively a primary alcohol in the presence of a secondary alcohol.

To simplify the synthesis further, the reagent should possess the degree of activation that is necessary in order to carry out the successive phosphorylations of two alcohols without the need to introduce any additional activating reagent in the course of the synthesis. In spite of this degree of activation, the reagent should not generate symmetrical phosphates as by-product:



The phosphorylating reagent must have, or it must develop during its application, a phosphate-blocking group, Z, which can eventually be removed, preferably in slightly alkaline aqueous solution, without any effect on the desired phosphodiester. Yet, Z, must be securely placed, otherwise it might be removed by transesterification with the alcohols.

The phosphorylating reagent should be able to perform two types of functions:

(1) Establish the oligonucleotide bond between the C3' and the C5' positions of a nucleoside pair.

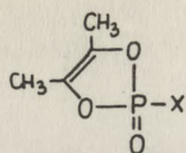
(2) Act as a protective-group for the C5' alcohol function of the first nucleoside of the oligonucleotide chain. In this manner, C5'-protection is combined with C5'-phosphorylation of one of the terminal nucleosides.

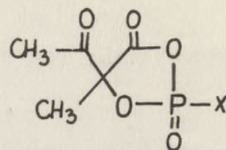
Finally, the reagent must be quite reactive, if it is going to operate on oligonucleotides having more than three or four nucleoside units, and it must be easy to prepare and convenient to store and to dispense.

Certain derivatives of the 5-membered cyclic enediol phosphoryl group, or CEP-X reagents[22], and of the 5-membered cyclic acyl phosphoryl group, or CAP-X reagents[22], meet all of these requirements, at least when applied to the synthesis of oligonucleotide models; Scheme 1.

Scheme 1

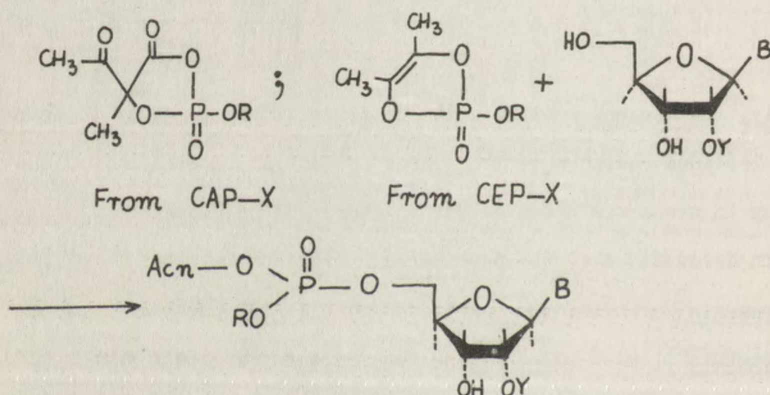
Scheme 2 is a hypothetical illustration of the protection-*cum*-phosphorylation of the C5'-OH group of the first nucleoside of an oligonucleotide chain. This illustration is based on the performance of some of these reagents in model compounds as described in Section 4. The CEP-X or CAP-X is first converted into the protective CEP-OR or CAP-OR reagent. The latter phosphorylates selectively the primary C5'-OH of a 2'-protected ribonucleoside in the presence of the unprotected C3'-OH, to give an alkylacetoinyl phosphotriester (*cf.* Section 3). At some later stage, the acetoinyl group (Acn) will be removed from the phosphotriester. The second blocking-group, R, is removed at the same time as the Acn-group, or in a subsequent step. One attractive variation would be to select an R-group capable of undergoing cleavage at the alkyl-oxygen bond, R—O-, by a



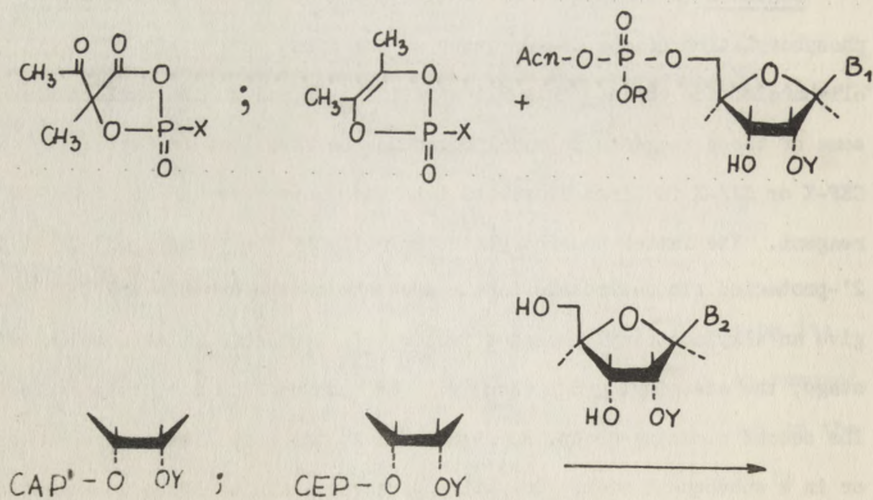


CEP—X CAP—X

Scheme 1



Scheme 2



Scheme 3

suitable de-blocking reagent, and to incorporate the same group, R, as its carbonate ester $-CO_2R$, in the protective-group, Y, at C2'-OH. In this manner de-blocking and de-protecting techniques operate simultaneously.

Scheme 2

The CEP-X or CAP-X is now used to CEP-ylate[22] or CAP-ylate[22] the secondary C3'-OH of a 2',5'-diprotected nucleoside; Scheme 3. The new CEP-OR or CAP-OR phosphorylates selectively the primary C5'-OH of another 2'-protected nucleoside in the presence of the unprotected secondary C3'-OH.

Scheme 3

The result of the last reaction is the dinucleotide shown in Scheme 4. This dinucleotide has only a free C3'-OH, which can again be CEP-ylated or CAP-ylated in order to continue the synthetic sequence by repetition of the reactions in Scheme 3 using a third C2'-protected nucleoside. Removal of the Acn- and R-blocking-groups, and of the Y-protective group, completes the oligonucleotide synthesis.

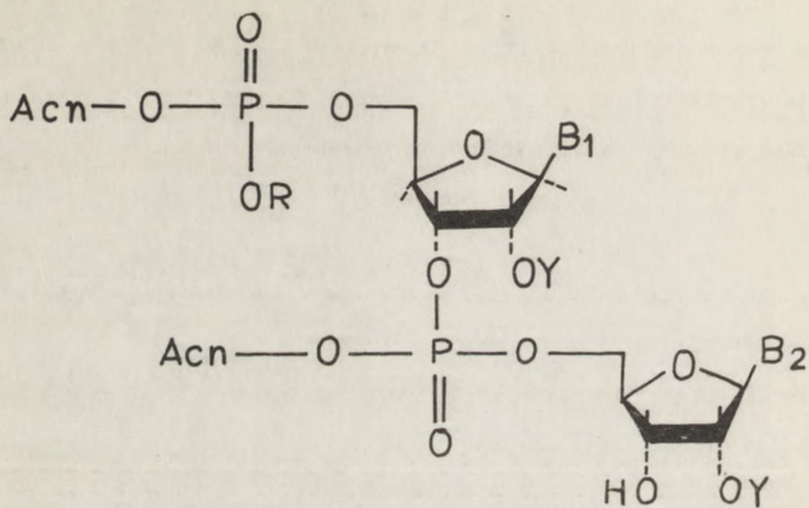
Scheme 4

3. Mechanistic Rationale For the Synthesis of Unsymmetrical Phosphodiesters By CEP-X and CAP-X Reagents

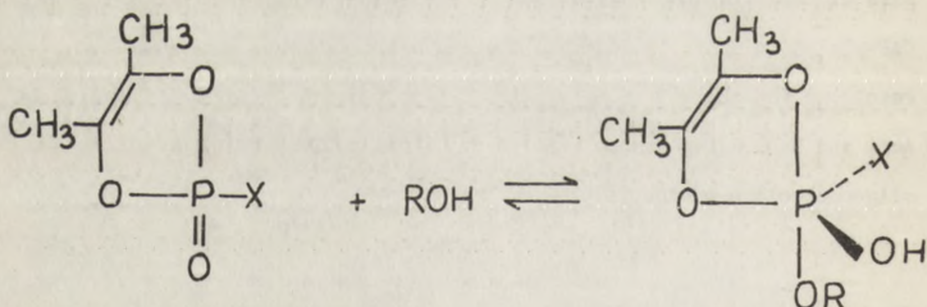
According to the oxyphosphorane concept, alcohols add to the 4-coordinate phosphorus of the CEP-X and CAP-X reagents to form the corresponding trigonal bipyramidal oxyphosphoranes shown in Scheme 5.

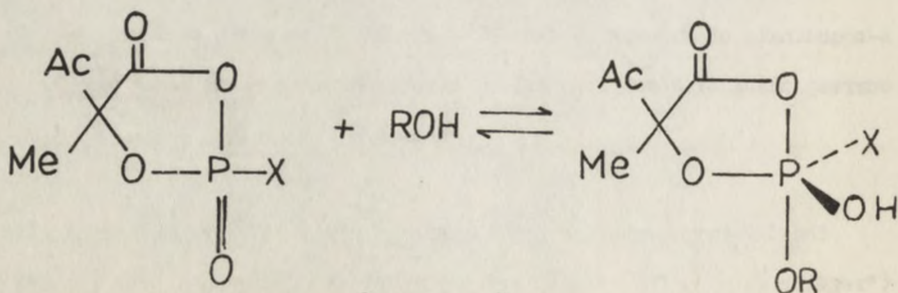
Scheme 5

The CEP-Oxyphosphorane should undergo rapid PI[22] by bond-deformations ("regular PI")[10], followed by apical departure of ligand-X from the new oxyphosphorane, to give CEP-OR, as shown in Scheme 6. This displacement



Scheme 4





Scheme 5

with ring-retention (CEP-ylation)[22] is favored by the high apicophilicity[10] and the good leaving-group properties of the strongly electron-withdrawing X-ligand. These features favor ring-retention vs ring-opening in the displacement. The regular PI is thought to take place by the single TR[22,23-25] mechanism with the ring functioning as the obligatory "ligand-pair".

Scheme 6

The analogous reaction of an alcohol with CAP-X is shown in Scheme 7. Now, the regular PI can occur by the double $(TR)^2$ mechanism, which leaves the apicophilic[10] acyloxy-ligand in an apical skeletal position, and moves the apicophilic X-ligand to the apex. However, the available experimental data (vide infra) show that the CAP-oxyphosphoranes have a higher tendency to undergo PI by bond ruptures and recombinations ("irregular PI")[10], than the CEP-oxyphosphoranes. The irregular PI has the same consequence as the regular PI by $(TR)^2$. In essence, the irregular PI is a manifestation of carboxyl-participation in reactions of phosphate esters of α-hydroxy-carboxylic acids. The net result is that displacement with ring-retention, rather than with ring-opening, is the general rule with the CAP-X reagents, as well as with the CEP-X reagents.

Scheme 7

The alcohol R'OH adds to the 4-coordinate phosphorus of CEP-OR and CAP-OR to give the corresponding oxyphosphoranes shown in Scheme 8, according to the oxyphosphorane concept.

Scheme 8

The CEP-Oxyphosphorane can collapse to the acyclic enol-phosphate (ring-opening), or it can undergo regular PI by the TR mechanism; Scheme 9.

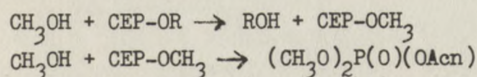
Scheme 6

Scheme 7

Scheme 8

The new oxyphosphorane can, again, undergo ring-opening, or it can eject the apical RO-ligand to give a new CEP-OR' (transesterification, or displacement with ring-retention). The acyclic enol-phosphate can tautomerize to the unsymmetrical dialkylacetoinyl phosphate, or it can engage in enol-participation, i.e., become part of the irregular PI.

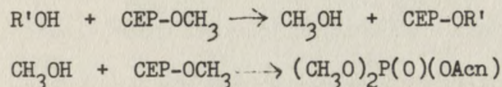
The main difference in the behavior of the CEP-OR oxyphosphorane pictured in Scheme 9, and that of the CEP-X oxyphosphorane pictured in Scheme 6, stems from the circumstance that the apicophilicities of both ligands, RO- and R'O- (in the first case) are approximately the same, while ligand X (in the second case) is more apicophilic than ligand RO-. Hence, the tendency for displacement with ring-retention is lower in the case of CEP-OR than in the case of CEP-X; or conversely, there should be more ring-opening in the reaction: R'OH + CEP-OR, than in the reaction: ROH + CEP-X. It will be seen in Section 4 that the reaction of relatively bulky alcohols, R'OH, with CEP-OR having relatively bulky R-groups gives exclusively the product of ring-opening, namely the unsymmetrical dialkylacetoinyl phosphate[26]. The reactions of relatively small alcohols, e.g., methanol, with CEP-OR tend to give some ring-retention, and hence some transesterification and formation of some symmetrical dialkylacetoinyl phosphate[26]:



CH_3OH + CEP-OR → ROH + CEP-OCH_3

CH_3OH + CEP-OCH_3 → $(CH_3O)_2P(O)(OAcn)$

Likewise, the reactions of most alcohols with CEP-OCH_3 produce some symmetrical dialkylacetoinyl phosphates, in particular the dimethyl ester[27]:



R'OH + CEP-OCH_3 → CH_3OH + CEP-OR'

CH_3OH + CEP-OCH_3 → $(CH_3O)_2P(O)(OAcn)$

Apparently, the bulk of both ligands RO- and R'O- affects the integrity of the oxyphosphorane, and its tendency to allow transesterification to occur, which is highly desirable from a synthetic standpoint. On

the other hand, one can choose X-ligands to give exclusive ring-retention in displacements on CEP-X[26].

Scheme 9

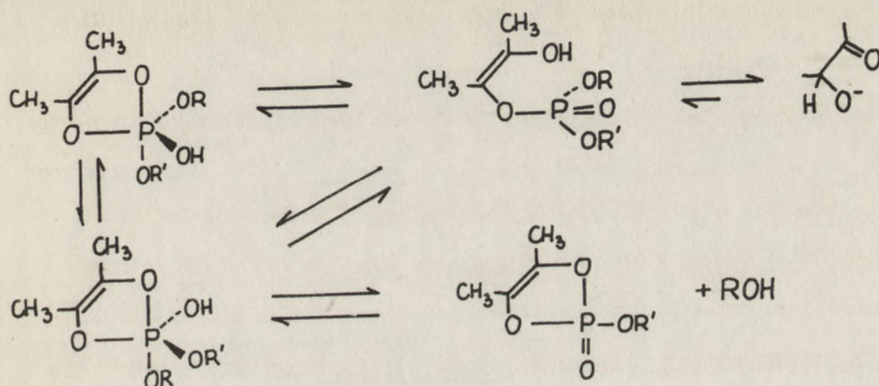
The behavior of the oxyphosphorane derived from the addition of the alcohol R'OH to CAP-OR is pictured in Scheme 10. The situation is somewhat different from that which obtains in the addition of R'OH to CEP-OR. The tendency for irregular PI is higher in the CAP-oxyphosphoranes than in the CEP-oxyphosphoranes[28,29]. The life-time of the intermediate α-hydroxy-β-ketoacid phosphate is much longer than that of the intermediate enol phosphate; the reaction: R'OH + CAP-OR is more prone to give symmetrical dialkylacetoinyl phosphates as by-products.

Scheme 10

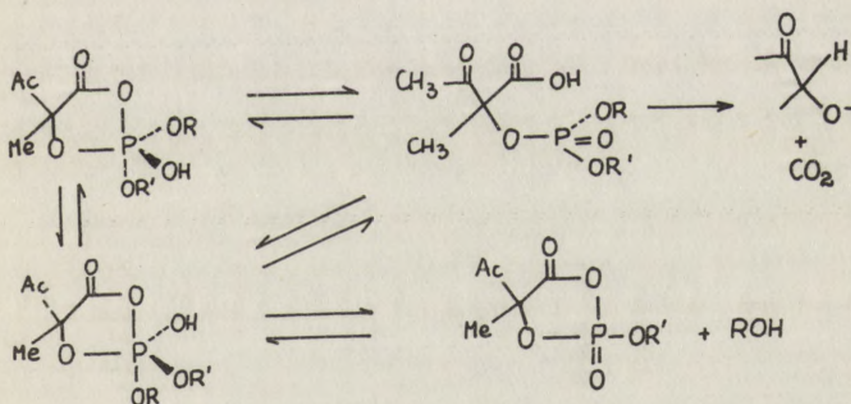
An effective way to avoid the formation of the symmetrical phosphates is to add catalytic amounts of triethyl amine[29] together with the alcohol R'OH to the CAP-OR; Scheme 11. The amine catalyzes the decarboxylation of the β-ketoacid and prevents carboxyl-participation, irregular PI, and transesterification.

Scheme 11

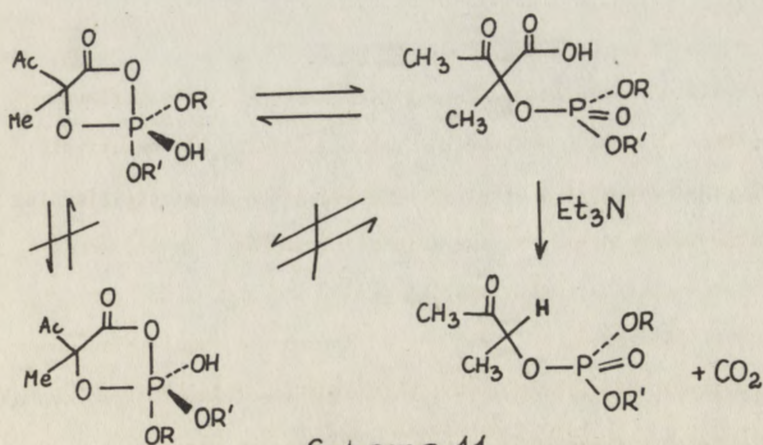
The validity of this reasoning is demonstrated by the experiment of Scheme 12. The addition of methanol to CAP-OCH_3 yields the relatively stable α-hydroxy-β-ketoacid dimethyl phosphate. Subsequent addition of ethanol to this intermediate, in the absence of triethyl amine, leads to a complex mixture containing methylethylacetoinyl phosphate, and possibly diethylacetoinyl phosphate, in addition to the expected dimethylacetoinyl phosphate. Apparently, carboxyl-group participation allows transesterification to take place[29,30,31].



Scheme 9



Scheme 10



Scheme 11

Scheme 12

When this experiment is repeated in the presence of triethyl amine, the only product is dimethylacetoinyl phosphate; Scheme 13. The amine catalyzes decarboxylation and prevents transesterification[29].

Scheme 13

Unsymmetrical dialkylacetoinyl phosphotriesters are hydrolyzed to the dialkyl phosphodiesters under mild alkaline conditions[32,33]; Scheme 14. This step completes the phosphorylative coupling of the alcohols ROH and R'OH by means of the CEP-X and CAP-X reagents. The interpretation of the extraordinarily fast hydroxide-ion catalyzed hydrolysis of dialkylacetoinyl phosphotriesters to dialkyl phosphodiesters was one of the first application of the oxyphosphorane concept to the displacement reactions of phosphate esters[32,33].

Scheme 14

4. Synthesis of Oligonucleotide Models by the CEP-X Reagent Acetoinenediolcyclopyrophosphate

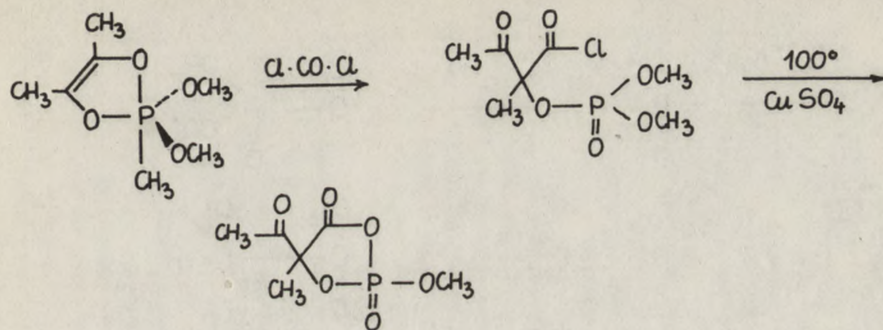
The readily available biacetyl-trimethyl phosphite oxyphosphorane[34] is easily transformed into CEP-OCH_3[35,36]; Scheme 15.

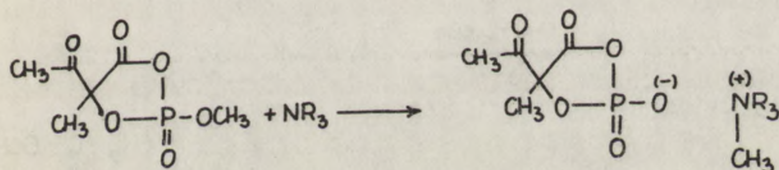
Scheme 15

The reaction of pyridine with CEP-OCH_3 gives the N-methylpyridinium CEPO-salt in 90% of the theory; Scheme 16[26].

Scheme 16

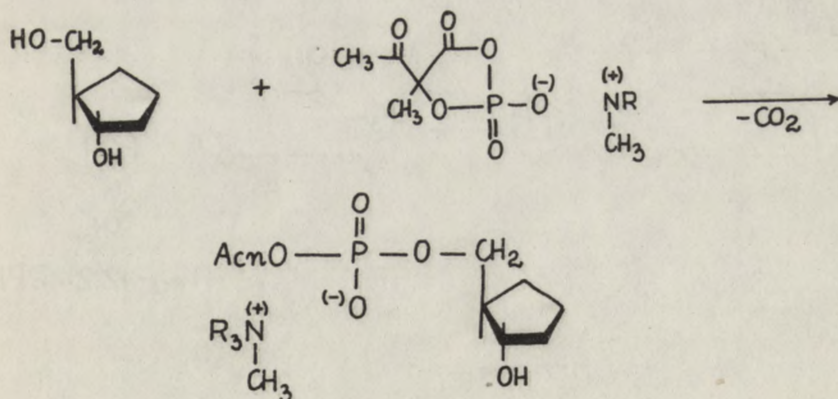
Phosgene converts the CEPO-salt into acetoinenediolcyclopyrophosphate in 85% of the theory; Scheme 17[26]. The cyclic pyrophosphate is a good



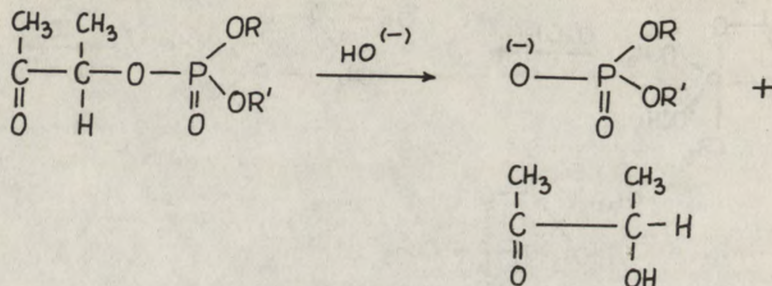


Scheme 32

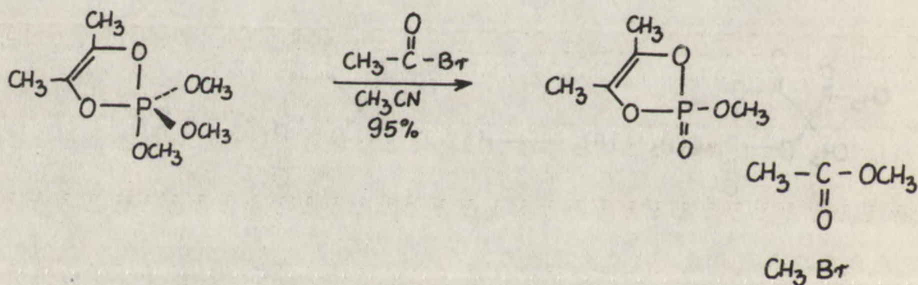
Scheme 33



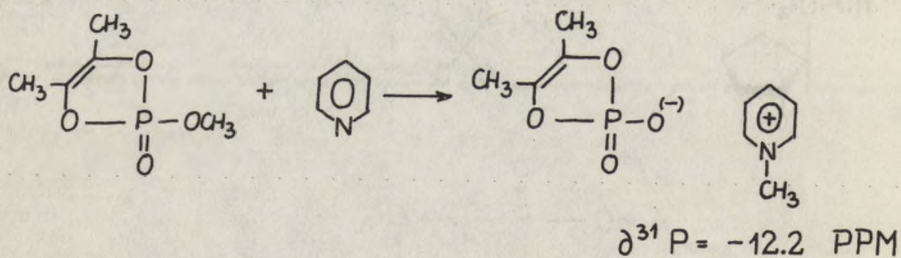
Scheme 34



Scheme 14



Scheme 15



Scheme 16

example of a CEP-X reagent where X=-OCEP; a convenient notation for it is CEP-OCEP.

Scheme 17

CEP-OCEP is formed regardless of the mole ratio of phosgene to CEPO-salt. Apparently, an intermediate CEP-OCOCl is formed and reacts faster than phosgene with the CEPO-anion; Scheme 18.

Scheme 18

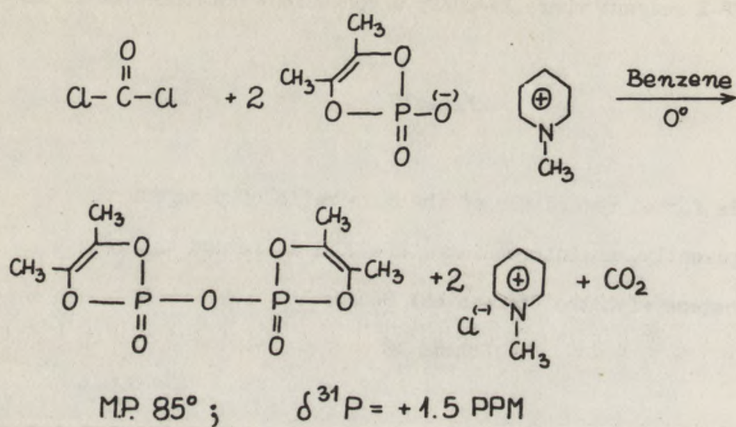
The oxyphosphorane concept leads to the picture in Scheme 19. The regular PI by the TR mechanism brings the ClCOO-ligand to the apex. The driving force for the formation of the high energy cyclic pyrophosphate is the loss of CO_2 and Cl^-.

Scheme 19

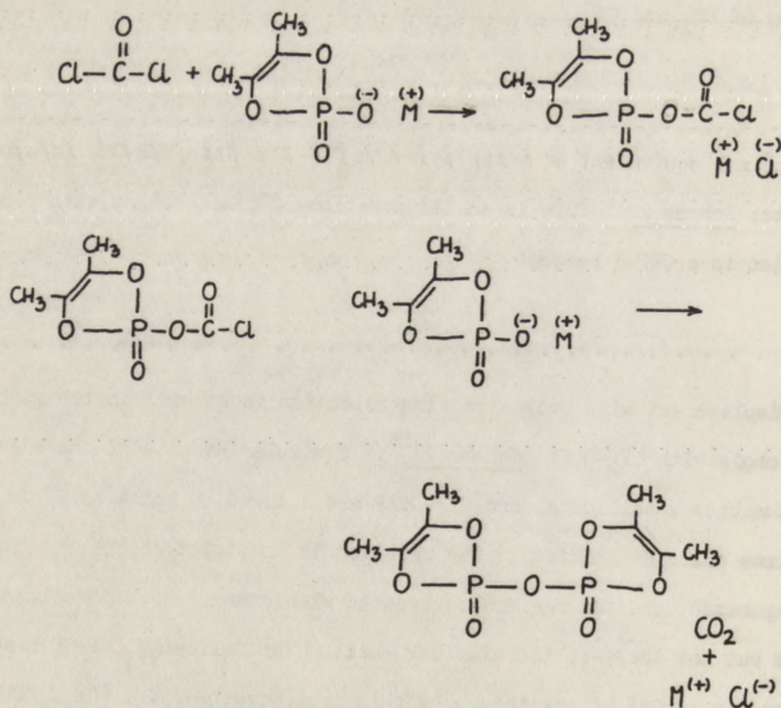
One mol equivalent of water converts CEP-OCEP into CEP-OH, in aprotic solvents; Scheme 20. This is an illustration of displacement with ring-retention in a CEP-X reagent.

Scheme 20

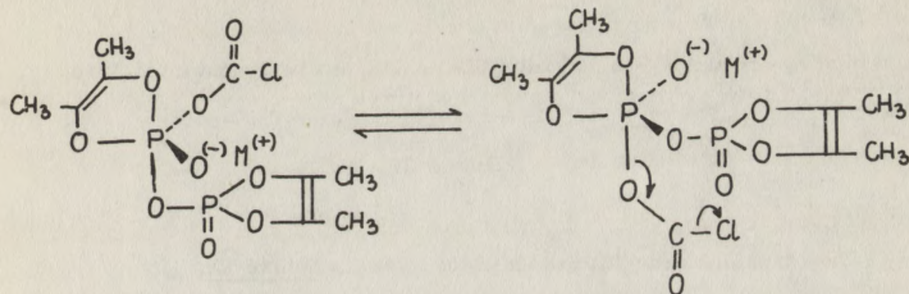
Displacement with exclusive ring-retention is general in the reactions of alcohols with CEP-OCEP; Scheme 21[26]. Equimolar amounts of the alcohol ROH, the base γ-collidine, and CEP-OCEP are allowed to react at 0° in methylene chloride solution. The reaction is completed at 20°, the solvent is evaporated, and the residue is treated with ether, which dissolves the CEP-OR but not the γ-collidinium CEPO-salt. The following CEP-OR have been prepared in 90-95% of the theory by this procedure: R=C_2H_5; $(CH_3)_2CHCH_2$; $C_6H_5CH_2$; CH_2BrCH_2; CCl_3CH_2; cyclo-C_5H_9; $(CH_3)_2CH$; $(CF_3)_2CH$; $(CCl_3)_2CH$;



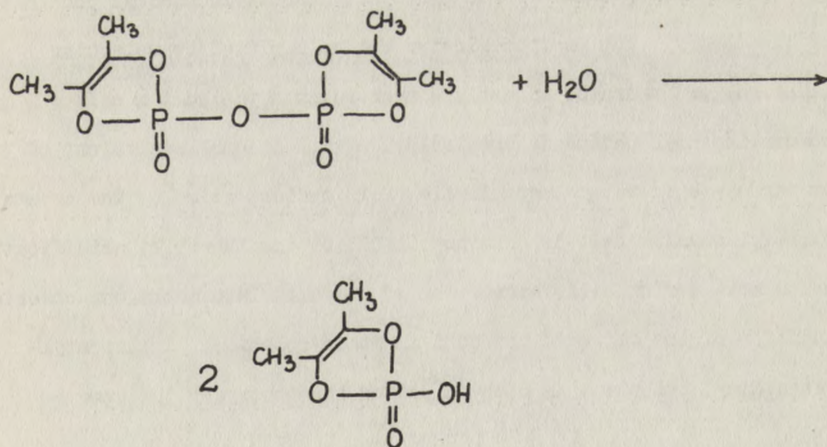
Scheme 17



Scheme 18



Scheme 19



Scheme 20

$(CCl_3)(CH_3)CH$; $(CH_3)_3C$. CEP-OAr, with Ar=C_6H_5; p. NO_2-C_6H_4, are made by the CEP-ylation of the phenols.

The by-product, γ-collidinium CEPO-salt, can be reconverted into CEP-OCEP, as in the case for the N-methylpyridinium CEPO-salt.

Scheme 21

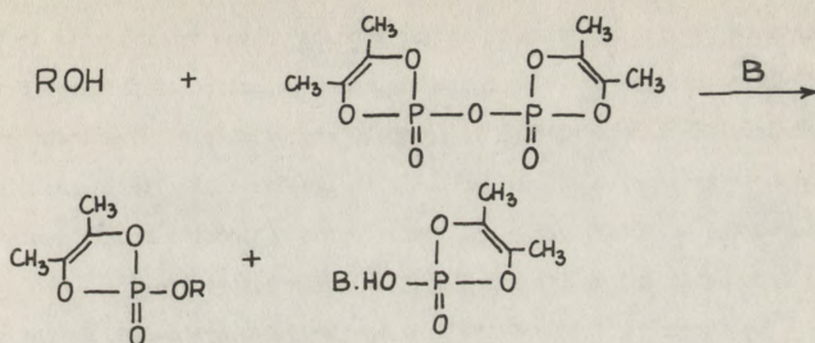
The oxyphosphorane intermediate is shown in Scheme 22. The displacement with ring-retention is attributed to the high apicophilicity of the CEPO-ligand.

Scheme 22

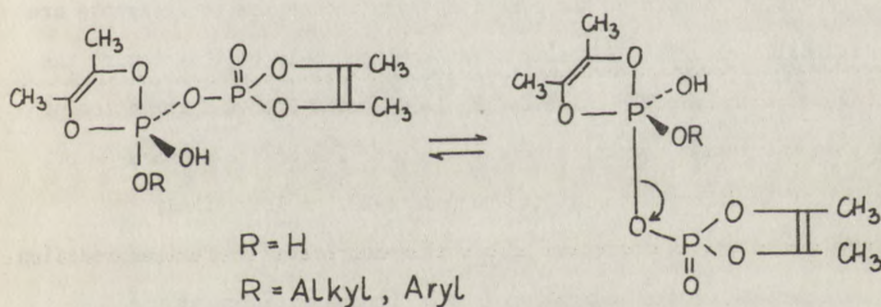
Displacement with exclusive ring-opening is observed when isobutanol is added to CEP-O.cyclo-C_5H_9 in methylene chloride at 20°; Scheme 23. Evaporation of the solvent affords cyclopentylisobutylacetoinyl phosphate in 95% of the theory. The phosphotriester can be purified by molecular distillation, or it can be directly converted into the sodium salt of cyclopentylisobutyl phosphate upon reaction with one mol equivalent of sodium carbonate in water-acetonitrile (2:1) medium, at 20°. The crystalline dicyclohexylammonium salt is obtained in 88% of the theory by acidification of the aqueous sodium salt, extraction of the acid into methylene chloride, and addition of the amine to the acid in ether solution. Cyclopentylbenzylacetoinyl phosphate is made in 95% of the theory by the same procedure.

Scheme 23

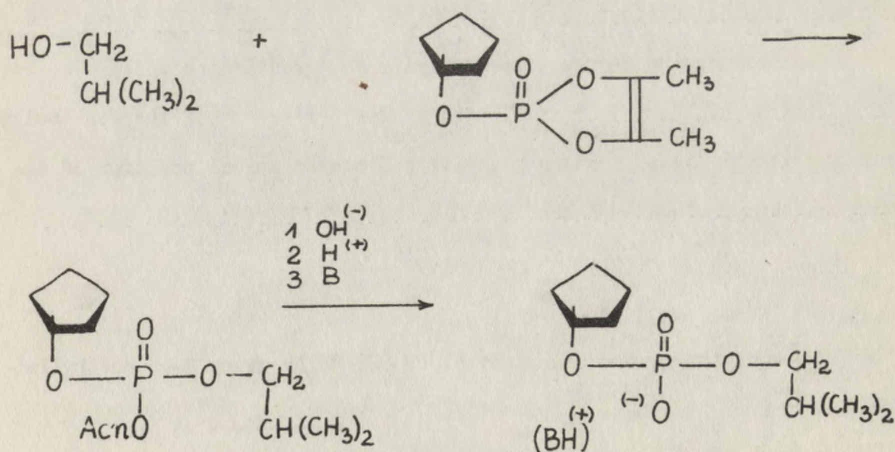
CEP-O.cyclo-C_5H_9 phosphorylates selectively the primary alcohol of *trans*-2-hydroxymethylcyclopentanol in the presence of its unprotected secondary alcohol; Scheme 24. A relatively small amount of phosphorylation at the secondary alcohol function of the diol is also observed. In



Scheme 21



Scheme 22



Scheme 23

reactions carried out with equimolar amounts of the two reagents in 0.3 M methylene chloride at 20° for 6 hours, the proportion of primary-OH to secondary-OH phosphorylation is as 90:10 approximately. Treatment of the phosphotriester with sodium carbonate in aqueous-acetonitrile (2:1) at 20° removes the acetoinyl group and leads to the nucleoside model, isolated as the free acid, and as its amine salt in satisfactory yield.

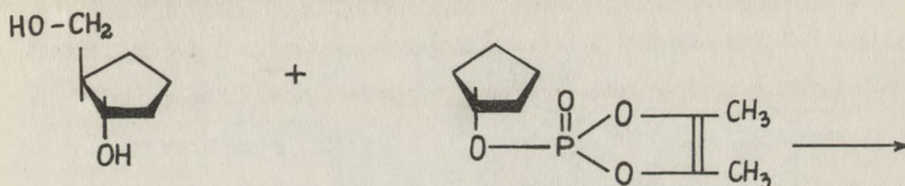
The phosphorylation of the diol by the CEP-O.cyclo-C_5H_9 is not as straightforward as it appears in Scheme 24, since ca. 5-10% of unreacted diol is present at the end of the reaction, even though the two reagents are used in equimolar amounts. A possible explanation could be that toward the end of the reaction some CEP-O.cyclo-C_5H_9 is diverted into the formation of the bis-phosphotriester, probably by further reaction of the primary-OH of the product of phosphorylation at the secondary-OH. A less likely alternative would be the formation of bis-phosphotriester by further reaction of the secondary-OH of the desired product of phosphorylation at the primary-OH. This phenomenon is being studied in detail, since it could reflect the hyperactivation of an alcohol function by a vicinal phosphate in a diol-monophosphotriester.

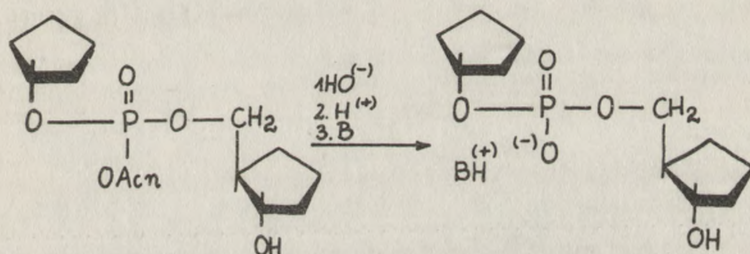
The encouraging degree of selectivity observed in the model dinucleotide synthesis, is attributed to the preference of the less bulky primary alkoxy-ligand for the sterically hindered apical position of the trigonal bipyramidal skeleton[37].

Scheme 24

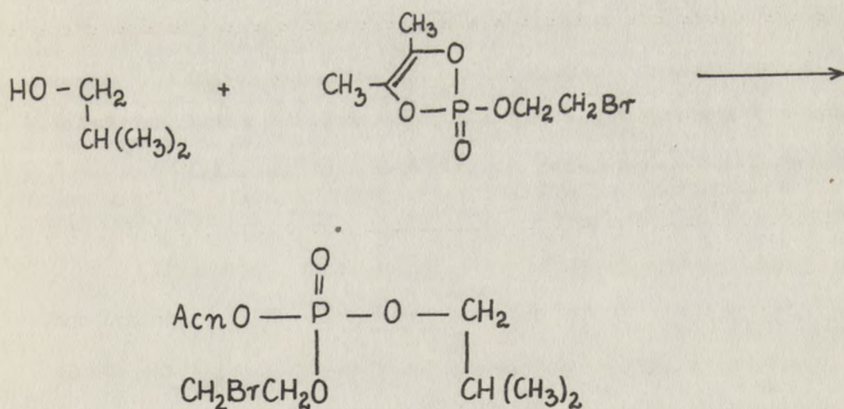
The reaction of isobutanol with CEP-OCH_2CH_2Br gives 2-bromoethyliso-butylacetoinyl phosphate in 95% of the theory; Scheme 25.

Scheme 25





Scheme 24



Scheme 25

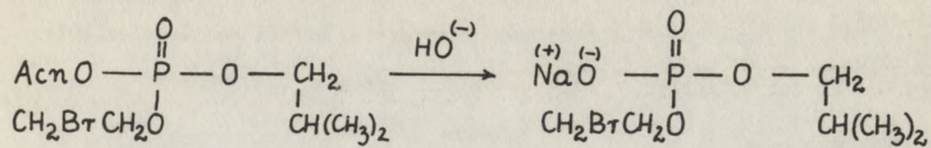
The acetoinyl group can be removed from the alkyl-(2-bromoethyl)-acetoinyl phosphate by mild alkaline hydrolysis, without any further effect on the resulting phosphodiester; Scheme 26. Work is now in progress to develop reagents capable of removing the 2-bromoethyl group from the phosphodiester, preferably by alkyl-oxygen bond fission, in order to adapt this procedure to the two-step removal of two phosphate-blocking groups from a phosphotriester.

Scheme 26

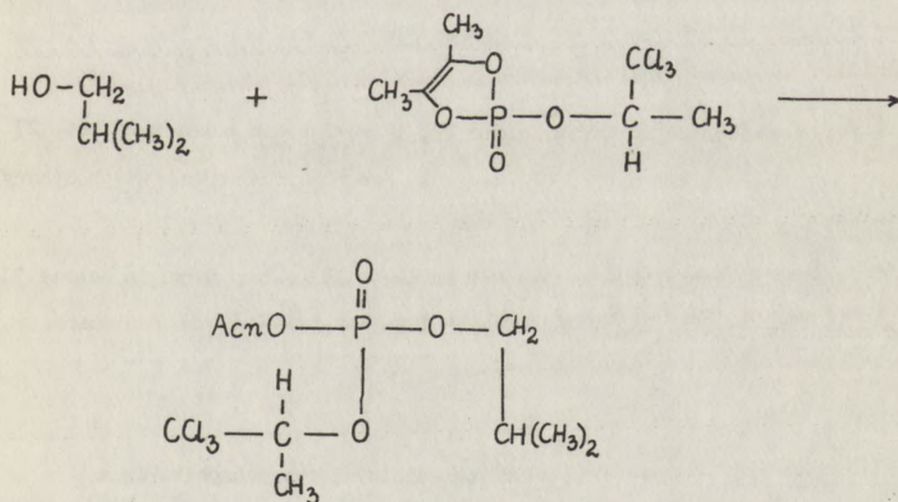
A related example is the reaction of isobutanol with CEP-OCH(CCl_3)(CH_3) which gives (1,1,1-trichloro-2-propyl)isobutylacetoinyl phosphate in 95% of the theory; Scheme 27. In these reactions there is no evidence for the formation of symmetrical phosphates[26].

Scheme 27

Treatment of the alkyl-(1,1,1-trichloro-2-propyl)acetoinyl phosphate with an excess of sodium carbonate yields the salt of the alkyl phosphomonoester as the major product; Scheme 28. Small amounts (less than 10%) of the alkylacetoinyl phosphodiester is formed as by-product. Apparently, the difference in the rates of hydrolyses of the acetoinyl and the trichloroisopropyl groups from the phosphotriester is not large enough to insure the complete initial removal of the acetoinyl group to give the alkyltrichloroisopropyl phosphodiester, which then loses its trichloroisopropyl group. The acetoinyl group is not removable from phosphodiesters by simple alkaline hydrolysis, and the relatively small amount of alkylacetoinyl phosphate initially produced remains as a by-product in the solution. Refinements of this procedure for the one-stage removal of two phosphate-blocking groups from a phosphotriester are under study. The CEP-ylation



Scheme 26



Scheme 27

method provides the required phosphotriesters for this study, e.g., those with hexachloroisopropyl-, hexafluoroisopropyl-, and other perhaloalkyl-groups as one of the two phosphate-blocking groups.

Scheme 28

CEP-OCH_2CH_2Br phosphorylates selectively the primary-OH of trans-2-hydroxymethylcyclopentanol, in the presence of the unprotected secondary-OH. The degree of selectivity, as well as the other features of the reaction, are analogous to those encountered in the phosphorylation with CEP-O.cyclo-C_5H_9.

Scheme 29

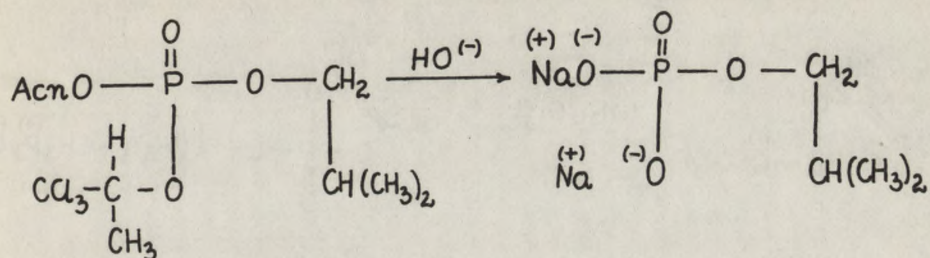
The reactions depicted in Scheme 29, as well as in Schemes 25, 26, 27 and 28, illustrate the use of CEP-reagents for the protection-cum-phosphorylation of the C5'-OH in nucleosides.

Another potential protective CEP is the silyl ester shown in Scheme 30, and made from the CEPO-salt and trimethyl chlorosilane[26].

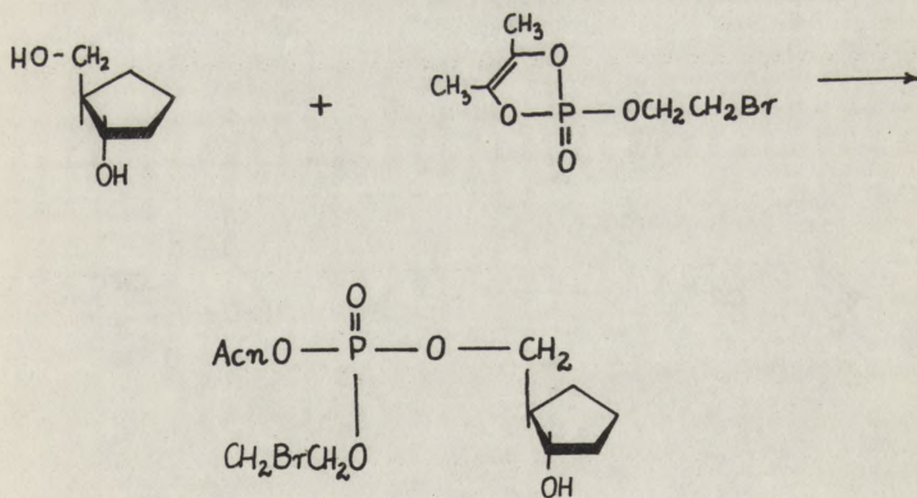
Scheme 30

It can be concluded that acetoinenediolcyclopyrophosphate is a promissing CEP-X reagent that can be used to carry out the sequence of reactions shown in Scheme 31. Work is now in progress to determine if this CEP-OCEP reagent performs satisfactorily in the more complex task of providing for C5'-protection and for the establishment of the oligonucleotide bond in actual nucleosides.

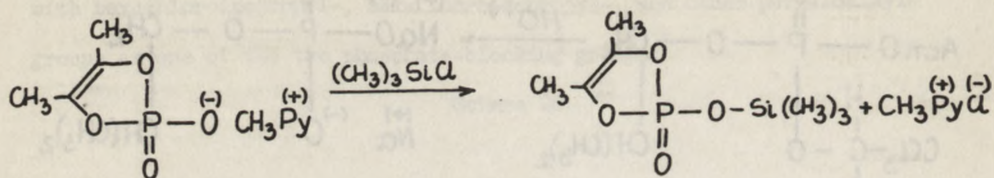
Scheme 31



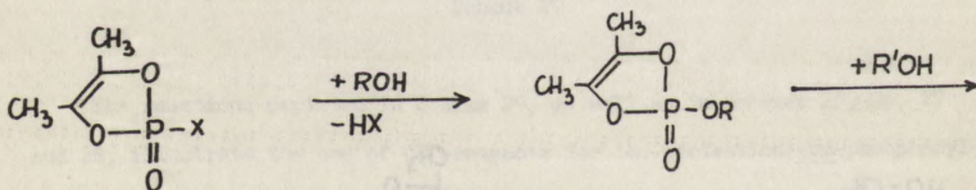
Scheme 28

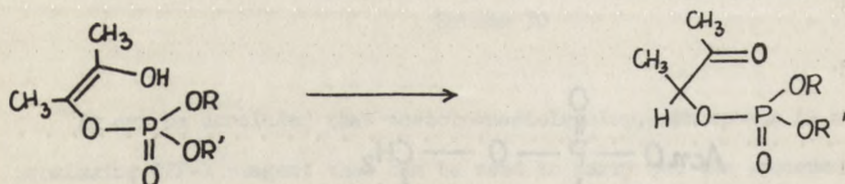


Scheme 29



Scheme 30





Scheme 31

5. Preparation and Some Reactions of Quaternary Ammonium Salts of Five-Membered Cyclic Acyl Phosphates (CAPO-Salts)

Phosgene performs a C-acylation of the biacetyl-trimethyl phosphite oxyphosphorane in hexane or in methylene chloride solution to give the phosphate of an α-hydroxy-β-ketoacid chloride; Scheme 32[28]. The acid chloride is converted into the two diastereomers of CAP-OCH_3. The diastereomer with cis-CH_3CO/O is isolated in crystalline form[38].

Scheme 32

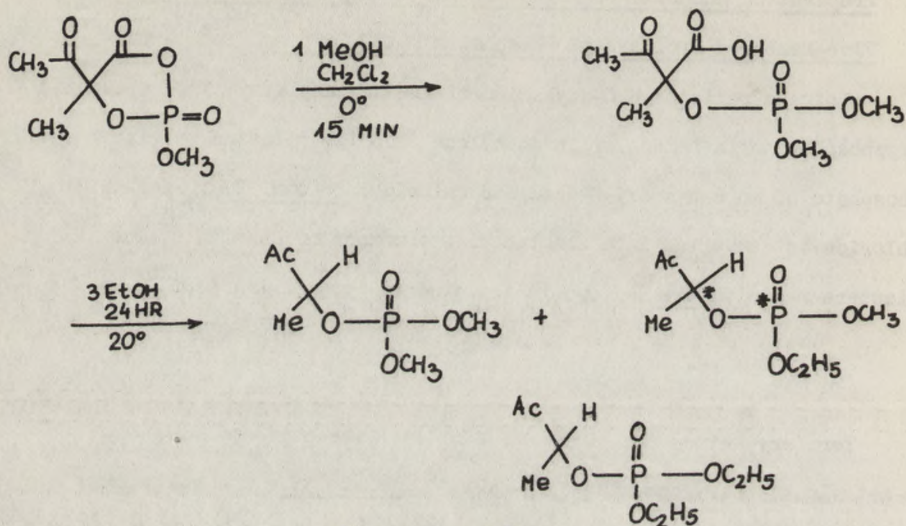
Tertiary amines transform CAP-OCH_3 into the corresponding N-methylquaternary ammonium CAPO-salts[28]; Scheme 33. The salts from triethyl amine, pyridine, γ-picoline and nicotinamide, have been reported[28].

Scheme 33

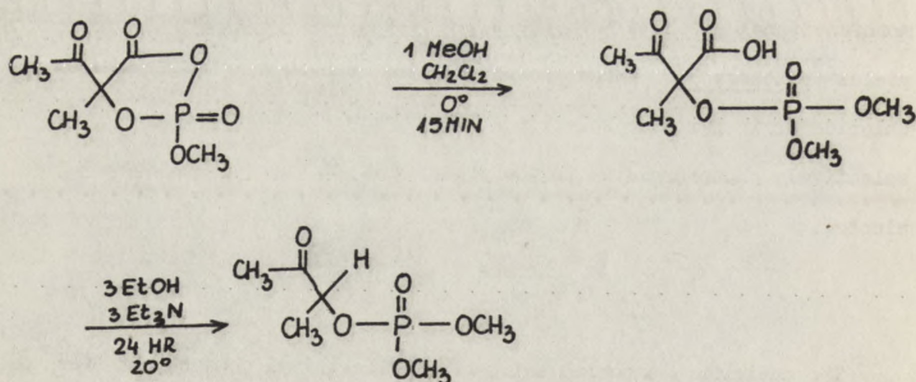
The CAPO-salts are excellent phosphorylating reagents, and convert monofunctional alcohols into the salts of alkylacetoinyl phosphates in high yields[39]; Scheme 34. These phosphorylations can be carried in methylene chloride or in pyridine solution. The primary alcohol of a diol is selectively phosphorylated in the presence of an unprotected secondary alcohol.

Scheme 34

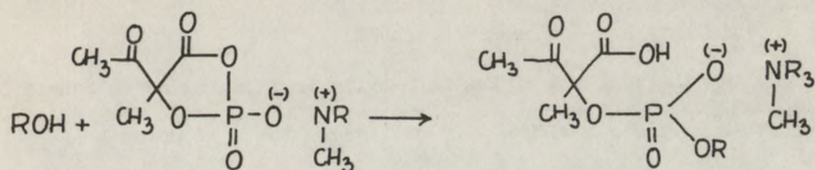
The quaternary ammonium salts of alkylacetoinyl phosphates offer some attractive synthetic possibilities. For example, a phosphate-activating reagent, such as mesitylsulfonyl chloride, acting on the phosphodiester shown in Scheme 34, gives the acetoinyl ester of a 6-membered cyclic phosphate analogous to cyclic-AMP. Hypothetically, if the secondary-OH is protected, or if the alcohol had been monofunctional, the phosphate-activating reagent, in conjunction with another alcohol, would represent

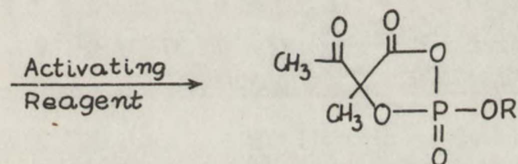


Scheme 12

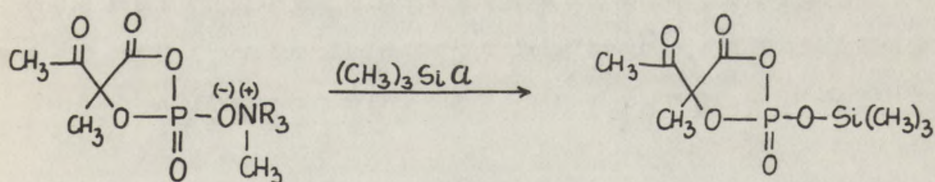


Scheme 13





Scheme 35



Scheme 36

another synthesis of unsymmetrical dialkylacetoinyl phosphates. However, this last possibility has not been researched.

Another hypothetical use of the CAPO-salts is illustrated in Scheme 35. The reaction of an alcohol with a CAPO-salt gives the salt of an α-alkylphosphato β-ketoacid, which usually decarboxylates to the salt of the alkylacetoinyl phosphate, at a relatively rapid rate. However, it is not inconceivable that the intermediate β-ketoaeid could be activated by a suitable carboxyl-activating reagent before it decarboxylates. The result would be a two-step CAP-ylation of the alcohol, ROH.

Scheme 35

No efficient way of removing the acetoinyl group from a phosphodiester has, so far, been found. Consequently, the phosphorylation of alcohols by CAPO-salts is not, at present, a general procedure for the synthesis of phosphomonoesters.

The CAPO-salts are sufficiently nucleophilic to yield the silyl ester shown in Scheme 36 upon reaction with trimethylchlorosilane[40]. The CAP-$OSi(CH_3)_3$ is, potentially, another protective phosphorylating reagent, analogous to CEP-$OSi(CH_3)_3$.

Scheme 36

6. Preparation of CAP-X Reagents

In homogeneous solution, a CAPO-salt and a carboxylic acid chloride are in equilibrium with the quaternary ammonium chloride and the CAP-O-C(=O)-R[29]; Scheme 37. In many cases, this equilibrium lies far to the left, which shows the extraordinary reactivity of these CAP-X reagents. Some CAP-anhydrides of this type can be isolated with considerable difficulty by the proper choice of quaternary ammonium salt and solvent, since the equilibrium is driven to the right when the ammonium chloride is less soluble

in a given solvent than the ammonium CAPO-salt.

Scheme 37

A similar equilibrium is established between a CAPO-salt and a sulfonyl chloride, on the one hand, and the ammonium chloride and CAP-O-SO_2Ar, on the other hand; Scheme 38. Some very reactive CAP-X reagents of this type can be isolated also with difficulty by the proper choice of ammonium salt and solvent.

Scheme 38

A more promissing approach to the synthesis of CAP-X reagents proceeds via the CAPO-sodium salt, which is obtained in quantitative yield from the reaction of CAP-OCH_3 with sodium iodide[41,42]. This route is now being intensively explored, since the CAP-X reagents complement the CEP-X reagents; the former are much more reactive than the latter, but are also more difficult to prepare and to use and more prone to give undesirable side reactions.

7. Acknowledgment

Dr. J.F. Marecek developed the synthetic chemistry of the CEP-X reagents. Drs. O.P. Madan and K. Tasaka are responsible for the earlier research on CEP-OCH_3. Drs. S. Glaser, P. Stern, H. Okazaki and J. Marecek (Stony Brook), and Dipl. Chem. P. Lemmen, E.V. Hinrich and R. Kamal (München), carried out the investigations on CAP-OCH_3, the CAPO-salts and CAP-X reagents. Dr. J. Firl (München) made valuable contributions to structural problems by NMR spectroscopy. Prof. C. Caughlan and Dr. G.D. Smith (Montana State University) carried out the X-ray crystallographic studies on CEP-OCH_3 and CAP-OCH_3. Prof. J. Ricci (Windham College and Brookhaven National Laboratory) did X-ray analyses of CEP-OCEP. We are grateful to the A. von Humboldt Stiftung for a U.S. Senior Scientist

Scheme 37

Scheme 38

$\xrightarrow[\text{Acetone}]{\text{NaI}}$

Scheme 39

Award to F. Ramirez at Technische Universität München. The support of this research by the National Institutes of Health, U.S.A. Public Health Service (Grant GM 20672 to F.R.) and the Petroleum Research Fund administered by the American Chemical Society (Grant 7136 to F.R.) is gratefully acknowledged.

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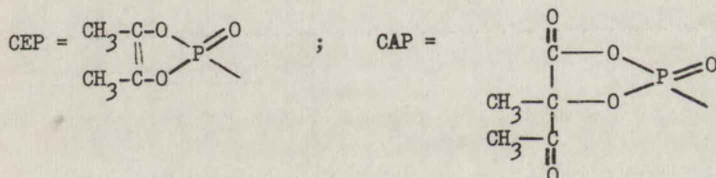
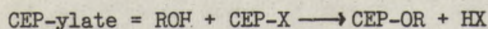
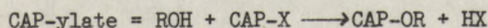
22. The following abbreviations will be used in this paper:







PI = Permutational isomerization or exchange of ligands among skeletal positions, e.g., in an oxyphosphorane.

TR = Turnstile Rotation.

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THE MECHANISM OF THE CHEMICAL SYNTHESIS OF OLIGONUCLEOTIDES

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The least elaborated step of the chemical synthesis of oligonucleotides remains up to now formation of the internucleotide diester or triester bond.The yields at this step in the most cases are far from quantitative even in the excess of either nucleoside (hydroxy) or nucleotide (phosphoryl) component,the final reaction mixture besides oligonucleotide prepared contains the unreacted starting compounds and a number of by-products.This results in a labourious separation procedures with unescapable losses of the product and consequently additional decrease of the yield.

Therefore,an important task of the chemistry of nucleic acids is the elucidation of the mechanism of the reactions leading to internucleotide bond formation,of the reasons of the accompanying by-processes,of the possibilities to prevent these processes in order to approach to quantitative,standard and suitable to automation procedure of a stepwise elongation of the oligonucleotide chain.

Today the most widespread method of the oligonucleotide synthesis is a condensation of the nucleoside and nucleotide components in the presence of carbodiimides (mainly dicyclohexylcarbodiimide,DCC) or aryl sulphonyl chlorides (mainly triisopropylbenzene sulphonyl chloride,TPS).In the present report the up-to-date state of our knowledge of the mechanism of these two methods of the internucleotide bond formation will be discussed.

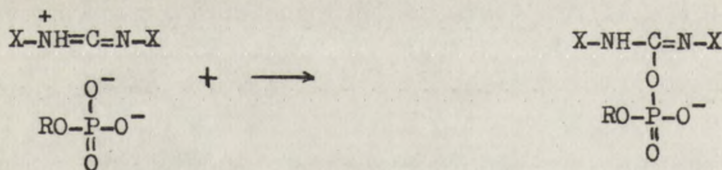
I. The nature of the active phosphorylating derivative of mononucleotides.

The role of both aryl sulphonyl chlorides and carbodiimides in the oligonucleotide synthesis is a production of an active phosphorylating intermediate from the nucleotide component. The most simple case we meet with mononucleotide as a nucleotide component.

It is reasonable to assume that the initial step of the reaction of aryl sulphonyl chloride ($ArSO_2Cl$) with nucleotide ($ROPO_3^{2-}$) is a formation of a mixed anhydride I

(I)

The initial step of the process in the case of carbodiimides is believed to be a formation of a O-phosphoryl isourea derivative II. The reaction is thought to proceed via attack of double ionised anion of a nucleotide at the electrophilic C atom of protonated carbodiimide

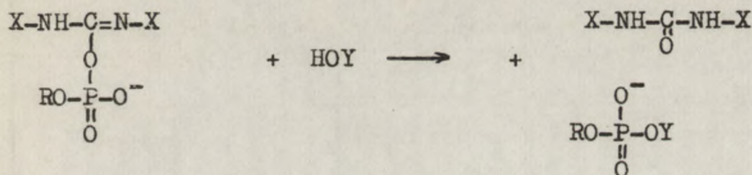


(II)

In accordance with this hypothesis, the internucleotide bond formation is suppressed in the presence of basic trialkylamines [1,2] Kinetic investigation of the reaction of cytidine-2'(3')-phosphate with N-cyclohexyl-N'-2-(4-methylmorpholinium)-ethylcarbodiimide in aqueous solution at various pH values resulting in intranucleotide phosphodiester bond formation demonstrated that the rate-limiting stage of the whole process is a bimolecular interaction between protonated carbodiimide and double ionised CMP [3] .

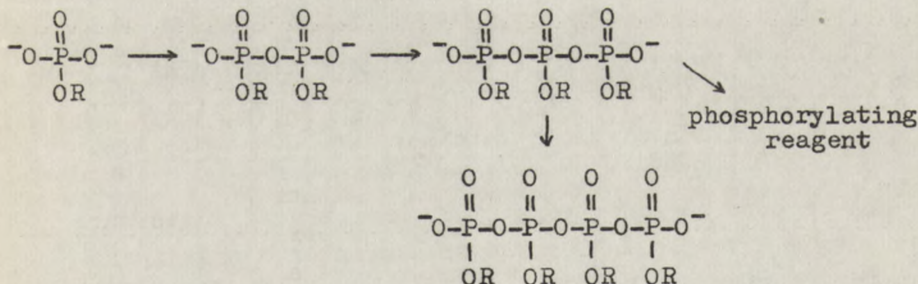
Although I and II seem to be powerful phosphorylating reagents, their reaction with hydroxy group of the nucleoside component may not be regarded as a main path leading to internucleotide bond formation. Khorana [1] supposed that direct

formation of internucleotide bond by interaction of II with hydroxy component



may be realized in a significant extent in a great excess of the hydroxy component. However with near by stoichiometric amounts usually employed this simple scheme is not realized.

Already in the early investigations of the phosphodiester bond formation it was found that pyrophosphates and longer polyphosphates are accumulated as the main products of the initial period of the reaction in the case of both DCC and aryl sulphonyl chlorides. Therefore, it was proposed that an active phosphorylating reagent is formed according to scheme [4]



Recenly the same sequence of the reactions was demonstrated in our laboratory [5,6] using pulse NMR spectroscopy at the ^{31}P nuclei. As it may be seen from fig.I, the starting mononucleotide (chemical shift relative 85% H_3PO_4 δ = -I.2) in the course of the reaction with TPS is transformed to symmetric pyrophosphate (δ = IO.3) and then to compound A with ^{31}P NMR spectrum consisting of two overlapping doublets centered at δ=II.5 ppm and a triplet (or doublet of doublets) centered at δ = 2I.6 similar to analogous spectrum of inorganic tripolyphosphate [7]. Some amount of a compound Ā with signals at II and 24 ppm was noticed. This was preliminary identified as a tetrasubstituted tetrapolyphosphate.

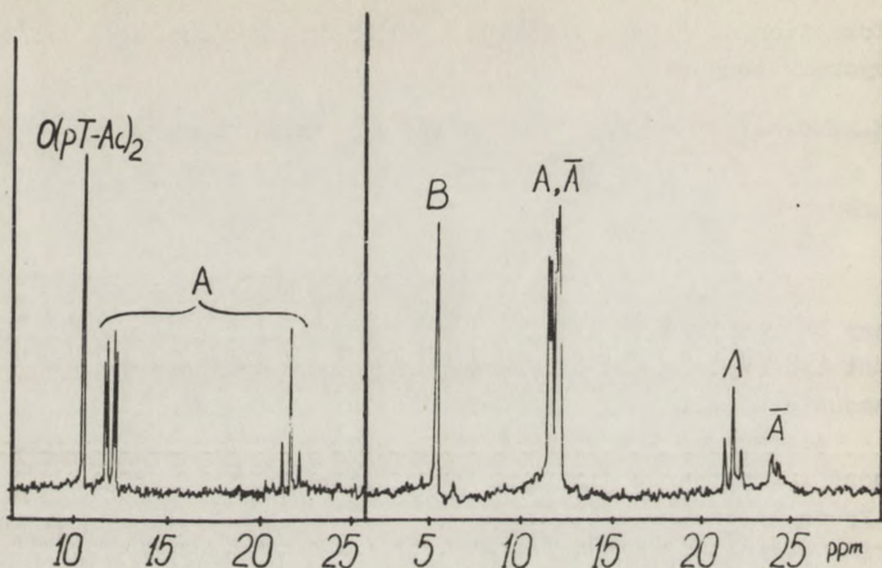
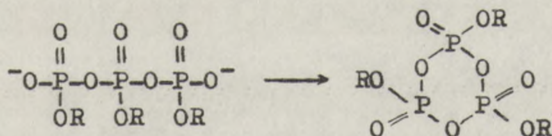


Fig.I. ^{31}P NMR spectra of the reaction mixture of 0.I5 M pT-Ac and TPS after a) II min, b) 90 min after addition of the second portion (0.5 eqv.) TPS.

All spectra represented at this and other figures were taken with a Brucker HX-90 pulse spectrometer operating at 36.43 MHz Fourier transform being performed after I00-500 accumulations. Unless otherwise stated spectra are recorded with heteronuclear spin-spin decoupling $^{31}P - \{^{1}H\}$.

A series of transformations leading to the phosphorus products with decreased nucleophilicity thus precedes the reaction with hydroxy group of the nucleoside component. This seems to be reasonable due to poor nucleophilicity of the hydroxy group of the ribose or deoxyribose residues making it incapable to compete with such a strong nucleophiles as monoesters of orthophosphoric acid or diesters of pyrophosphoric acid.

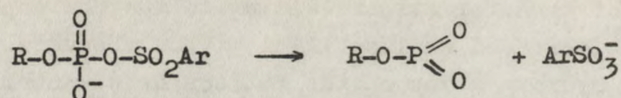
The structure of the phosphorylating reagent accumulated in the reaction mixture after complete consumption strong nucleophiles was discussed by several authors. Weimann and Khorana [4] proposed this reagent to be a cyclic trisubstituted trimetaphosphate III which may be regarded as a product of the reaction of tripolyphosphate with a condencing reagent with subsequent cyclisation

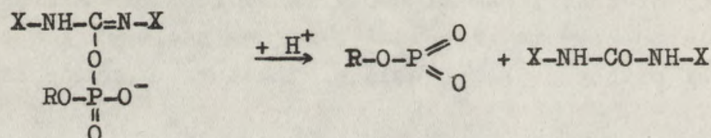


(III)

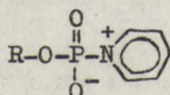
The attempts to find III in the reaction mixture of DCC with mononucleotide, cyanoethyl phosphate or ethyl phosphate were unsuccessful. This was explained by an extreme lability of III. In the same time, authors succeeded in preparation of inorganic trimetaphosphate by interaction of DCC with inorganic phosphate as well as with benzyl phosphate in pyridine at elevated temperature. In the latter case according to authors of [4] the appearance of inorganic trimetaphosphate may be regarded as a result of an attack of a base at tribenzyl trimetaphosphate preformed. Another explanation, namely hydrolysis of benzyl phosphate followed by cyclisation of phosphate was not discussed.

Another point of view was ptoposed by Todd and Michelson [8,9]. According to these authors, compounds I and II are believed to be unstable and to eliminate forming metaphosphate IV and aryl sulphonate anion or disubstituted urea





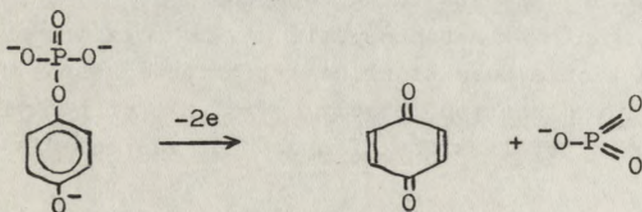
Metaphosphate IV is expected to be a powerful phosphorylating reagent for anions, amines and alcohols. The existence of a monomeric phosphorylating reagent in the form of nucleotidyl pyridinium V is not excluded [9]



(V)

The latter may be regarded as a product of addition of pyridine molecule to double bond of IV.

No direct evidence for the existence of some monomeric phosphorylating reagent was obtained till the last time. However some experimental data are readily explained by this assumption. For instance it was demonstrated that quinol phosphates behave as phosphorylating reagents in the presence of such oxidants as bromine and cerium sulphate. These data are well understood by postulating a reaction



The similar oxidative phosphorylation takes place with monosubstituted but not with disubstituted esters of quinol phosphate in accordance with capability of the former and inability ot the latter to produce metaphosphate [IO]. Phosphorous acid but not its benzyl ester is known to produce nucleoside phosphites in the presence of the condensing reagents [II]. The reason of this difference is supposed to be the ability of phosphorous acid but not of its esters to form metaphosphite $\mathrm{H{-}\overset{\overset{\displaystyle O}{\|}}{P}{=}O}$.

The usual analytical procedures did not permit one to elucidate the structure of the active phosphorylating intermediate or even to discriminate between structures III, IV or V proposed.

The appearence of the pulse NMR spectroscopy at the ^{3I}P nuclei provides new possibilities to investigate intermediate reactions of the oligonucleotide synthesis. This method permitted us to study some central stages of the process and to obtain new data about structure and properties of the intermediates participating in the internucleotide bond formation.

The ^{3I}P NMR spectroscopy was already used to elucidate the structure of the intermediate in the course of the oligonucleotide synthesis via triesters[I2]. These data will be discussed later. It is however only the pulse spectroscopy that permitted one to provide kinetic measurements with suitable concentrations of the components (circa O.I M).

It was already mentioned that using pulse ^{3I}P NMR spectroscopy we succeeded in demonstration of a stepwise transformation of mononucleotide in the presence of TPS to pyrophosphate and then to longer polyphosphates. The next step of the process is the reaction of TPS with tripolyphosphate resulting in the formation of a compound B with ^{3I}P NMR spectrum being a singlet with δ = 5.I ppm. The spectrum of the reaction mixture containing compound B as well as some amounts of unreacted A and $\bar{A}$ is represented in fig.2.

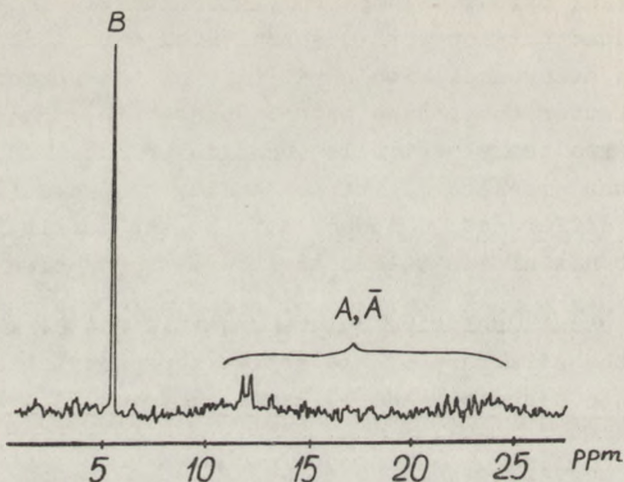


Fig.2. ^{31}P NMR spectrum of the pyridine solution of 0.I5 M pT-Ac treated three times with 0.5 eqv. TPS.

The kinetic curves of the whole process in the case of a stepwise addition of TPS (3 times per 0.5 eqv.) to solution of pT-Ac in pyridine are represented in fig.3. It is seen that addition if 0.5 eqv. of TPS results in the formation of 80% symmetric pyrophosphate $O(pT\text{-}Ac)_2$ as a single product. The reaction is complete within 20 min. The next addition of 0.5eqv. of TPS to the same reaction mixture results in a rather rapid transformation of $O(pT\text{-}Ac)_2$ to tripolyphosphate A. The third portion of TPS reacts with A significantly slower forming in a few hours compound B. The same sequence of transformations proceeds when I.5 eqv. of TPS are added to pyridine pT-Ac solution in one portion. Corresponding kinetic data are represented in fig.4. Additional amount of TPS does not lead to the appearance of any new signals in ^{31}P NMR spectrum. Therefore, compound B may be regarded as a final product of the reaction of pT-Ac with aryl sulphonyl chloride.

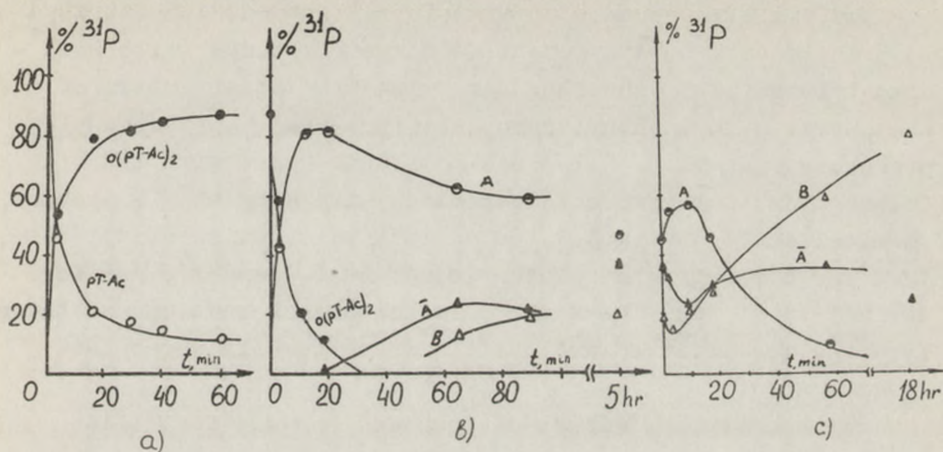


Fig.3. The kinetic curves of the reaction of 0.15 M pT-Ac with TPS: a) with 0.5 eqv. TPS; b) after next addition of 0.5 eqv. TPS; c) after third addition of 0.5 eqv. TPS. Each next portion of TPS was added after reaction reached plateau.

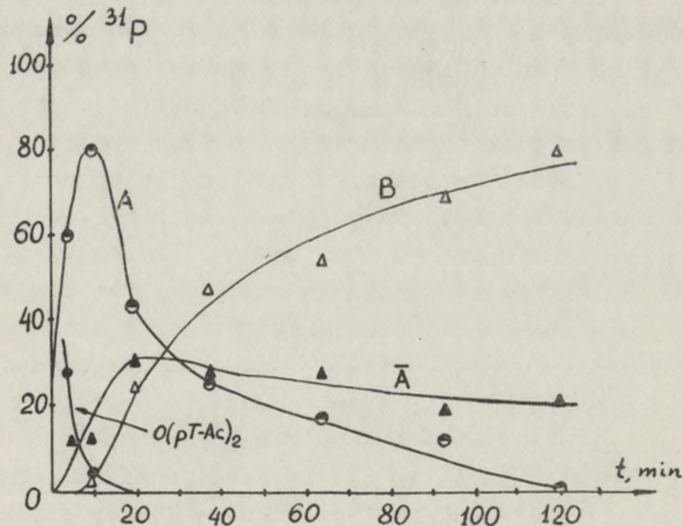


Fig.4. The kinetic curves of the reaction of 0.15 M pT-Ac with 1.5 eqv. TPS.

The similar sequence of transformations was demonstrated to proceed with other protected deoxynucleotides, with 2',3'-diacetyluridine-5'-phosphate and with some other esters of phosphoric acid - phenyl phosphate (pPh) and p-nitrophenyl-phosphate ($pPh-NO_2$). All these compounds react with TPS forming as final products compounds with singlet ^{31}P spectra, chemical shifts being 5.4 for derivative of diisobutiryl-dGMP, 12.2 for derivative of $pPhNO_2$ (compound B_I) and 7,2 ppm for derivative of pPh. The time course of transformation and therefore the reactivities are quite similar for all compounds investigated.

The singlet NMR signal of compound B means that this compound contains one P atom or several completely equivalent P atoms, that may be the case for metaphosphate as well as for cyclic trimetaphosphate of the type III. However III must exist as a mixture of two isomers, one of them with OR radicals on both sides of the cycle. In the latter P atoms are not completely equivalent and some splitting of the signal has to take place. Moreover, the chemical shift of P atoms of III participating in two anhydride bonds is expected to be of the order of 20 ppm, analogous to inorganic trimetaphosphate. Therefore, neither form nor position of the signal of the compound B agree with the structure III.

To elucidate the number of P atoms in compound B, a mixture of the equivalent amounts of pT-Ac and $pPhNO_2$ was treated with TPS in pyridine solution. Due to similar reactivities of both phosphoric esters it should be expected that the significant part of the compounds containing several B atoms should contain both pT-Ac and $pPhNO_2$ residues. Such mixed compounds may be readily seen in ^{31}P NMR spectrum due to spin-spin coupling between unequivalent P atoms (δ value of pT-Ac -1.4 ppm, of $pPhNO_2$ + 4.2 ppm). As it may be seen in fig.5, mixed pyrophosphates and mixed tripolyphosphates do really appear in the reaction mixture. However, the spectrum of the final mixture represents the sum of signals of compounds B with δ = 5.1 ppm and B_I with δ = 12.2 without any signals corresponding to the similar mixed structures. In the same time it is obvious that

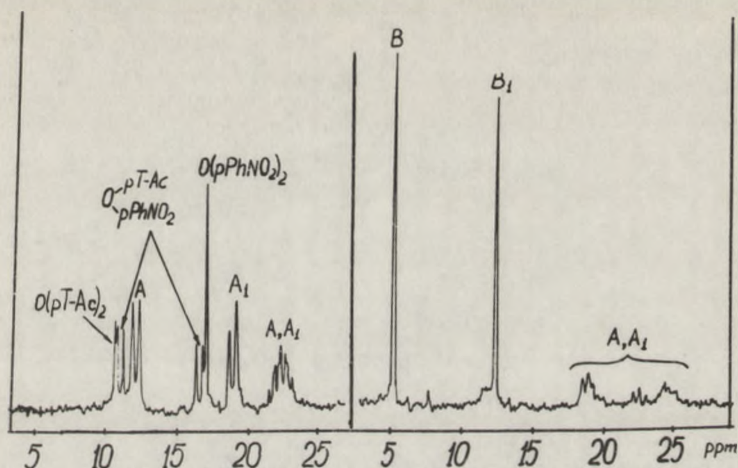


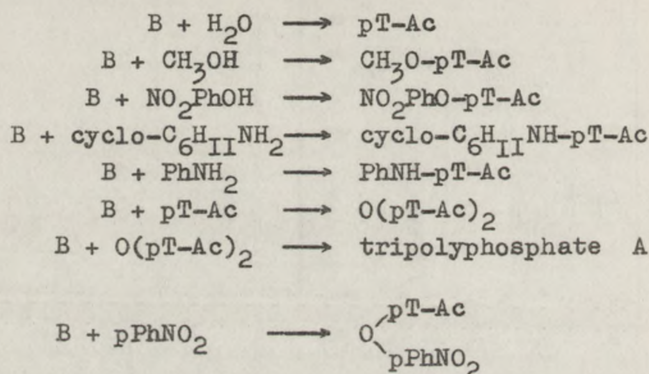
Fig.5. ^{31}P NMR spectra of the reaction mixture after treatment of 0.075 M $pPhNO_2$ + 0.075 M pT-Ac with TPS: a) Two times per 0.5 eqv TPS; b) three times per 0.5 eqv. TPS.

cyclisation of the mixed tripolyphosphates shoud result in the formation of the mixed trimetaphosphates with ^{31}P NMR signals splitted due to P-O-P spin-spin coupling.

Therefore it may be concluded that compound B is a final product of the reaction of mononucleotide with TPS and contains one P atom.

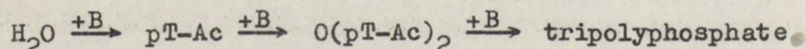
Chemical properties of B agree completely with monomeric structure. The following reactions were demonstrated to

proceed after addition of various nucleophiles to solution of the compound B by recording ^{31}P NMR spectra or by usual chemical analysis of the reaction mixture



All these reactions proceed rapidly, no signal of B being recorded one minute after addition of the nucleophile. Therefore compound B may be regarded as an active phosphorylating reagent.

The addition of small quantities of water to compound B solution leads to accumulation of $O(pT\text{-}Ac)_2$ as well as of pT-Ac. The traces of water transform B mainly to tripolyphosphate A. These data may be regarded as a result of the sequential transformations



The existence of two last reactions as was already mentioned was proved in separate experiments. These data demonstrate that isolation of trinucleoside tripolyphosphate from the reaction mixture containing active derivative of the nucleotide in [4] does not prove formation of trimetaphosphate III.

All above data do not exclude the possibility that a final product of the reaction of mononucleotide with TPS is a mixed anhydride I. To discriminate between structure I and structures IV or V the reaction of pT-Ac with polymeric crosslinked sulphonyl chloride was studied [13]. The latter was obtained by treatment of cross-linked polysterene with chlorosulphonic acid. It was found that a compound with the same δ value 5.I is obtained and may be separated from the polymer. This proves that compound B does not contain aryl sulphonyl residue.

To discriminate between the structures IV and V, the spectra of B (derivative of pT-Ac) and B_1 (derivative of $pPhNO_2$) were recorded without heteronuclear spin-spin decoupling $^{31}P-\{^1H\}$ used in the most part of the measurements. The data are represented in fig.6. It is seen that the spectrum of B is a triplet with J=8.5Hz characteristic for spin-spin coupling P-O-C-H with two equivalent protons of the 5'-CH_2 group.

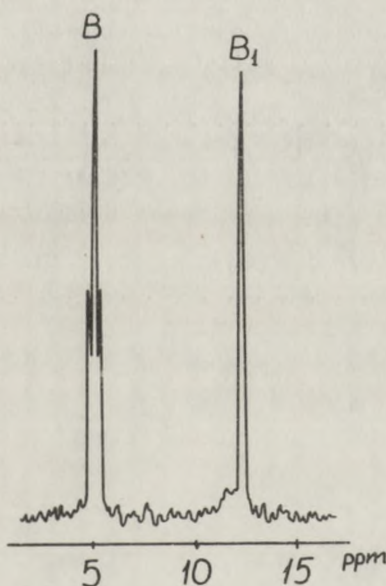
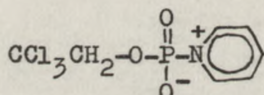


Fig.6. ^{31}P NMR spectra of the active monomeric derivatives of pT-Ac (B) and of $pPhNO_2$ (B_1) recorded without heteronuclear spin-spin decoupling $^{31}P-\{^1H\}$

Spectrum of B_1 remains unsplitted, although spin-spin interaction with about 10Hz should be expected for P-$\overset{+}{N}$=C-H system in the case of the structure V. Such splitting was found for compounds with P-Ph bond with isoelectronic P-C=C-H system [14]. The possibility of the formation of the structure V was earlier

investigated by Eckstein and Rizk [12]. The authors found no difference between the spectra of $CCl_3CH_2OPO_2Cl$ in pyridine and tetrahydrofuran solutions. In the case of the formation of phosphoryl pyridinium compound



of the type V, the spectra in these two solvents should differ significantly.

Therefore the active monomeric derivatives of mononucleotides and some other phosphates are most probably monomeric metaphosphates.

It should be noted that this is not the case for all esters of phosphoric acid. In the course of investigation of the reaction of TPS with phosphates bearing less spacious radicals, namely ethyl phosphate and cyanoethyl phosphate it was found that in the late period of the reaction, significant amounts of the compound with signal at $\delta \approx 25$ ppm was accumulated besides the compound of type B. The spectrum of the final reaction mixture is shown in fig.7.

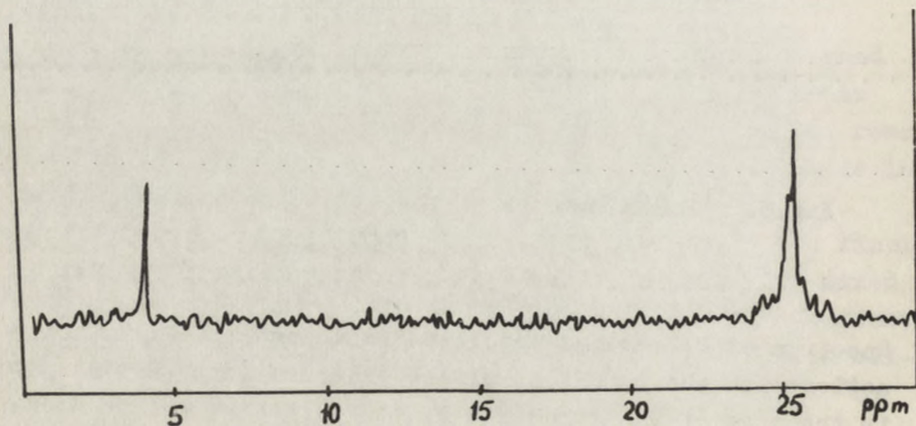
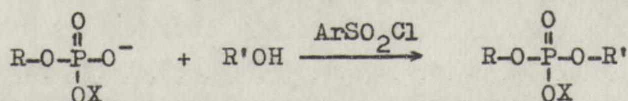


Fig.7. ^{31}P NMR spectrum of the reaction mixture of O.II M ethylphosphate with 0.3 M TPS in 40 min after beginning of the reaction.

Probably cyclic trimetaphosphates are formed in the reaction mixture in these cases.

2. Active phosphorylating derivative of the nucleotide component in the triester method of the oligonucleotide synthesis.

The last years the triester approach appears to be widely used in the oligonucleotide synthesis. In this method phosphory residue of a nucleotide component is esterified with some protecting group stable to all intermediate procedures used till the final oligonucleotide chain is obtained. This permits to prevent by-processes at the internucleotide phosphoryl residues in the course of the next steps of the synthesis. Aryl sulphony chlorides are mainly used as condensing reagents in the trieste method. The net reaction may be represented as follows



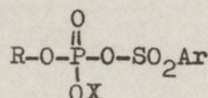
R,R' - nucleoside or oligonucleotide residues

X - protecting group $N{\equiv}C{-}C_2H_4-$, Cl_3CCH_2-, CH_3SPh-, $PhCH_2-$

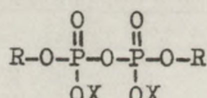
Interaction of aryl sulphonyl chloride with phosphodiester group is the first step of the process.

This reaction is also important in connection with usual diester approach to the oligonucleotide synthesis. In this case the reaction of aryl sulphonyl chloride with internucleotide phosphodiester group of oligonucleotide used as a nucleotide or a nucleoside component may accompany the main reaction of a new internucleotide bond formation resulting in some by-processes. It was found that oligonucleotide chain is partially splitted by treatment of oligonucleotides with aryl sulphonyl chlorides [15, 16] . However , the structure and properties of the intermediates formed till the last time remained unestablished.

The active reagents of the reaction of the phosphoric acid diesters with $ArSO_2CI$ may be most probably compounds with structure VI or VII

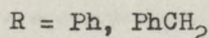


(VI)

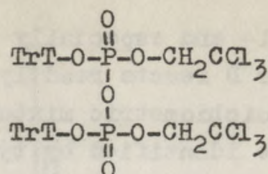


(VII)

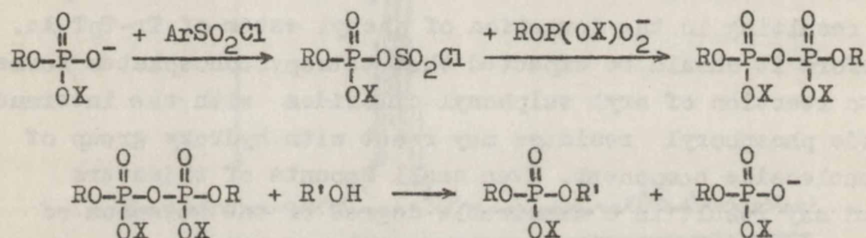
The reaction of lithium diphenylphosphate with p-toluene sulphonyl chloride was investigated by measuring the electric conductivity in dimethylformamide solution [17]. The amount of LiCl formed as revealed by the increase in conductivity corresponds to the consumption of one mole of $ArSO_2Cl$ per two moles of the diester. This corresponds to accumulation of VII as a main product. It may be thought that the mixed anhydride VI formed as a primary product is immediately attacked by second diester molecule forming more stable tetrasubstituted pyrophosphate. Tetraphenyl pyrophosphate and tetrabenzyl pyrophosphate were obtained independently by the reaction of the corresponding diesters with their chlorides



Eckstein and Rizk investigated the reaction of β,β,β-trichloroethyl ester of 5'-O-tritylthymidine-3'-phosphate with TPS using ^{31}P NMR spectroscopy [12]. It was found that a compound with $\delta = 33$ ppm is formed as a single product capable of phosphorylation of the hydroxy group of a nucleoside component. The chemical shift of this phosphorylating compound was found to be similar to that of tetra-(β,β,β-trichloroethyl)-pyrophosphate ($\delta = 34.5$ ppm), prepared starting from bis-(β,β,β-trichloroethyl)-phosphate and its chloride. Therefore, the phosphorylating intermediate was identified as a tetrasubstituted pyrophosphate



The complete scheme of the internucleotide bond formation via triester method may be consequently represented as follows



The formation of tetrasubstituted pyrophosphate in the course of the reaction of dinucleoside phosphate Tr-TpT-Ac with TPS was demonstrated by us using ^{31}P NMR pulse spectroscopy [18]. A single new signal with δ= 13.8 ppm was found in the NMR spectrum of the reaction mixture of Tr-TpT-Ac and TPS in pyridine. The range is typical of pyrophosphate group. The same signal appeared in the case of the reaction of Tr-TpT-Ac with cross-linked polymeric sulphonyl chloride thus proving that compound formed (compound D) does not contain aryl sulphonyl residue [13].

Tetrasubstituted pyrophosphates were found to differ significantly in their phosphorylating ability. Tetrabenzylpyrophosphate capable to phosphorylate amines is known to be poorly reactive towards hydroxy groups [19]. In the same time tetra-(p-nitrophenyl)-pyrophosphate readily phosphorylates various alcohols [20]. The reactivity of tetraesters of pyrophosphoric acid correlates with a partial positive charge of P atoms influenced by the nature of substituents. In the case of unsymmetric pyrophosphates, reaction proceeds mainly at P atom of weaker acid thus eliminating anion of stronger acid.

Phosphorylating ability of tetrasubstituted pyrophosphate derived from Tr-TpT-Ac (compound D) was investigated using pulse ^{31}P NMR spectroscopy. This compound should be less reac-

tive than tetraphenyl- and especially tetra-(p-nitrophenyl)-pyrophosphate. However D reacts readily with NH_3 and cyclohexylamine forming stoichiometric mixture of Tr-TpT-Ac and its amide. The latter were identified by typical values of the chemical shifts (-II.5 for amide and -9.0 for cyclohexylamide) and zero electrophoretic mobility.

The reaction of D with excess of phenol was shown to proceed resulting in the formation of phenyl ester of Tr-TpT-Ac. Therefore it should be expected that tetrapyrophosphates formed due to reaction of aryl sulphonyl chlorides with the internucleotide phosphoryl residues may react with hydroxy group of the nucleoside component. Even small amounts of triesters formed may result in a measurable degree of the scission of the oligonucleotide chains in the course of the subsequent treatments of the reaction mixture especially with long oligonucleotides.

3. The reaction of the active phosphorylating derivative of mononucleotide with hydroxy group of a nucleoside component and with phosphodiester groups

To elucidate the role of the active phosphorylating derivative B of mononucleotide in the oligonucleotide synthesis, the reaction of this compound with 5'-O-tritylthymidine (TrT) was investigated [2I]. The addition of 0.5 moles of TrT per mole of B within several minutes results in significant decrease of the signal at $\delta = 5.I$ ppm in ^{3I}P NMR spectrum of the reaction mixture with simultaneous appearance of a multiplet in the range II-I3 ppm consisting of five lines. The constancy of the ratia of the intensities of these lines and the typical spin-spin splitting permit to relate this multiplet to one compound C. The ^{3I}P NMR spectrum of the reaction mixture after 80 min of the reaction is represented in fig. 8. The kinetic curves of accumulation of C and of consumption of B are represented in fig.9.

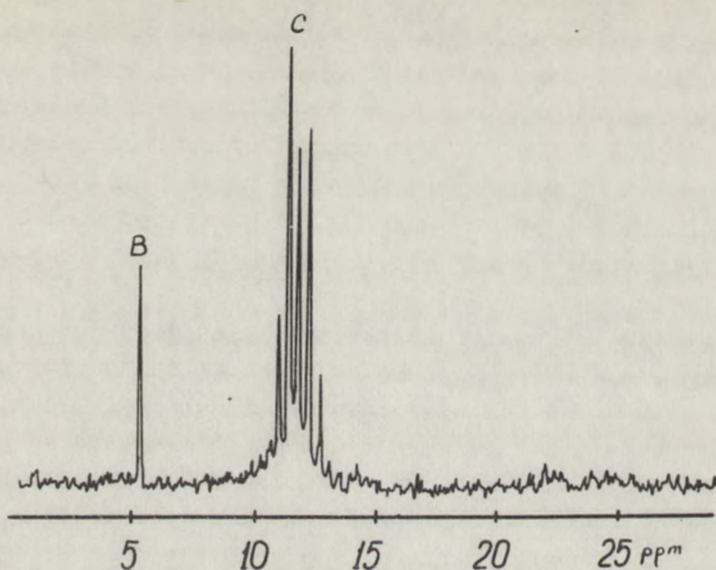


Fig.8. ^{31}P NMR spectrum of the reaction mixture of compound B + 0.5 eqv. TrT (in 80 min after Tr-T addition).

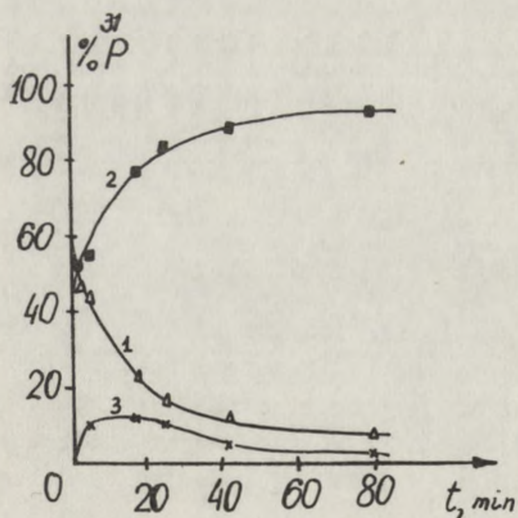


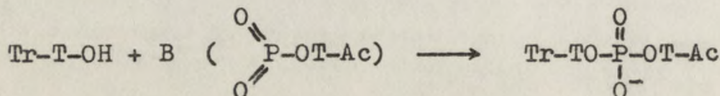
Fig.9. Kinetic curves of the reaction of compound B (0.I5 M) with (0.075 M) Tr-T
I)consumption of B, 2)accumulation of C,
3)accumulation of additional signals nearly 24 ppm.

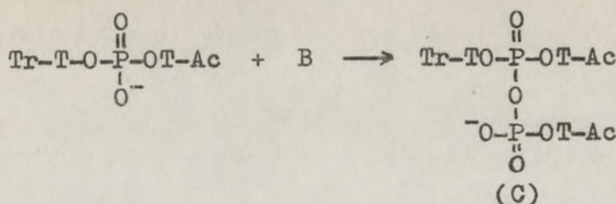
Treatment of the solution of C with water results in immediate conversion of C to equimolar amounts of Tr-TpT-Ac and starting pT-Ac. Therefore, C contains Tr-TpT-Ac as a fragment. The occurence of the C signal in the range typical of substituted pyrophosphates and spin-spin splitting typical of P-O-P group with unequivalent P atoms permit to identify compound C as P^{I}-5'-O-tritylthymidine-P^{I},P^{2}-bis-(3'-O-acetylthymidine)-pyrophosphate.

The detailed structure of the multiplet does not contradict this assumption. Taking in account the chemical shift values of Tr-TpT-Ac (δ = I.0 ppm) and of pT-Ac (δ = -I.2 ppm) and the difference between δ values of pT-Ac and corresponding pyrophosphate $O(pT\text{-}Ac)_2$ $\Delta\delta$= II.5 ppm, the chemical shifts of P^{I} and P^{2} atoms of the structure proposed are expected to be δ_{I}=I2.5ppm, δ_{2} = I0.3 ppm. The difference $\delta_{I} - \delta_{2}$ expressed in Hz is of the same order of magnitude as the spin-spin coupling constant of P-O-P group. Therefore, the spectrum of the proposed structure should be that of AB system with four lines with intensities equal in pairs for outer and inner lines. The more complicated structure of the signal is readily explained by occurence of two diastereoisomers due to assymetric P^{I} atom and assymetric atoms of fixed configuration in ribose residues.

Therefore, it may be concluded that the first compound accumulated in significant amouts sufficient for ^{31}P NMR investigations in the reaction mixture containing B and Tr-T is trisubstitu ted pyrophosphate C.

In separate experiments it was demonstrated that addition of Tr-TpT-Ac to B solution results in immediate formation of C. Therefore, it is reasonable to assume that Tr-TpT-Ac formed as a primary product of the reaction of B with TrT converts immediately to C due to reaction with a second B molecule. The complete scheme of the reaction may be represented as follows





According to this scheme, two moles of B are consumed per mole of pT-Ac.

The similar reactions were found for a variety of other phosphoric acid derivatives. For instance, the treatment of β-cyanoethyl-methylphosphate with the active monomeric derivative of β-cyanoethylphosphate results in the appearance in the ^{31}P NMR spectrum of the reaction mixture of multiplet containing four lines (fig. IO) typical of AB system. The spectrum agrees with that

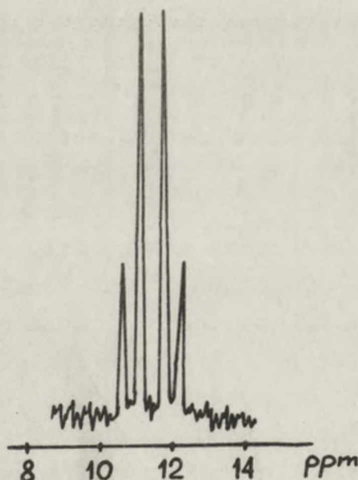
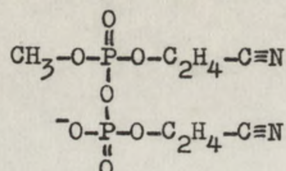


Fig. IO. ^{31}P NMR spectrum of the product of the reaction of methyl-β-cyanoethylphosphate with the monomeric phosphorylating derivative of β-cyanoethylphosphate.

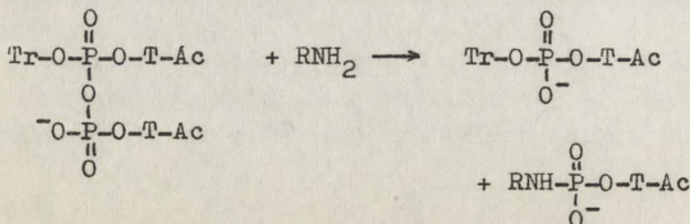
expected for trisubstituted pyrophosphate of the structure



in this case without any complications connected with diastereoisomerism.

In the synthesis of dinucleosidephosphates, the phosphodiester group formed is the single group of this type. In the course of the synthesis of longer oligonucleotides by diester method diesterified phosphoryl residues are already present in nucleoside or nucleotide components. Therefore, the formation of trisubstituted pyrophosphates of the type C should be unescapable process in the oligonucleotide synthesis by diester approach and the knowledge of their properties is of a great interest in this connection.

The compound C was shown to contain rather reactive mononucleotide residue. It was already mentioned that it reacts with water converting to stoichiometric amounts of Tr-TpT-Ac and pT-Ac. The reaction with mononucleotide pT-Ac results in the formation of equal amounts of Tr-TpT-Ac and of $O(pT\text{-}Ac)_2$. Cyclohexylamine converts C to equal amounts of Tr-TpT-Ac and C_6H_{11}NH-pT-Ac. These reactions are readily seen by immediate changes of the ^{31}P NMR spectrum of the reaction mixture as well as by usual chemical means. For instance, cyclohexylamide of pT-Ac demonstrate a typical singlet shifted to lower field ($\delta = -4.8$). It is seen that all reactions of C with nucleophiles proceed at ionised phosphoryl group with elimination of the anion of stronger acid. For example



The question of especial interest is the possibility of the compounds C to participate in the formation of new internucleotide bonds. To answer this question the reaction of C with additional amount of Tr-T was investigated. In 20 hours only 30% of mononucleotide residues converted to dinucleosidephosphate with excess of Tr-T. Consequently, C may participate in the oligonucleotide synthesis; however it is low reactive towards nucleoside component.

The data presented demonstrate that compound C is a phosphorylating reagent similar to P^I,P^I-diphenyl-P^2-nucleoside-5'-pyrophosphates widely used to prepare amines and nucleotide anhydrides [22].

Two mechanisms were suggested for the reaction of trisubstituted pyrophosphates with nucleophiles: (I) the attack of a nucleophile at monosubstituted P^2 atom of the reagent or (2) reversible dissotiation of the reagent to diester and monomeric metaphosphate, the latter being actually a phosphorylating compound.

It is reasonable to expect that with longer nucleoside components containing phosphodiester groups the monomeric phosphorylating reagent should be consumed in the first line by the reaction with these groups thus converting to significantly less reactive trisubstituted pyrophosphates. Therefore, to obtain high yields of oligonucleotides the sufficient excess of phosphorylating reagent has to be used. In the general case with nucleoside component with n internucleotide bonds one must use no less than n + 2 moles of the reagent per one mole of the nucleoside component (n moles to attack all phosphodiester groups , one to form a new internucleotide bond and one mole to react with new formed bond).

In accordance with this conclusion A.S.Levina and T.N.Shubina in our laboratory performed the syntheses of dinucleotides using $(NCEt)_2pT$ and (NCEt)pT as nucleoside component with correspondingly 2.2 and 3.3 eqv. of the compound B. In both cases the ^{31}P NMR spectra of the reaction mixtures represented complicated multiplets in the range typical of trisubstituted pyrophosphates. Decomposition of the reaction mixtures with water resulted in the first case in the formation of equimolar amounts

of $(NCEt)_2$pTpT-Ac and pT-Ac and in the second case in the formation of (NCEt)pTpT-Ac and pT-Ac in I:2 ratio. The results agree with the structures IX and X expected for the products of the reactions of $(NCEt)_2$pT and (NCEt)pT with B

```
             O        O                          O        O
             ‖        ‖                          ‖        ‖
N≡CEt-O-P-O-T-O-P-O-T-Ac          N≡CEt-O-P-O-T-O-P-O-T-Ac
             |        |                          |        |
   N≡CEt-O            O                          O        O
                      |                          |        |
                   ⁻O-P-O-T-Ac                ⁻O-P=O   ⁻O-P=O
                      ‖                          |        |
                      O                          O-T-Ac   O-T-Ac

          (IX)                                     (X)
```

In both cases nearly quantitative yields of dinucleotide were obtained.

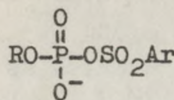
4. <u>Interconversions of the active derivatives of mononucleotide in the excess of aryl sulphonyl chloride in the presence of a nucleoside component.</u>

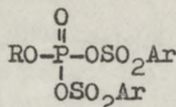
It is known that the reaction rate of the internucleotide bond formation is significantly greater with aryl sulphonyl chlorides than with DCC. According to authors of trimetaphosphate hypothesis this may be explained by subsequent reaction of trimetaphosphate with the excess of $ArSO_2Cl$ resulting in the formation of the structures

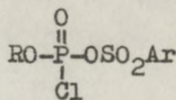
```
              O                                  O   OR
              ‖                                   ⸌P⸍
  O       O⸍  P  ⸌O      O                       O   O
   ⸌P⸍        |       ⸌P⸍                  RO    |   |  OSO2Ar
 RO   Cl      OR     O    OR                 ⸌P     +P⸍
                     |                      O⸍  ⸌O⸍   ⸌OR
                     SO2Ar

          (XI)                                  (XII)
```

XI [23] or XII [24].

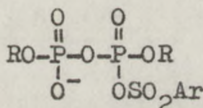
Structure XI is believed to disproportionate to several reactive derivatives with one or two nucleotide residues

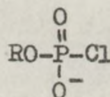






(I) (XIII) (XIV)





(XV) (XVI)

The main reason for the latter assumption was that the treatment with water of the reaction mixture of mononucleotide with 2-2.5 eqv. of TPS resulted in the formation of the starting mononucleotide as a single product.Metaphosphate has to convert in the same conditions via tripolyphosphate to symmetric pyrophosphate and mononucleotide in I:I ratio.

As it may be seen from the data represented in the first section the main idea of these explanations is wrong. It was demonstrated, that the final product of the reaction of mononucleotide with TPS is a monomeric derivative [5,6] without any arylsulphonyl residues [I3].

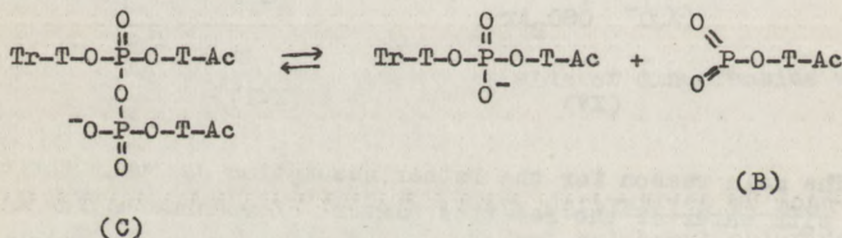
Therefore, the reason of the enhanced reactivity of aryl sulphonyl chlorides is not connected with the appearance of some additional reactive derivatives of mononucleotide but rather with some additional processes in the course of the oligonucleotide synthesis proceeding in the excess of aryl sulphonyl chloride.

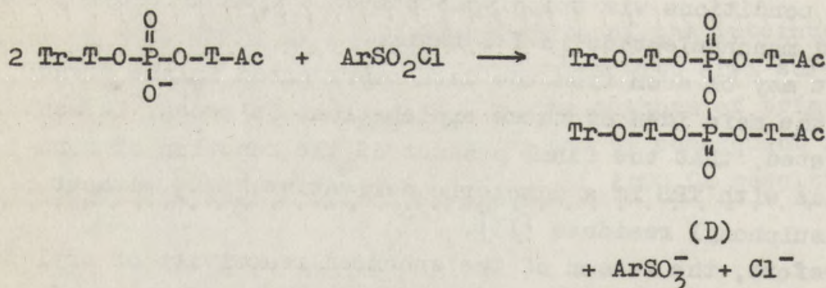
It was already demonstrated that in the presence of a nucleoside component a significant part of the active derivative of mononucleotide converts to trisubstituted pyrophosphate (C) thus decreasing the reactivity of the nucleotide component. Therefore, it was reasonable to look for some reactions of C

with aryl sulphonyl chloride.

It was found [I8] that the addition of TPS to the C solution results in one hour in the conversion of the main part of C to stoichiometric amounts of the compounds B and D.

The mechanism of this conversion may be represented as follows. Compound C exists probably in the equilibrium with dissociation products - Tr-TpT-Ac and compound B displaced almost completely to the left. In the presence of aryl sulphonyl chloride the conversion of Tr-TpT-Ac to compound B proceeds thus displacing the equilibrium to the decomposition of C according to scheme





These data permit to expect that the high yields of the internucleotide bond formation may be obtained using the excess of TPS as well as the excess of the reactive derivative of the nucleotide component, as it was mentioned in the third section of the report. In the excess of TPS reactive compound B is continiously regenerated from compound C thus making possible further reaction with hydroxy group of a nucleoside component.

In accordance with these considerations nearly quantita-

tive yield of dinucleotide $(NCEt)_2$pTpT-Ac was obtained using equimolar amounts of $(NCEt)_2$pT and pT-Ac with 4-fold excess of TPS.

However this approach to high yield in the oligonucleotide synthesis is inevitably connected with the formation of tetra-substituted pyrophosphates capable of the phosphotriester production. The latter may be the reason of the scission of the internucleotide bonds at following treatments of the reaction mixture.

5. The nature of the active phosphorylating derivatives of dinucleotides

When di- or longer oligonucleotides are used as nucleotide components, it should be expected that internucleotide phosphoryl residues of these components have to participate in transformations already in the early stages of the process. No significant difference was found between reaction rates of TPS with pT-Ac and Tr-TpT-Ac. Therefore, one may expect that in the dinucleotide molecule both monoester and diester phosphoryl residues should be attacked by aryl sulphonyl chloride with similar rates. Due to high reactivity of both primary products rapid intramolecular reaction with another untouched phosphoryl residue should be expected , for instance for pTpT-Ac (the mixed anhydride at phosphomonoester P atom is believed to convert immediately to metaphosphate and aryl sulphonic acid)

$$^{-}O-\overset{O}{\underset{O^-}{P}}-O-T-O-\overset{O}{\underset{O^-}{P}}-O-T-Ac \quad + \quad ArSO_2Cl$$

$$\swarrow \qquad \searrow$$

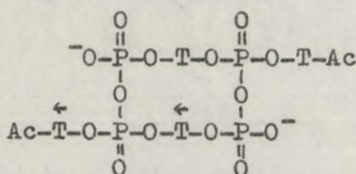
$$\begin{matrix} O_2P-O-T-O-\overset{O}{\underset{O^-}{P}}-O-T-Ac & \qquad & ^{-}O-\overset{O}{\underset{O^-}{P}}-O-T-O-\overset{O}{\underset{OSO_2Ar}{P}}-O-T-Ac \\ \text{(XVII)} & & \end{matrix}$$

$$\searrow \qquad \swarrow$$

$$T-O-\overset{O}{P}-O-T-O-Ac \text{ (cyclic, with } O-P(=O)(O^-)-O \text{ bridging T and P)}$$

(XVIII)

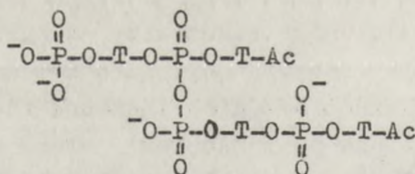
One mole of aryl sulphonyl chloride per mole of dinucleotide is consumed in this reaction. With less amounts of TPS the non-reacted dinucleotide present may react with both XVII or XVIII to produce symmetric pyrophosphate. At rather high concentrations usually employed in the oligonucleotide synthesis some other compounds containing trisubstituted pyrophosphate fragments (fragments of the type C) may accumulate due to intermolecular reactions. These may be compounds of the cyclic structure XIX



(XIX)

($\overleftarrow{T}$ indicates thymidine residues with 3' end at the left and 5' end at the right side)

as well as of the linear structures XX



(XX)

with two or more pTpT-Ac residues. In the structures of the latter type the monoesterified phosphoryl residue most probably does not exist in a free form but rather participates in the formation of disubstituted pyrophosphate or longer polyphosphate fragments.

Therefore, a complicated mixture of the compounds containing fragments of the type C should be expected.

The ^{31}P NMR investigations of the reaction of TPS with pTpT-Ac proved the above considerations. As it may be seen in fig.II the spectrum of the reaction mixture of 0.I2 M pTpT-Ac

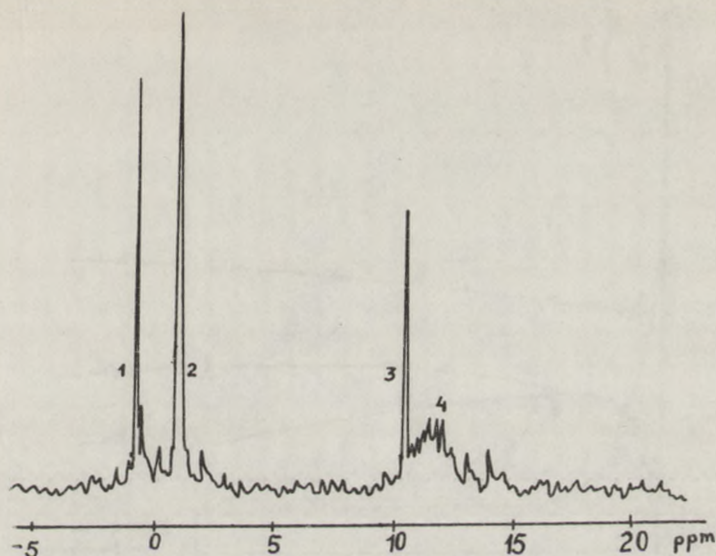


Fig. II. ^{31}P NMR spectrum of the reaction mixture of pTpT-Ac + + 0.5 eqv. of TPS I) unreacted end phosphate, 2) internucleotide phosphate, 3) symmetric pyrophosphate, 4) signal of the type C.

with 0.5 eqv. of TPS besides the singlet at δ = I0,3 ppm corresponding to symmetric pyrophosphate O(pTpT-Ac)$_2$ contains a multiplet in the range of I0,5-I3,5 ppm typical of the fragments of the type C present in the structures XVIII, XIX, XX. Kinetic curves of the reaction (fig.I2) demonstrate parallel accumulation of symmetric pyrophosphate and of fragments of the type C. In the later stage of the reaction some conversion of these fragments to O(pTpT-Ac)$_2$ may be noticed.

With greater amounts of TPS the formation of the fragments of tetrasubstituted pyrophosphate (fragments of the type D) may be expected. The signals of these fragments in the ^{31}P NMR spectrum of the reaction mixture may partially overlap those of the fragments of the type C. The NMR spectrum in this range is rather complicated and up-to-now can not be interpreted unequivocally. However, the sum of C + D fragments may be esti-

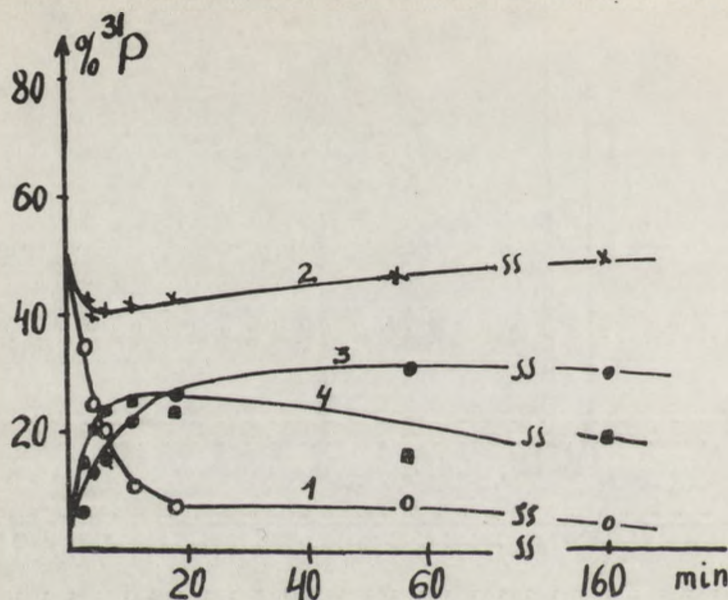


Fig. I2. The kinetic curves of the reaction of pTpT-Ac with 0.5 eqv. of TPS I) unreacted end phosphate, 2) internucleotide phosphate, 3) symmetric pyrophosphate, 4) signal of the type C.

mated by measuring the integral intensity of the lines in the range of I0,5-I5 ppm. With excess of TPS more than 2 eqv. per mole of dinucleotide there appears a signal at 5.2 ppm typical of metaphosphate (fragment of the type B). The spectrum of the reaction mixture obtained by reaction of 4.6 eqv. of TPS with pTpT-Ac is represented in fig. I3.

To simplify the interpretation of the data obtained, the reaction mixtures may be treated with amines. It was already mentioned that trisubstituted pyrophosphates react with amines converting exclusively to amides of the monoester component. The other half of C is converted to the nonmodified diester fragment. Keeping in mind that the fragments of the type B must also convert to monoester amides (MEA) the amount of the latter has to be equal to the sum $MEA = B + \frac{1}{2}C$

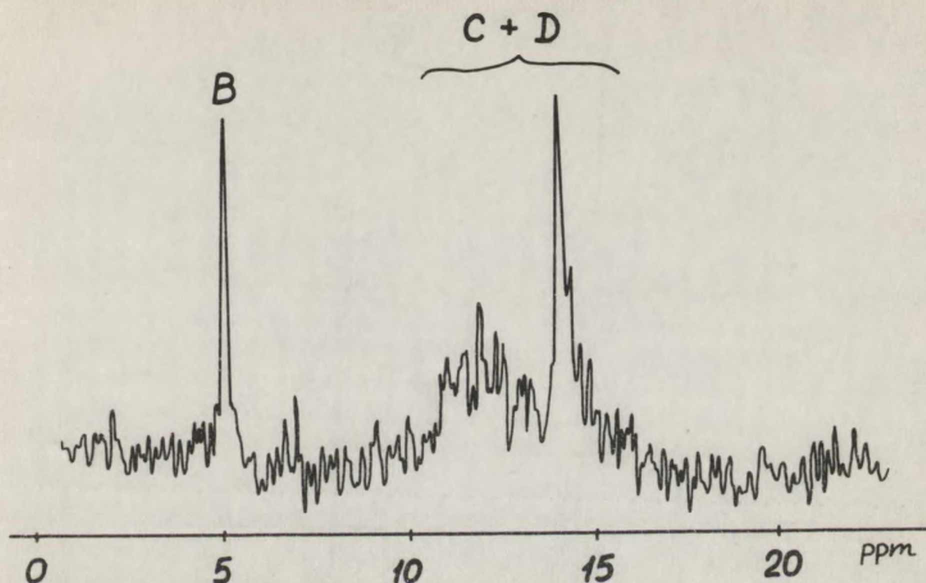


Fig.13. ^{31}P NMR spectrum of the reaction mixture of pTpT-Ac + 4.6 eqv. of TPS

Tetrasubstituted pyrophosphates are converted by amines to equimolar amounts of diester amide (DEA) and diester (DE). Therefore

$$DEA = \frac{1}{2}D , \quad DE = \frac{1}{2}(C + D)$$

The ^{31}P nmr spectrum of the reaction mixture obtained by the treatment of pTpT-Ac with excess of TPS and then with cyclo hexylamine is represented in fig.14.A signal at $\delta \approx -9.0$ ppm similar to that of cyclohexylamide of Tr-TpT and two signals at -5.8 and -5.0 ppm in the range typical of monoester cyclohexylamide are seen in the spectrum.Only the signal correspon-ding to monoester amide appears after amine treatment of the reaction mixture obtained with stoichiometric amount of TPS. The changes in the NMR spectra agree with the above conside-rations.The reason of the occurence of two distinct signals in the monoester amide range remains up-to-now unclear.

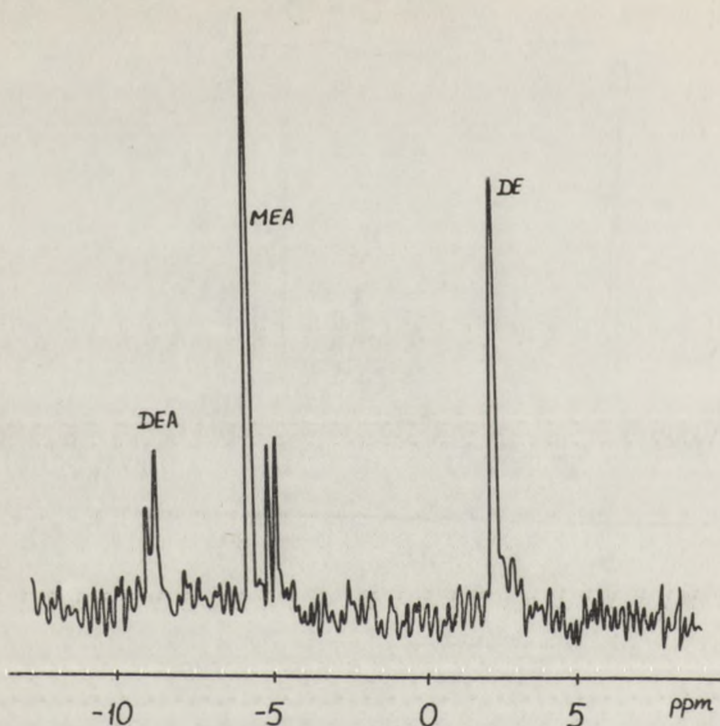


Fig.14. ^{31}P NMR spectrum of the reaction mixture of pTpT-Ac + 4.6 eqv.of TPS after addition of cyclohexylamine. MEA - phosphomonoester amide; DEA - phosphodiester amide; DE - internucleotide phosphate.

The results of some experiments are represented in the table. In the same table in parantheses are given the expected values for the composition of the final mixture calculated from the composition of the mixture before addition of amine. To calculate these values it was assumed that the numbers of P atoms participating in B and D fragments were equal. This assumption seems to be reasonable for the reaction mixtures

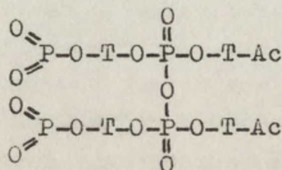
containing three types of fragments namely B,C and D, for the total number of the end and internucleotide phosphoryl residues are equal as well as the number of these residues participating in the C fragments. The agreement between the calculated and experimentally found composition of the final reaction mixture is within the limit of the accuracy of integration of the spectra.

T a b l e

Percent of various types of phosphoryl residues in the reaction mixtures obtained by treatment of pyridine solution of pTpT-Ac with TPS before and after addition of cyclohexylamine (CHA)

pTpT-Ac / TPS	CHA addition	symm. pyrophosph.	B	C + D	DE	MEA	DEA
I:I	-	25	-	44	3I	-	-
	+	27	-	-	50	23	-
I:2	-	-	7	93	-	-	-
	+	-	-	-	43(46)	48(50)	8(4)
I: 7.6	-	-	3I	69	-	-	-
	+	-	-	-	30(34)	48(50)	22(I6)

Therefore, it may be concluded that two types of the active derivatives of dinucleotides accumulate in the reaction mixture. Derivatives of the type C are formed already at low amounts of TPS with the reactive monoester group. Derivatives of the type BD for instance



(XXI)

with both metaphosphate and tetrasubstituted pyrophosphate fragments are accumulated at high excess of TPS capable of producing derivatives of the end as well as of the internucleotide residues.

Similar to compound C, the first type of the derivatives may be thought to be poorly reactive in the oligonucleotide synthesis It was found that the reaction of the active derivative of pTpT-Ac of the type C with twofold excess of Tr-T resulted in the conversion of only 30% of the derivative to Tr-TpTpT-Ac. Therefore, even with the excess of the nucleoside component the yield of trinucleoside diphosphate is rather poor.

In the same time using the reaction mixture obtained with pTpT-Ac to TPS ratio I:4.6 near 90% yield of Tr-TpTpTAc was reached with the same excess of Tr-T. Consequently the derivatives of the BD type are highly reactive in the oligonucleotide synthesis.

Therefore, two severe complications may accompany the oligonucleotide synthesis by diester method with dinucleotide or longer oligonucleotide as the nucleotide components.

The first is that an active form of the monoester phosphoryl residue may accumulate only with simultaneous accumulation of the same amounts of the fragments of the type D with reactive internucleotide residues. The latter may compete with the fragments of the type B for hydroxy group of the nucleoside component producing triesters. The hydrolysis of the triester groups in the course of subsequent treatments of the reaction mixture may produce oligonucleotides of wrong lengths and even with a wrong type of the internucleotide bond (3'→ 3').

The second is that a great excess of aryl sulphonyl chloride must be used to convert dinucleotide to reactive BD form. This excess exceeds significantly stoichiometric amount (I.25 eqv. of TPS is necessary theoretically to convert one mole of pTpT-Ac to XVII). Therefore, the most part of TPS remains most probably nonreacted and may attack the internucleotide phosphoryl residues of a nucleoside component providing scissions of phosphodiester bonds. Of course, only detailed kinetic investigation may permit to estimate the real scale of these complications.

The represented considerations agree with the observations of J.Hachmann and H.G.Khorana [I6]. These authors demonstrated that the significant yield of octanucleotide Tr-T-$(pT)_7$-Ac may be obtained from Tr-T-$(pT)_5$and pTpT-Ac using either a moderate excess of aryl sulphonyl chloride and I0-20 fold excess of pTpT-Ac, or with twofold excesses of dinucleotide and I0 fold excess of the condensing reagent.

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SOME RECENT IMPROVEMENTS IN THE SYNTHESIS OF DEOXYOLIGONUCLEOTIDES

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Despite the significance of deoxyribonucleic acids, the number of laboratories where bihelical polydeoxynucleotides with defined sequence and meaningful genetic information are in synthesis or have been synthesized is still less then five [1]. The reason for this fact lies in the tremendous difficulties encountered, and the expenditure of work required during the chemical synthesis of the short oligodeoxyribonucleotides suitable for the enzymatic joining which is catalyzed by polynucleotide ligase.

I may sum up the main problems with which one is faced using the method of Khorana and his coworkers [2], which is the most reliable procedure developed to date:

1) To synthesize a long bihelical polydeoxynucleotide it is essential to synthesize a large number of different building blocks. A rather complicated chemistry of protecting groups is necessary in order to establish the right 5'-3'-internucleotidic linkage. The expenditure of work to fulfil this task is very great.
2) The condensation reaction is not quantitative. In order to get reasonable yields, a strong activation of the protected mono-, di- or trinucleotides is necessary. It may be added that yields between 40 and 70 % which are good for low molecular weight organic chemical

reactions are not good enough for the synthesis of a macromolecule, as several successive reaction steps have to be employed. During the strong activation of the phosphate component, the sugar- and heterocyclic moiety, and the internucleotidic linkage are unfortunately not inert. This leads to several side products.

3) A time-consuming chromatographic purification is therefore necessary. In consequence of the possible similarities of the compounds in the reaction mixture, and the low separation capacity of DEAE-cellulose, the purification of larger amounts of the desired condensation product and the recovery of the nonreacted starting compounds is often very difficult to achieve.

The time-consuming chromatography can be very much reduced by using polymeric carriers [3], but the condensation yield and the reduction of side-products has to be improved by a better condensation technique. Although work is in progress along these lines in our laboratory, I want to concentrate in this lecture on some improvements of Khorana's method.

We developed these improvements during synthesis of a bihelical polydeoxynucleotide with the genetic information for the peptide hormone angiotensin II. For brevity, this short DNA will be referred to as the angiotensin gene.

The strategy used for the synthesis of the oligonucleotide chains for the angiotensin gene is to prepare the tri- or tetranucleotides using mononucleotide units and then to extend these short chains with blocks usually dinucleoside diphosphates or trinucleoside triphosphates. This has the advantage of one being able to use the much more easily obtainable protected mononucleotides during that phase of the chain synthesis when large quantities have to be used. Besides this, the condensation yields using mononucleotides are always higher than those using oligonucleotides. Moreover we can use the dinucleoside monophosphates and trinucleoside diphosphates, which are intermediates in the chain synthesis, for the synthesis of dinucleoside

diphosphates and trinucleoside triphosphates by the direct phosphorylation procedure which I will discuss later on. This way is therefore the most economical. It has, however, the disadvantage of difficult chromatographic purification using large amounts, because in the synthesis of trinucleoside diphosphates the product, the phosphate component, and its pyrophosphate have almost the same charge, and hence are rather difficult to separate using anion-exchange chromatography [4].
This is shown by figure 1.

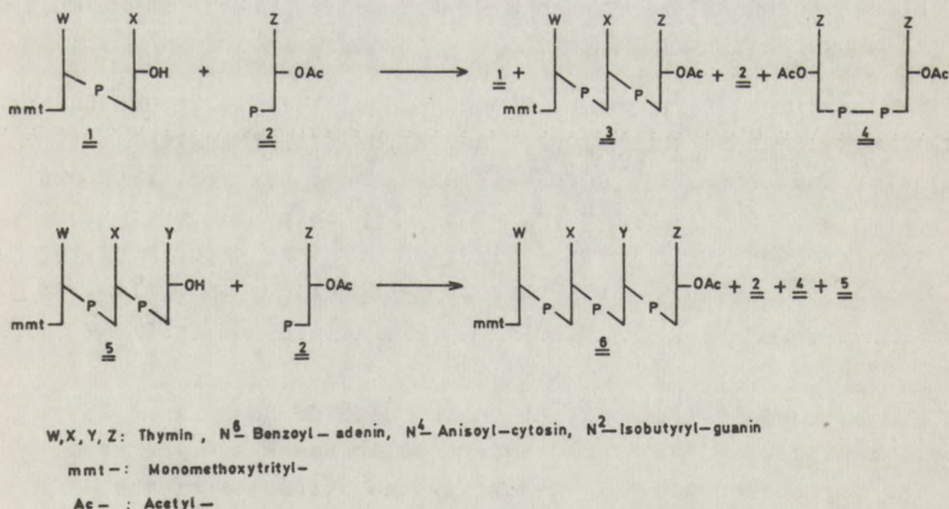


Figure 1

Khorana introduced the use of tritylated cellulose to do this job. In this case, however, one has to use three different column chromatographies if one wants to get the product <u>and</u>

the non-reacted starting materials in a pure form, which of course would be desirable.

We decided to develop a separation technique to do this with only one column chromatography.

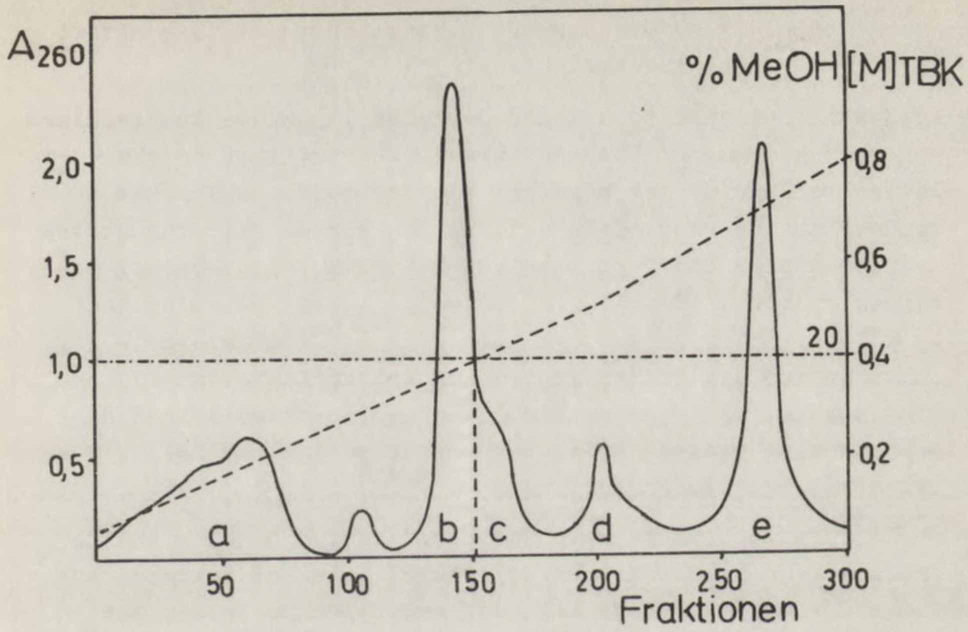
We first chose DEAE-Sephadex A 25 for it is known that Sephadex has a pronounced affinity for aromatic compounds, and moreover anion-exchange chromatography is possible with this matrix. Therefore adsorption phenomena are superimposed on ion-exchange chromatography, and it should be possible to discriminate between compounds having the same charge and differing only in the presence or absence of a 5'-monomethoxytrityl group.

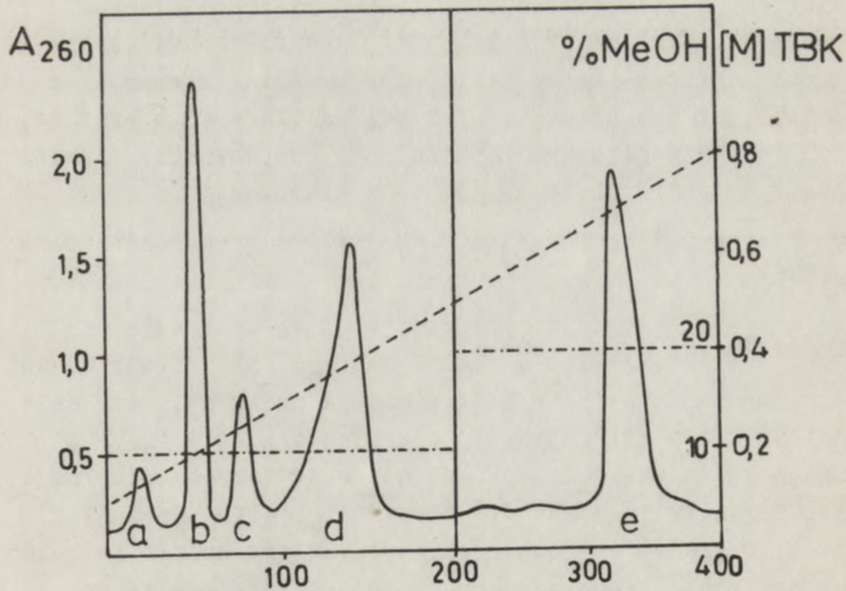
We tried this idea during synthesis of MMTr-TpTpTpT+) by a block condensation between MMTr-TpT and pTpT(Ac). The result is shown in figure 2.

Taking first the upper part one can see that, although having almost the same charge, pTpT and MMTr-TpTpTpT are well separated using 20 % methanol throughout. However, pTpT and MMTr-TpT are only separated using 10 % methanol, as is shown in the lower part of figure 2. By this experiment, we learnt the delicate influence of methanol concentration, as with 20 % methanol no separation of pTpT and MMTr-TpT is possible.

The strength of this effect in the case of Sephadex A 25 is remarkable. As all the heterocyclic bases are the same, the phenomenon can only be due to the stickness of the aromatic monomethoxytrityl group compensating for about two charges. At low alcohol concentrations the effect can be used to great advantage. At alcohol concentrations sufficiently high to overcome adsorption, the separation will be according to

+) The prefix 'd' for deoxy is omitted throughout this paper as it deals only with deoxyoligonucleotide chemistry.





a: TIPS-OH b: pTpT c: MMTr-TpT
d: $O(pTpT)_2$ e: MMTr-TpTpTpT

Figure 2

charge only. It is obvious that the strength of this effect is a function of the chain length.

Although the capacity of DEAE-Sephadex is three- to fivetimes as great as that of DEAE-cellulose, we went back to the latter because of the high molarity of buffer which has to be used in the case of Sephadex A 25. Fortunately the above-mentioned chromatographic principle holds, although not so strongly, for DEAE-cellulose as well. It may be added that in all our later chromatographic work we used triethylammonium acetate buffer instead of triethylammonium bicarbonate, as by using the latter we observed a loss of N-protecting groups during column chromatography and evaporation of the fractions.

Now I want to come back to the purification problem in the synthesis of 5'-monomethoxytritylated tri- and tetranucleotides. If one considers all four heterocyclic bases, the picture is a little more complicated by the different hydrophobicities of C^{An}, A^{Bz}, G^{iBu} and T. The different hydrophobicities can be dealt with by using mixtures or gradients of two alcohols with very different polarities, which is the case using methanol and isopropanol. I shall illustrate the technique with some examples.

The trinucleotides are first prepurified by an extraction procedure.

Figure 3 shows the elution profile after synthesis of MMTr-A^{Bz}pTpA^{Bz}. At low methanol content, pA^{Bz} (peak C) and its pyrophosphate (peak E) are eluted using a linear salt gradient. Then the methanol concentration is increased rapidly while the salt concentration is increased using a shallow linear gradient. If the trinucleotide (peak F-K) begins to elute, the ionic strength of the buffer is held constant. This technique has proved very effective in separating rather similar compounds or in isolating the desired compounds in a very high purity. In this experiment, the dinucleoside monophosphate (peak A) could

be separated very efficiently by an extraction procedure.

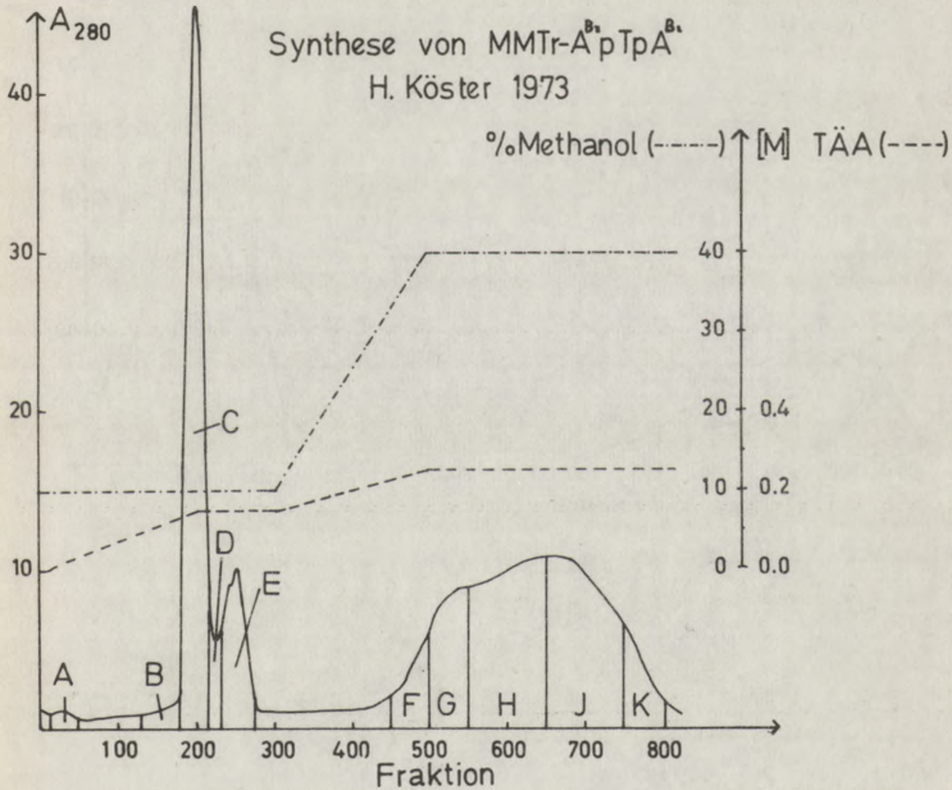


Figure 3

If this is not the case, it is advisable to elute the dinucleoside monophosphate before the phosphate component using a very low ionic strength in the buffer - low enough not to elute the phosphate component - and a high alcohol concentration to overcome the adsorption phenomena. The choice of the alcohol mixture depends on the base composition of the oligonucleotides. Figure 4 gives an example.

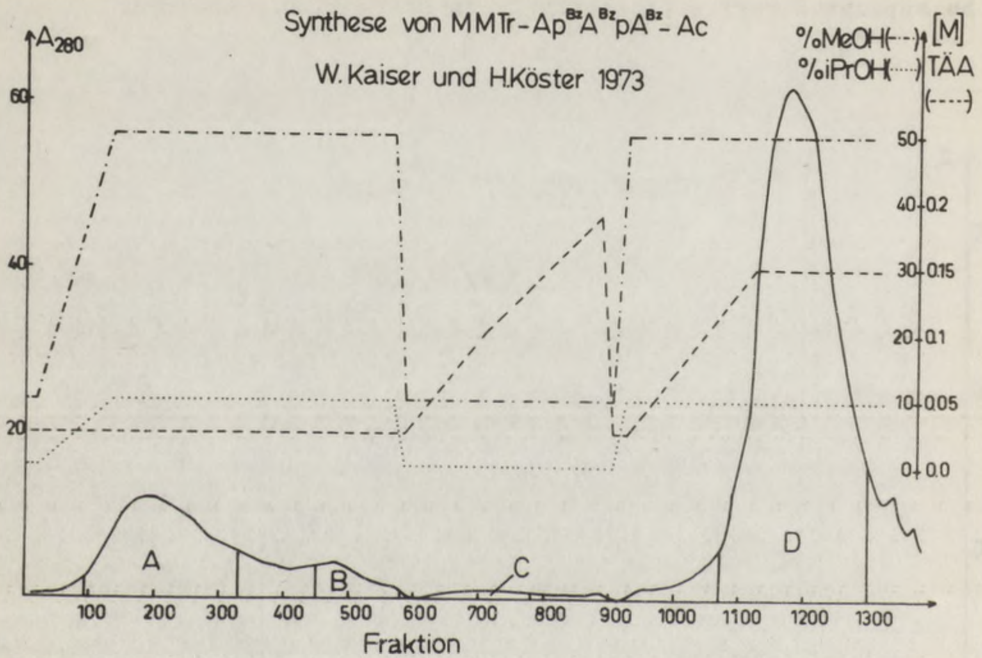


Figure 4

In peak A MMTr-$A^{Bz}pA^{Bz}$ elutes, in B pA^{Bz}(Ac), and in C its pyrophosphate; peak D contains the trinucleotide MMTr-$A^{Bz}pA^{Bz}pA^{Bz}$(Ac).

The case of a more complicated sequence is shown in figure 5.

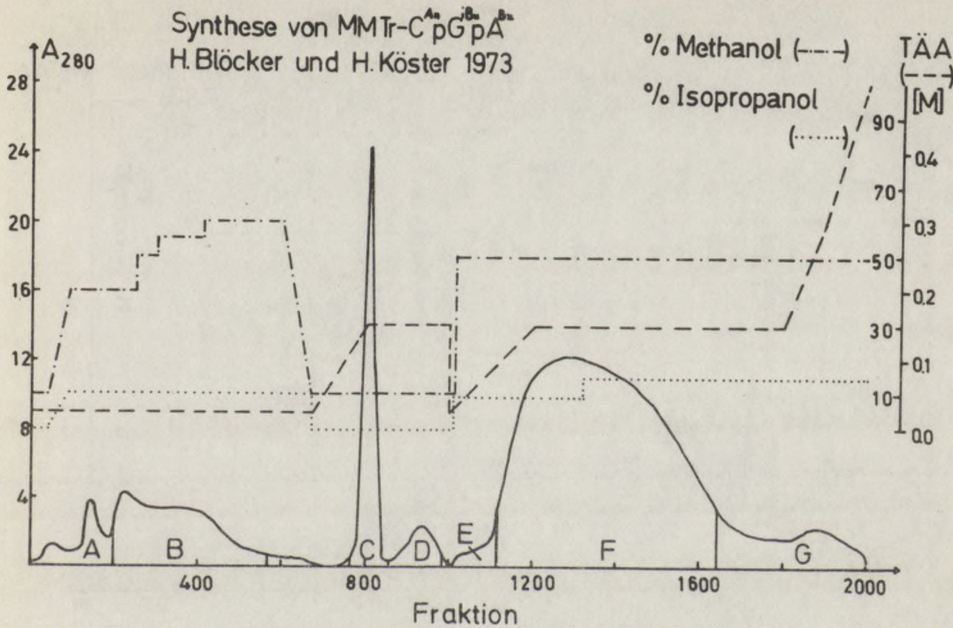


Figure 5

B contains MMTr-$C^{An}pG^{iBu}$, C contains pA^{Bz}, D is its pyrophosphate and F is the desired MMTr-$C^{An}pG^{iBu}pA^{Bz}$ in a very pure state. G contains the trinucleotide which has lost the N-isobutyryl group. As we use triethylammonium acetate as eluting buffer this cleavage must have occured during the selective 3'-de-O-acetylation using sodium hydroxide.

Figure 6 shows the elution pattern after synthesis of MMTr-$A^{Bz}pTpA^{Bz}pT$.

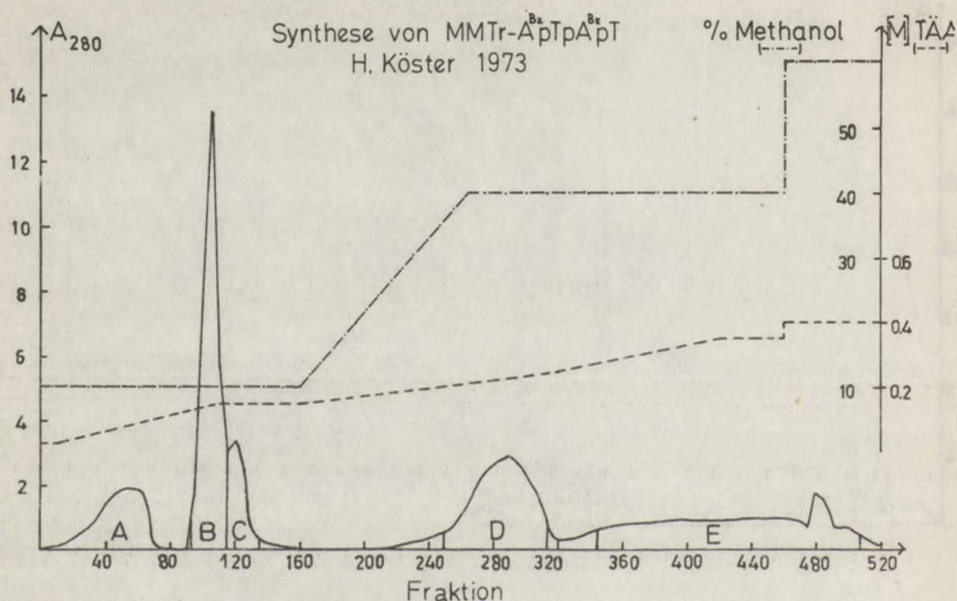


Figure 6

At low ionic strength pT (peak B) and its pyrophosphate (peak C) elute. Then follows a steep gradient in methanol and a shallow gradient in the buffer. By this the trinucleotide MMTr-A^{Bz}pTpA^{Bz} (peak D) is well separated from the tetranucleotide (peak E). The tetranucleotide elutes in a very broad peak due to the use of methanol alone. Using methanol-isopropanol-mixtures would bring about a sharpening of this peak. This chromatographic technique can also be used very conveniently for chains longer than tetranucleotides, as is shown in figure 7. The hexanucleotide is synthesized by a block condensation

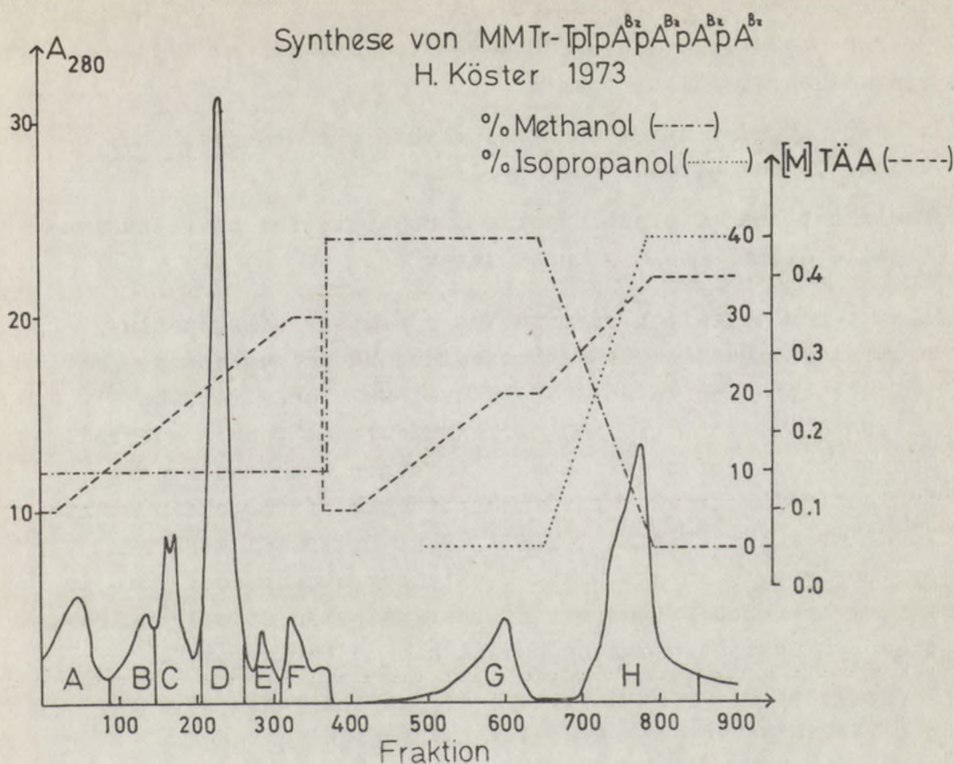


Figure 7

between MMTr-TpTpA^{Bz}pA^{Bz} and pA^{Bz}pA^{Bz}(Ac). Peak A contains the sulfonic acid, D is pA^{Bz}pA^{Bz}, and F is its pyrophosphate; G is the tetranucleotide and H the desired hexanucleotide. The column is washed first with 100 mM triethylammonium acetate (TAA), containing 10 % methanol; then follows a gradient from 100 mM to 350 mM TAA with 10 % methanol, as we know that the tetranucleotide will not elute at that high molarity using only 10 % methanol. Then the column is washed with 100 mM TAA containing 40 % methanol, as we know that at this low molarity, even with 40 % methanol, the tetranucleotide will not elute. It is eluted with a gradient from 100 mM to 250 mM TAA in the presence of 40 % methanol. The gradient is stopped as the tetranucleotide starts to elute, and thereafter the hexanucleotide is eluted using a gradient

of 250 mM TAA containing 40 % methanol to 400 mM TAA containing 40 % isopropanol.

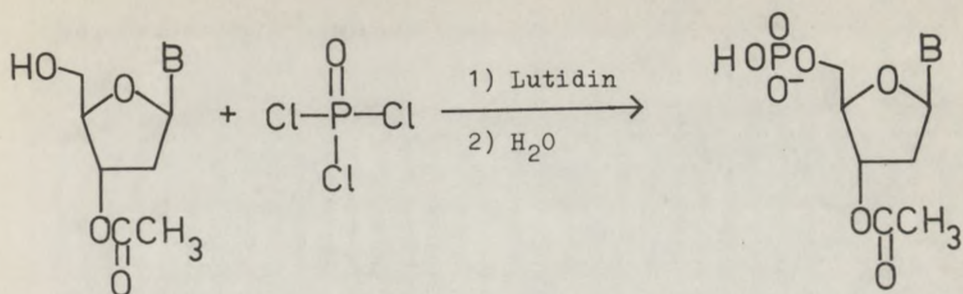
A short communication describing this chromatographic technique has already been published [5].

Purifications of even longer chains up to the undecanucleotide level will be discussed later on.

I want now to switch over to the synthesis of suitable protected dinucleoside diphosphates and trinucleoside triphosphates which have to be used as blocks for the extension of the tri- and tetranucleotides (chain starts). The synthesis of these blocks, together with the mononucleotide units used for the synthesis of the chain starts, leads to a large number of different intermediates when synthesizing a long bihelical polydeoxynucleotide. This is a very uneconomical aspect of the synthesis of polynucleotides of defined sequence, which had to be improved.

It would be of great advantage if one could use some key intermediates for the synthesis of the chain, and simultaneously for the synthesis of the protected mono-, di- and trinucleotides used for the elongation of the chain.

We have developed a procedure which fulfils this task. It is very simple and it depends on our having been able to work out conditions for the direct phosphorylation of 3'-O-acetylated deoxynucleosides, dinucleosidemonophosphates and trinucleosidediphosphates with phosphorus oxychloride. The principle of this reaction is shown in figure 8.



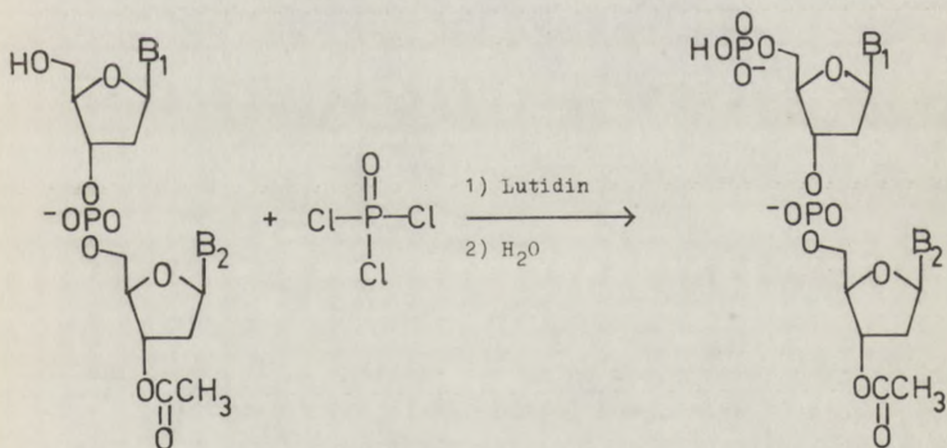
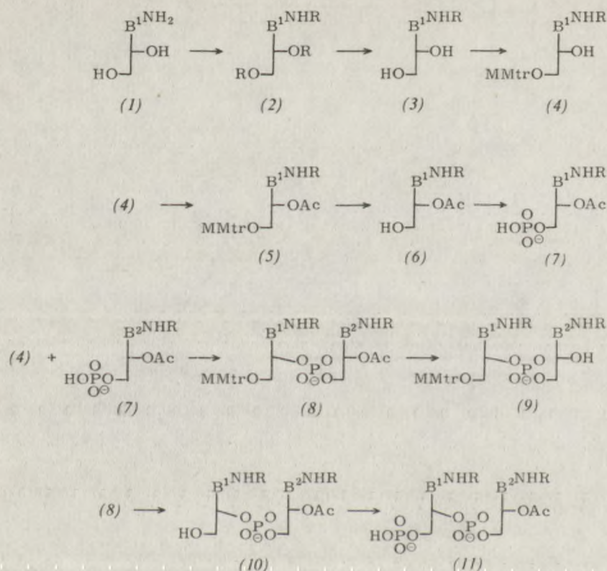


Figure 8

Figure 9 illustrates how this very simple procedure reduces



Synthesis of oligodeoxyribonucleotides. R = benzoyl, anisoyl, isobutyl; Ac = acetyl; MMtr = monomethoxytrityl; B = adenine, guanine, cytosine, thymine (without NH_2).

Figure 9

the synthetic expense. Using the strategy of Khorana, the synthesis of an oligonucleotide chain starts with a 5'-monomethoxytritylated deoxynucleoside of type 4. Simple acetylation and detritylation - two reactions which can be performed in the same reaction flask with quantitative yields lead to compound 6, which is subjected to phosphorylation to give the fully protected mononucleotide ready for chain elongation. All four deoxynucleosides of type 6 are crystalline compounds and can be synthesized without difficulty in large amounts.

The first step of chain elongation leads to a fully protected dinucleosidemonophosphate (compound 8), which can easily be purified by extraction procedures. After de-O-acetylation, this compound is ready for the next chain elongation step. However, after de-O-tritylation, it is ready

for phosphorylation giving a fully protected dinucleoside-diphosphate which can be used as a block for the extension of the chain.

The phosphorylation of the 3'-O-acetylated deoxynucleosides in tetrahydrofuran as a solvent and in the presence of 2.6-lutidine can be accomplished in half an hour with a yield of more than 95 % using 1.5 equivalents of phosphorus oxychloride.

The phosphorylation of the 3'-O-acetylated dinucleoside monophosphates takes place in a heterogeneous medium using 2.5 equivalents of phosphorus oxychloride (in the presence of 2.6-lutidine) in 1 hour with yields between 40 and 75 %. Of critical importance is the suspension medium. The work-up after phosphorylation of the dinucleoside monophosphates starts by removing the excess of phosphorus oxychloride by washing the precipitate with ether, in which the phosphorylation complex is soluble; then follows a chromatographic step on an anion-exchanger to give very pure products - a prerequisite for avoiding wrong sequences during chain elongation. The chromatographic purification is simple and quick by using steep gradients and can be done with rather large amounts, as the chromatographic difference between the starting material and the product is very large. Figure 10 shows a typical separation pattern of the reaction mixture after synthesis of $pA^{Bz}pT(Ac)$. The starting material has already been aluted with 25 mM TAA containing 40 % methanol and is not shown. The fully protected dinucleoside diphosphate is eluted in the main peak. After precipitation it is ready for a condensation reaction. The phosphorylation of 7 to 8mmoles was done with several examples and encountered no problems. Figure 11 sums up some yields.

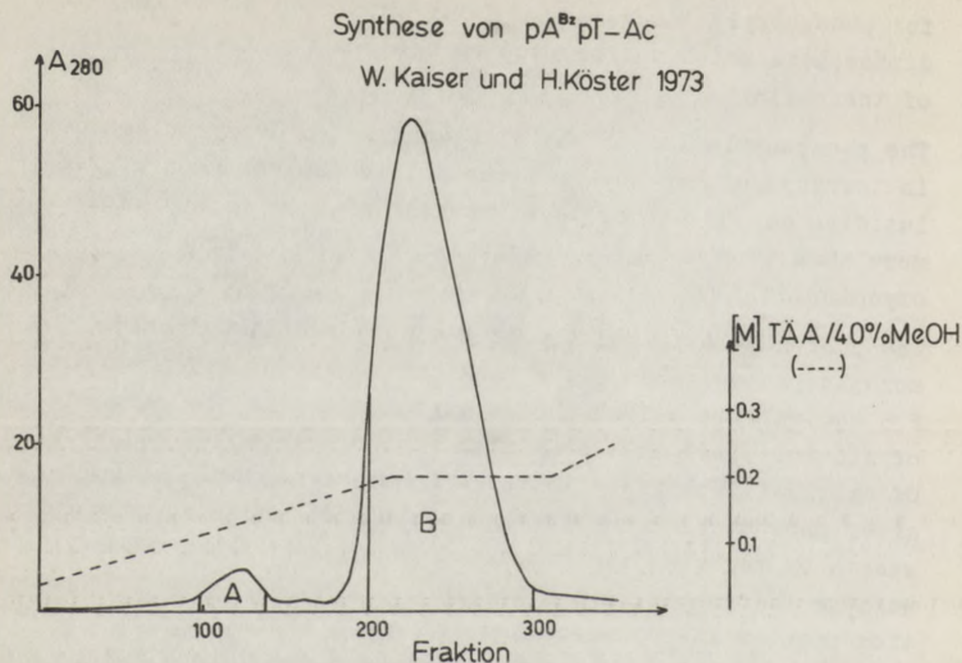


Figure 10

Table 1. Phosphorylation of dX(Ac) and dX—dY(Ac) with $PZCl_3$ (Z = O, S).

Cpd. [2]	Z	Phosphorylation [%]	Solution or suspension medium
dT(Ac)	O	95	tetrahydrofuran
dT(Ac)	S	75	tetrahydrofuran
$dbz^6A(Ac)$	O	97	tetrahydrofuran
dT—dT(Ac)	O	54	tetrahydrofuran
dT—$dan^4C(Ac)$	O	72	diethyl ether with 20% tetrahydrofuran
dbz^6A—dT(Ac)	O	51	diethyl ether with 20% tetrahydrofuran
dan^4C—$dibu^2G(iBu)$ [3]	O	57	diethyl ether with 10% petroleum ether
$dibu^2G$—$dibu^2G(iBu)$ [4]	O	63	diethyl ether with 40% petroleum ether

Figure 11

The extraction method for the synthesis of dinucleoside diphosphates using hydrophobic phosphoramidates proposed by Agarwal and Khorana [6] we were not able to reproduce. The resulting reaction mixture could not be purified by the extraction procedure, moreover it was too complicated to be able to purify by DEAE-cellulose chromatography.

The 3'-O-acetylated trinucleoside diphosphates are phosphorylated with 3 to 5 equivalents of phosphorus oxychloride in tetrahydrofuran as suspension medium, in the presence of 2.6-lutidine, for 1 to 2 hours with yields between 20 and 50 %. Figure 12 shows the separation pattern in the synthesis of $pA^{Bz}pA^{Bz}pA^{Bz}(Ac)$. Peak A contains the recovered trinucleo-

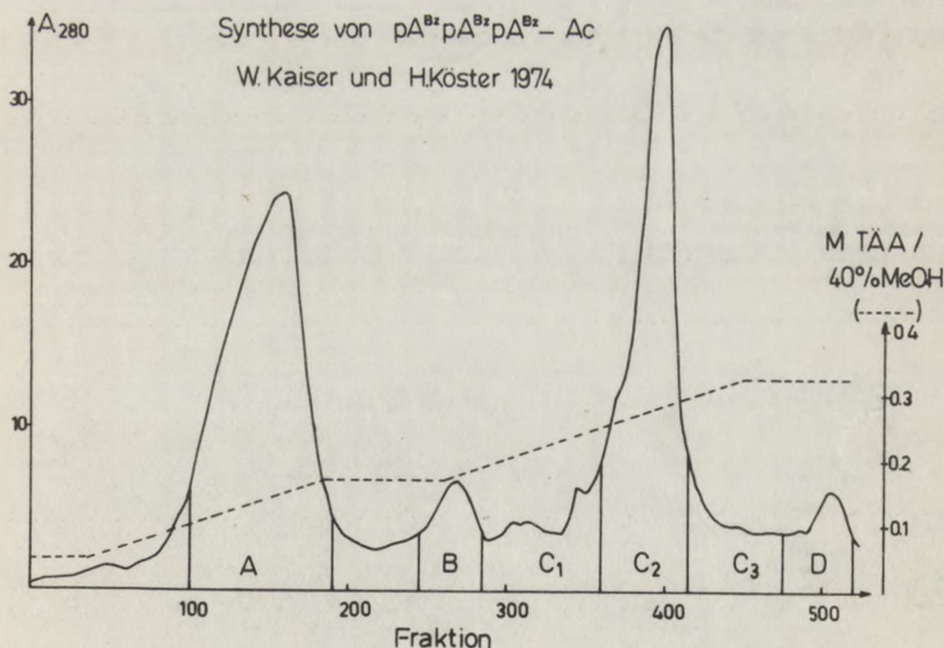


Figure 12

side diphosphate, C_1 is the desired trinucleotide block, which after precipitation is ready for condensation.

We have synthesized several trinucleoside triphosphates including $pG^{iBu}pG^{iBu}pG^{iBu}(iBu)$ by this method.

This concept makes possible the synthesis of rather large quantities of protected mononucleotides, dinucleoside diphosphates and trinucleoside triphosphates. Thus the synthesis of polynucleotides is possible by using the deoxynucleosides only.

A short communication of this concept has been published [7].

Coming to the end I want to discuss the plan of the synthesis of the angiotensin gene in more detail.

The total gene with stop and start codons consists of 33 base pairs and is shown in figure 13.

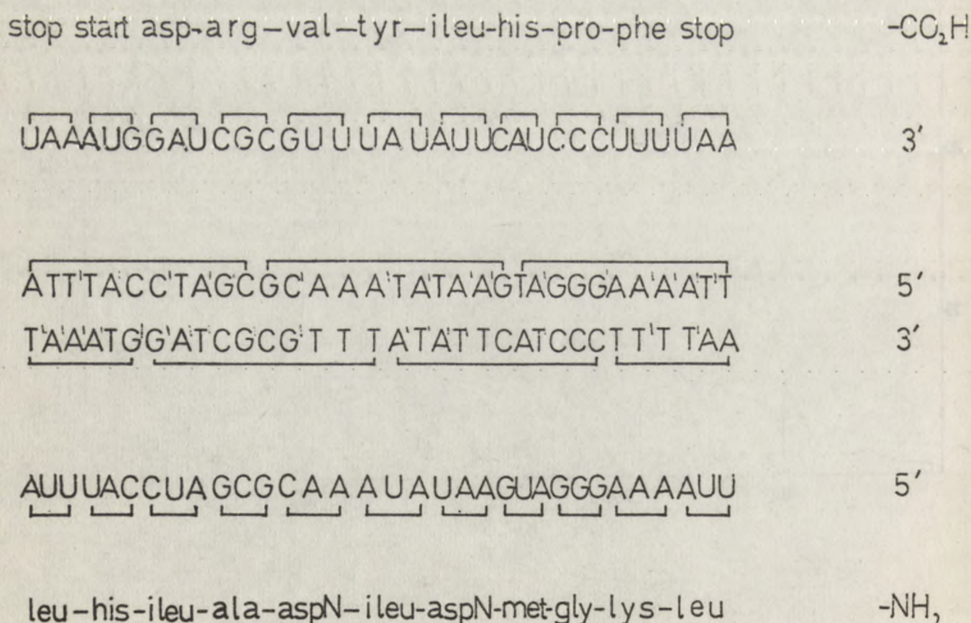
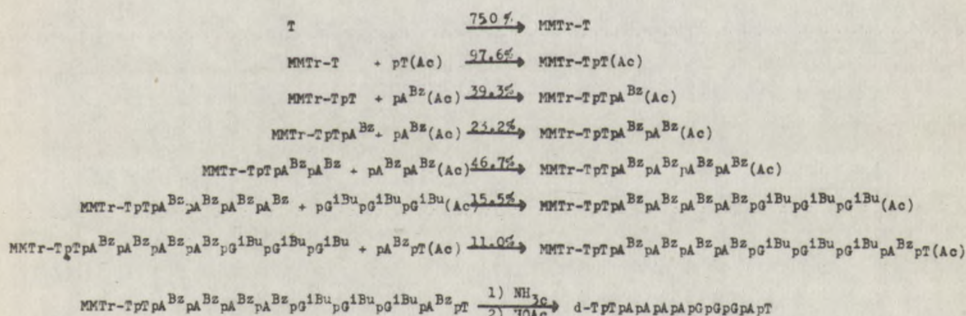


Figure 13

Four undeca-, one deca- and two hexanucleotides have to be synthesized. The chemical synthesis of these chains is almost completed. To give an impression of the synthetic problems I shall discuss the synthesis of two undecanucleotides.

One of the biggest difficulties had to be overcome during synthesis of the oligonucleotide TpTpApApApApGpGpGpApT which has eight purine nucleotides, including three guanosine units, following one after the other. Figure 14 gives a summary



MMTr-	:	Monomethoxytrityl-
Bz	:	Benzoyl-
iBu	:	Isobutyryl-
Ac	:	Acetyl-
T	:	Desoxythymidin
A	:	Desoxyadenosin
G	:	Desoxyguanosin

Figure 14

of the synthetic plan and yields. We used the strategy 4 + 2 + 3 + 2. The yields are - as expected - rather low, as condensations are unfavourable if OH- and phosphate component both belong to purine nucleotides. Most unfavourable, however, is the use of a trinucleotide

consisting of G-units alone.

The characterization of the nona- and undecanucleotides turned out to be rather difficult due to the ability of G to form very stable secondary structures. The elution profile of the last condensation step is shown in figure 15.

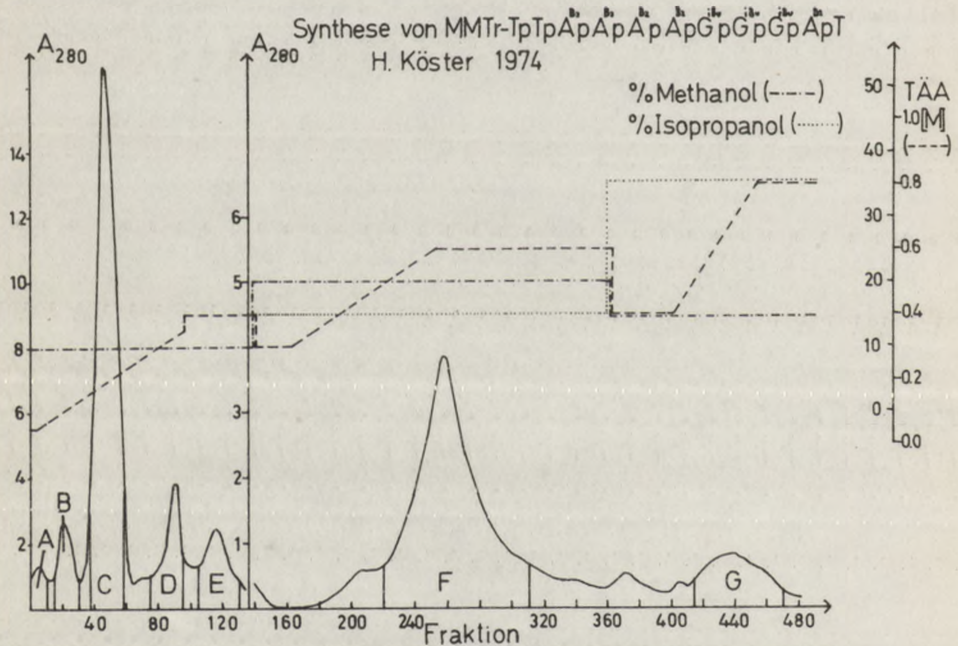


Figure 15

Chromatography of MMTr-TpTpA^{Bz}pA^{Bz}pA^{Bz}pA^{Bz}pG^{iBu}pG^{iBu} pG^{iBu}pA^{Bz}pT. Peak C contains pA^{Bz}pT, D its pyrophosphate, F is the nonanucleotide and G the undecanucleotide.

The overall yield of the fully protected undecanucleotide starting from T is 0.14 %.

With the experience we have now, it would have been better to use the strategy 4 + 2 + 2 + 3, especially since the trinucleotide has the sequence of the chain start of the decanucleotide.

Figure 16 shows the synthetic plan and the yields of the undecanucleotide ApTpApTpTpCpApTpCpCpC. The strategy was

$$\text{A} \xrightarrow{44\%} \text{MMTr-A}^{Bz}$$

$$\text{MMTr-A}^{Bz} + \text{pT(Ac)} \xrightarrow{78\%} \text{MMTr-A}^{Bz}\text{pT(Ac)}$$

$$\text{MMTr-A}^{Bz}\text{pT} + \text{pA}^{Bz}\text{(Ac)} \xrightarrow{35\%} \text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{(Ac)}$$

$$\text{MMTr-A}^{Bz}\text{pTpA}^{Bz} + \text{pT(Ac)} \xrightarrow{67\%} \text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{pT(Ac)}$$

$$\text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{pT} + \text{pTpC}^{An}\text{(Ac)} \xrightarrow{41\%} \text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{pTpTpC}^{An}\text{(Ac)}$$

(Hexanucleotid)

$$\text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{pTpTpC}^{An} + \text{pA}^{Bz}\text{pT(Ac)} \xrightarrow{29\%} \text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{pTpTpC}^{An}\text{pA}^{Bz}\text{pT(Ac)}$$

(Oktanucleotid)

$$\text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{pTpTpC}^{An}\text{pA}^{Bz}\text{pT} + \text{pC}^{An}\text{pC}^{An}\text{pC}^{An}\text{(Ac)} \xrightarrow{41\%} \text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{pTpTpC}^{An}\text{pA}^{Bz}\text{pTpC}^{An}\text{pC}^{An}\text{pC}^{An}\text{(Ac)}$$

$$\text{MMTr-A}^{Bz}\text{pTpA}^{Bz}\text{pTpTpC}^{An}\text{pA}^{Bz}\text{pTpC}^{An}\text{pC}^{An}\text{pC}^{An} \xrightarrow[\text{2) HOAc}]{\text{1) NH}_{3c}} \text{ApTpApTpTpCpApTpCpCpC}$$

MMTr- : Monomethoxytrityl-
Bz- : Benzoyl-
An- : Anisoyl-
Ac- : Acetyl
A : Desoxyadenosin
T : Desoxythymidin
C : Desoxycytidin

Figure 16

4 + 2 + 2 + 3. It is pyrimidine rich, therefore the yields in the different steps are better. The characterization is easier. Figure 17 shows the purification of the octanucleotide and the following figure that of the undecanucleotide.

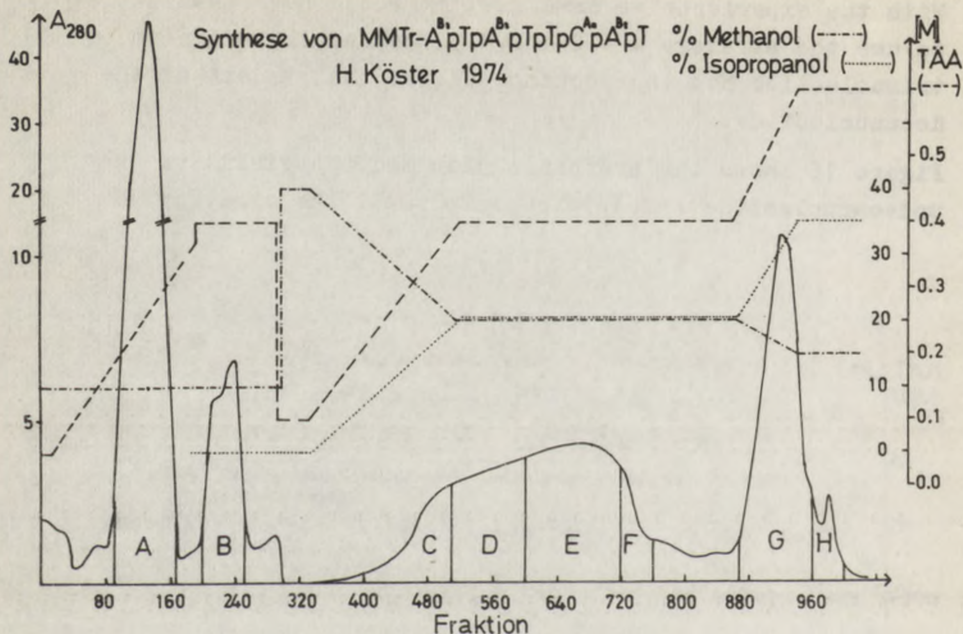


Figure 17: Chromatography of MMTr-$A^{Bz}pTpA^{Bz}pTpTpC^{An}$ $pA^{Bz}pT$. Peak A contains the $pA^{Bz}pT$, B its pyrophosphate, D, E pure MMTr-$A^{Bz}pTpA^{Bz}pTpTpC^{An}$, peak G the octanucleotide.

The overall yield of the fully protected undecanucleotide starting from deoxyadenosine is 0.4 %. That is 37 umoles with a molecular weigth of 5,313 (as triethyl ammonium salt). Although the chromatographic technique used leads to rather pure products even of the longer chains, we routinely make an anion-exchange chromatography of the fully deprotected chains in the presence of 7 M urea and 20 % methanol, as is shown for the last mentioned undecanucleotide in figure 19.

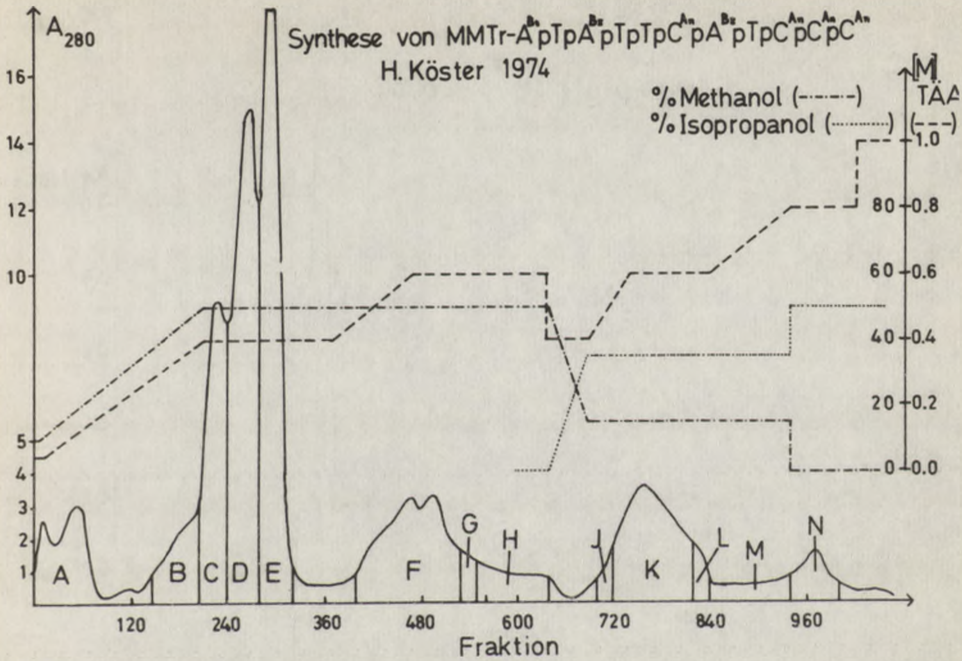


Figure 18

Chromatography of MMTr-$A^{Bz}pTpA^{Bz}pTpTpC^{An}pA^{Bz}pTpC^{An}pC^{An}pC^{An}$. Peak C,D,E contains the trinucleotide triphosphate, which in part is de-N-anisoylated, peak F contains its pyrophosphate and the octanucleotide, K the undecanucleotide, N presumably the tetradecanucleotide carrying another three C^{An} residues.

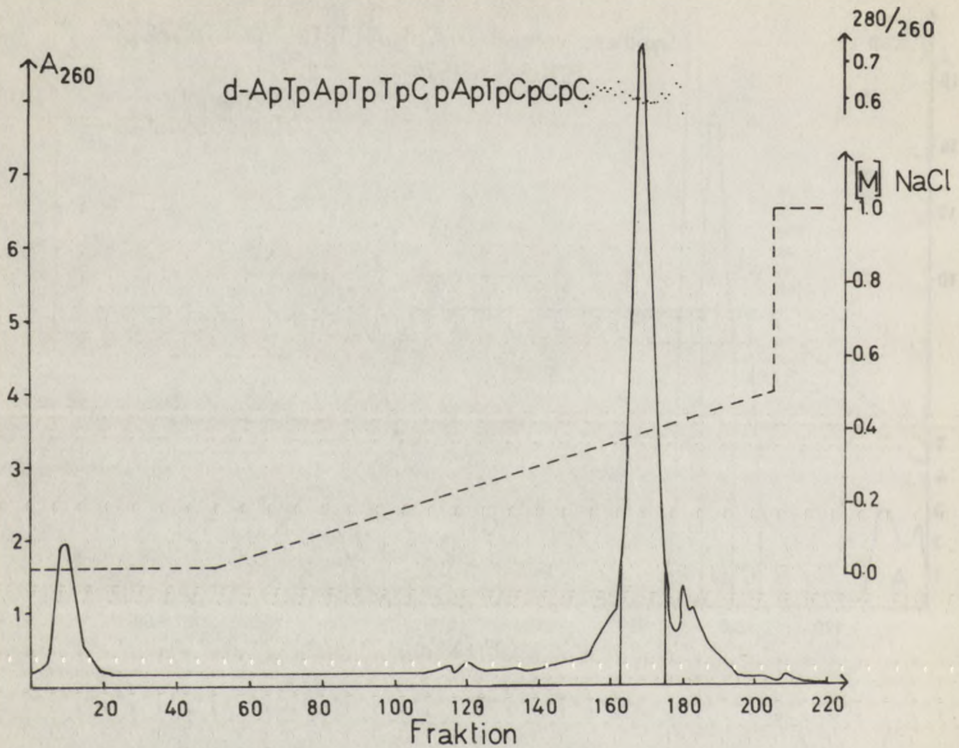


Figure 19

The degeneracy of the genetic code makes it possible to choose the most favourable nucleotide sequence for the angiotensin gene. Our choice was governed by - amongst other factors - the minimization of the preparative work. There are a lot of repeating dinucleoside diphosphates and some identical chain starts. Almost all chain starts could be used for the synthesis of trinucleoside triphosphates by the direct phosphorylation method. Another factor has been the facilitation of the isolation of the expressed and translated genetic information.

We decided to choose angiotensin II for it is an oligopeptide with only 8 amino acids and with very strong biological activity; picomoles of the substance are detectable.

Angiotensin II has the task of controlling the blood pressure and the tension of the blood-vessels in mammals [8]. Its biochemical synthesis occurs via several steps which in part are shown in figure 20. From angiotensinogen - a tetradeca-

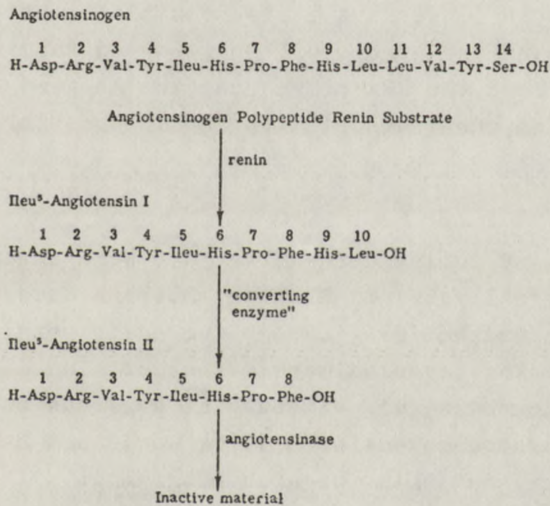


Figure 20

peptide - a tetrapeptide is cleaved off by renin to give the decapeptide angiotensin I. This in turn is transformed by the so called 'converting enzyme' to angiotensin II, which is the only peptide with the above mentioned hormonal acitivity in this pathway. Angiotensinase cleaves angiotensin II to give inactive products.

We are especially interested in studying the mechanism of transcription and translation of protein genes. The transcription of t-RNA and ribosonal RNA must be controlled in a different way from the transcription of protein genes.

As the control cannot depend on the sequence of the structural gene, but on special initiation and termination signals, it should be rather unimportant how long the structural gene is. Only meaningful and easily detectable biological information is of importance. The synthesis of this gene should give us a well-defined model system for in-vitro experiments, with the advantage of being able to modify it very quickly by attaching artificial sequences at one end or the other. Perhaps it is possible to attach isolated or synthesized promoter, operator or terminator sequences to direct the RNA-polymerase to the gene start and to force it to do the transcription job in the biologically asymmetric manner.

I want to thank my colleagues who in part were engaged in this work, especially Helmut Blöcker, Stephan Geussenhainer, Walter Heidmann und Wolfgang Kaiser for their persistence and enthusiasm. The technical assistance of Alan J. Sparrow is gratefully acknowledged. Without the generous support of the Deutsche Forschungsgemeinschaft it would not have been possible to carry out this research programm.

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A SYSTEM FOR SEPARATION AND IDENTIFICATION OF THE NATURALLY OCCURRING CYTOKININS: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - FIELD DESORPTION MASS SPECTROMETRY

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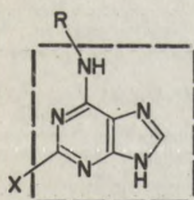
Introduction

Cytokinins, plant growth promoters capable of increasing rates of cell enlargement and division and inducing morphogenesis, have been observed to regulate or influence seed germination, root development, budding, flowering, fruit formation, and cell mitotic activity (Skoog and Armstrong, 1970). The most common natural cytokinins known to date share a modified adenine structure, carrying in all cases an alkyl substituent at N^6 and in some instances a second substituent at C-2. The work of determining whether or not this diversity of structure functions in the plant to furnish correspondingly diverse regulatory activities has proceeded slowly. The problem is being approached through synthesis and biological testing of both the natural hormones and structural analogs (Leonard, 1974), including compounds which can act as cytokinin antagonists (Hecht, *et al.*, 1971). Of great importance are the search for as yet unknown natural cytokinins and attempts to correlate growth characteristics with those cytokinins actually present in a given tissue.

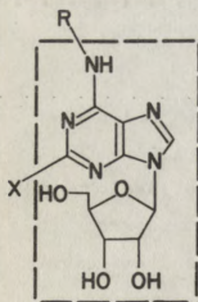
A prime requirement for this work is a means of rapidly separating and reliably identifying cytokinins obtained from natural sources. Numerous chromatographic techniques have been applied to provide solutions to this problem. Paper chromatography (Letham, 1973) and gas chromatography (Muni and Altschuler, 1974; Upper, *et al.*, 1970) have been used to separate some cytokinins, providing tentative identification through comparison with standards. Even more difficult to scale up to a semi-preparative level, but capable of distinguishing geometric isomers, thin layer chromatography on silica gel has proven useful (Vreman, *et al.*, 1974). Rapid and efficient

screening of a plant extract for the relatively large number of known cytokinins is time consuming with these methods, however. Column liquid chromatography offers advantages in terms of separation efficiency and capacity over these other methods, ion exchange chromatography in particular having been found useful for the separation of nucleic acid components (Uziel, et al., 1968). Since, however, the major known natural cytokinins appear to have one of three N^6-alkyladenine-type structures and thus share one of three sets of pK_a values, ion exchange must be supplemented by some additional chromatographic process to effect complete separation. We have found that by proper manipulation of elution conditions to take advantage of liquid - solid partioning phenomena, this may be accomplished on the ion exchange column itself. A high performance liquid chromatography system capable of both analytical and preparative separation of the natural cytokinin free bases and their nucleosides shown in Table I has been developed.

NATURAL CYTOKININS



	R	X	FDMS, M^{+}
1:	$-CH_2CH{=}C(CH_3)_2$	$-H$	203
2: CIS / 3: TRANS	$-CH_2CH{=}C(CH_3)CH_2OH$	$-H$	219
4:	$-CH_2CH{=}C(CH_3)CH_2OH$	$-OH$	235
5:	$-CH_2CH_2CH(CH_3)CH_2OH$	$-H$	221
6:	$-CH_2CH_2C(CH_3)(OH)CH_2OH$	$-H$	237
7:	$-CH_2CH(OH)C(CH_3)(OH)CH_2OH$	$-H$	253



	R	X	FDMS, M^{+}
8:	$-CH_2CH{=}C(CH_3)_2$	$-H$	335
9:	$-CH_2CH{=}C(CH_3)_2$	$-SCH_3$	381
10: CIS / 11: TRANS	$-CH_2CH{=}C(CH_3)CH_2OH$	$-H$	351
12: CIS / 13: TRANS	$-CH_2CH{=}C(CH_3)CH_2OH$	$-SCH_3$	397
14:	$-CH_2CH_2CH(CH_3)CH_2OH$	$-H$	353
15:	$-CH(CO_2H)CH_2CO_2H$	$-H$	383
16:	$-CH(CO_2H)CH(OH)CH_3$	$-H$	369

Table I

Chromatography, however, is a separation method useful in the actual identification of compounds only if standards are available. Even when the retention volume of an unknown compound may be directly compared with that of

a known substance and shown to be identical, additional proof of structure must be obtained. Frequently, only small amounts of material are available for this work. For this reason, mass spectrometry has been widely used to characterize further materials isolated by chromatographic methods. Conventional mass spectrometry suffers numerous drawbacks in this regard, however. Samples must be completely solvent free before introduction into an electron impact ion source via solids probe, and if only microgram amounts are available special loading techniques are required, necessitating additional handling steps. Electron impact ionization further requires that samples be at least somewhat volatile. In analysis of highly polar nucleic acid components this often means that tedious derivatization and further purification must be carried out (Most, et al., 1968). The resulting electron impact mass spectrum with its large number of fragment ions can be very difficult to interpret if complete purity is not obtained in the chromatographic step. Such problems of interpretation are greatly compounded when the material under investigation does not yield a molecular ion, as is often the case with the cytokinins, especially those occurring as ribonucleosides or ribonucleotides, of interest in the present work. These difficulties may be avoided by use of the field desorption (FD) ionization technique. Samples, including non-volatile materials, eluted from the high performance liquid chromatographic column may be analyzed immediately in the mass spectrometer without preliminary work-up or preparation, even in the case of salt-containing ion exchange eluates. The "soft" ionization effected by the field desorption source tends to give a very pronounced molecular ion, even for labile and non-volatile compounds (Beckey, 1969), thus providing rapid identification of substances assigned tentative structures from chromatographic retention volumes. The number of molecular ion peaks observed at low field emitter temperatures is a direct indication of the number of compounds present in the sample, providing an excellent criterion of purity. For compounds eluting at positions not corresponding to standards, knowledge of the molecular weight and - if data are collected at high resolution on a photoplate - molecular formula, contributes greatly to subsequent identification, confirmable by synthesis.

Materials and Methods

Apparatus. Chromatronix model CMP-1 chemically inert pumps fitted with variable speed feedback controlled DC motors were used for operation at column inlet pressures up to 700 psi (47 atm). For gradient elution, two pumps

were connected via a mixing tee to a single column, their individual flow rates being controlled by a digital linear gradient programmer of local construction and design. The ultraviolet absorbance of the column effluent was continuously monitored by a Chromatronix model 220 or model 200 absorbance detector, using a flow cell of 1 cm pathlength and operating at 254 and/or 280 nm. A Hewlett-Packard model 680 six-inch strip chart recorder operated at 10 mv full scale deflection was used for obtaining the chromatograms. The recorder was fitted with limit switches activated by pen movement to the extremes of the chart. At either point, a signal is fed to an offset device of our design which adds or subtracts ten mv, as appropriate, from the signal to the pen drive servo motor. True peak shape is preserved by this technique, while chromatographic peaks having a height of up to 4.7 meters (thirty-one chart widths) may be directly and quantitatively compared to peaks only slightly above recorder noise levels (approximately 2 mm in height). The recorder is fitted with an electric writing pen to avoid loss of chromatographic information during rapid pen travel. Conductivity of column effluents was monitored continuously during desalting operations by a conductivity monitor using a Chromatronix flow cell placed in the liquid line just ahead of the UV flow cell. Conductivity data were charted by a Varian A-25 recorder. Samples were introduced to the liquid stream without stopping flow or opening the system to atmospheric pressure by use of a Chromatronix chemically inert three-way injection valve. Liquid connections were made using Altex tube end fittings and 0.035 inch I.D. Teflon tubing, obtained from Cope Plastics. Field desorption mass spectra were obtained with a Varian MAT 731 mass spectrometer fitted with a Varian MAT field desorption/electron impact combination ion source. Column fractions to be analyzed were concentrated in vacuo to a volume as near 20 μl as possible and the field emitter wire was dipped directly into the solution to load the sample. For sample quantities less than 20 μg, loading is more effectively accomplished by direct application to the emitter wire (Cook and Olson, 1974).

Columns. Chromatographic columns were constructed of heavy wall glass tubing of 0.5 inch inside diameter. Adjustable height bed supports were constructed and inserted in both ends of the column to provide a seal leak-proof to pressures in excess of 1000 psi (67 atm), retention of resin particles smaller than five microns diameter, and uniform radial distribution of incoming sample over the resin bed. All liquid contact parts were glass or Teflon. Columns were temperature controlled by water flowing through concentric glass jackets from a Haake circulator.

Packings. Cation exchange resin used was Bio-Rad Aminex A-5, a sulfonic acid cation exchanger based on a polystyrene-divinylbenzene polymer matrix. Total bed length of the column was 29 inches (73.7 cm). Also employed were Porapak Q, an ethylvinylbenzene-divinylbenzene copolymer from Waters Associates (200-325 mesh), and Partisil 5 micron silica gel from Reeve-Angel. These materials were slurry-packed in the intended eluent from a pressurized stainless steel stirred reservoir. Bio-Rad Bio-Gel P-2, a polyacrylamide gel of minus 400 mesh, was poured in small portions as a slurry in 50% aqueous ethanol (after preshrinking in this solvent) to a bed length of 36 inches (91.4 cm). Maximum inlet pressure for this column was 120 psi (6.2 atm).

Chemicals. Dimethylformamide was vacuum distilled before use and stored in the dark. Water and ethanol were distilled before use. Ammonium formate stock solutions of known concentration were prepared by titrating formic acid with ammonia. Eluants were degassed before use. Samples were prepared by dissolving pure synthetic nucleosides and free bases in a small volume of eluant. Injected volumes were approximately 200 μl.

Evaluation of Chromatograms. Adjusted retention volumes are reported (Horvath and Lipsky, 1969), V_R', calculated from the retention time, t_R, the liquid hold-up time, t_0 (as determined by injection of a liquid sample differing slightly in DMF concentration from the eluent), and the flow rate, F, according to the equation

$$V'_R = (t_R - t_0) F \cdot \tag{1}$$

For incompletely separated peaks, a separation factor (SF) was calculated according to the equation

$$SF = \frac{K_b - K_a}{W_a + W_b} = \frac{\Delta K}{\Delta W} , \tag{2}$$

where K is peak position and W the peak width at half-height (Uziel, et al., 1968).

Results and Discussion

We turned initially to ion exchange as the best method for chromatographic separation of a large number of compounds related to nucleic acids. While these resins have been used with eluants composed entirely of organic liquids (Gilmer and Pietrzyck, 1971; Larson, et al., 1973), salt elution is much more common. In the case of cation exchange chromatography, chosen for the present work, the ammonium ion has been found to be particularly effective as counterion with respect to minimizing peak width (Larson, et al.,

1973). Ammonium formate was chosen for reasons to be outlined later. Since acidic ammonium formate appears to undergo an interaction with stainless steel, leading to changes in pH of solutions in contact with the metal (Horvath and Lipsky, 1969), we have used heavy walled glass tubing exclusively. Adjustable column bed supports combine with the transparency of these columns and water jackets to allow the chromatographer to eliminate dead volume on the inlet side of the resin bed as it occurs. This capability is vital for achievement of high resolution at minimal sample dilution. With steel columns lacking these features, one is limited to the use of completely rigid packings which do not change particle size in response to pressure or elution conditions. Heavy wall glass columns have lower maximum inlet pressures (though these are well in excess of 1000 psi, or 67 atmospheres) than stainless steel columns, but the totally porous resins of interest as packings in this work must be used at pressures below this limit.

Previous efforts directed at "achieving fast separation of complex mixtures by liquid chromatographic techniques similar in speed and quantitative range to gas chromatography" have in fact been successful with respect to speed and quantitative range, the former, as in gas chromatography, being high and the latter limited (Horvath, et al., 1967). The need in high speed (pressure) liquid chromatography to achieve rapid mass transport between stationary and mobile phases by use of pellicular packing materials (Horvath, et al., 1967) limits quantitative range, and absolute resolution is sacrificed for speed. (Chromatographers interested in maximizing elution velocity invoke "resolution per unit time" as the quantity most suitable for defining the quality of a given chromatographic separation: Kirkland, 1972.) Low capacity pellicular column packings, necessary at high carrier velocities (Horvath et al., 1967), become less attractive when actual biological materials (as opposed to contrived mixtures) are to be analyzed, due to the broad range of component concentrations likely to be encountered in a given sample.

Aminex A-5, a cation exchange resin of small mesh size and narrow range (13 micron ± 2 microns) with a totally porous structure was chosen for the present work. Eight percent crosslinking of the bead structure gives sufficient rigidity to withstand inlet pressures in excess of 40 atmospheres without irreversible compression of the resin.

Beginning our investigation with the naturally occurring cytokinin free bases (Figure 1), we initially noted that all compounds had very large elution volumes and gave broad peaks over a wide range of buffer salt concentration and pH values. It has been shown that ion exchange resins, par-

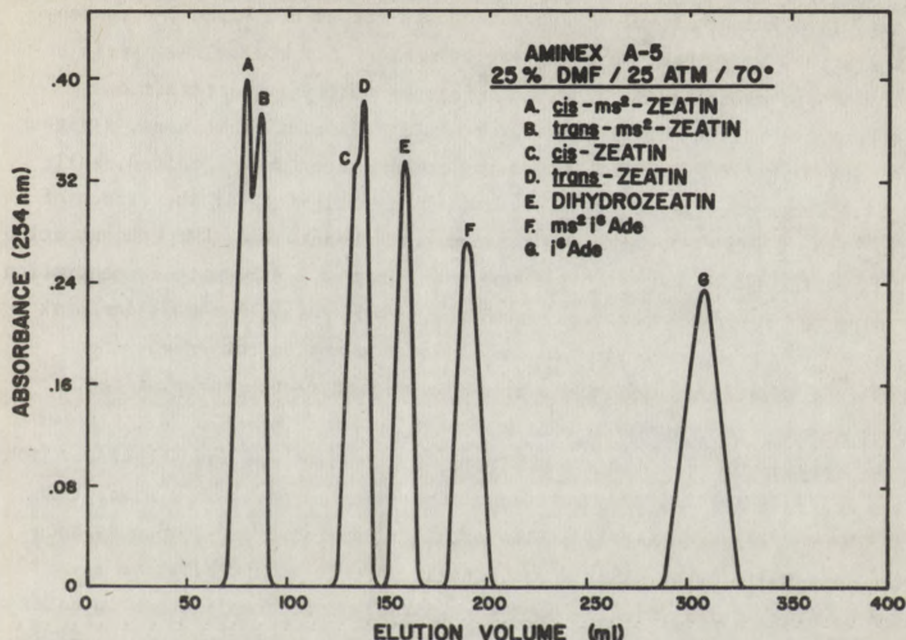


Figure 1. Cation exchange separation of cytokinin free bases. Column 1.3 cm x 76.2 cm, 0.2 M formate, pH 4.1, 0.65 ml/min. Separation factor for A,B = 0.3. Compound 19 is free base corresponding to ribonucleoside 9: F.

ticularly those such as Aminex based on polystyrene, can act as solid solvents for organic compounds, especially those having aromatic character (Larson, et al., 1973). This absorption phenomenom can be exploited for such lipophilic compounds if the distribution coefficient can be shifted in favor of the mobile phase. We have shown that addition of a water-miscible organic liquid to the salt-containing buffered eluant is effective in this regard.

Ethyl alcohol was initially evaluated as an organic additive, but tended to cause rapid elution of compounds with poor separation and was abandoned in favor of dimethylformamide (DMF). Optimization of ion exchange elution conditions through systematic investigation of the effect of varying single eluant components was well illustrated by Uziel, Koh and Cohn (1968). We found the elution characteristics of the cytokinins to be rather sensitive

as a class to small changes in eluant composition.

Initially, formate total concentration was set at 0.5 M and DMF concentration at 20% by volume, pH being varied. At pH 3.7 to 3.8, all peaks became broad and exhibited tailing due to strong absorptive interactions. Since our goal was obtaining Gaussian peaks at all elution volumes, a higher pH was investigated. At pH 4.0, Gaussian peak shapes were obtained, while at pH 4.5 separation deteriorated. Similar investigation of the effect of varying eluant ionic strength was carried out. Maintaining DMF concentration at 20% and the pH at 4.0, it was found that below 0.2 M formate concentration all compounds interacted strongly with the resin causing non-Gaussian peak shapes and overlarge retention volumes. Formate concentration was then raised to a level just sufficient to restore symmetrical peak profiles. Holding formate concentration constant at 0.25 M and the pH at 4.1, the effect of varying DMF concentration between 10% and 40% was investigated. DMF, 25% in water, proved to give well separated Gaussian peaks in minimal time.

It should be noted that in an ion exchange separation of compounds such as the lipophilic cytokinins, where a polar organic eluant additive is needed, system optimization becomes considerably more complex than in cases in which simple salt elution may be used (Horvath and Lipsky, 1969). Resin bead size and porosity change with dimethylformamide concentration, a factor which in turn affects cation exchange site availability (Larson, *et al.*, 1973). Changes in DMF or other organic component concentration also cause changes in pH of buffered eluents and in pK_a values of solutes as well as the obvious changes in solute distribution coefficients based on mobile phase solvating ability relative to that of the resin polymer matrix. Somewhat more trial and error is thus involved in final separation optimization than has previously been required for high performance ion exchange chromatography (Uziel, *et al.*, 1968; Blattner and Erickson, 1967).

Elution conditions for the cytokinin ribonucleosides were developed by using the free base optimal eluant as a starting point. Under these conditions the highly modified nucleosides elute very early. (If nucleosides are found thus during analysis of cytokinin free base content, they may be collected, desalted, and rechromatographed as described.) The DMF content of this initial eluant was systematically decreased until the chromatogram of the ribonucleosides had been "spread" to give satisfactory resolution of all compounds. Slight empirical adjustment of pH and ionic strength completed the optimization of elution conditions for both ribonucleosides and free bases.

Figure 1 shows a chromatogram of compounds 1, 2, 3 and 5 from Table I as well as the free bases corresponding to naturally occurring ribonucleosides 9, 10, and 11, developed under optimal free base elution conditions. It may be seen that, except for the geometric isomer pairs, all compounds are sufficiently resolved for chromatographic identification.

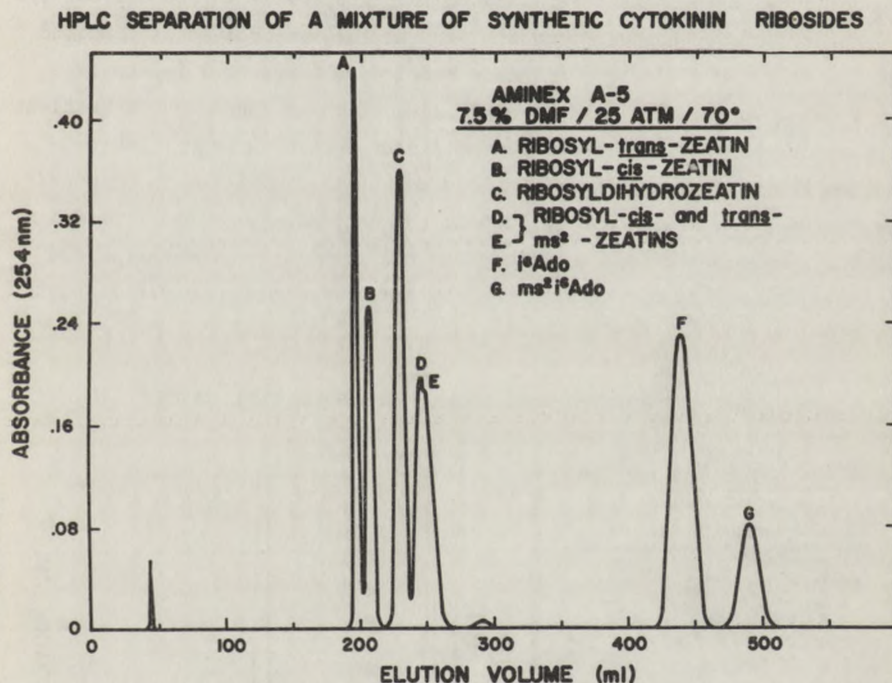


Figure 2. Cation exchange separation of cytokinin ribonucleosides. Column 1.3 cm x 76.2 cm, 0.15 M formate, pH 4.2, 0.8 ml/min. Separation factor for A,B = 1.1.

Figure 2 shows the chromatogram obtained for a mixture of cytokinin ribonucleosides, 8 - 14. Good analytical separation has been achieved here also. Of particular interest, and illustrative of the power of chromatography on ion exchange resins when the full range of their interaction with solutes is exploited, is the excellent separation of the cis and trans isomers of zeatin ribonucleoside (10 and 11).

Somewhat less effort has been expended in the search for cytokinins occurring free in the cell than for those which are components of transfer RNA. The plant pathogen Corynebacterium fascians has been investigated in this

regard, however (Helgeson and Leonard, 1966; Frihart, 1973; Vreman, *et al.*, 1974), and the immature kernels of sweet corn (*Zea mays*) have recently been analyzed using conventional chromatographic techniques (Letham, 1973). The latter work revealed the existence of five new natural cytokinins, compounds 4, 6, 7, 15, and 16. Two of these, 4 and 6, had previously been synthesized and shown by bioassay to be active cytokinins (Leonard, *et al.*, 1969). When these additional cytokinins were added to those separable in the solvent systems described above, new compound 6 eluted at the same volume as compound 5, and new compound 4 almost completely overlapped compound 1 in the free base mixture. In order to achieve separation of these compounds, a gradient elution system was devised, based on the linear system in hand. DMF concentration and pH were unchanged and held constant. Variation in compound elution volumes was achieved by a formate concentration gradient from 0.1 *M* to 0.5 *M*. Complete elution conditions and the resulting chromatogram are shown in Figure 3. It can be seen that by initially holding ionic strength

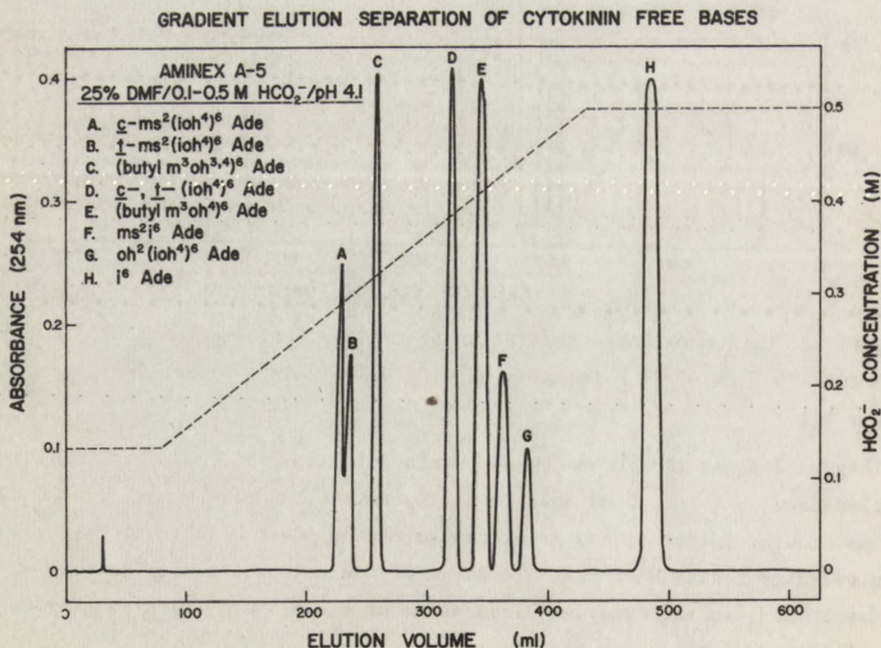
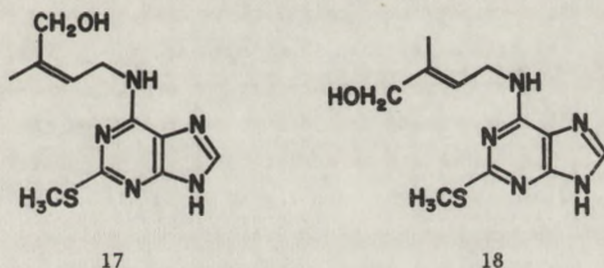


Figure 3. Gradient elution cation exchange separation of cytokinin free bases. Column 1.3 cm x 76.2 cm, linear gradient run over 9 hours after 2 hour initial delay, 0.65 ml/min., 70° C.

at a constant low level followed by a moderate salt gradient, the new free bases are completely resolved. The resolution of cis- and trans-2-methylthiozeatin (17 and 18) has also been improved, the difference in side-chain



geometry affording a remarkable difference in chromatographic behavior on the ion exchange resin.

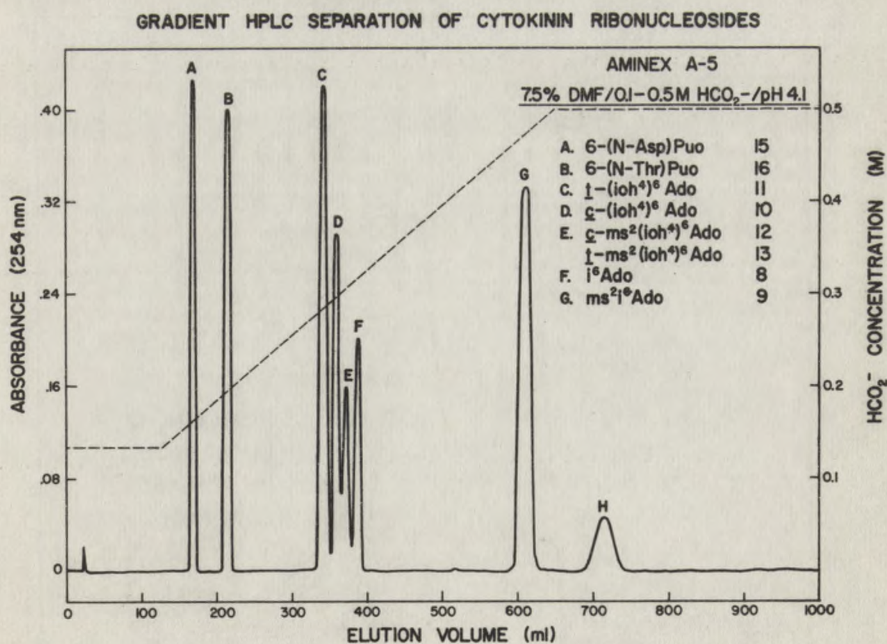


Figure 4. Gradient elution cation exchange separation of cytokinin ribonucleosides. Column 1.3 cm x 76.2 cm, linear gradient run over 9 hours following 2 hour initial delay, 0.85 ml/min., 70° C.

A gradient elution modification of the cytokinin ribonucleoside linear elution scheme (Fig. 2) was also investigated. The new *Zea mays* cytokinins 15 and 16 were found to be separable using the ionic strength gradient system shown in Figure 4.

Chromatographic separation of the naturally occurring cytokinins involves resolution of three *cis-trans* isomer pairs. This task has been accomplished in the past largely by silica gel thin layer chromatography (Vreman *et al.*, 1974). Incomplete recovery from the plate may render TLC a poor preparative method, however. High performance liquid chromatography, on the other hand, offers the advantage of being both an analytical and a preparative method. Geometric isomers 17 and 18 and the isomer pair 10 and 11 are quite well separated by cation exchange under the conditions presented here. Compounds 2 and 3, however, and the isomer pair 12 and 13 show only slight separation under these and other ion exchange conditions investigated.

A gravity-flow chromatographic system for the separation of *cis*- and *trans*-zeatin (2 and 3) and the corresponding ribonucleosides (10 and 11) has

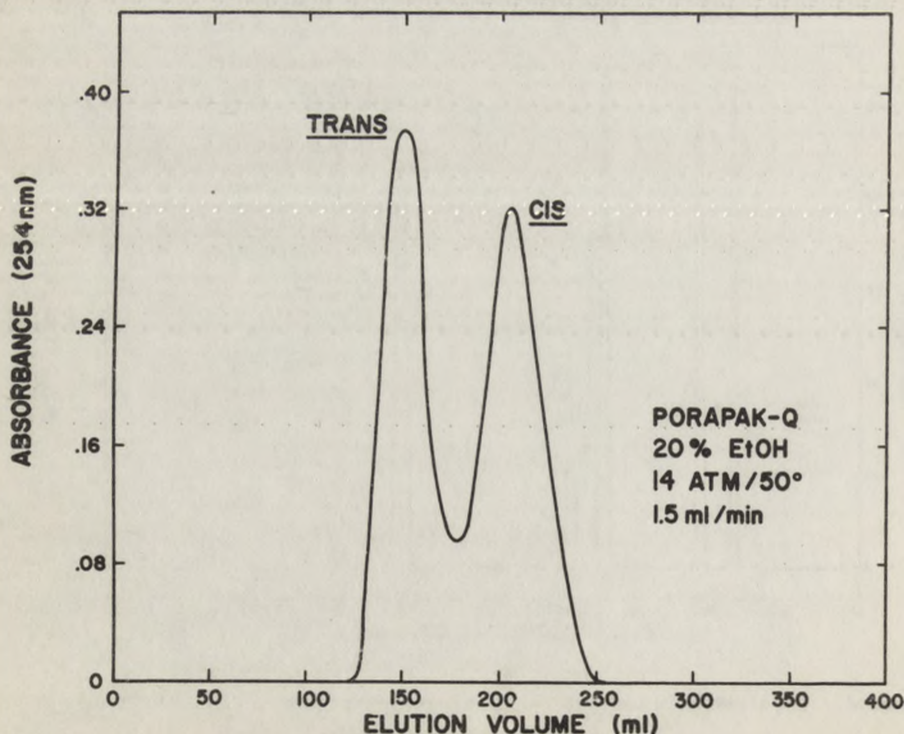


Figure 5. Porapak Q separation of *cis*- and *trans*-zeatin ribonucleosides. Column 1.3 cm x 96.5 cm, separation factor = 0.9.

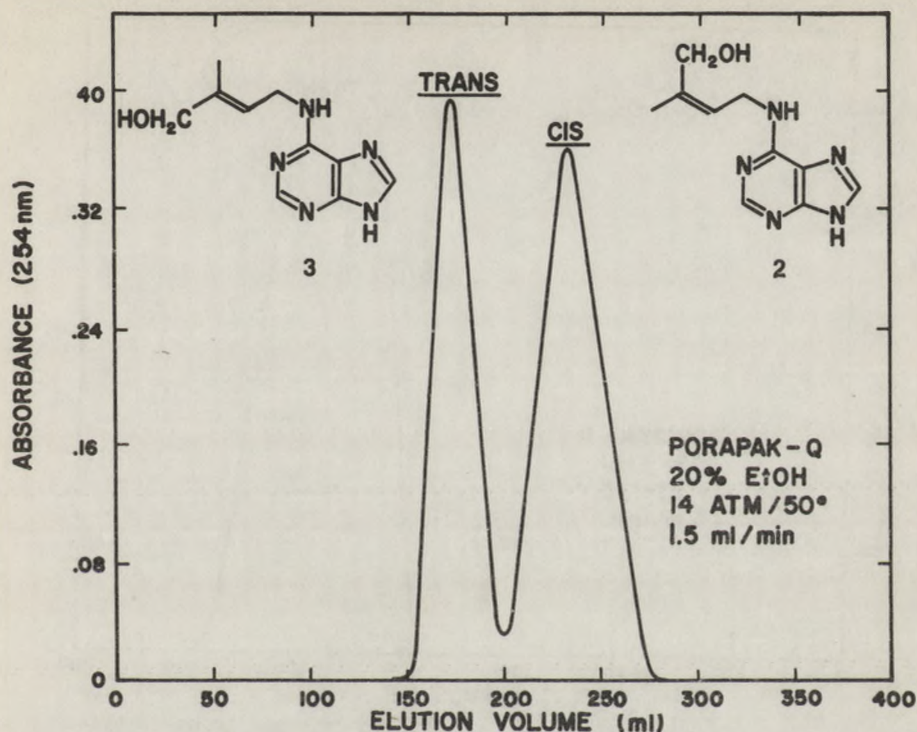


Figure 6. Porapak Q separation of 2 and 3. Column 1.3 cm x 96.5 cm, SF = 1.1.

recently been developed by Armstrong and Skoog (1974). This method utilizes an organic polymer bead, ethylvinylbenzene crosslinked with divinylbenzene, originally developed as a gas chromatography packing under the name Porapak Q (Hollis, 1966). The material has previously seen limited use in conventional liquid column chromatography as its adsorption properties offer an alternative to the common silica gel and alumina (Martinu and Janak, 1972).

In agreement with Armstrong and Skoog's findings, we were able to separate cis- and trans-zeatin ribonucleosides (10, 11) on Porapak Q (Figure 5) as well as the corresponding free bases (2 and 3), the latter pair not having been resolved in the cation exchange system (Figure 6). We have extended the Porapak system to the separation of 2-methylthio-cis- and trans-zeatins (17 and 18) as an adjunct to the good separation attainable by gradient elution cation exchange (Figure 7).

The 2-methylthio-cis- and trans-zeatin ribonucleosides (12 and 13) unfortunately proved to be only very slightly separable in both the cation exchange and Porapak Q absorption systems. Since thin layer chromatography on silica gel in 9:1 chloroform-methanol separates these isomers (Vreman,

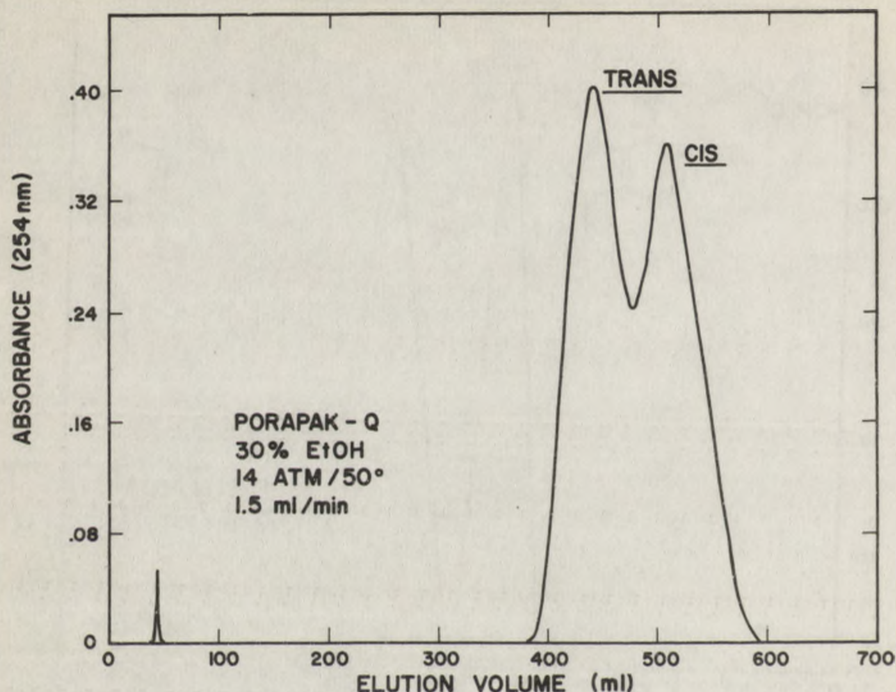


Figure 7. Porapak Q separation of 2-methylthio-cis- and trans-zeatins. Column 1.3 cm x 96.5 cm, separation factor = 0.6.

et al., 1974), we turned to HPLC on five-micron silica gel (Partisil) to effect their separation (Figure 8).

Rechromatography of materials eluted from ion exchange columns on either Porapak Q or Partisil requires that the sample first be desalted. Removal of salt from ion exchange eluates has long reduced the utility of this otherwise very attractive chromatographic method. Solutions to the problem have included use of relatively volatile buffer salts (Blattner and Erickson, 1967), charcoal adsorption chromatography (Walker, 1974), and multiple further ion exchange steps (Khym and Uziel, 1968). We have found that gel filtration chromatography is far superior to any of these methods for desalting organic compounds, and we are able to remove both ammonium formate and DMF from cation exchange eluates using this technique. After concentration *in vacuo* the solutions are injected onto a column of Bio-Gel P2 and eluted at room temperature with 50% aqueous ethanol (This technique was developed in this laboratory in conjunction with Dr. L. H. Kirkegaard. The scope of the method is being investigated further at this time.) The procedure is applicable to both cytokinin free bases and ribonucleosides and gives complete

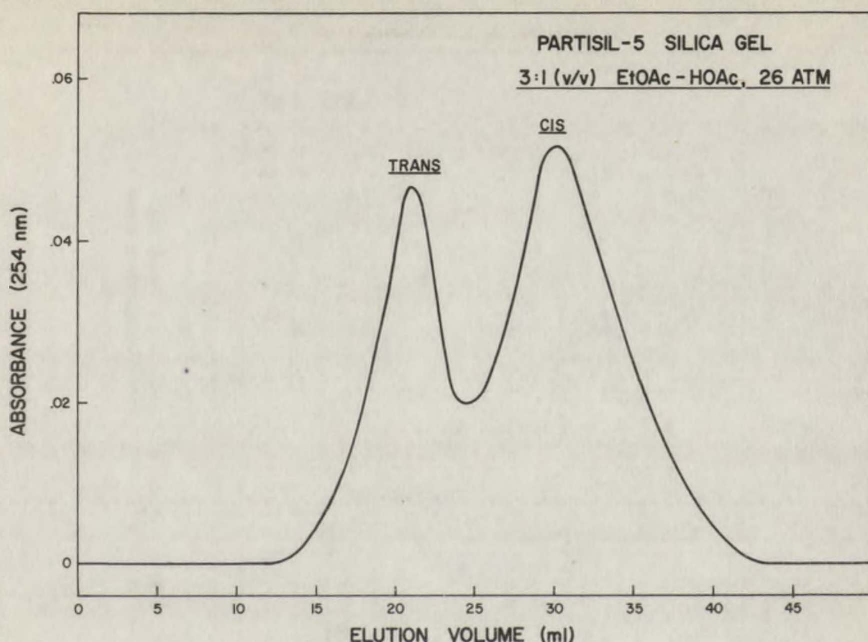


Figure 8. Separation of 2-methylthio-cis- and trans-zeatin ribonucleosides. Column 0.63 cm x 38.1 cm, 50° C, separation factor = 0.57.

removal of salt as shown by continuous monitoring of eluate UV absorbance and conductivity (see Figure 9 for representative chromatograms).

Extraction of actual biological material to obtain cytokinins may obviously also yield other substances (Helgeson and Leonard, 1966), including highly modified nucleic-acid related compounds other than those shown in Table I. It is also quite possible that such compounds would elute at the same volumes as known cytokinins: corroborating non-chromatographic evidence must be obtained before unknown materials are assigned known structures.

Additional light may be shed on the identity of eluted materials by observing their ultraviolet absorption spectra. Crude information in this regard is available directly from the column monitor since 254 nm and 280 nm absorbance may be simultaneously monitored and recorded and the ratio of the two compared to spectral characteristics of known compounds. If sufficient sample is available, of course (ca. 10 μg for most purine derivatives), the ultraviolet spectrum of the collected material may be determined on a scanning instrument. The most important non-chromatographic structure elucidation technique for small samples, however, has been mass spectrometry; this

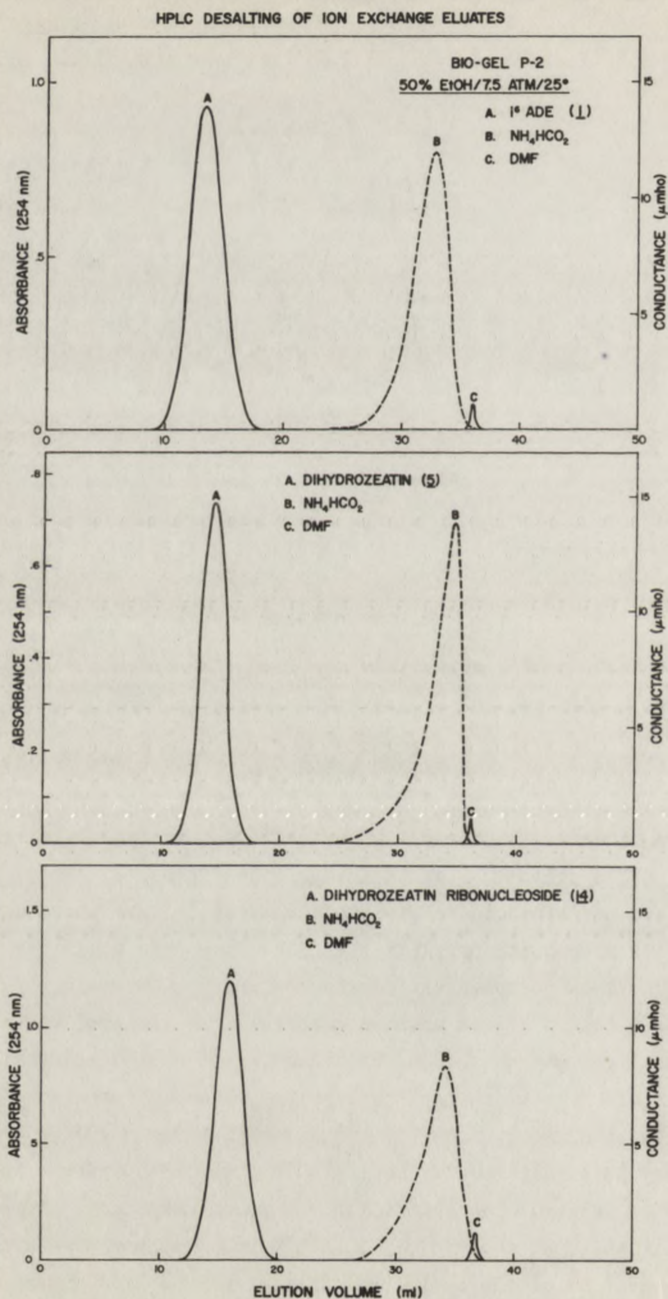


Figure 9. Bio-Gel P-2 desalting. Column 1.27 cm x 71.1 cm of -400 mesh, 0.15 ml/min.

is particularly true of cytokinin-related research (Vreman, et al., 1974).

Conventional mass spectrometry has some serious drawbacks in this regard, however. Bioassay and chromatographic techniques allow detection of cytokinin activity and isolation of very small amounts of the plant hormones. It is quite difficult, though, to obtain a good quality mass spectrum of an unknown compound using electron impact-induced ionization when sample quantity falls below 3 μg. Tedious evaporative loading of the solids probe is required, and after insertion into the ion source a rapid search for the proper probe temperature must be successfully made before the small sample is consumed. The suitable temperature range for a cytokinin sample of 1 μg may be only five degrees in breadth. Further, electron impact ionization yields high energy molecular ions which ordinarily fragment rapidly before leaving the source, so that the "molecular ion" will make up only a fraction of the ion current at the detector. This is a serious problem when the molecular weight of a small amount of sample must be determined and is worsened in the case of ribonucleosides by the fact that many prominent ions in their mass spectra will also appear in the spectrum of the free base: it is sometimes impossible to say with certainty when sample is limited whether or not a compound was a nucleoside or a free base before ionization.

The problem of obtaining mass spectral information from small quantities of labile compounds is now being approached by field desorption, an alternative method of ionization which yields molecular ions of very low energy (Beckey, 1969). The resulting lack of post-ionization fragmentation means that nearly all the ion current may be concentrated at a single mass value at the detector, thus allowing direct determination of the molecular weight of small quantities of compounds which do not yield molecular ions under electron impact ionization.

Good quality field desorption mass spectra may be obtained routinely from 10 to 20 μg of compounds of average molecular weight. Actual sample consumption is much less than this, but obtaining FD spectra from smaller amounts has been difficult for mechanical reasons connected with dipping the emitter wire into the sample solution. Common practice has been to dip the wire in a minimal volume (usually 20 μl) of a solution of the compound of interest in a volatile solvent. Only a small portion of the total sample remains on the wire when it is withdrawn from the solution. A technique has now been developed in these laboratories, however, which extends field desorption mass spectrometry into the nanogram range. Direct loading of sample onto the emitter may now be carried out so that the sample quantity

necessary to obtain a good field desorption spectrum reflects actual sample consumption (Cook and Olson, 1974).

In a report of field desorption mass spectrometry being indirectly coupled with high speed liquid chromatography to give a combined separation - identification capability (Schulten and Beckey, 1973), it is pointed out that the utility of the system is limited by the amount of material which

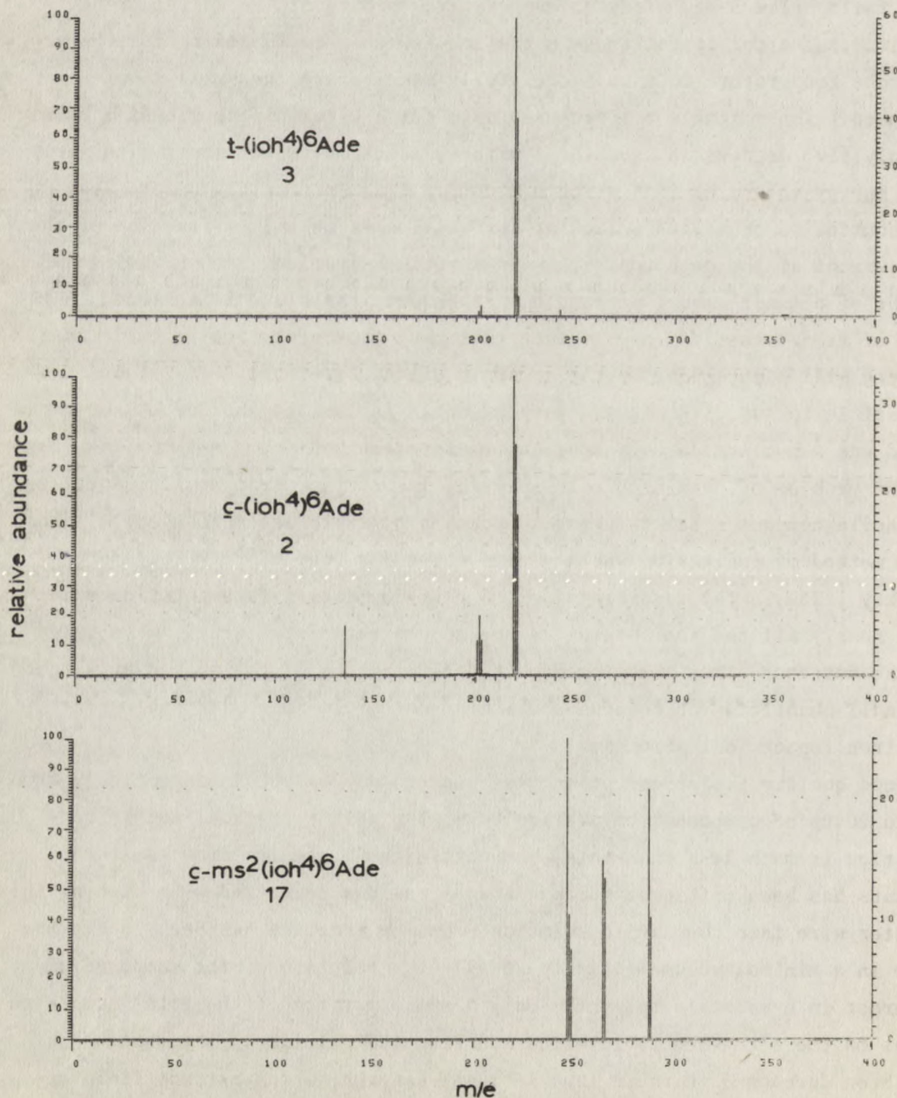


Figure 10a. Field desorption mass spectra. Emitter currents: upper panel, 13 ma; middle, 15 ma; lower, 17 ma.

may be transferred from a very dilute LC eluate to the emitter wire by the dip technique. Direct sample application offers the solution to this problem and makes the lower detection limit of combined HPLC - FDMS dependent on the sensitivity of the chromatography flow monitor.

It has been observed that inorganic salts, particularly those of the

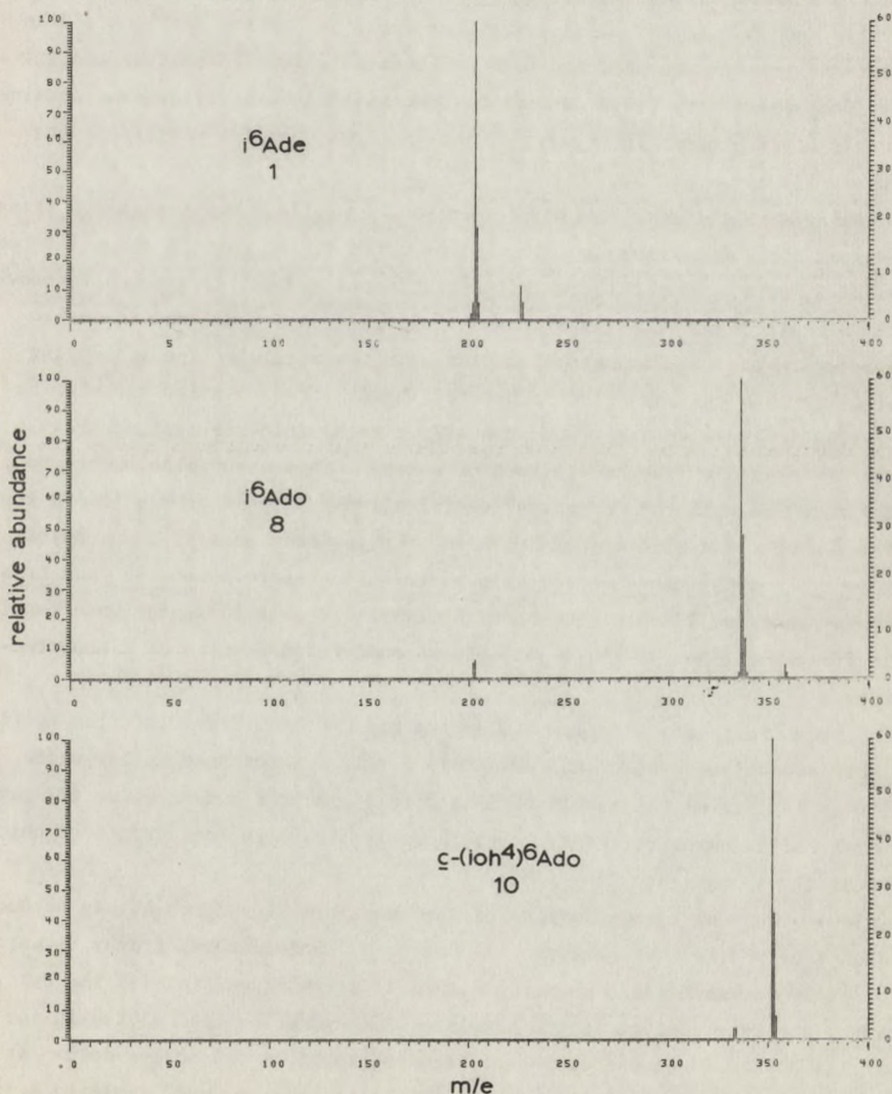


Figure 10b. Field desorption mass spectra. Emitter currents: upper panel, 10 ma; middle, 17 ma; lower, 15 ma.

alkali metals, desensitize the field emitter. It is not surprising, then, that initial investigations of the possibility of coupling FDMS to HPLC were made with silica gel columns and did not involve ion exchange methods. We have found, however, that by use of a volatile buffer salt, such as ammonium formate, the field emitter wire may be dipped directly into the HPLC eluate fractions after concentration in vacuo. A weak spectrum of the salt is initially seen but it soon disappears; the field desorption spectrum of the solute of interest is obtained with no noticeable loss in emitter sensitivity. Representative field desorption spectra of several cytokinins obtained in this way are shown in Figures 10a and 10b. Sample quantities were arbitrarily set at 40 μg.

Features exhibited by these FD spectra (Figure 10) are typical of those observed for the cytokinins. It will be noted in the spectra shown for compounds 3, 1, 8, and particularly 17, that a peak occurs 23 mass units above the molecular ion. This is a commonly observed phenomenom and is due to association of trace amounts of sodium with the molecular species during flight through the mass discriminator. Thermal fragmentation may also be observed in these spectra. Compound 17, 2-methylthio-cis-zeatin, shows a loss of 18 mass units, due to loss of water. Dehydration also occurs to a lesser degree with the other zeatin derivatives. A peak at m/e 135 in the spectrum of cis-zeatin (2) is presumed to arise from loss of the complete sidechain and may prove useful in discriminating between the cis and trans isomers on a small scale. Compound 8 illustrates the loss of ribose to give the free base (m/e 203) as a possible thermal fragmentation of ribonucleosides.

System limits of detection are determined by the ultraviolet flow monitor electronic noise level of approximately 5×10^{-4} absorbance units under flowing conditions. A sample of 5 μg of cis-zeatin ribonucleoside (2) gave a peak height equal to twenty times the noise level in the chromatographic system shown in Figure 2.

Separation and identification of the known cytokinins, then, may be accomplished by the following steps. 1) Cation exchange chromatography is carried out under the conditions shown in Figure 1, allowing analysis of the cytokinin free base content of the sample. 2) Any nucleosides eluted in the initial 50 ml of solvent may be rechromatographed on the Aminex column as shown in Figure 2. 3) The molecular weight of the compounds isolated by ion exchange may be checked by concentration of fractions to minimal volume, dipping of the FD emitter wire into the solution, and determination of the

spectrum. 4) If compounds possessing the zeatin sidechain are detected, the corresponding ion exchange eluate fractions are evaporated in vacuo to minimal volume, applied to a Bio-Gel P-2 column, and desalted by elution with 50% ethanol. The stereochemistry is then assigned by chromatography on the Porapak Q column, or on the Partisil column in the case of 2-methylthiozeatin ribonucleoside (12 and 13).

Summary

A high performance liquid chromatographic system allowing separation of the known naturally occurring cytokinins and assignment of their stereochemistry has been developed. Additional confirmation of structure is afforded by indirect coupling to a field desorption mass spectrometer, leading to direct determination of the molecular weight of the compound of interest, even for the labile cytokinin ribonucleosides.

Acknowledgement

This work was supported by research grant GP 41507X from the National Science Foundation and grant GM 05829 from the National Institutes of Health. The mass spectrometer and the MS data processing equipment employed in the present study were provided by Research Grants CA 11388 and GM 16864 from the National Cancer Institute and the National Institute of General Medical Sciences, respectively, of the National Institutes of Health.

The conductivity monitor, recorder offset device, and gradient elution programmer used in this study were designed and constructed by Mr. Ronald W. Anderson, Director of Electronics Services, School of Chemical Sciences, University of Illinois. The Authors with to thank Dr. L. H. Kirkegaard for many enlightening discussions on chromatographic theory and methodology.

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USE OF MODIFIED tRNA'S IN BIOCHEMICAL STUDIES

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In this paper, I would like to report and to comment on four different results which were obtained in the Göttingen laboratory or in cooperation with other laboratories during this year, and which are dealing with modified nucleotides, either naturally occurring or artificially introduced into tRNA.

I. Structural Studies, Using the Methyl and Methylene Proton Resonances of Yeast $tRNA^{Phe}$

(with L.S. Kan and P.O.P. Ts'O, Johns Hopkins University and F. von der Haar, A. Maelicke and M. Sprinzl, Göttingen[1]).

Nuclear magnetic resonance has been used for structural and conformational studies of tRNA, using mainly the low field proton resonances of the hydrogen bonding base pairs[2-7]. Potentially, the methyl signals of the modified bases could also be used for structural studies, however, the unambiguous assignement of these signals in the macromolecular structure of tRNA poses enormous difficulties. In yeast $tRNA^{Phe}$ one could expect the following methyl or methylene signals (number of nucleotide in tRNA sequence in brackets): $m^2G(10)$, hU(16,17), $m^2_2G(26)$, Cm(32), Gm(34), Y(37), $m^5C(40)$, $m^7G(46)$, $m^5C(49)$, T(54) and $m^1A(58)$ (Fig. 1). Using a number of suitable fragments, for example A-Cm-U-Gm-A-A-Y-A-Ψ-m^5C-U-Gp[8] and T-Ψ-C-G[9], we were able to assign all methyl resonances in $tRNA^{Phe}$. This was done by comparing the particular signal in the monomer, in various oligomers of increasing chain length and in the tRNA, all at different temperatures. As an example, the oligonucleotides used for the assignment in the anticodon region are:

Yp, Y>p	Gm-A-A-Y-Ap
Y-Ap	Gm-A-A-r-A-Ψp
Gm-Ap	Gm-A-A-Y-A-Ψp
pA-Ψp	A-Cm-U-Gm-A-A-r-A-Ψ-m^5C-U-Gp
Gm-A-A-Y	A-Cm-U-Gm-A-A-Y-A-Ψ-m^5C-U-Gp

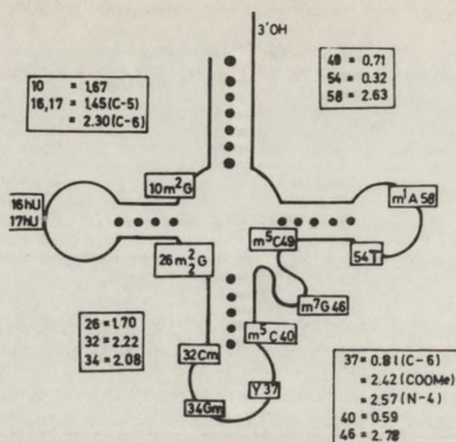


Fig. 1: Assignement of NMR methyl and methylene signals in yeast $tRNA^{Phe}$. The chemical shift values are given in ppm (standard:t-butanol) at 71°C.

The methyl resonances in tRNA are sharp at 71°C and have been assigned at that temperature. At lower temperature, however, most of them are shifted to higher field, become broad and gradually disappear, indicating an involvement of these bases in secondary or tertiary structure. The responses of these signals to the temperature perturbation can be divided into three categories where: (1) The chemical shifts and the linewidths of the resonances are not sensitive to temperature. The two Y $COOCH_3$ groups (6 protons) belong to this category. (2) The chemical shifts of the resonances are slightly affected by the decrease in temperature (either shifting upfield or downfield), but the linewidths of these resonances are greatly broadened at temperatures below the cooperative structural transition. This category includes m^2G, two m^5C, m^1A, C_6-CH_3 and N_4-CH_3 of Y, Gm, Cm, and perhaps m^7G. Basically, the chemical shifts of the methyl groups in the anticodon loop are not greatly affected during the thermal transition. (3) The chemical shifts and the linewidths of the resonances in this category are both sensitive to temperature. Near the transition region, an abrupt upfield shift together with a large linewidth braodening of these resonances occurs with decreasing temperature. The methyl resonances of m_2^2G and T, as well as the methylene resonances of hU (C_5 or perhaps C_6), belong to this category (Fig. 2).

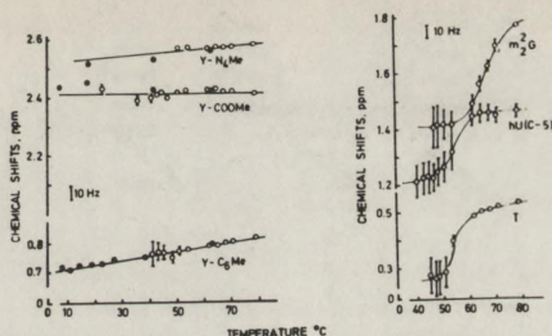


Fig. 2: Chemical shifts of NMR methyl and methylene signals in yeast $tRNA^{Phe}$ dependent on temperature.

The following conclusions can be derived from this work:

1.) The anticodon loop in the intact $tRNA^{Phe}$ does not possess more structure than the corresponding single-stranded dodecanucleotide already has, or, in other words, this loop is not involved in tertiary structure, but is partially stacked. Most of the methyl resonances of the tRNA became broadened at temperatures below the transition (~45°C) as compared to the fragment, but the chemical shifts are rather similar to each other. The Y $COOCH_3$ groups can be observed as a narrow line even at 10°C, suggesting a large rotational freedom of this group. The data suggest that the sidechain of the Y base protrudes out into the solvent and the anticodon loop does not associate with other components of the molecule. A detailed study of the Y base signal, together with previous spectroscopic results[8], therefore suggests a structure for the anticodon region as depicted in Fig. 3.

The Y, being a long flat system suitable for stacking, is attached towards the 3'-side in the normal half-helical way to the adenine ring of A38. The continuation of this helix is, however, interrupted in the other direction. The A36 of the anticodon stacks onto the external end of the Y base and thereby protrudes into the solution. In this conformation, the anticodon is maximally exposed to achieve interaction with the messenger and at the same time it is held rigidly in position by stacking interaction with Y. We would like to name this the "balcony-structure" of the anticodon.

Fig. 3: Proposed conformational structure of the anticodon region of yeast $tRNA^{Phe}$. Left: The original suggestion by Fuller and Hodgson[10]. Middle: Y serving as a "balcony" for the anticodon. Right: "Shrinking" of the stack, when Y is excised[1].

Another feature for the anticodon loop emerges from this study: When Y is excised by mild acid treatment, the A36 and A38 clearly form a new and shorter stack, the helix shrinks. In this structure, the remaining ribose 37 must form a small loop of the length of one ribose phosphate unit. This clearly shows, that loops or imperfections in helical structures are possible, in which only one single nucleotide is looped out. This seems to us a new structural feature, which has often been postulated, and here has been found experimentally for the first time.

2.) The chemical shifts of the methyl resonances of m^5C and m^2G in the helical duplex are not very sensitive to temperature at the transition range (about 0.1 ppm from 45°-80°C), even though the linewidths of these resonances are narrow at high temperature and are broad at low temperature. The linewidth data indicate the involvement of m^5C and m^2G in the conformational transition. This insensitivity of chemical shifts may be due to the locations of these methyl protons which are too far from the helical duplex to be influenced by the diamagnetic effect of the stacked bases. Similarly, the chemical shift, but not the linewidth, of the methyl group of m^1A in the T-Ψ-C loop is insensitive to temperature. All these results suggest that the methyl groups of

these four bases are not near any diamagnetic regions in the native tRNA.

3.) There are dramatic changes in both chemical shifts and linewidths of methyl and methylene groups of m_2^2G, T and hU at the transition region. The profile of the chemical shifts versus temperature indicate a cooperative phenomenon: The T_m^{nmr} on the curves of T and hU are nearly equal, i.e., around 55°C with the transition starting at 48°C and ending at about 67°C. The T_m^{nmr} of the m_2^2G curve is higher, about 67°C, and the transition does not start until 53°C and has not ended near 80°C. These four bases are not located in the helical, double-stranded region as predicted by the generally accepted cloverleaf model. Therefore, the NMR studies of the methyl and methylene protons may provide additional information about the conformational change of tRNA, especially in the non-basepaired regions.

II. Investigation of the Structure of the -C-C-A End with Fluorescence and Spin Labels

(with H. Sternbach, M. Sprinzl, A. Maelicke and F. von der Haar and D. Stehlik and E. Krämer, MPI für med. Forschung, Heidelberg)

The -C-C-A end of tRNA can be regenerated with the enzyme tRNA nucleotidyl transferase according to the scheme given in Fig. 4. Because the enzyme will

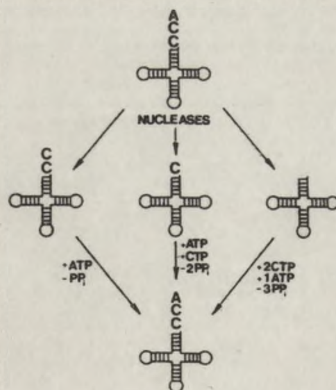
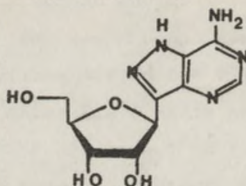


Fig. 4: Degradation by nucleases and restoration by tRNA nucleotidyl transferase of the 3' and of tRNA.

also accept certain modified ATP's and CTP's, one can introduce altered or substituted bases into the -C-C-A end of tRNA in this way(11).

When the fluorescent nucleotide formycin monophosphate is incorporated into the terminal position of yeast $tRNA^{Phe}$ [12], its fluorescence is reduced to



25 % of its original value by quenching (Table 1). This quenching must be attributed to the stacking interaction between formycin and the neighbouring

Table 1: Fluorescence quantum yields of formycin derivatives before and after opening of the ribose ring by periodate and borohydride treatment related to the fluorescence of quininium sulfate [12].

	FTP	C-A-C-C-F	$tRNA^{Phe}$-C-C-F
unmodified	5.2	1.4	1.2
oxi-red	14.0	15.0	13.0

-C-C. This quenching is also observed in the oligomer. If, however, the ribose ring is opened with periodate and subsequently reduced to the open chain diol with borohydride the fluorescence of the monomer, oligomer and tRNA is equal. Thus, in tRNA the full fluorescence is restored by this chemical treatment. This emphasizes the important role of an intact ribose ring and of a conformationally stable backbone for the stacking interaction. These results are well paralleled by biochemical findings: $tRNA^{Phe}$-C-C-$A_{oxi\text{-}red}$ is still fully chargeable[13] and so is $tRNA^{Phe}$-C-C-F[12]. In contrast, $tRNA^{Phe}$--C-C-$F_{oxi\text{-}red}$[12] is not a substrate for the aminoacyl-tRNA synthetase. When two parameters at the -C-C-A end are changed (A into F and ribose into $ribose_{oxi\text{-}red}$), the system becomes conformationally unstable, unstacks and at the same time looses its substrate properties. We therefore postulate that a stacked -C-C-A terminus is required for aminoacylation in tRNA. This is depicted in Fig. 5.

Position 75 in $tRNA^{Phe}$ can in a similar fashion be substituted with 2-thiocytidine instead of cytidine[14] and this product be restored to $tRNA^{Phe}$--C-s^2C-A. This tRNA is undistinguishable in its properties in aminoacylation

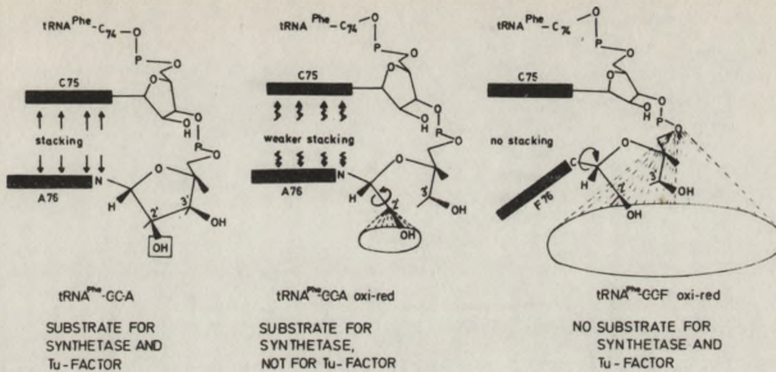


Fig. 5: Schematical display of the structure of the -C-C-A end of three $tRNA^{Phe}$ species and the possible motion area of the terminal 2'-hydroxyl group.

from normal $tRNA^{Phe}$-C-C-A. Interestingly, the s^2-sulfur behaves like an SH-group in its reaction with iodoacetamide; the reaction is quantitative and pseudofirst order at $tRNA^{Phe}$-C-s^2C-A (Fig. 6). This reaction has now been

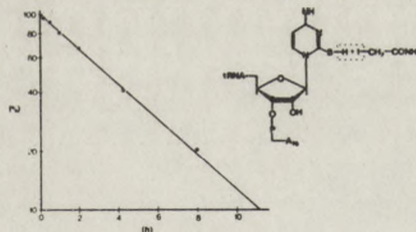


Fig. 6: Time dependence of the reaction of $tRNA^{Phe}$-C-s^2C-A with $[^{14}C]ICH_2CONH_2$.

used to introduce a spin label into the tRNA molecule. The nitroxide label is fixed to the sulfur atom of C75 (Fig. 7)[15]. The product, $tRNA^{Phe}$-C-(s.l.)s^2C-A, can still be aminoacylated by phenylalanyl-tRNA synthetase. EPR spectra (a typical spectrum at room temperature is shown in Fig. 7) obtained between 10 and 70°C for $tRNA^{Phe}$-C-(s.l.)s^2C-A and the pentanucleotide C-A-C-(s.l.)s^2C-A, a model compound, were evaluated for the correlation time, τ_c, of the

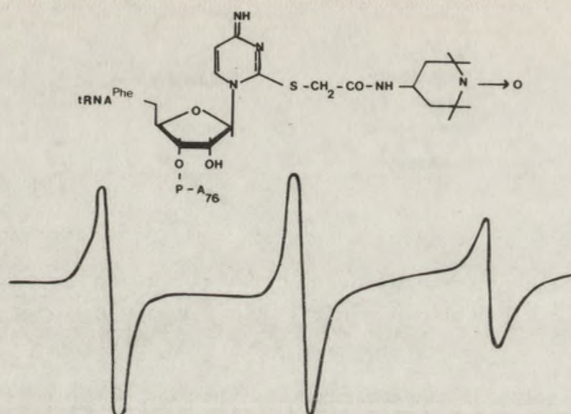


Fig. 7: Structure and EPR spectrum of spin labeled $tRNA^{Phe}$-C-s^2C-A.

reorientation of the spin label in its environment and for the motional anisotropy parameter, ε. Spin-labeled pentanucleotide is characterized by a spin label motion with an activation energy, E_A, of 7.0 kcal/mol and a strongly anisotropic motion with a long axis of the rotational ellipsoid along the N-O bond of the radical. This motion is temperature independent. In comparison spin label bound to the tRNA molecule is more immobilised and its motion is more isotropic. In addition, the slope of log $\tau_c(T)$ and the nature of the motion changes at a critical temperature T_{cr} which, in the absence of magnesium, varies with ionic strength. In the presence of magnesium T_{cr} is 47.5°C, whereas the midpoint temperature of the optical melting curve, T_m, is 75°C. The melting transition monitored by the spin label is a cooperative process associated with the melting of a higher-ordered structure of the $tRNA^{Phe}$ molecule. These results again show, that the -C-C-A end of tRNA is rigidly bound to the main structure of the molecule by stacking interactions.

This is further confirmed by a combined EPR and NMR study[16]. A free radical will broaden the nuclear magnetic resonances in its immediate environment. High resolution NMR of the low field protons in the base pair region[17] has revealed that only a few base pairs in the acceptor stem are influenced by the spin label. Thus the -C-C-A end (together with its spin label) is clearly not loosely wiggling around the 3' end of the molecule but held in a

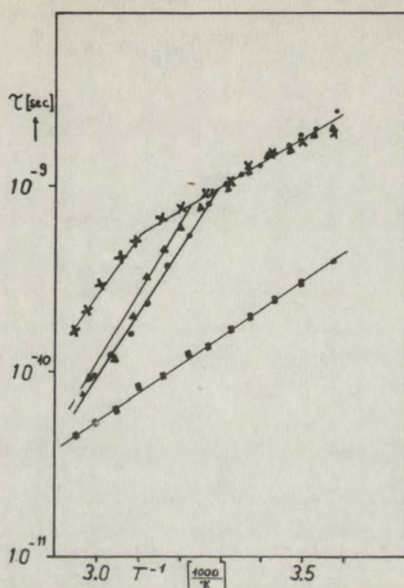


Fig. 8: The rotational correlation time, τ_c, as function of the inverse temperature. The three top curves are spin labelled $tRNA^{Phe}$ at various buffer and Mg^{++} concentrations, the lower curve is the spin labelled oligonucleotide at high salt and Mg^{++} concentration (for details see original paper[15]).

defined and rigid position, disturbing only the one closest base pair. This finding can serve as final proof of our previous postulate[18] about the structure of the -C-C-A end in solution.

III. Incorporation of Deoxyadenosine and Position of the Aminoacylation of tRNA

(with M. Sprinzl, K.H. Scheit, H. Sternbach and F. von der Haar).

The question has been asked since ten years, whether tRNA is aminoacylated at its 2' or 3'-hydroxyl[19-21]. Rapid acyl migration between 2' and 3'-hydroxyl prevents an unambiguous decision, since any isolation procedure of the aminoacyl-tRNA would have to go through conditions, where an isomerisation could occur. With yeast $tRNA^{Phe}$ this could finally be decided in the following way. The two deoxy ATP's, 2' and 3'-dATP were prepared and incorporated into $tRNA^{Phe}$-C-C (Fig. 9)[22]. These ATP derivatives are however poor substrates and their incorporation is not complete. Therefore the remaining $tRNA^{Phe}$-C-C

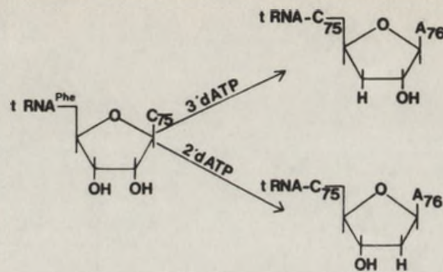


Fig. 9: Preparation of $tRNA^{Phe}$-C-C-3'dA and $tRNA^{Phe}$-C-C-2'dA.

was removed on a boronic acid column (Fig. 10) making use of the fact that the $tRNA^{Phe}$-C-C-dA cannot form an ester with boronic acid[23]. In this way

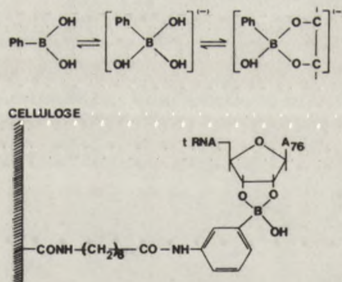


Fig. 10: Formation of boronic acid complexes with cis-diols.

both $tRNA^{Phe}$-C-C-dA can be obtained in pure form free of contaminants as shown in Fig. 11.

When these tRNA's were studied in aminoacylation experiment[24] it turned out that the 2'-OH group of $tRNA^{Phe}$-C-C-3'dA was the only OH-group that could

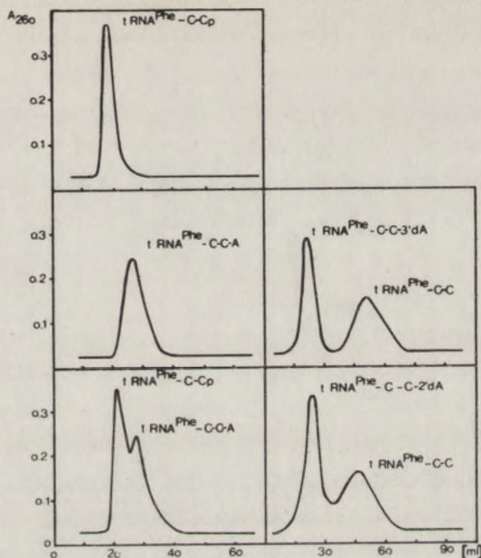


Fig. 11: Separation of modified $tRNA^{Phe}$ species on cellulose-bound phenylboronic acid.

accept phenylalanine. Fig. 12 shows the enzyme kinetics. It accepted 1 420 pmol [^{14}C]phenylalanine per A_{260} unit tRNA, whereas $tRNA^{Phe}$-C-C-A accepts

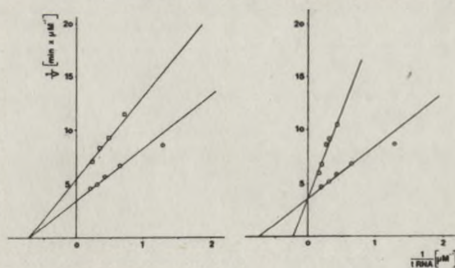


Fig. 12: Lineweaver-Burk plot for aminoacylation of yeast $tRNA^{Phe}$-C-C-A (o-o), $tRNA^{Phe}$-C-C-3'dA (□-□, left) and $tRNA^{Phe}$-C-C-A in presence of $tRNA^{Phe}$ -C-C-2'dA (□-□, right).

1 480 pmol [^{14}C]phenylalanine per A_{260} unit tRNA. Oxidation of $tRNA^{Phe}$-C-C-A with sodium periodate abolishes its aminoacylation capacity completely. In

contrast $tRNA^{Phe}$-C-C-3'dA is resistant to this reagent and periodate treatment does not influence its aminoacylation. The K_m for $tRNA^{Phe}$-C-C-A is identical to that for $tRNA^{Phe}$-C-C-3'dA (2.8 μM); the V_{max} of the latter is slightly reduced (perhaps reflecting loss in nucleophilicity of the 2'-OH group because of the absence of the neighbouring 3'-OH group). $tRNA^{Phe}$-C-C-2'dA, which is not aminoacylated, is at the same time a competitive inhibitor of phenylalanyl-tRNA synthetase having a K_i of the same order as the K_m of the neutral substrate $tRNA^{Phe}$-C-C-A (K_i = 2.16 μM; K_m = 2.86 μM). In this connexion it is noteworthy that at least in the yeast-phenylalanine system no modified inhibiting tRNAs have been found so far. Even such structurally similar molecules as the periodate-oxidized $tRNA^{Phe}$, $tRNA^{Phe}$-C-C-A_{oxi}, or $tRNA^{Phe}$-C-C are not competitive inhibitors. The $tRNA^{Phe}$-C-C-2'dA, however, being a competitive inhibitor, can be regarded as a "correct substrate" with respect to the active site of the enzyme. We therefore conclude that the -C-C-A end and especially the terminal ribose moiety must have a highly defined position during the aminoacylation reaction. Its 2'-hydroxyl group is so strictly localized that no "mischarging" at the 3'-hydroxyl group can occur.

We had found previously that also $tRNA^{Phe}$-C-C-$A_{oxi-red}$ can still be aminoacylated[13]. We assume that this tRNA also carries the phenylalanine residue at the 2'-hydroxyl. Because of the opened ribose ring, this residue can no longer migrate. The 2'-phenylalanylester has indeed recently been isolated and characterized by NMR[25]. When 3'-deoxy-3-amino-ATP is used as a substrate for tRNA nucleotidyl transferase one obtains a $tRNA^{Phe}$ with an aliphatic amino group at the 3'-position of the 3'-end. This tRNA also accepts phenylalanine[26]. In this case the phenylalanine will end up in an amide linkage on the 3'-amino group by rapid and irreversible isomerisation. The various derivatives thus obtained are summarized in Fig. 13.

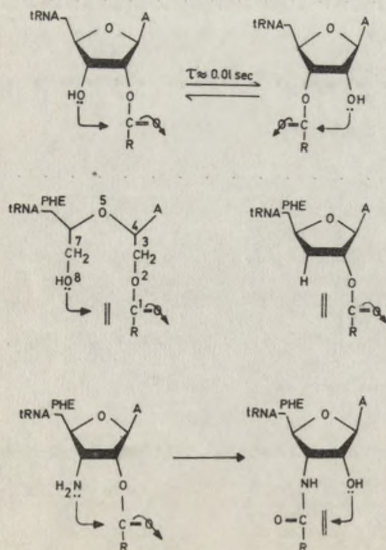


Fig. 13: Various aminoacyl derivatives of modified $tRNA^{Phe}$ and their possible 2'-, 3'- isomerisation.

IV. Modified tRNA's in Studies of the Mechanism of Protein Biosynthesis

(with G. Chinali, M. Sprinzl and A. Parmeggiani, Gesellschaft für Molekularbiologische Forschung, Stöckheim and Max-Planck-Institut für experimentelle Medizin, Göttingen).

With the tRNA's thus obtained, studies on the sterical requirements of aminoacyl-tRNA's for protein biosynthesis could be carried out. The results are briefly summarized in Table 2. For details see the original paper[27]. The

Table 2: Function of various modified tRNA's in single steps of the ribosomal protein synthesis.

Phe-$tRNA^{Phe}$ Species	Function in Ribosomal Site EFT-Directed Binding	GTPase Activity	A Acceptor (Aminoacyl)	P Donor (Peptidyl)
-C-C-A	+	+	+	+
-C-C-3'dA	+	+	(+)	-
-C-C-NH_2A	-	-	+	-
-C-C-$A_{oxi-red}$	-	-	(+)	-

principal steps of this enzymatic mechanism are depicted in Fig. 14. The first

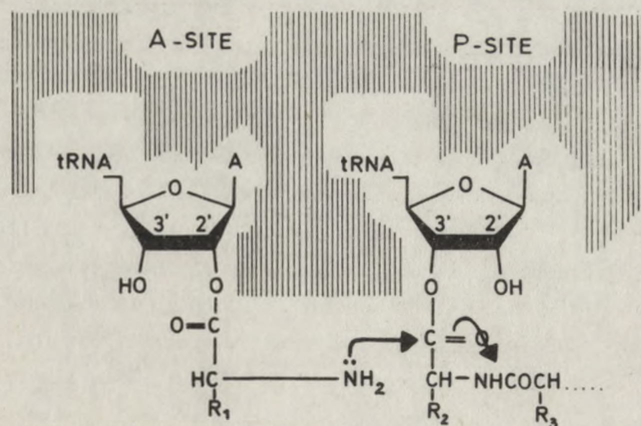


Fig. 14: Scheme of peptide bond formation (position of aminoacyl residues in 2' or 3' are arbitrary).

step in ribosome peptide synthesis, the EF-T directed binding of the aminoacyl-tRNA, seems to require an aminoacyl residue at the 2' position of the ribose and/or an intact ribose ring. As we have seen before, the intactness of the ribose ring can have a decisive influence on the structure of the -C-C-A end. Both the "non-isomerisable" 2'-aminoacylated Phe-$tRNA^{Phe}$-C-C-3'dA and Phe-$tRNA^{Phe}$-C-C-$A_{oxi-red}$ are active as acceptors of the peptidyl residue. Using these substrates bound to the A-site of E.coli ribosomes and an Ac-Phe-$tRNA^{Phe}$ bound to the ribosomal P-site in both cases a formation of Ac-Phe-Phe was observed. The formation of the dipeptide proceeds, however, with a much slower rate as compared to a case when the native Phe-$tRNA^{Phe}$-C-C-A is used as acceptor. Phe-$tRNA^{Phe}$-C-C-3'dA bound enzymatically to the ribosomal A-site can even be replaced by native Phe-$tRNA^{Phe}$-C-C-A in a EF-T catalysed reaction. This shows that this 2'-aminoacyl-tRNA being a substrate for EF-T is still not able to interact effectively with the A-site. Judging from the structure of puromycin, which is a 3'-analog of aminoacyl-tRNA, and from the results obtained by investigation of specifically aminoacylated tRNA fragments in a fragment reaction[(28)], the 3'-isomer of aminoacyl-tRNA is most probably the substrate required for reaction with the peptidyl-tRNA on the peptidyl transferase center. Transacylation from 2' to 3'-position must therefore occur during the process of EF-T dependent binding of aminoacyl-tRNA to the ribosomal A-site. Only the native aminoacyl-tRNA possessing a 3'-terminal ribose, and thus being able to isomerise, can be transported as a 2'-isomer by the elongation factor. On the other hand only the 3'-isomer is tightly bound to the ribosomal A-site. We suggest therefore that the isomerisation from 2' to 3'-position is required to lock the aminoacyl-tRNA into the ribosomes.

Despite the fact that Ac-Phe-$tRNA^{Phe}$-C-C-3'dA and Ac-Phe-$tRNA^{Phe}$-C-C-$A_{oxi-red}$ bind to the ribosomal P-site as tightly as Ac-Phe-$tRNA^{Phe}$-C-C-A, they are completely devoid of donor activity. Ac-Phe-$tRNA^{Phe}$-C-C-3'NH_2A also does not function as a donor[(26)] but this can be due to the stable amide linkage by which the peptidyl residue is bound to this tRNA. Although the ester bond in the isomerisable 2'-aminoacyl-tRNA species is slightly more stable than in native Phe-$tRNA^{Phe}$ such an enhanced stability is probably not the reason for the complete failure of this analog to act as a donor. More likely the 3'-aminoacyl-tRNA is the active isomer also on the ribosomal P-site, or a free vicinal hydroxyl is required for the release of the peptidyl residue from the 2'-hydroxyl group.

One final comment should be made on the surprising finding that both the 2' and the 3'-aminoacyl derivative of $tRNA^{Phe}$ can function as acceptors in the A-site. This apparent lack of specificity is against the general experience of the high selectivity and narrow spatial requirements in enzymatic reactions. How could it be explained, that in the center of the peptidyl transferase, both derivatives do function? If one assumes the sterically most favoured conformation of the amino acid side chain at the tRNA, one can accomodate the reacting amino group in almost the same position, no matter whether it is a 2' or a 3' aminoacyl derivative (Fig. 15). From our results we there-

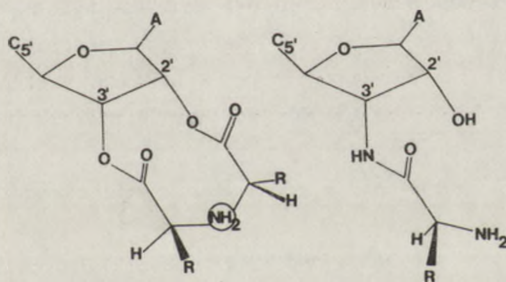


Fig. 15: Left: Stereochemically possible conformations for the aminoacyl residues in 2' or 3' position of a tRNA with identical position of the amino group. Right: Conformation of puromycin[29-30].

fore would like to postulate that the amino group of the aminoacyl residue is accomodated in the position indicated (circle, Fig. 15, left) in the center of the peptidyl transferase, and it is from this position, that the amino group performs the nucleophilic attack on the activated ester of the peptide. From X-ray analysis of puromycin crystals the same conformation for the side chain of the substituted tyrosine has been confirmed[29-30].

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CHARACTERIZATION AND BIOSYNTHESIS OF MODIFIED NUCLEOSIDES IN tRNA

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1. INTRODUCTORY REMARKS

One of the typical characteristics of tRNAs is that they contain a variety of modified nucleosides. Approximately 50 modified nucleosides have been isolated and characterized to date, and these are shown in Table I. Until the early 1960s, most of the methylated nucleosides, pseudouridine and other simple modified nucleosides were discovered and characterized by Hall, Dunn, Lane, Cohn, Allen and their coworkers. In 1964 Holley and his coworker obtained the total sequence of yeast $tRNA^{Ala}$ and after this studies on modified nucleoside progressed greatly as information accumulated on the primary structures of tRNAs.

Results have shown that modified nucleosides are not randomly distributed in tRNA, but are located in specific positions in the clover-leaf structure which vary with the type of modification. This suggested that modified nucleosides have specific roles in tRNA functions, and indeed it now seems likely that several unique modified nucleosides located in the first position of the anticodon must play a role in recognizing specific codon sequences.

During studies on the primary structure of tRNA, more new modified nucleosides have been found. For example, even simple modified nucleosides, such as dihydrouridine (49) and $\underline{N}^4$-acetylcytidine (50) were not detected until the nucleotide sequences of tRNAs containing these modified nucleosides were studied.

Table I

Modified nucleosides isolated from tRNA

Nucleosides	References
Purine Nucleosides	
Methylated derivatives	
1-Methyladenosine	1
2-Methyladenosine	2,3,4
7-Methylguanosine	5,6
1-Methylguanosine	7,8
N^6-Methyladenosine	2,9
N^6,N^6-Dimethyladenosine[a]	3
N^2-Methylguanosine	7,8
N^2,N^2-Dimethylguanosine	8
2'-O-Methylated derivatives	
2'-O-Methyladenosine	10,11
2'-O-Methylguanosine	11
Deaminated derivatives	
Inosine	12
1-Methylinosine	12
Adenosine derivatives containing the isopentenyl group	
N^6-(Δ^2-Isopentenyl)adenosine	13,14
2-Methylthio-N^6-(Δ^2-isopentenyl)adenosine	15,16,17
N^6-(cis-4-Hydroxy-3-methylbut-2-enol)adenosine	18,19,20,21
N^6-(cis-4-Hydroxy-3-methylbut-2-enol)-2-methylthio-adenosine	20,21
Others	
2-Ribosylguanosine[a]	22,23
9-[2'(3')-O-Ribosyl-β-D-ribofuranosyl]adenine	24
Base "Y"	25
Base "Yt"	26
Base "Peroxy Y"	27
N-[(9-β-D-Ribofuranosylpurin-6-yl)carbamoyl]threonine	28,29
N-[(9-β-D-Ribofuranosylpurin-6-yl)carbamoyl]glycine	30
N-[(9-β-D-Ribofuranosylpurin-6-yl)-N-methylcarbamoyl]-threonine	31
N-[N-[(9-β-D-Ribofuranosylpurin-6-yl)carbamoyl]-threonyl-trihydroxymethylmethylamide[c]	32
Nucleoside "Q"	33

Pyrimidine Nucleosides	
Methylated derivatives	
Ribothymidine	3,34
5-Methylcytidine	35,36
3-Methyluridine	37
3-Methylcytidine	37
2'-O-Methylated derivatives	
2'-O-Methyluridine	11
2'-O-Methylcytidine	11
2'-O-Methylribothymidine	38
2'-O-Methylpseudouridine	39
4-Methyl-2'-O-methylcytidine[a)]	40
Sulfur-containing derivatives	
4-Thiouridine	41
2-Thiouridine-5-acetic acid methyl ester	42
5-Methylaminomethyl-2-thiouridine	43
5-Methyl-2-thiouridine	44
2-Thiocytidine	42,45
Others	
Pseudouridine	46,47,48
Dihydrouridine	49
N^4-Acetylcytidine	50
5-Carboxymethyluridine[b)]	51
5-Carboxymethyluridine methyl ester	51,52
5-Hydroxyuridine[a)]	53
Uridin-5-oxyacetic acid	54
3-(3-Amino-3-carboxypropyl)uridine	55

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a) It is not certain whether this compound is derived from tRNA.

b) This might be a degradation product formed during the drastic isolation procedure.

c) The trihydroxymethylmethylamide moiety of this compound is derived from Tris-buffer added to the medium for growth of *E. coli*.

It is very difficult to isolate a modified nucleoside that is present in only one or a few species of amino acid specific tRNA, using unfractionated tRNA as a source of the modified nucleoside, because the content of this modified nucleoside in the total unfractionated tRNA will be less than 0.05%, and it is extremely difficult to isolate so small a quantity of material. Moreover, use of pure species of tRNA as a source of modified nucleoside is possibly the only way to be certain that the component is a part of the tRNA and not an impurity carried through the isolation procedures. In addition, it is essential to know the location of the modified nucleoside in the tRNA molecule to understand its function and *vice versa*.

Since 1967, we have been interested in the structures and functions of modified nucleosides in *E. coli* tRNA. For this purpose, we have obtained quantities of purified tRNAs by combinations of several column chromatographic procedures (56). Using these procedures, most of the tRNAs from *E. coli* can be obtained in sufficient amounts for characterization of their modified nucleosides, and studies on their structures, and locations in the tRNA molecule. It has also been possible to correlate the presence of particular modified nucleosides with codon recognition of tRNA.

This article, reports details of modified nucleosides that were characterized very recently in our laboratory.

2. RESULTS and DISCUSSION

2.1. Modified nucleoside, Q.

An unidentified modified nucleoside, designated as Q, was first discovered in the first position of the anticodon of *E. coli* $tRNA^{Tyr}$ (57,58). Later it was found that Q was also present in the same position of *E. coli* $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ (59). Namely all four *E. coli* tRNAs containing Q recognize the codon sequence, XA^{u}_{c}. It is thought that Q is derived from G (58). It was shown that modification of G to Q resulted in increase in the efficiency of recognition of codon sequences ending with U as compared with those ending with C, *in vitro* (59) (Fig. 1). However, it is unlikely that replacement of G by Q in the first position of the anticodons has any drastic affect on *in vivo* protein synthesis,

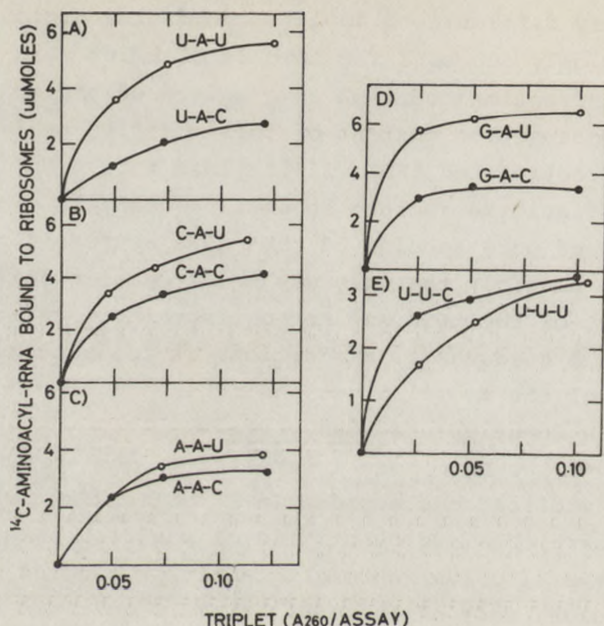

Fig. 1. Stimulation of binding of E. coli [^{14}C]tyrosyl-tRNA$_2^{Tyr}$, [^{14}C]histidyl-tRNA$_1^{Hil}$, [^{14}C]asparaginyl-tRNA^{Asn}, [^{14}C]-aspartyl-tRNA$_1^{Asp}$ and [^{14}C]phenylalanyl-tRNA^{Phe} to ribosomes by the corresponding codon triplets in the presence of 0.02 M magnesium ion. The reaction mixture (0.05 ml) contained: (A) [^{14}C]tyrosyl-tRNA$_2^{Tyr}$ (12.5 pmoles), (B) [^{14}C]histidyl-tRNA$_1^{His}$ (15.6 pmoles), (C) [^{14}C]asparaginyl-tRNA^{Asp} (13.3 pmoles), (D) [^{14}C]aspartyl-tRNA$_1^{Asp}$ (12.2 pmoles) and (E) [^{14}C]phenylalanyl-tRNA^{Phe} (13.7 pmoles).

since there was only slight preferential recognition of code words ending with U. Thus the real function of Q in E. coli tRNA is not yet understood. In 1973 White et al. (60) made the interesting observation that Q or its derivative was found in Drosophila tRNAs corresponding to the same four amino acids, and that the amount of Q varied during the life cycle of this organism. They suggested that Q plays a role in the regulatory function of tRNA rather than in protein synthesis. It has been found that inhibition of the enzymatic activity of tryptophan pyrrolase by Drosophila tRNA^{Tyr} from a vermilion mutant depends on whether the tRNA^{Tyr} contains Q or guanosine in the first position of the anticodon (61).

To understand the function of Q, it is necessary to characterize its structure. We have been working on this for the last five years collaboration with Dr. James A. McCloskey and his colleagues, and we are now able to propose the structure of Q shown in Fig. 2. (33). Q has a very unique structure in that it

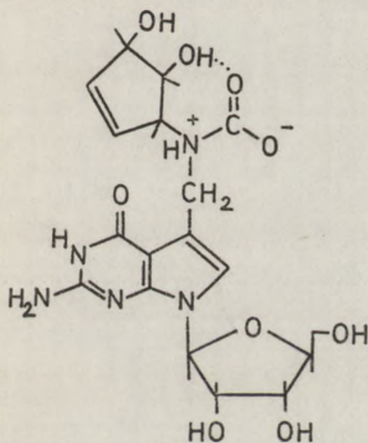

Fig. 2. Structure of nucleoside Q.

is the only modified nucleoside so far isolated in which the purine skelton is modified to a 7-deaza structure. Another interesting feature is that it contains a cyclopentene diol group as a side chain.

2.1.1. Isolation

The presence of Q in tRNA is very easily detected by two-dimensional thin-layer chromatography of a RNase T_2 digest of the tRNA. As shown in Fig. 3, chromatograms of digests of each tRNA showed that 1 mole of modified nucleotide, Qp was found in *E. coli* $tRNA_2^{Tyr}$, $tRNA_1^{His}$, $tRNA^{Asn}$ and $tRNA_1^{Asp}$. Qp is clearly separated from the spot of Gp. As shown in Fig. 4, the UV absorption spectra of the samples of Qp isolated from the four different tRNAs were identical in all respect. The spectra are rather similar to those of Gp, except that the profile is shifted to a higher wavelength, having a λ_{max} at 260 nm at neutral pH, and there is a second λ_{max} at 220 nm.

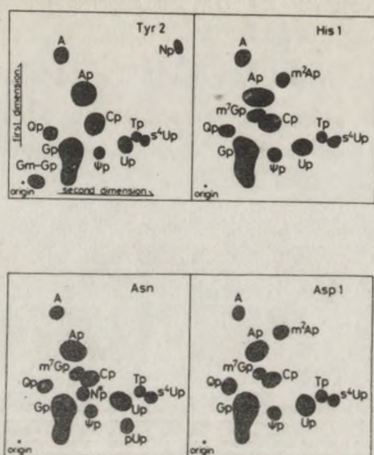

Fig. 3. Two-dimensional chromatograms of RNase T_2 digest of *E. coli* tRNAs containing Q.

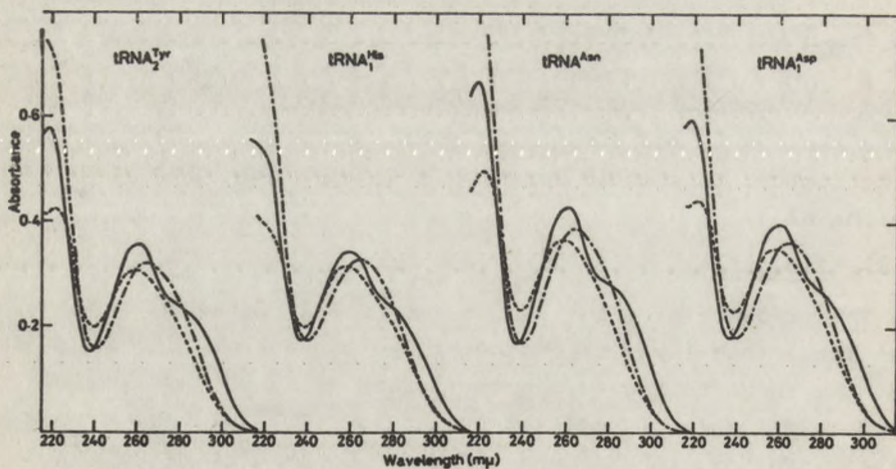

Fig. 4. UV-absorption spectra of Qp, isolated from *E. coli* tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn} and tRNA$_1^{Asp}$: 1 *N* HCl, ·······; pH 2.0 and 7.0, ————; pH 12.0, —·—·—.

For isolation of Q on a large scale, unfractionated tRNA was used instead of pure individual tRNAs. Generally 100,000 A_{260} units (approximately 5 g) of unfractionated E. coli tRNA were extensively hydrolyzed by pancreatic RNase, and fractionated by DEAE-Sephadex A-25 column chromatography (5 cm x 100 cm) in the presence of 7 M urea. As shown in Fig. 5, Q was recovered as

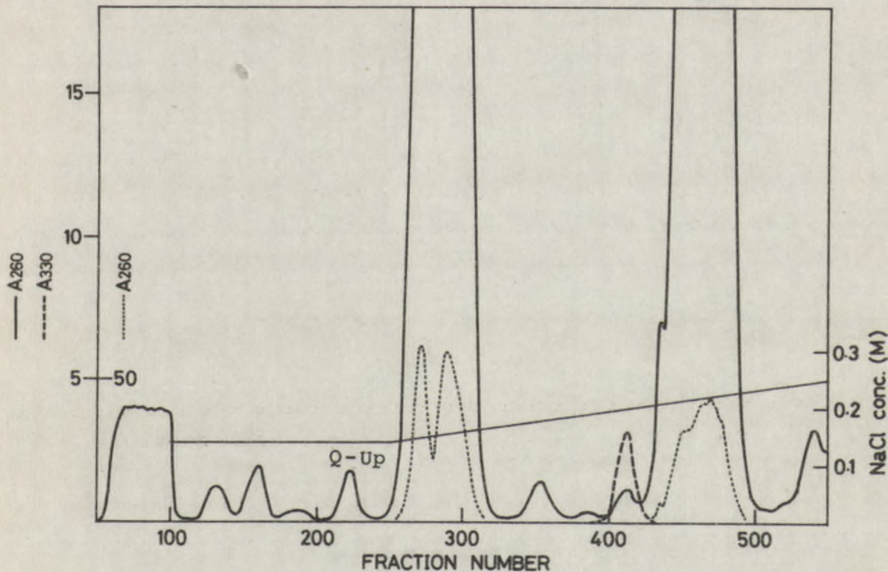

Fig. 5. Isolation of Q-Up from pancreatic RNase digest of E. coli unfractionated tRNA by DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea.

Q-Up, and Q-Up was eluted from the column before most mononucleotides. The Q-Up obtained was completely hydrolyzed by RNase T_2, and Qp was purified by Dowex 1 column chromatography (Fig. 6). The Nucleoside, Q, was obtained from Qp by treatment with E. coli alkaline phosphomonoesterase, followed by Dowex 1 column chromatography. Approximately 100 A_{260} units of Q were obtained from 100,000 A_{260} units of unfractionated E. coli tRNA. Frequently we also isolated Q on 4 times this scale fractionating 400,000 A_{260} units of tRNA hydrolyzate on a 10 cm x 100 cm column.

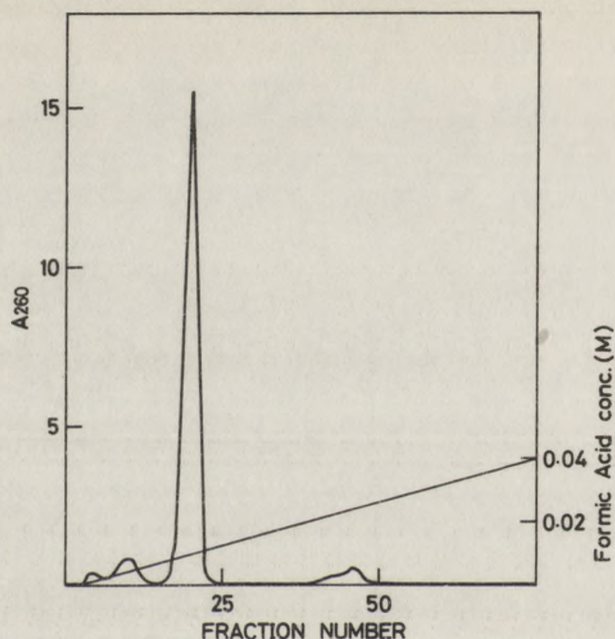

Fig. 6. Isolation of Qp by Dowex 1 column chromatography.

2.1.2. Elucidation of the Structure of Q

2.1.2.1. General properties of Q

As mentioned above the UV absorption spectra of Q are rather similar to those of guanosine, but its λ_{max} is shifted to higher wavelength, and there is a second λ_{max} at 220 nm. The phosphodiester bond of Q is resistant to pancreatic RNase, but is ruptured by RNase T_2. Q behaves as if it has a positive charge on DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea or on Dowex 1 column chromatography. This apparent basic character of Q is lost after treatment with cyanogen bromide or periodate. The UV spectra of Q change on periodate oxidation, as discussed later. Q is resistant to acid treatment (100°C for 1 hr in 1 N HCl), indicating that the glycosidic bond of Q is stable. This is a characteristic property of all 7-deazapurine compounds. It should also be mentioned that the shift of the λ_{max} in the UV spectrum of Q to a higher wavelength than that of guanosine is also generally observed with 7-deaza compounds.

2.1.2.2. Mass spectrometry of Q

The most valuable information on Q was obtained by mass spectroscopic analysis. Attempts to record the mass spectrum of free Q directly were unsuccessful because of its high polarity. Therefore, it was subjected to the following treatments to convert it to more volatile derivatives; (a) acetylation by acetic anhydride treatment followed by permethylation; (b) acetylation followed by trimethylsilylation; (c) direct trimethylsilylation; (d) cyanogen bromide treatment followed by trimethylsilylation; and (e) periodate oxidation followed by trimethylsilylation.

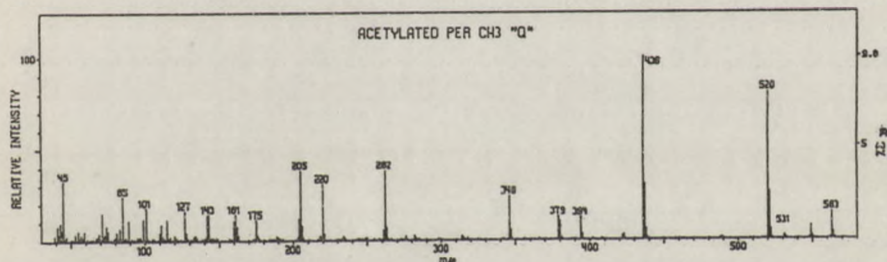

Fig. 7. Mass spectrum of Q_{Ac-Me}.

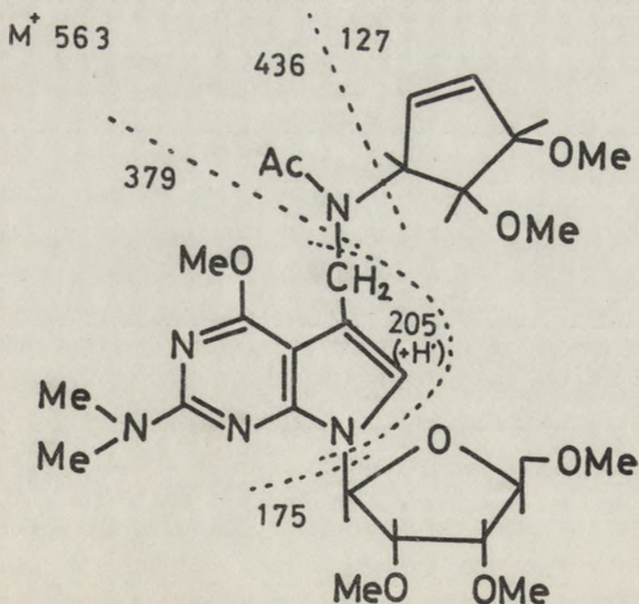

Fig. 8. Structure of Q_{Ac-Me} and assignment of the fragment peaks.

Fig. 7 shows the low-resolution mass spectrum of Q obtained by procedure (a). The spectrum exhibits a molecular ion peak of m/e 563 (Q_{Ac-Me}), which is known to contain 8 methyl groups and one acetyl group. This established the molecular weight of Q_{Ac} as 409. High-resolution data of the molecular ion and fragmentation peaks were also recorded and are shown in Table II. These data indicate that the skelton of Q contains only four nitrogen atoms, and that the side chain may contain a cyclopentene diol group. From these results and those on the NMR discussed later, the structure of Q_{Ac-Me} was concluded to be as shown in Fig. 8.

Table II

Elementary composition of Q_{Ac-Me} deduced by high-resolution analysis

Mass	Composition	Error (m mass)
563.2978	$C_{27}H_{41}N_5O_8$	2.19
520.2767	$C_{25}H_{38}N_5O_7$	-0.46
531.2724	$C_{26}H_{37}N_5O_7$	3.21
436.2204	$C_{20}H_{30}N_5O_6$	0.84
380.2043	$C_{18}H_{28}N_4C_4$	-1.62
379.1964	$C_{18}H_{27}N_4O_5$	-1.70
346.1904	$C_{17}H_{24}N_5O_3$	2.49
262.1327	$C_{12}H_{16}N_5O_2$	2.28
220.1226	$C_{10}H_{14}N_5O$	2.81
205.1090	$C_{10}H_{13}N_4O$	0.10
175.0985	$C_8H_{15}O_4$	1.44
143.0708	$C_7H_{11}O_3$	-0.04
127.0766	$C_7H_{11}O_2$	0.11

Mass spectrum taken by procedure (c), i.e. direct trimethylsilylation, gave a molecular ion of m/e 1029 with 8 silyl groups, thereby indicating that the molecular weight of Q is 453. This is 44 mass units higher than that obtained by procedure (a). It is

very likely that acetylation resulted in loss of a CO_2 group from Q. It should be mentioned that a strong fragmentation peak of m/e 656 (5 TMS) was seen.

To obtain the mass spectrum of Q by procedure (e), the nucleotide, Qp was first treated with periodate, and then its phosphate was removed by treatment with E. coli alkaline phosphomonoesterase. The modified nucleoside, Q^*, was then trimethylsilylated. The mass spectrum of this Q^*_{TMS} gave a molecular ion of m/e 749 (5 TMS)

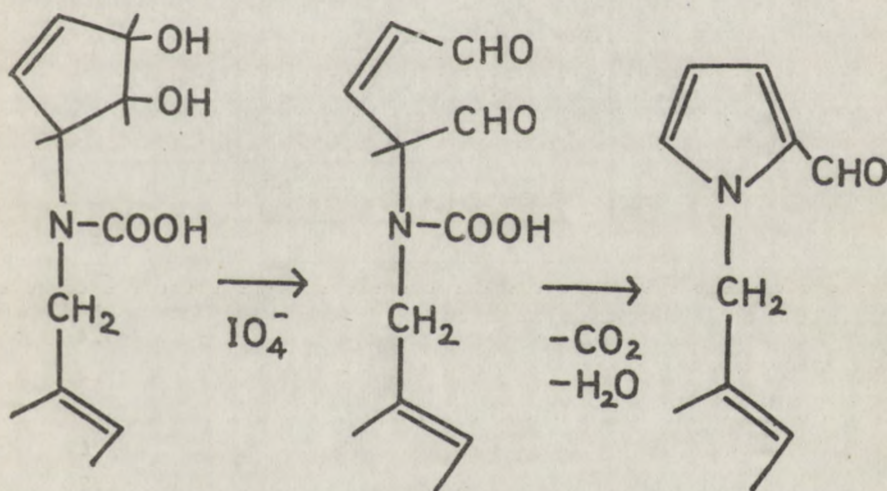

Fig. 9. Formation of pyrrole ring by periodate oxidation.

in addition to a strong fragmentation peak of m/e 734 (5 TMS), indicating that the molecular weight of Q^* is 389. These results indicate that periodate reacts with a cis-diol in a cyclopentene group, thereby forming N-substituted pyrrole aldehyde as shown in Fig. 9. Change of the UV spectra of Q by modification with periodate can reasonably be explained by this chemical conversion (see Fig. 10), since N^1-methyl-2-pyrrole-aldehyde has UV absorption spectrum with λ_{max} of 279 nm and ε of 4.25. Mass spectrum of Q taken by procedure (d) showed that treatment with cyanogen bromide liberates CO_2 with incorporation of a CN group into Q, since a molecular ion of m/e 938 was obtained (Fig. 11). The

presence of fragmentation peaks of m/e 656 and 696 also indicates that the nitrogen attached to the cyclopentene diol is chemically active (Fig. 11).

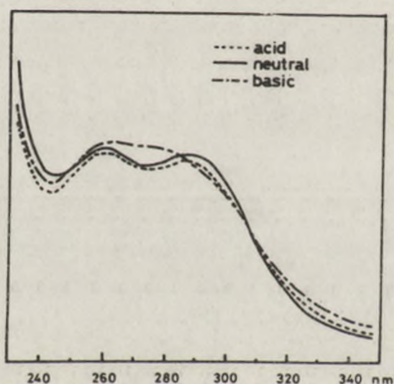

Fig. 10. UV absorption spectra of Q modified with periodate (Q^*).

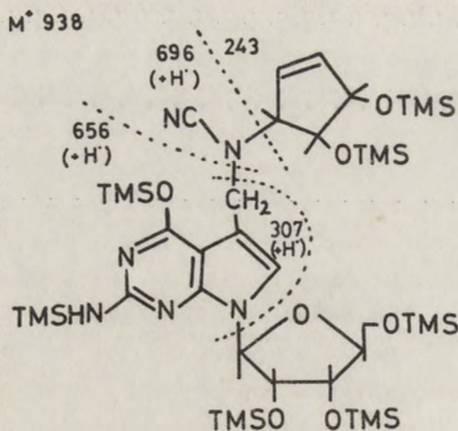

Fig. 11. Structure of $Q_{BrCN-TMS}$ and assignment of the fragment Peaks.

2.1.2.3. Confirmation of the structure of Q by study of the NMR

The NMR of Q was taken in D_2O using a 220 MHz instrument (Fig. 12). The signals were assigned and were shown in Fig. 13.

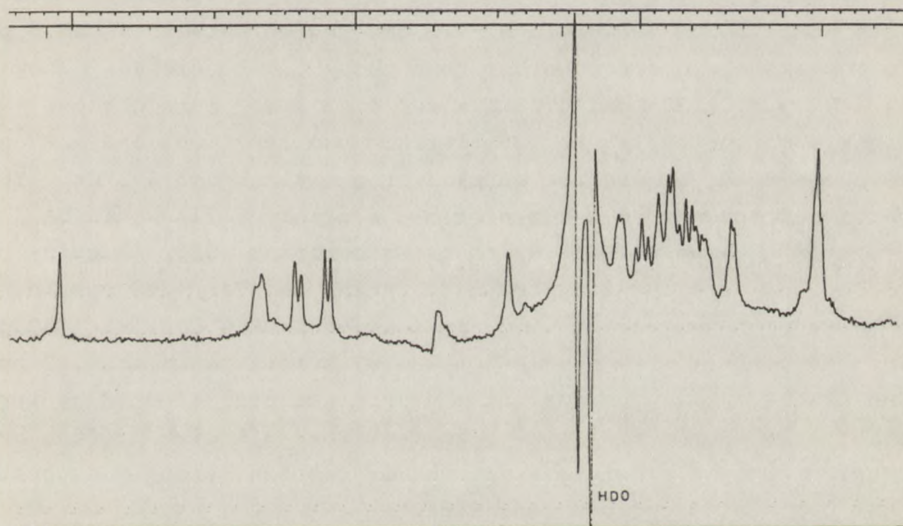

Fig. 12. NMR spectrum of Q.

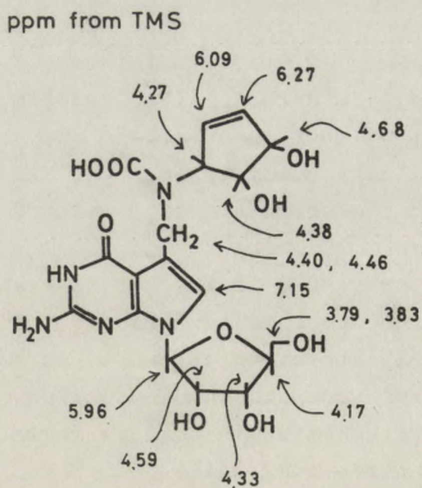

Fig. 13. Assignment of proton NMR signals of Q.

The presence of unmodified ribose was deduced from the presence of NMR peaks in 5.96 ppm (doublet, C1'H), 4.59 ppm (triplet, C2'H), 4.33 ppm (triplet, C3'H), 4.17 ppm (quartet, C4'H) and 3.83 ppm (A-B pattern, C5'H) that are commonly observed with other usual nucleosides. In the region between 4 and 5 ppm, four peaks are observed other than those due to ribose. They are at 4.27 ppm, 4.38 ppm, 4.68 ppm and 4.43 ppm. In addition, signals corresponding to two olefinic protons (6.09 and 6.27 ppm) were observed. These are coupled at a distance of 6.5 Hz. This value corresponds to a cis olefin. A trans olefin would be expected to have a 10-14 Hz coupling constant (62). A spin-decoupling experiment indicated that the peak at 4.68 ppm is coupled with the peak at 6.27 ppm, but not with that at 6.09 ppm. The NMR peak at 4.27 ppm is coupled with both peaks at 6.09 ppm and at 6.27 ppm. The peak at 4.43 ppm was not affected by irradiation at 6.09 and 6.27 ppm. The fact that all five protons are coupled to each other in a sequential fashion strongly suggested that Q contains cyclopentene diol.

The sharp singlet at 7.15 ppm is also unique. This has been assigned to an 8-H proton, since it is seen with other 7-deaza compounds, such as tubercidin and toyocamycin. The doublet at 4.43 ppm can be assigned to protons in methylene linking 7-deazaguanosine with cyclopentene diol group. The doublet of methylene protons can be explained as due to bulkiness of the side chains attached to the methylene carbon. [For example, the NMR peak of the methylene at C_{21} of cortisone acetate appeared as a doublet (63)]. Thus all the data obtained from the NMR study conform with, and strongly support the structure of Q deduced from mass spectral analysis.

An unusual property of Q is that it is stable under acid conditions even though it contains carbamic acid. This stability of Q can be reasonally explained as due to formation of a chelate-ring with the hydroxyl group in the β-position, as shown in Fig. 2. For example, hydroxyethylcarbamic acid is known to be stabilized by formation of a chelate-ring (64).

Other possible structure of Q can not be excluded as far as the data obtained by mass spectroscopy and NMR are concerned. For instance, a 3-deaza structure with a side chain at position C-8

might be possible. However such structure is very unlikely, because 3-deazapurine nucleosides are easily hydrolyzed to the corresponding bases. It will be interesting to synthesize Q chemically for final confirmation of its structure and for studies on its physiological effects.

2.2. 3-(3-Amino-3-carboxypropyl)uridine [acp^3U]

2.2.1. Isolation and characterization

The primary sequence of *E. coli* $tRNA^{Phe}$ was previously reported by Barrell and Sanger (65). It was shown that an unknown modified nucleoside, designated as X, was located in the extra-region. X was later found in the same region of several other *E. coli* tRNAs (66,67). The exact chemical structure of X has not been determined.

We became interested in the structure of X because of its unusual properties (66). Attempts to isolate X from unfractionated *E. coli* tRNA were unsuccessful, due to lack of a suitable isolation procedure. Therefore, as a source of X, more than 100 mg of pure *E. coli* tRNA were isolated, employing several column chromatographic procedures. For isolation of X, *E. coli* $tRNA^{Phe}$ was extensively hydrolyzed by pancreatic RNase, and the digest was fractionated by paper electrophoresis. The fraction, m^7G-X-Cp, isolated was then completely hydrolyzed by RNase T_2, and the nucleotide Xp was separated by two-dimensional paper chromatography. Xp was converted to nucleoside X by treatment with *E. coli* alkaline phosphomonoesterase. Approximately 10 A_{260} units of X were obtained from 1,600 A_{260} units of *E. coli* $tRNA^{Phe}$.

The UV absorption spectra of X are shown in Fig. 14. The λ_{max} of X in both alkaline and acidic conditions is at 263 nm, and its molecular extinction coefficient is not affected by the pH value. The spectra are similar to those of 3-methyluridine (68), suggesting that X has an alkyl substituent at position N-3 of uridine. X reacted with ninhydrin, giving a reddish-brown color, suggesting that it contains an α-amino, α-carboxy group. The most valuable information on the structure of X was obtained by mass spectral analysis of its trimethylsilylated derivative. As shown in Fig. 15, the spectrum exhibited a molecular ion peak of m/e 633,

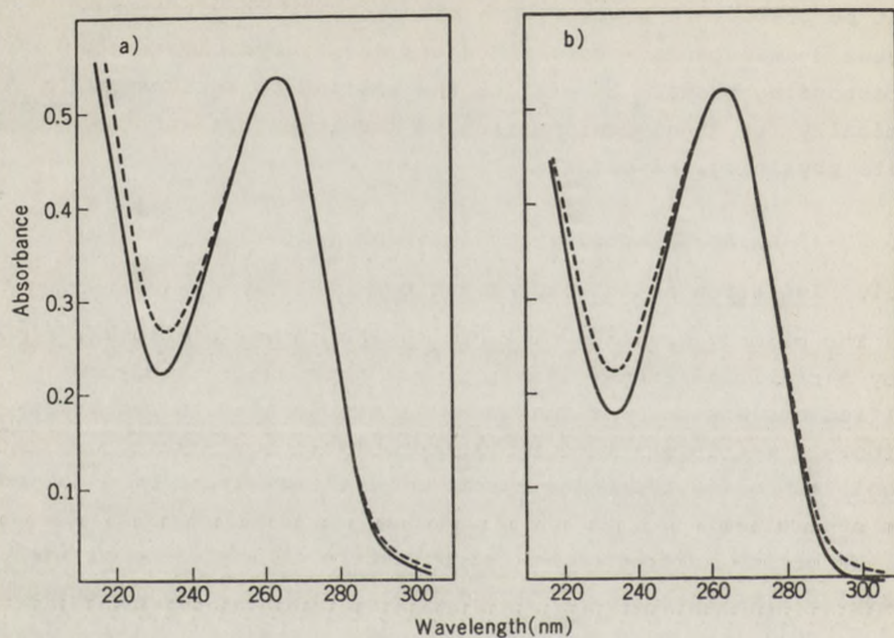

Fig. 14. UV absorption spectra of (a) X and (b) authentic acp^3U; pH 6 and 2, ————; pH 12, -------.

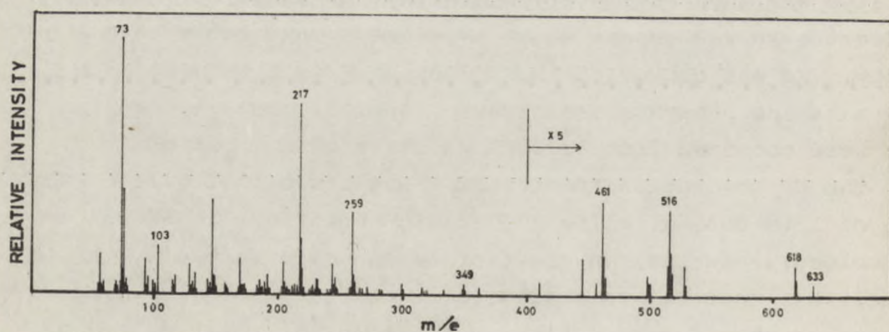

Fig. 15. Mass spectrum of the trimethylsilylation product of X.

the identity of which was confirmed by the presence of m/e 618, due to the characteristic loss of a methyl group from the trimethylsilyl residue. The spectrum of the corresponding silyl-d_9 derivative

showed a molecular ion shift of 36 mass units, indicating the presence of four silyl groups, which established the molecular weight of free X as 345. Since the side chain is known to contain a carboxyl and an amino group, the derivative can reasonably be postulated to be $C_4H_7NO_2SiMe_3$. To determine whether the side chain of X contains a $-CH_2-CH_2-$ or $-CH(CH_3)-$ group, the NMR spectrum was taken using a 100 MHz FT spectrometer, with 18,000 times accumulation, using 3 A_{260} units of the nucleotide, Xp. The NMR spectrum indicated the presence of a multiplet at 2.1 ppm, which can only be expected when the side chain of X contains the $>CH-CH_2-CH_2-$ group. Thus the total structure of X can be deduced as 3-(3-carboxypropyl)uridine (acp^3U) (Fig. 16).

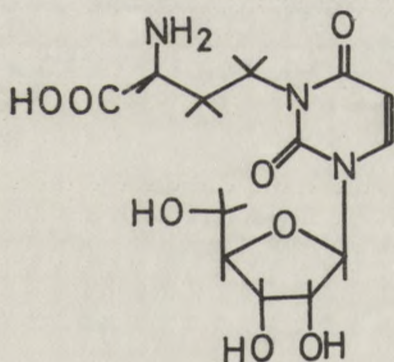

Fig. 16. Structure of 3-(3-amino-3-carboxypropyl)uridine.

This structure of X was unambiguously confirmed by comparison of X with synthetic acp^3U. For this purpose, acp^3U was synthesized chemically by the route shown in Fig. 17. The synthetic acp^3U exhibited λ_{max} (pH 1, 7.2, and 13), 263 nm; ε (pH 1, 7.2, and 13), 8.5×10^3; mp, 161-163. The synthetic material was identical with X with respect to its ultraviolet absorption spectra, thin-layer chromatographic mobilities and mass spectrum. Recently our results have been independently confirmed by Friedman et al. (70). They deduced the structure of acp^3U by analyzing the phenoxyacetylated derivative of acp^3U isolated from unfractionated E. coli tRNA.

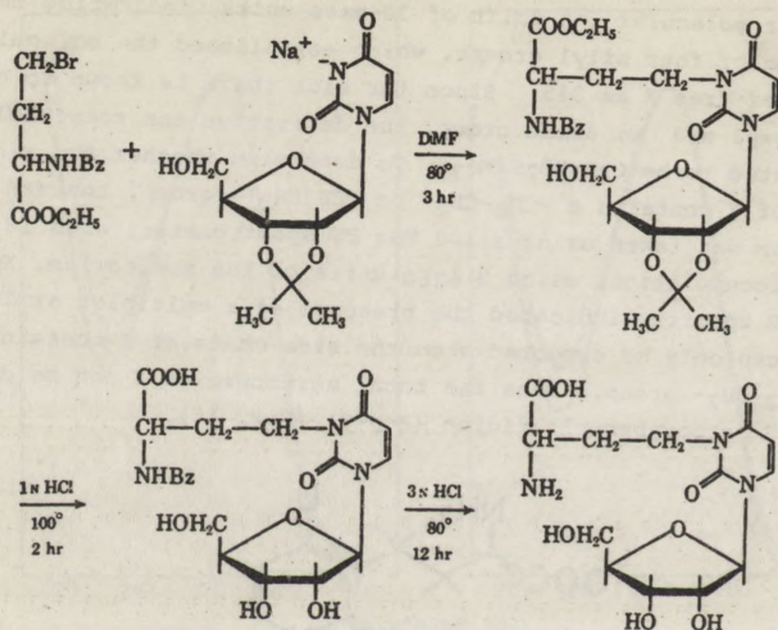

Fig. 17. Route of chemical synthesis of acp^{3}U.

2.2.2. Biosynthesis

After elucidation of the structure of X as acp^{3}U the next question was its mechanism of biosynthesis. The simple route considered is attachment of the 3-amino-3-carboxypropyl group from S-adenosylmethionine to a uridylate residue. It was found that purified methyl-deficient tRNA^{Phe} lacked acp^{3}U as well as almost all the 7-methylguanosine and ribothymidine present in normal tRNA^{Phe}. This strongly suggested that S-adenosylmethionine is involved in the synthesis of acp^{3}U as well as methylated modified nucleosides. In fact, we were able to synthesize acp^{3}U in a cell-free extract of E. coli, using S-adenosylmethionine as a donor, and methyl-deficient tRNA^{Phe} or tRNA^{Arg} as an acceptor (71). The radioactivity was incorporated into methyl-deficient tRNAs either from S-adenosyl-[carboxyl-^{14}C]methionine or from S-adenosyl-[2-^{3}H]-methionine, suggesting that incorporation of radioactivity is due to transfer of the 3-amino-3-carboxypropyl moiety of S-adenosyl-

methionine.

To characterize the product synthesized in vitro, the ^{14}C-labelled tRNA was completely hydrolyzed with RNase T_2, and the digest was subjected to two-dimensional thin-layer chromatograph: As shown in Fig. 18, the position of the radioactive spot completely coincided with that of the 3'-phosphate of acp^3U, indica ing that the product synthesized was in fact acp^3U. It should b

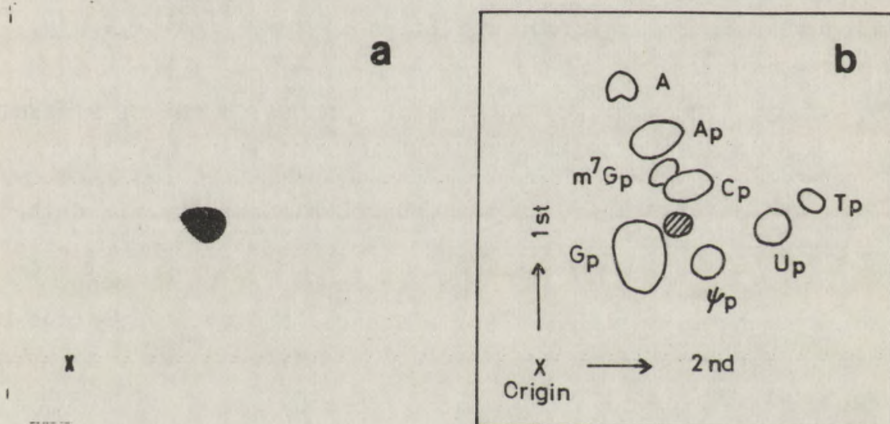

Fig. 18. Characterization of acp^3U synthesized in E. coli cell-free system by radioautogram of RNase T_2 digest of ^{14}C labelled product: (a) Autoradiogram of the chromatogra (b) Composite tracing of (a) in relation to the locati of the spot with UV absorption.

noted that possible degradation products of S-adenosylmethionine i.e. methionine, S-adenosylhomocysteine, homoserine and adenosyl-methionine were located in completely different positions from that of the 3'-phosphate of acp^3U. The enzyme responsible for the synthesis of acp^3U was further purified by DEAE-cellulose, hydroxylapatite and DEAE-Sephadex A-50 column chromatographies. Magnesium ion was found to be necessary for the reaction. The most notable feature of the reaction is the requirement for ATP. Addition of ATP had no effect on synthesis of acp^3U with crude enzyme extract, but it strongly stimulated synthesis of acp^3U us: enzyme purified by column chromatography.

In the biosynthesis of spermidine and spermine in bacteria,

yeast, rat prostate and regenerating rat liver, S-adenosylmethionine is first decarboxylated, and then this decarboxylated compound serves as a propylamine donor (72,73,74,75,76). On the contrary, in the synthesis of acp^3U at the level of tRNA described here, S-adenosylmethionine serves directly as the donor molecule without prior decarboxylation. In this sense, synthesis of acp^3U is a novel transfer reaction involving S-adenosylmethionine. S-Adenosylmethionine is probably cleaved to form methylthioadenosine in the reaction resulting in formation of acp^3U.

3. CONCLUDING REMARKS —— GENERAL DISCUSSION ON THE BIOSYNTHESES AND FUNCTIONS OF MODIFIED NUCLEOSIDES

As reported here, we have thoroughly characterized the structures of two novel modified nucleosides, X and Q, which are present in *E. coli* tRNAs. The structures of both compounds were found to be very unique. For instance, X, i.e. 3-(3-amino-3-carboxypropyl)uridine (acp^3U) can be defined as an α-amino acid. No 3-amino-3-carboxypropyl group has previously been found in a nucleoside in nucleic acid. Characterization of the structure of X raises several interesting problems, such as its chemical modification. acp^3U should be a very useful target for selective chemical modification of tRNA containing acp^3U, since this could be done with several chemical reagents used for modification of amino acids. In fact, we have found that acp^3U in *E. coli* $tRNA^{Phe}$ can be selectively modified by dancyl reagent (M. Saneyoshi and S. Nishimura, unpublished results). Modified $tRNA^{Phe}$ can also be charged with phenylalanine. The modified tRNA can be used for studies on the relation between the conformation of tRNA and its biological function. It was found that acp^3U in tRNA can also be modified by fluorescamine (Z. Ohashi and S. Nishimura, unpublished results). The nucleoside, Q should also be good target for selective modification of tRNA, since it reacts with periodate or cyanogen bromide.

The nucleoside, acp^3U, seems to be widely distributed in tRNAs from other sources besides *E. coli*. Randerath *et al*. (77) reported that mammalian tRNA contains a large quantity of acp^3U. Their identification was based on the chromatographic mobility of the

oxidized product labelled with ^{3}H-labelled sodium borohydride. By applying this procedure, we found that rat liver tRNA and ascites hepatoma 7974 tRNA also contain acp^{3}U. Saponara and Enger (78) also isolated a modified nucleoside, which they tentatively identified as acp^{3}U, from 4S RNA of Chinese hamster cells. Friedman (79) showed that certain species of rat liver tRNA reacted with N-hydroxysuccinimide ester of phenoxyacetic acid. This also suggests the presence of acp^{3}U in rat liver tRNA. Thus it seems that acp^{3}U is present in a variety of mammalian tRNAs.

There are two main problems on the biosynthesis of modified nucleosides in tRNA, namely, the specificity of the synthesis and the biosynthetic pathway. All modified nucleosides are thought to be synthesized by modification of a parental nucleotide residue after synthesis of the polynucleotide linkage of the tRNA molecule, either at the precursor level or in the mature form (80). Modified nucleosides are not randomly distributed in tRNA but are located in specific positions in the clover-leaf structure, which vary with the type of modification. Thus enzymes for modification must have a strict specificity, recognizing only the particular nucleotide residue to be modified. The mechanism of this recognition specificity is not fully understood. There is evidence that tRNA-methylase recognizes the short oligonucleotide sequence surrounding the nucleotide residue to be methylated (81), as well as the three-dimensional structure of tRNA (82). To examine this pure species of enzymes for modification of tRNA must be isolated and their interaction with pure species of tRNA must be studied at a molecular level.

The biosynthetic pathways of many modified nucleosides are not yet clearly elucidated. Only tRNA methylases have been highly purified and studied extensively (80). The syntheses of pseudouridine (83,84) and N-[9-β-D-ribofuranosyl)purin-6-ylcarbamoyl]-threonine (85) have recently been demonstrated *in vitro*, but the synthetic mechanisms are mostly unknown and not even the syntheses of simple modified nucleosides, such as N^4-acetylcytidine and dihydrouridine, have yet been demonstrated *in vitro*. In *in vivo* experiments the "Y" base was found to be derived from the guanosine residue (86,87). The main difficulty in these experiments is the lack of a proper substate, namely unmodified tRNA or tRNA precursor.

Isolation of mutant bacteria in which the enzymes involved in the modification of tRNA are temperature-sensitive may be useful in isolation of unmodified tRNA as well as in studies on the function of modified nucleosides in tRNA (88,89).

It seems likely that the modified nucleosides in tRNA have specific roles in tRNA function, because of the complexities of their structures, and because of their presence in specific locations in the tRNA molecule. However, the functions of most modified nucleosides except those located in the anticodon region are still unknown. Methylated nucleosides or dihydrouridine may serve to stabilize the correct two- or three-dimensional structure by preventing incorrect base-pairing. Evidence supporting this possibility has been obtained (90). Roe _et al._ (91) recently reported that _N_ ^{2}-methylguanosine, which is only present in eukaryote tRNA, is related to recognition of homologous aminoacyl-tRNA synthetase. Tψ, present in the GTψC loop in most tRNAs, may play a role in the general functions of tRNA, such as binding to ribosomes or interaction with tRNA nucleotidyl transferase. In fact, recently the specific interaction of the T-ψ-C-G sequence in tRNA with ribosomes, possibly through 5S RNA, has been demonstrated (92,93). We have also found that 5-methyl-2-thiouridine (m^5s^2U) was present in the sequence of G-m^5s^2U-ψ-C-G in tRNA isolated from an extreme thermophilic bacterium, _Thermus thermophilus_, indicating that most of the ribothymidine normally present in the GTψC region is replaced by m^5s^2U in the tRNA of this thermophile (94). The m^5s^2U is probably important for the capacity of the tRNA to synthesize protein at high temperature. On the contrary, tRNA completely lacking ribothymidine has been isolated from various sources. These tRNAs are known to be completely active in functions such as aminoacylation, formylation, code recognition, amino acid transfer, and interaction with initiation factor (95-99).

Striking regulations were observed in the presence of particular modified nucleosides adjacent to the anticodon (100). Transfer RNAs that recognize codons starting with U almost always contain _N_ ^{6}-isopentenyladenosine, one of its derivatives or the hydrophobic compound "Y". Most tRNAs that recognize codons starting with A contain _N_-[(9-β-_D_-ribofuranosyl)purin-6-ylcarbamoyl[threonine or one of its derivatives. It seems possible that these modified

nucleosides may facilitate the formation of precise codon-anticodon base-pairs by stabilizing the three-dimensional structure of the anticodon loop.

The first letter of the anticodon is frequently occupied by a modified nucleoside. Unlike the modified nucleosides discussed before, the function of these modified nucleosides is more or less understood. Inosine is the first modified nucleoside, distinguished as a "wobbling base", pairing with U, C, or A in the third position of the codon sequence (101, 102). Uridin-5-oxyacetic acid, that was isolated from E. coli tRNAs, functions to recognize A, G and U, its function differing from that of inosine (103,104). The crystal structure of uridin-5-oxyacetic acid has recently been determined (105). A number of appreciable differences were found between the bond lengths and base planarity of this molecule and those of other uracil derivatives. The 2-thiouridine derivatives function to achieve strict base-pairing with A, but not with G, in the third letter of the codon sequence (106,107). Another possible function of 2-thiouridine derivatives is to prevent mispairing with U or C, which would be lethal to cells. We have recently isolated an unidentified modified nucleoside, N^+, in the first position of the anticodon of minor species of E. coli $tRNA^{Ile}$ (108). This tRNA recognizes AUA, but not AUU, AUC or AUG. Elucidation of the structure of N^+ is interesting, because this modified nucleoside must function to prevent mispairing with G in the third position of the codon sequence.

The most important and fascinating problem on modified nucleosides of tRNA for future study is their involvement in the regulatory function of tRNA. Ames and his coworkers showed that $tRNA^{His}$ isolated from a mutant of Salmonella (His T), in which histidine biosynthesis does not occur on addition of histidine, contained the U-U sequence in the region of the anticodon stem and loop, while $tRNA^{His}$ from wild-type cells contained the ψ-ψ sequence in the same location (109). Presumably the histidyl-$tRNA^{His}$ of the mutant is inactive in control of function of the histidine operon, indicating that the ψ-ψ sequence is important for this function. Thus other modified nucleosides may also be involved in the regulatory function of tRNA. As discussed in the Results section, the modified nucleoside Q in Drosophila tRNA may be re-

lated to differentiation of cells (60). New tRNA species are often detected from changes in the chromatographic profiles of isoaccepting tRNA under different growth conditions, in different tissues, in cancerous cells and during differentiation or transformation. It is likely that their appearance is mostly due to the degree of modification of tRNA. Hyper-modified nucleosides, such as Q or acp^3U as discussed here, must have a significant effect on the distribution of charge or metal ion in the structure of tRNA. In addition, the bulky side chain present in these modified nucleosides may facilitate specific interactions with protein components that are related to regulation. Modified nucleosides with unique structures and specific functions in this sence may be present in eukaryote cells, so the identification of modified nucleosides in tRNAs from mammals should be studied further.

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SUGAR-MODIFIED PYRIMIDINE NUCLEOSIDES AND NUCLEOTIDES

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The rapid development of the nucleic acid chemistry in the last ten years has now culminated in solution of the genetic code in the proteosynthesis with the aid of all 64 possible combinations of naturally occurring trinucleoside diphosphates as well as in the chemico-enzymatic synthesis of the first polydeoxyribonucleotide related to the naturally occurring DNA, a gene for Ala-tRNA. These two great achievements simultaneously represent a milestone in development of this field of chemistry; the future trends might consist in methodical investigations on suitable protecting groups and condensation processes. The economical aspects of these processes are discussed in numerous papers on triester condensations. Each improvement brings an instantaneous profit to those who are engaged in the synthesis of polynucleotides of the required sequence related to biologically active molecules of this type.

The aim of the present lecture is to survey another field of the nucleic acid chemistry which is investigated in our Laboratory, namely, the analogues of nucleosides and nucleotides and the effect of modified nucleic acid components on the biochemical behaviour of these analogues. There are several aspects of such an investigation, e.g., mechanism of the action of biologically active nucleoside analogues _in vivo_ and _in vitro_[1,2], the influence of modification on the physicochemical interactions with a special respect to the biological processes (codon-anticodon interaction in the proteosynthesis[3-5]), or finally, the problem of specificity, i.e., structural requirements of enzymes in nucleic acid metabolism. Particularly the

last mentioned field has been paid considerable attention in this Laboratory. The knowledge on the character of interactions between the enzyme and the substrate or the inhibitor could be helpful in the search for resistant biologically active analogues or specific enzyme inhibitors or in comparisons of analogous enzymes from different or related organisms[6-8].

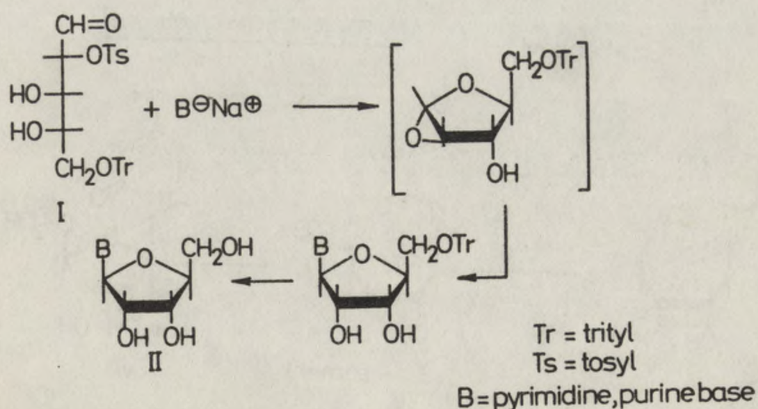
The results from investigations on analogues of nucleic acid components might be then applied to the synthesis of substances of predestined properties. In the preparation of modified nucleotides, however, the classical methods have to be often replaced by novel procedures taking into account the special properties of the analogues. These methods, in turn, have often found a general application. In some cases, labelled analogues of nucleic acid components were required in the investigation, it was therefore necessary to search for suitable methods for this purpose. When compared with the chemistry of naturally occurring nucleosides and nucleotides, the preparation of the modified analogues mainly differs in the low accessibility of the starting material which must be in most cases produced by multistep procedures. In view of the minute amount of the starting material, it is necessary to apply the micropreparative methods or selective methods which do not require the presence of protecting groups (e.g., in phosphorylations). The emzymatic methods[9] may also be advantageously used in this field. The progress in the synthesis of modified nucleotides is closely connected with improvements in the synthesis of nucleosides and in their phosphorylations as well as with new findings in investigations on suitable enzymatic processes. It is noteworthy that also the opposite procedure, namely, the analysis of modified nucleotides requires sometimes the application of special methods.

The modification of nucleic acid components may relate to all the three portions of the molecule, i.e., the base, the sugar residue, and the phosphate grouping. Nucleotides modified on the basic moiety are usually prepared by procedures which do not substantially differ from general methods; some limitations are due to the higher instability of intermediates or products in some cases[10-13]. Modifications of the phosphate residue do not usually require any special protection of functional groups;

in most cases, the syntheses in this field start from the naturally occurring nucleosides (cf. refs[14,15] and additional references quoted therein). Therefore, the preparation of nucleotide derivatives modified in the sugar moiety might be regarded as the most difficult since the fully-synthetic material is not easily accessible, the introduction of blocking groups must be selective, the phosphorylation must be performed by special procedures, and the isolations require special techniques; moreover, there are some different features in the analysis of relationships between the structure and the response to enzymes.

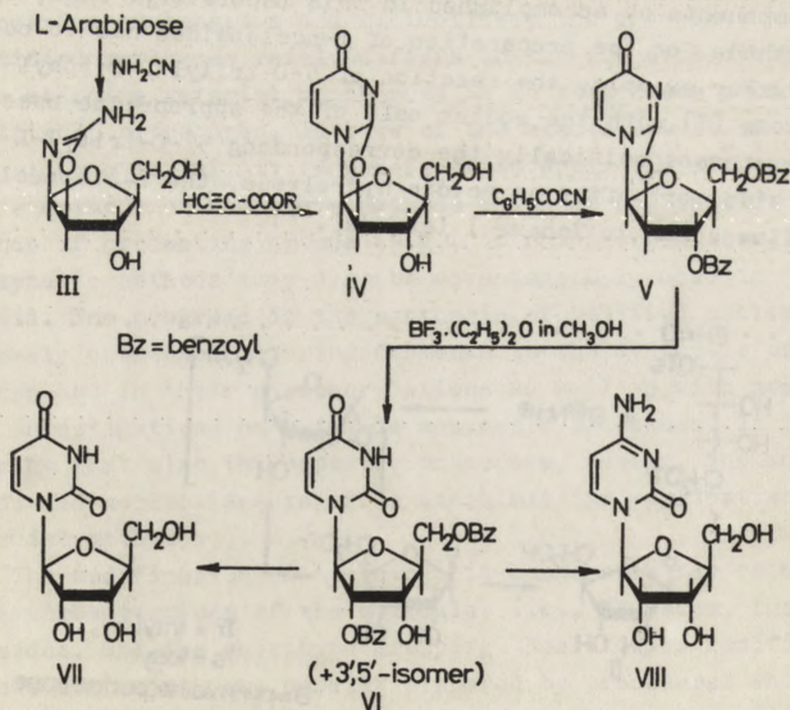
Fortunately, the aim of most syntheses in this field is the preparation of substrate analogues on the level of monomers or short oligonucleotides.

The investigations in this field of chemistry might be exemplified by the research of the L-enantiomers of nucleic acid components as accomplished in this Laboratory. Thus, special methods for the preparation of L-nucleosides had to be developed. For example, the reaction of 5-O-trityl-2-O-tosyl-L-arabinose (I) with the sodium salt of the appropriate base affords stereospecifically the corresponding 5′-O-trityl-L-nucleoside, or, after an acidic hydrolysis, the L-ribonucleoside II as shown in Scheme 1 (cf. ref.[16]).



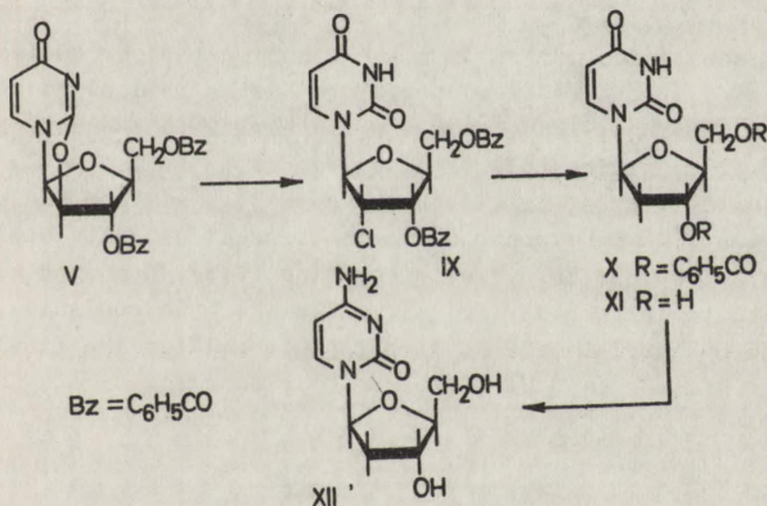
Scheme 1

Another route suitable for pyrimidine L-ribonucleosides, consists in the reaction of L-arabinose with cyanamide and alkyl propiolate to afford $O^{2,2'}$-anhydro-L-uridine which is benzoylated and the resulting 3′,5′-dibenzoate V treated with boron trifluoride etherate in methanol to yield a mixture of 2′,5′- and 3′, 5′-di-O-benzoyl-L-uridine (VI). The subsequent debenzoylation leads to L-uridine (VII) which may be benzoylated and the benzoyl derivative subjected to thiation and then ammonolysis to afford L-cytidine[17] (VIII) as shown in Scheme 2.



Scheme 2

From 3′,5′-di-O-benzoyl-$O^{2,2'}$-anhydro-L-uridine, the pyrimidine 2′-deoxy-L-ribonucleosides may be obtained. Thus the reaction of the 3′,5′-dibenzoate V with hydrogen chloride in dimethylformamide affords the 2′-deoxy-2′-chloro derivative IX which is dehalogenated with tri-n-butyltin hydride to 3′,5′-di-O-benzoyl-2′-deoxy-L-uridine (X). Compound X may be converted either to 2′-deoxy-L-uridine (XI) or (by thiation and ammonolysis) to 2′-deoxy-L-cytidine[18] (XII) as shown in Scheme 3.

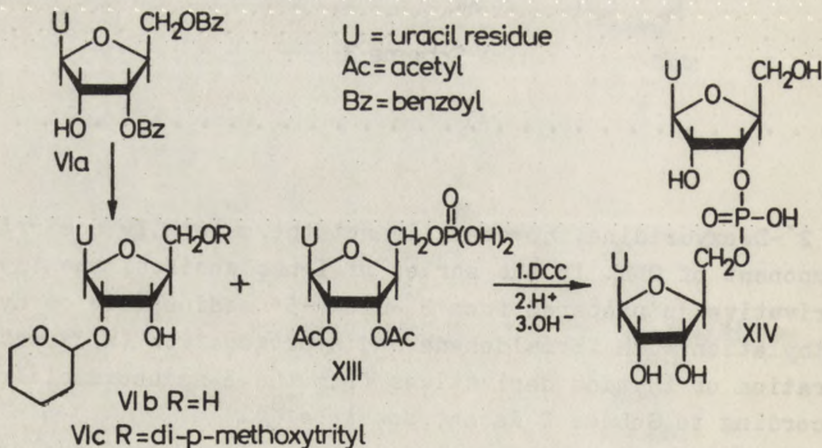


Scheme 3

2′-Deoxyuridine, however, is not the naturally occurring component of DNA. In the series of L-nucleosides, the thymine derivative is prepared from 2′-deoxy-L-uridine (XI) by hydroxymethylation with formaldehyde and hydrogenation (a direct preparation of thymine derivatives from the 2-aminooxazoline III according to Scheme 2 is not possible[18]).

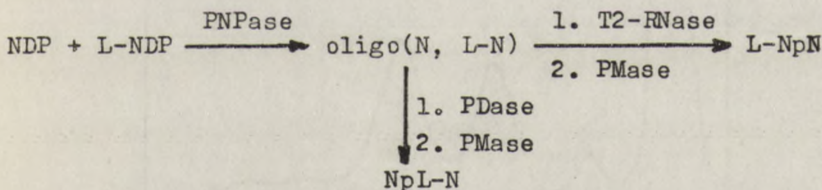
The preparation of L-nucleotide monomers from L-nucleosides is rather simple since the two enantiomers are not different with respect to their chemical properties. Consequently, the selective phosphorylation methods of the D-series such as the triethyl phosphite-hexachloroacetone method[19] or the phosphorus

oxychloride-trialkyl phosphate route[20] may also be used in the L-series. Thus, there have been successfully prepared 5′-L-nucleotides[21], 2′(3′)-L-nucleotides, and 2′,3′-cyclic L-nucleotides[16]. Some difficulties have been encountered in the synthesis of oligonucleotides of the L-series. The stepwise synthesis as developed in the Laboratory of Khorana and in this Laboratory[22] starts from 3′-nucleotides and depends on the possibility of 2′,3′-cyclic nucleotide splitting with ribonucleases. This route cannot be used in the L-series since the L-nucleotides are not substrates for ribonucleases and most other nucleases[23]. For this reason, a reversed procedure has to be used as developed in Cambridge. Thus, from a mixture of 2′,5′- and 3′,5′-di-O-benzoyl-L-uridine (VI) obtained according to Scheme 2, there is isolated by crystallisation the pure 3′,5′-isomer VIa which is reacted with dihydropyran and the product debenzoylated to afford 2′-O-tetrahydropyranyl-L-uridine (VIb). Compound VIb is converted to the trityl derivative VIc which is condensed with 2′,3′-di-O-acetyl-L-uridine 5′-phosphate (XIII); the final deblocking affords L-(UpU) (XIV, ref.[24]); Scheme 4.



Scheme 4

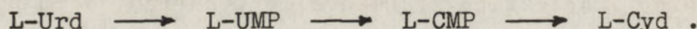
The poly-L-ribonucleotides cannot be prepared by polymerisation of the corresponding L-ribonucleoside 5′-diphosphates with polynucleotide phosphorylase. In contrast, the L-5′-diphosphates inhibit the polymerisation of naturally occurring 5′-diphosphates as well as the phosphorolysis of polymers. It is, however, possible to effect the copolymerisation of the two enantiomers. The resulting oligonucleotide was subjected to enzymatic hydrolysis with ribonuclease T2 or the snake venom phosphodiesterase; both these enzymes cleave exclusively the bonds attached to the D-nucleotides[23]. After the hydrolysis, the mixture was dephosphorylated with nonspecific phosphatase[23] and analysed. Dinucleoside phosphate was present in a small amount in the mixture as the single oligonucleotide. The D-nucleotide sequence contains therefore at most one L-nucleotide:



It has been demonstrated by means of the labelled 5′-L-diphosphate that polynucleotide phosphorylase M. lysodeicticus affords a mixture of homooligonucleotides; after the dephosphorylation, there was identified[24,25] (Fig. 1) the doublet L-(NpN) and the triplet L-(NpNpN). This observation is in accordance with the idea on the mechanism of polynucleotide phosphorylase action and the polymer release; the growing chain remains in a complex with the enzyme until a certain chain length is attained[26]. In the case of the L-oligonucleotide, the existence of the complex depends on stereochemical requirements of the enzyme with respect to the shape of the chain. As soon as a sufficiently long chain loop stabilises by intramolecular forces the reverse helix of the L-oligonucleotide, the chain is liberated from the complex, and the polymerisation is interrupted, most probably in the stage of a dimer or at most a trimer.

The resistance of L-nucleosides and L-nucleotides towards most enzymes of nucleic acid metabolism and catabolism makes it possible to reveal in vivo those processes which remained un-

known because the changes of the naturally occurring nucleosides *in vivo* are too rapid. Thus, e.g., in rat liver, L-uridine is converted to L-cytidine on the monophosphate level[27,28]:



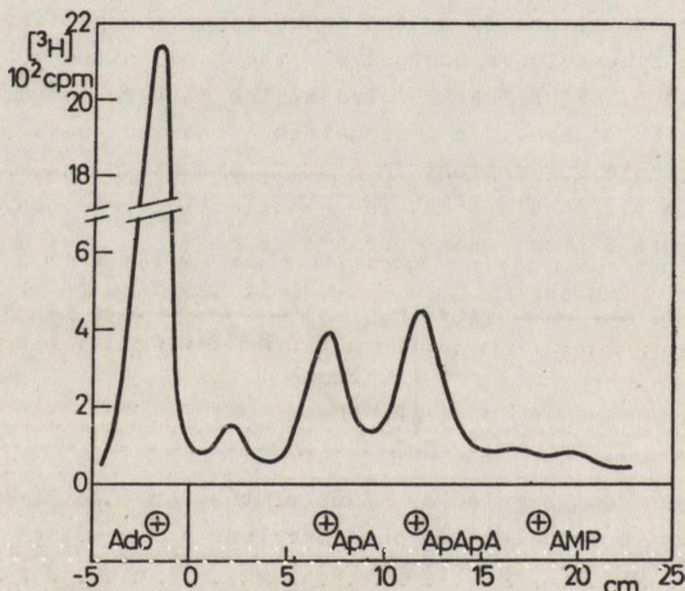
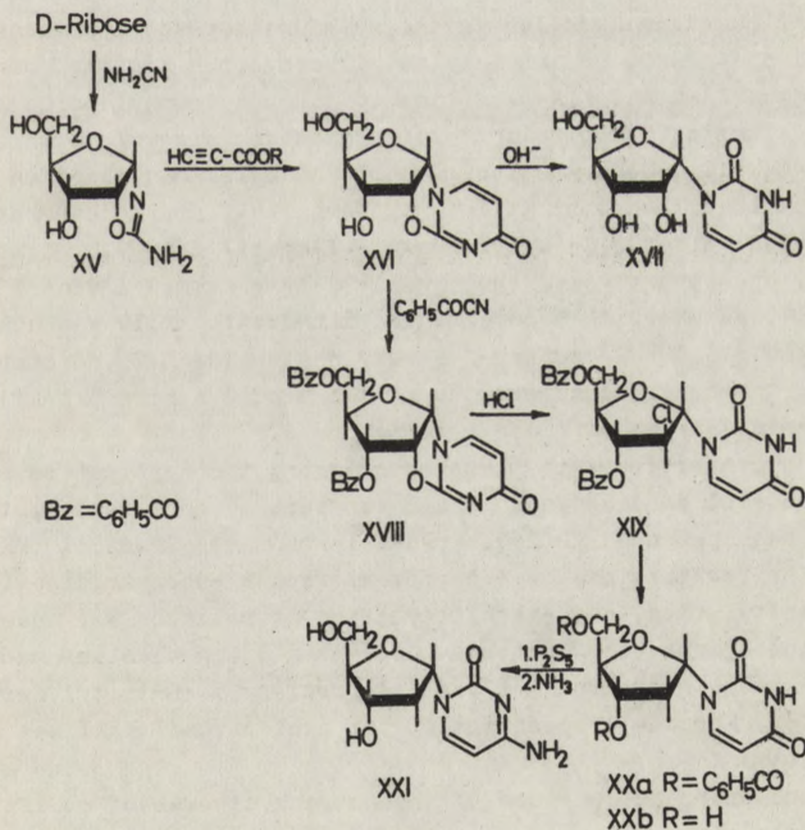


Fig. 1 - Electrophoresis of the dephosphorylated oligonucleotide mixture after polymerisation of L-ADP with polynucleotide phosphorylase *E. coli* (0.1M triethylammonium hydrogen carbonate, pH 7.5, 20 V/cm, 1 hour).

The pyrimidine α-ribonucleosides and 2′-deoxy-α-ribonucleosides[29] may be obtained from D-ribose analogously to the preparation (Scheme 2) of L-nucleosides from arabinose. Thus, the reaction with cyanamide affords the 2′-amino-1,2-oxazoline XV which is converted to $O^{2,2'}$-anhydro-α-uridine (XVI) on treatment with ethyl propiolate. Hydrolysis of compound XVI affords α-uridine (XVII) while the benzoylation leads to the 3′,5′-dibenzoate XVIII which is converted on treatment with hydrogen chloride in dimethylformamide to the 2′-deoxy-2′-chloro deriva-

tive XIX. Reduction of compound XIX affords the protected nucleoside XXa which is then converted either to 2′-deoxy-α-uridine (XXb) or to 2′-deoxy-α-cytidine (XXI) (by thiation and ammonolysis), see Scheme 5.



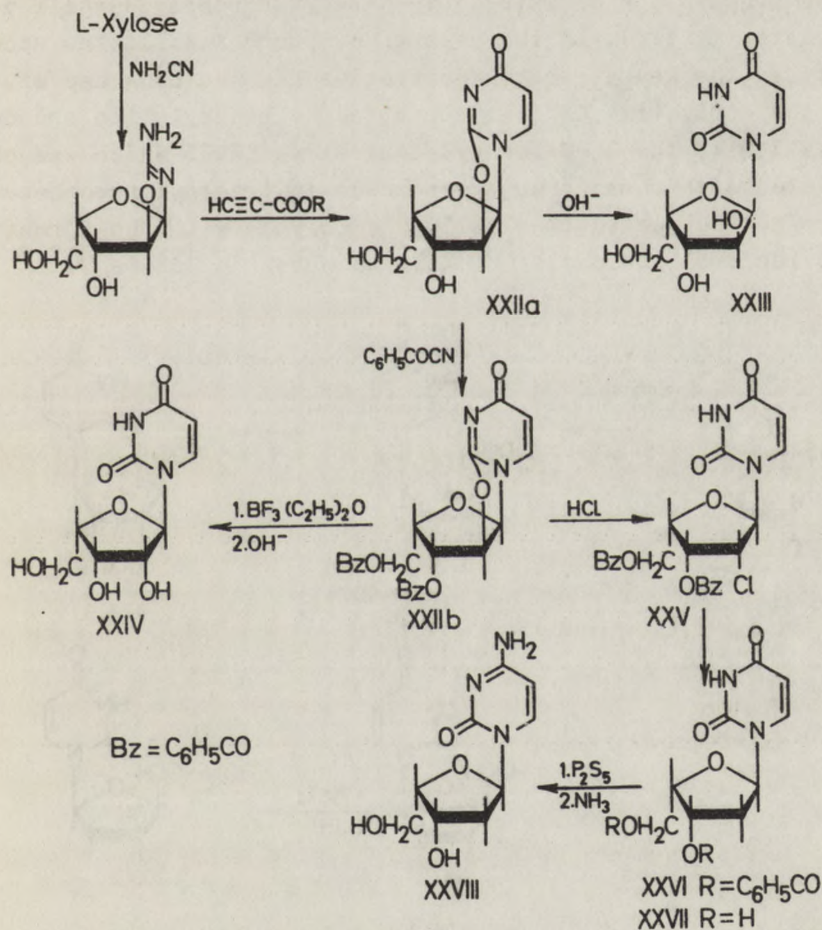
Scheme 5

The α-nucleosides are transformed to the corresponding nucleotides by means of usual selective methods[29]. It should be mentioned that the syntheses afford mixtures of anomers from which the α-nucleosides are isolated by laborious processes. With the use of α-nucleotides, some controversial prcblems on the nuclease specificity and substrate-enzyme bondings have been elucidated[29].

Finally, the formation of uracil $O^{2,2'}$-anhydronucleosides from sugar 2′-amino-1,2-oxazolines and ethyl propiolate (or alkyl β-alkoxyacrylates and alkyl β-chloroacrylates)[30] has been used in the preparation of D- and L-enantiomers of nucleosides and 2′-deoxynucleosides in the xylofuranose and lyxofuranose series[31]. Thus, D- and L-xylose is converted via the corresponding 2′-amino-1,2-oxazoline to the anhydronucleoside XXIIa, the alkaline hydrolysis of which affords 1-(α-xylofuranosyl)uracil (XXIII). Benzoylation of compound XXIIa and reaction of the resulting dibenzoate XXIIb with boron trifluoride etherate in methanol affords 1-(α-lyxofuranosyl)uracil 2′(3′),5′-dibenzoate and, by methanolysis, the free 1-(α-lyxofuranosyl)uracil[17] (XXIV) as shown in Scheme 6. The dibenzoate XXIIb was also converted via the 2′-chloro-2′-deoxy nucleoside XXV and compound XXVI to the free nucleosides of the uracil series (XXVII) or cytosine series (XXVIII), see Scheme 6.

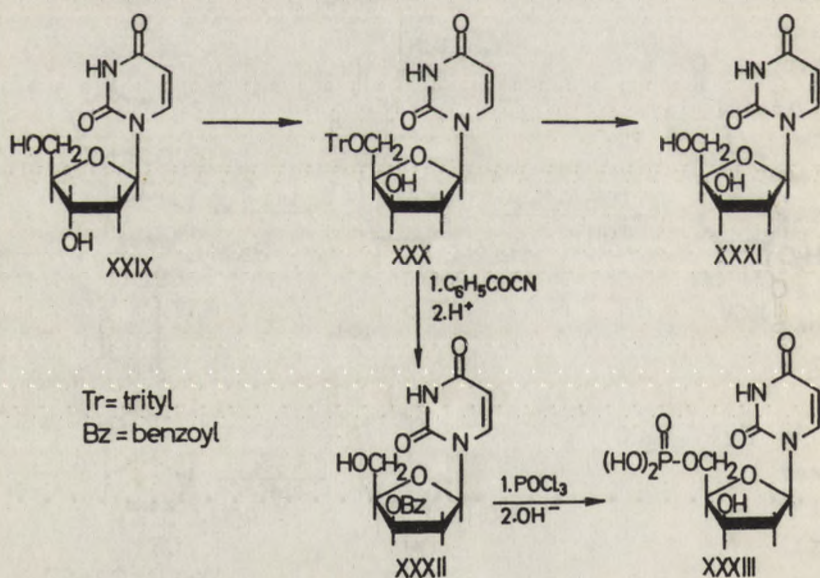
In connection with these substances, the application of which will be discussed in a later part of this review, there has been prepared 1-(2-deoxy-β-D-lyxofuranosyl)uracil (XXXI). The preparation has been performed from 2′-deoxyuridine (XXIX) by tritylation, methanesulfonylation at position 3′, inversion of the hydroxylic function at position 3′ in alkaline media, and acidic hydrolysis of the intermediary 5′-O-trityl derivative XXX. For the preparation of the nucleoside[32] XXXI see Scheme 7.

The principal purpose of the above syntheses of modified 2′-deoxynucleosides was a detailed investigation on thymidylate synthetase. This enzyme transforms dUMP to dTMP and represents the single source of 2′-deoxythymidine *de novo* in living systems. In this connection it was necessary to prepare analogues of dUMP, i.e., 5′-nucleotides derived from the above mentioned uracil pentafuranosyl and 2-deoxypentafuranosyl derivatives.



Scheme 6

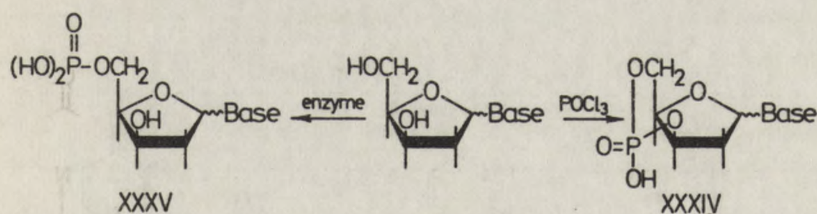
While the 5′-phosphorylation in the series of L-ribonucleosides and α-ribonucleosides was relatively easy, considerable difficulties have been encountered in the xylo and lyxo series except for the preparation of 1-(2-deoxy-β-D-lyxofuranosyl)uracil 5′--phosphate (XXXIII). In the case of compound XXXIII, the accessibility of the key 5′-trityl derivative XXX was made use of. Thus, the derivative XXX was converted by benzoylation and detritylation to the 3′-O-benzoyl derivative XXXII which was phosphorylated with phosphorus oxychloride in triethyl phosphate and the product subjected to alkaline hydrolysis with the formation of the required phosphate[32] XXXIII as shown in Scheme 7.



Scheme 7

Selective phosphorylation of the primary hydroxylic function in nucleosides with phosphorus oxychloride in triethyl phosphate[20] has been successfully used by several teams as well as in this Laboratory. This reaction, however, completely fails with nucleosides where the 3′-hydroxylic function and the 4′--hydroxymethyl group are in cis configuration, namely, with the lyxofuranosyl and xylofuranosyl derivatives XXIIa, XXIV, XXVII, XXVIII, XXXI, and the like. In all these cases, there is ex-

clusively formed the 3′,5′-cyclic phosphate of the corresponding structure (XXXIV), see Scheme 8. Such a behaviour is not surprising but considerably interferes with the preparation of pure 5′-nucleotides of the type XXXV. The hydrolysis of cyclic nucleotides XXXIV may result in destruction of the skeleton or afford a mixture of 3′- and 5′-nucleotides in the most favourable case. Except for rare occasions, a specific protection of the molecule is difficult. Consequently, we have attempted the enzymatically catalysed phosphorylation which (in the presence of carrot phosphotransferase and phenyl phosphate as donor) occurs exclusively on the primary hydroxylic function regardless the stereochemistry of the surrounding[32-34]. By this enzymatic route, there have been simply prepared the 5′-phosphates XXXV of numerous nucleosides[2,32] including the above mentioned analogues (Table 1). By this example a situation is exemplified when the enzymatic (though rather low) conversion is more advantageous from the preparative point of view than an apparently more effective multi-step chemical synthesis.



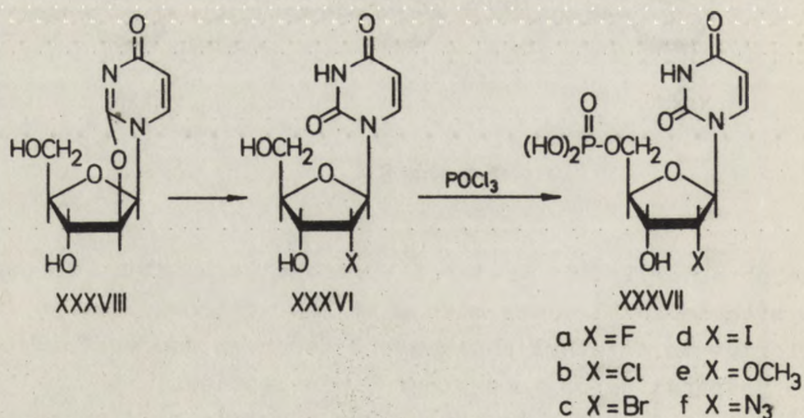
Scheme 8

The phosphorylation of the 5′-hydroxylic function in nucleosides with modified sugar moiety by the action of phosphorus oxychloride in triethyl phosphate[20] has been successfully used in the preparation of some other 5′-nucleotides with the <u>ribo</u> situated 3′-hydroxylic function. For the selective phosphorylation of 2′-modified nucleosides XXXVI (performed in connection with investigations on thymidylate-synthetase[32]) see Scheme 9. Also in the arabino series the reaction is selective and affords a fair yield of the 5′-phosphate[32].

Table 1. Enzymatic Phosphorylation of Modified Nucleosides in the Presence of Carrot Phosphotransferase[a]

Compound	Nucleotide yield, %
Uridine	45
2′-Deoxythymidine	37
L-Uridine	28
2′-Deoxy-L-uridine	32
1-(2-Deoxy-α-L-lyxofuranosyl)uracil	22
1-(α-L-Lyxofuranosyl)uracil	16
1-(α-D-Lyxofuranosyl)uracil	18
1-(β-D-Xylofuranosyl)uracil	19

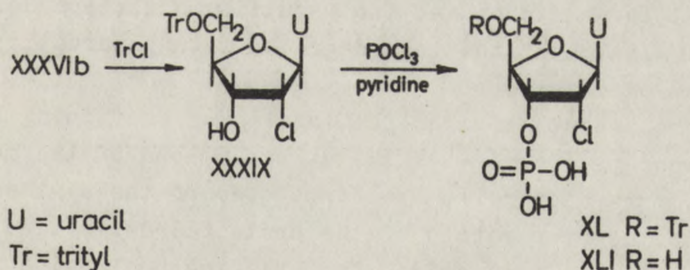
[a] Incubation mixture (200 μl): 10 μmol nucleoside, 100 μmol sodium phenylphosphate, 100 μmol sodium acetate (final pH 5.5), 60 e.u. enzyme; incubated at 37°C for 5 hours.



Scheme 9

The phosphorus oxychloride-triethyl phosphate phosphorylation method is especially advantageous with the 2′-deoxy-2′-halo derivatives XXXVIb-d which could be hardly subjected to other phosphorylation procedures*.

Of a special interest is the preparation of the isomeric 2′-chloro-2′-deoxyuridine 3′-phosphate (XLI). It is not possible to apply in this case methods which require in whatever steps an alkaline work-up. In the attempted phosphorylation of the trityl derivative XXXIX (readily obtained from compound XXXVIb) with phosphorus oxychloride in triethyl phosphate, the trityl group was quantitatively split off by the action of hydrogen chloride which is formed in the course of the reaction. Finally, use was made of the almost forgotten phosphorylation method consisting in the action of phosphorus oxychloride in pyridine. With the use of excess reagent and a long reaction period of time, there was obtained a high yield of the tritylated intermediate XL which was subjected to acid processing to afford the product XLI free of by-products[37] (see Scheme 10).

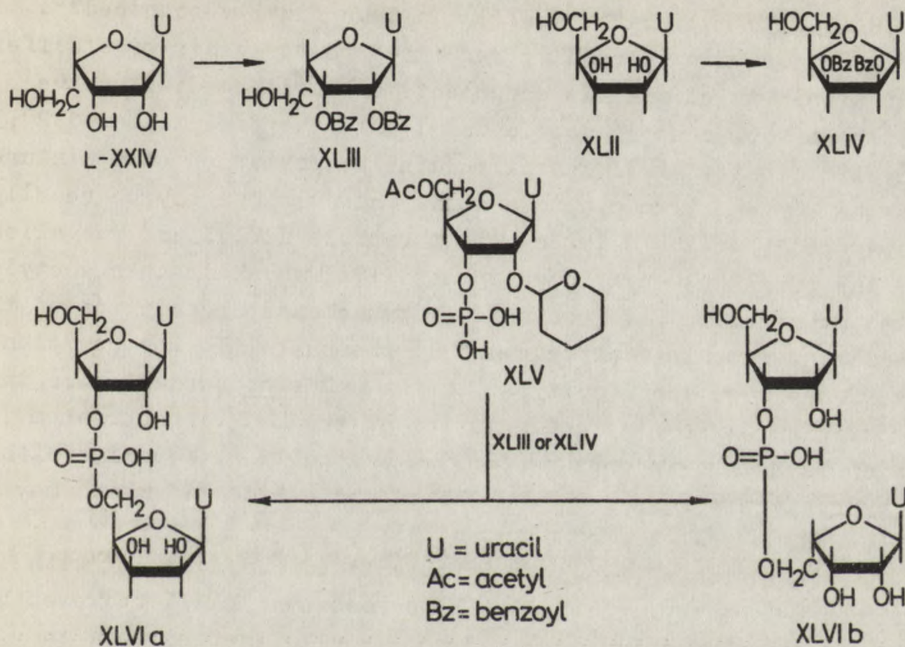


Scheme 10

* The starting nucleosides are preferably prepared from $O^{2,2'}$-anhydrouridine (XXXVIII) by reaction with anhydrous hydrogen halide in dimethylformamide[35]. When compared with other techniques[36], the use of dimethylformamide is particularly advantageous since the deionisation may be then performed on ion exchange resins which do not affect the sensitive products.

In the series of uracil lyxofuranosyl derivatives, we also were interested in the preparation of analogues of the dinucleoside phosphate UpU in which one or two uridine residues are replaced by a modified nucleoside. Somewhat easier is the preparation of those analogues where the modified nucleoside is placed at the 3′-end of the molecule. In such a case, 5′-O--acetyl-2′-O-tetrahydropyranyluridine 3′-phosphate[38] (XLV) is used as the nucleotide component; the pyridinium salt of compound XLV is then condensed in the presence of N,N′-dicyclohexylcarbodiimide with the 2′,3′-di-O-benzoyl derivatives (XLIII and XLIV, resp.) of 1-(α-L-lyxofuranosyl)uracil or 1-(β-D-lyxofuranosyl)uracil. The latter benzoyl derivatives were prepared from the parent nucleosides L-XXIV and XLII by tritylation, benzoylation, and detritylation; in order to circumvent the migration of the benzoyl group from position 3′ to position 5′, there was used the more sensitive bis-p-methoxytrityl group which can be removed under mild conditions. The condensation was then followed by removal of the alkali-labile groups (acetyl, benzoyl) and finally, the tetrahydropyranyl group was deblocked in acidic media. The resulting dinucleoside phosphates XLVI were quantitatively degraded with pancreatic ribonuclease[39] (Scheme 11).

The preparation of those UpU analogues containing 1-(α-L--lyxofuranosyl)uracil (L-XXIV) at the 5′-end of the molecule is somewhat more difficult. The first step of the synthesis consisted in the preparation of the protected derivative of the corresponding 3′-nucleotide. The starting nucleoside[17] L-XXIV was phosphorylated by reaction with triethyl phosphite and hexachloroacetone[19]. This reaction has been developed as the selective method for the preparation of 2′,3′-cyclic phosphates from ribonucleosides. The selectivity is based on the formation of phosphorous acid triesters with participation of the cis-diol system under thermodynamic control. Hydrolysis of the intermediate affords a mixture of monophosphites which is then subjected to an oxidative cyclisation by reaction with hexachloroacetone with the direct formation of the 2′,3′-cyclic phosphate. In the case of lyxofuranosides (e.g. XXIV), also the hydroxylic functions at positions 3′ and 5′ may be involved in the formation of the cyclic phosphite. A mixture of 2′,3′- (XLVII) and

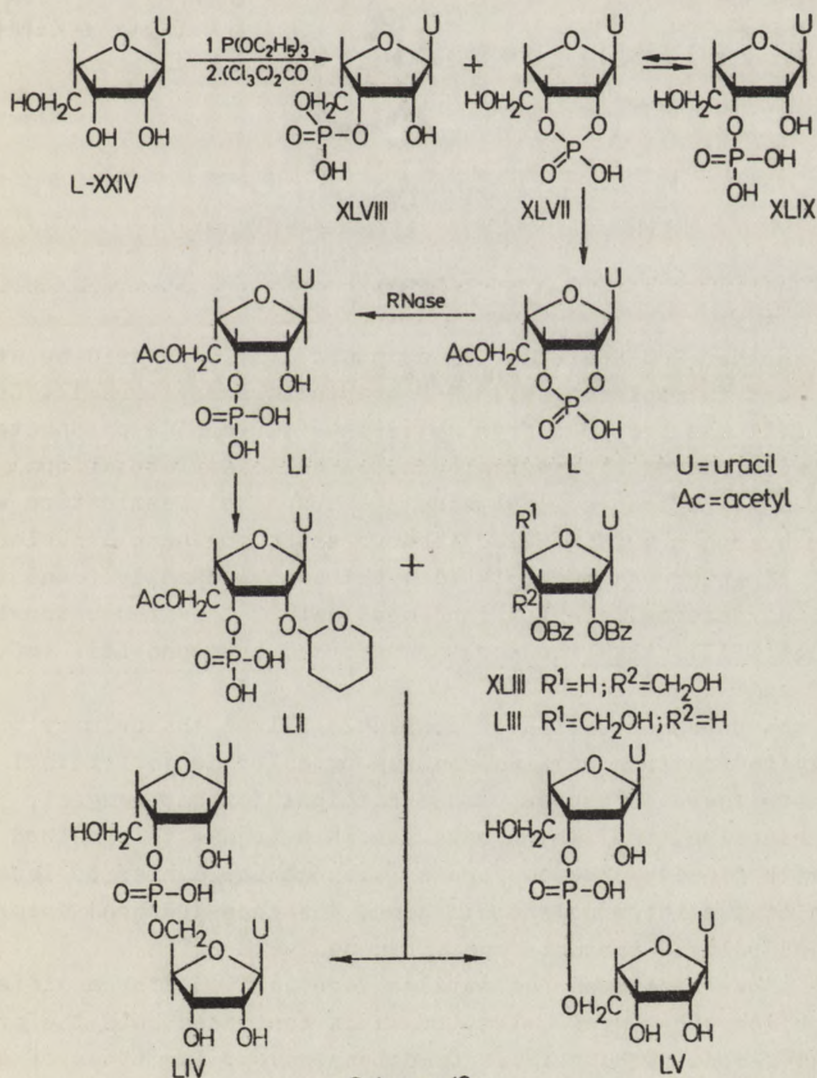


Scheme 11

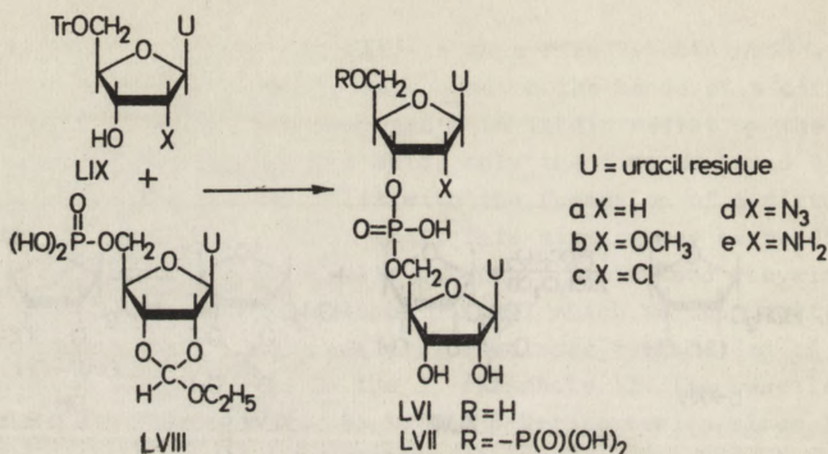
3′,5′-cyclic phosphate (XLVIII) is thus finally obtained[40]. This mixture may be readily separated on the basis of a different stability of the two components in acidic media: by the action of 50% aqueous acetic acid, only the five-membered 2′,3′-cyclic diester XLVII is split with the formation of a mixture of the 2′- and 3′-nucleotide XLIX. This mixture may be readily separated from the 3′,5′-cyclic phosphate XLVIII and recyclised to the pure 2′,3′-cyclic phosphate XLVII which is then acetylated at position 5′. Pancreatic ribonuclease degradation of the resulting acetate L affords the 3′-phosphate LI. The reaction is of the same specificity as in the β-D-ribo series since the two series differ in configuration at the $C_{(4')}$ carbon atom which is not of importance for the enzyme[41,42]. Reaction with dihydropyran afforded the protected nucleotide LII which may be regarded as analogue of compound XLV.

Condensation of the pyridinium salt of compound LII with 2′,3′-di-O-benzoyluridine (LIII) or compound XLIII followed by removal of protecting groups first in alkaline and then in acidic media yielded[39] the dinucleoside phosphates LIV and LV, resp., as shown in Scheme 12. The present combination of protecting groups excludes the cleavage of the internucleotidic bond in alkaline media in such cases when the hydroxylic function at positions 2′ and 5′ of the 3′-nucleotide would be free.

In connection with investigations on the specificity of some ribonucleases and cyclic phosphodiesterases[43] it was necessary to synthesize some analogues of uridylyl-(3′→5′)-uridine and pUpU modified at position 2′ of uridine 3′-phosphate. For this purpose, compounds of the general formula LVI and LVII were used. The UpU analogues LVI were prepared with the use of a general method excluding the alkaline work-up and consisting in condensation of 2′,3′-O-ethoxymethyleneuridine 5′-phosphate (LVIII) with a 5′-O-p-methoxytrityl derivative LIX of the corresponding nucleoside (compounds LIX were obtained by the usual tritylation procedure). The condensation mixture was processed in acidic media to afford products LVI which were isolated by column chromatography[43] (Scheme 13). The 2′-amino-2′-deoxy derivative LVIe was obtained from 2′-azido-2′-deoxy derivative LVId by hydrogenation in acidic medium. Compound LVIe is considerably unstable towards the chemical hydrolysis.



Scheme 12



Scheme 13

In another UpU analogue, in compound LXII, the uridine at the 5′-end is replaced by 1-(β-D-arabinofuranosyl)uracil. Compound LXII was prepared from cytidine 2′,3′-cyclic phosphate which was treated with trimethylsilyl chloride[44] to afford 1-(β-D-arabinofuranosyl)cytosine 3′-phosphate. Deamination with sodium hydrogen sulfite gave the corresponding uracil nucleotide[32] LX which was benzoylated with benzoyl cyanide[45] and the resulting intermediate LXI condensed with 2′,3′-di-O-benzoyluridine (LIII). Alkaline work-up afforded compound LXII (cf. ref.[43] and Scheme 14).

In the phosphorylation of compounds LVI on the primary hydroxylic function with phosphorus oxychloride in triethyl phosphate there have been used conditions (excess reagent, short reaction time) which make possible to use this method also with dinucleoside phosphates without any danger of isomerisation of the internucleotidic bond. The thus-prepared compounds LVII and related products are shown on Table 2.

The above mentioned nucleotides were derived from modified nucleosides, the sugar moiety of which contained both the primary and secondary hydroxylic functions, i.e., functions of a rather different character. Some analogues, however, may contain

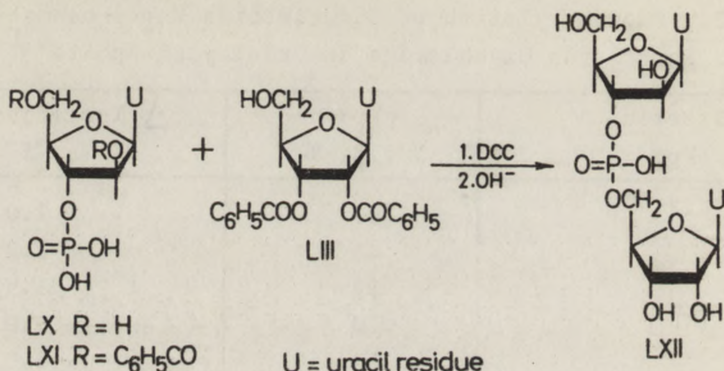
Table 2. Phosphorylation of Dinucleoside Monophosphates with Phosphorus Oxychloride in Triethylphosphate[a,b]

Starting XpY	pXpY Yield, %	△ Isomerisation, %
UpU	37	1.0
UpA	41	1.2
CpU	29	1.5
ApU	20	1.0
GpA	18	1.0
GpG	24	0
(2′→5′)-UpU	46	–
UpαU	36	0.5
Up5-Me_2N-U	41	1.0
araUpU	47	–
2′-Cl-dUpU	37	–
2′-MeO-dUpU	47	–
CpX	21	0.7
d(UpU)	29	–
d(CpU)	42	–
GparaU	32	1.5
Gp2′-Cl-dU	28	0.9

[a] 10 µmol XpY and 25 µl $POCl_3$ in 500 µl triethyl phosphate; temperature, 0°C; time, 4 hours; [b] abbreviations: αU... α-uridine, 5-Me_2N-U... 5-dimethylaminouridine, araU... uracil arabinoside, 2′-Cl-dU... 2′-chloro-2′-deoxyuridine, 2′-MeO-dU... 2′-O-methyluridine.

only the secondary hydroxylic functions such as the nucleosides derived from β-D-ribopyranose. These isomers of ribonucleosides may be readily obtained by a stereospecific reaction of 2-O--tosyl-D-arabinose[16] (LXIII) with the sodium salt of the corresponding heterocyclic base. The reaction course strongly depends on the character of the solvent (Table 3). The best results are obtained in dimethylformamide as solvent[46] (Scheme 15).

The β-D-ribopyranosides LXIV contain in their sugar moiety three almost equivalent secondary hydroxylic functions which



Scheme 14

Table 3. Effect of Solvents in the Reaction of Adenine Sodium Salt with Compound LXIII[a]

Solvent	Nucleoside yield, %	Ratio Pyranoside/Furanoside
Methanol	20	3.0
Ethanol	23	4.6
Dioxane	19	1.7
Acetonitrile	28	2.5
Dimethylformamide	45	3.1
Dimethylsulfoxide	43	6.2

[a] 1 mmol of LXIII, 2 mmol of adenine sodium salt, 6 ml of the solvent; reaction time, 16 hours at room temperature.

are mutually cis oriented. The phosphorylation with phosphorus oxychloride does not afford encouraging results. On the other hand, the triethyl phosphite-hexachloroacetone procedure[19] proved to be the method of choice in this case. In view of the two cis-diol systems in the molecule of LXIV, two cyclic phosphates are obtained[47], namely, the 2′,3′- (LXV) and the 3′,4′-cyclic phosphate LXVI. As expected, this pair is difficult to

CH=O
TsO
OH
OH
CH_2OH
LXIII

+ $B^{\ominus}Na^{\oplus}$ →

LXIV

1. $(C_2H_5O)_3P$
2. $(Cl_3C)_2CO$

Ts = tosyl group
B = pyrimidine or purine

LXV

LXVI

LXVII

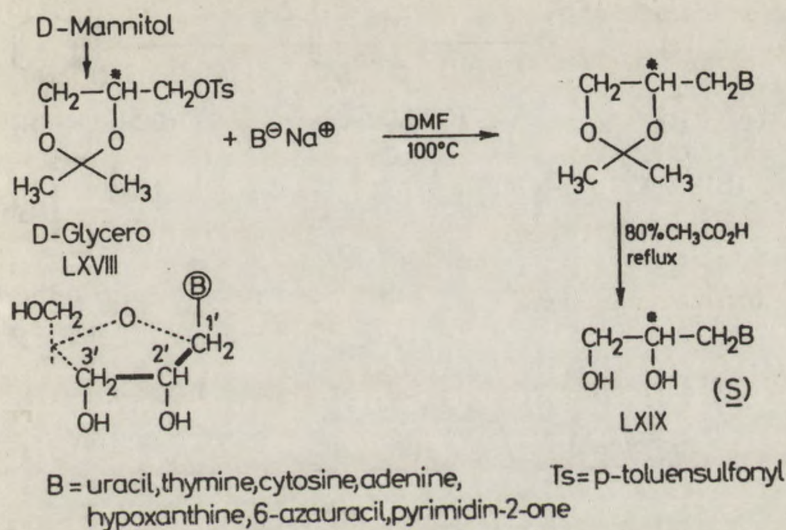
Scheme 15

separate. The acidic hydrolysis leads to a mixture of three isomeric mononucleotides from which only the 3′-nucleotide does not contain the _cis_-diol system and may be thus separated from the remaining two components of the mixture in borate-containing systems. A similar situation may be observed with other polyfunctional molecules such as nucleosides derived from hexopyranoses[47]. The above mentioned procedure exemplifies the approach to the synthesis of additional nucleotides of this type which might be of interest, especially in investigations on enzymatic reactions.

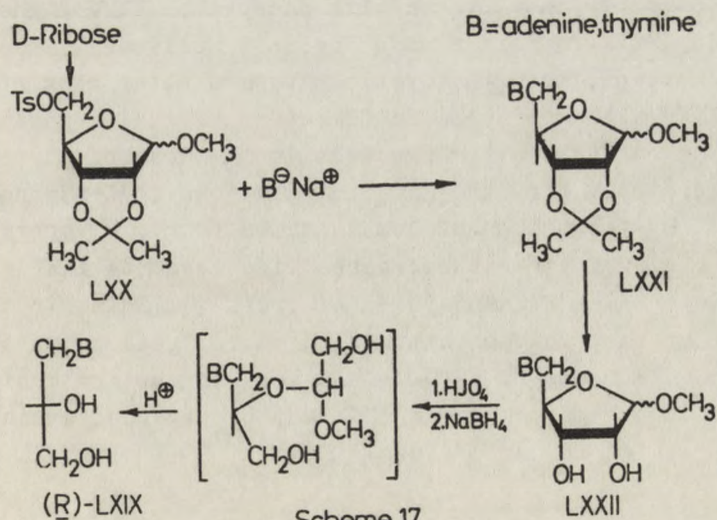
As the last group of nucleoside analogues with a modified sugar moiety I should like to mention the aliphatic nucleoside analogues, namely, derivatives of heterocyclic bases carrying a polyfunctional hydroxylated aliphatic residue. The typical representatives of such analogues, the 2,3-dihydroxypropyl derivatives LXIX, may be regarded as modified ribonucleosides with a degraded portion of the sugar skeleton[48,49]. The aliphatic chain contains a chirality center; it is therefore necessary to perform an asymmetric synthesis and to avoid reactions which could lead to racemisation. The (S)-enantiomers LXIX were prepared by condensation of 1-O-tosyl-2,3-O-isopropylidene-D-glycerol (LXVIII) with the sodium salt of the corresponding base followed by acidic cleavage of the 2,3-dioxolane group[48,49] as shown in Scheme 16.

The (R)-enantiomers LXIX were prepared by condensation of 5-O-tosyl-2,3-O-isopropylidene-1-O-methyl-β-D-ribofuranoside (LXX) with the sodium salt of the corresponding base, cleavage of the dioxolane function of the intermediate LXXI, periodate oxidation, and the sodium borohydride reduction of the intermediate LXXII (cf. ref.[49] and Scheme 17).

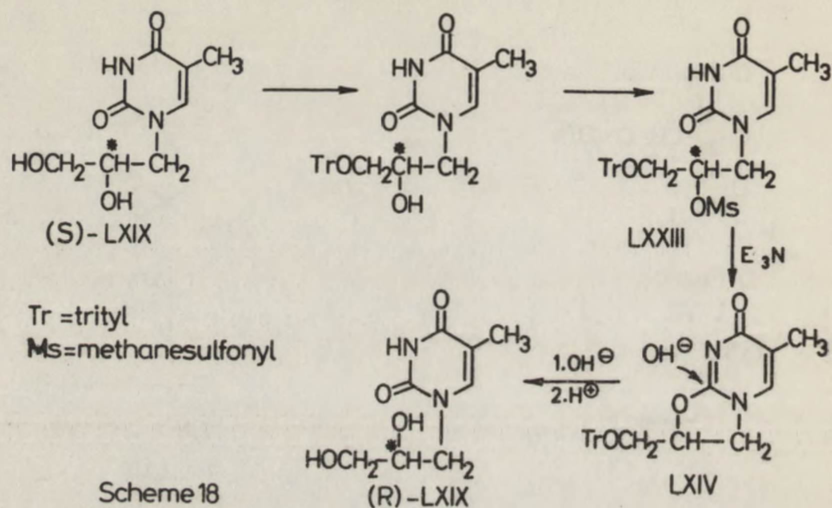
An alternative route is based on the inversion. Thus, the thymine derivative (S)-LXIX was tritylated and selectively methanesulfonylated at position 2′ to afford the derivative LXXIII which was then converted by the action of a base into the $O^{2,2'}$-anhydronucleoside analogue LXXIV. The stereospecific opening of the anhydro bond in an alkaline medium consists in an attack at position 2 of the uracil ring. The final removal of the trityl group afforded an optically pure (R)-enantiomer of compound LXIX (cf. ref.[49] and the Scheme 18).



Scheme 16

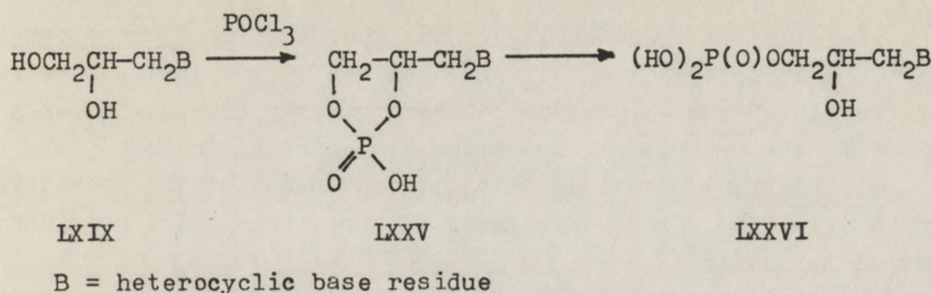


Scheme 17



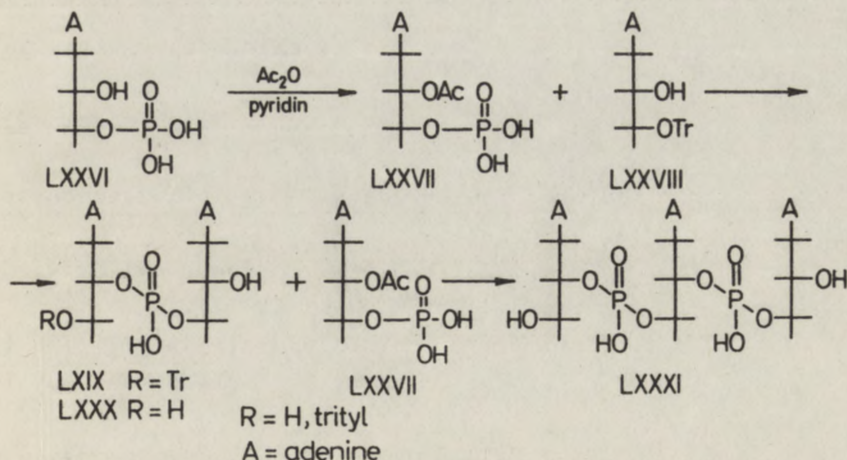
Scheme 18

The CD spectra of compounds LXIX are very similar to those of nucleosides. Particularly in the pyrimidine series, the extrema positions as well as the molar elipticity values are similar. Compounds LXIX thus appear to prefer in aqueous solutions conformations closely related to those of nucleosides[52]. Phosphorylation of compounds LXIX with phosphorus oxychloride affords directly the 2',3'-cyclic phosphates LXXV (Scheme 19). Compounds LXXV are not degraded by most nucleases. Some nucleases, however, open the cyclic phosphodiester ring of compounds LXXV with the specific formation of the 3'-phosphates LXXVI. Such a degradation proceeds in the case of the (S)-enantiomers only (cf. the genetic relations to D-ribonucleosides[48]). By the action of some enzymes (e.g., P. brevicompactum or P. claviforme ribonucleases) the racemate LXXV was resolved into (S)-LXXVI and (R)-LXXV (ref.[48]). This finding is of special interest in connection with investigations on the substrate requirements of various enzymes. In another context, the (S)-enantiomers of compounds LXIX may be regarded again as closely related to nucleosides.



SCHEME 19

In view of the above observations, our syntheses were extended by the preparation of oligonucleotide analogues derived from compounds LXIX. Acetylation of the 3′-phosphate (S)-LXXVI yielded the 2′-O-acetyl derivative LXXVII; tritylation of the nucleoside analogue (S)-LXIXb afforded the 3′-O-trityl derivative LXXVIII. Condensation of both these components and ammonolysis led to compound LXXIX (the protected ApA-dinucleoside phosphate analogue) and the free analogue LXXX. Repeated condensation of compound LXXIX with the protected nucleotide analogue LXXVIII and the subsequent removal of protecting groups afforded compound LXXXI (the ApApA-analogue); see Scheme 20.



Scheme 20

The structure of these analogues was confirmed by P. brevicompactum ribonuclease degradation which was quantitative; moreover, the result of the enzymatical degradation may be regarded as a proof of the optical purity of the condensation products[49].

The growing interest in aliphatic analogues of the above type might stimulate further syntheses of more complicated polyfunctional derivatives.

Investigation on modifications of the sugar moiety in nucleotides, from modifications consisting in simple configurational changes through the pyranoside analogues to the aliphatic analogues, along with modifications of the basic moiety or of the phosphate grouping may markedly contribute to the knowledge on the relationship between the structure and properties (especially biochemical properties) of nucleotides. A further development of this field is therefore desirable from the standpoint of both organic chemistry and molecular biology.

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2'-MODIFIED NUCLEOSIDES AND NUCLEOTIDES

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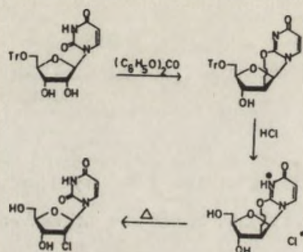
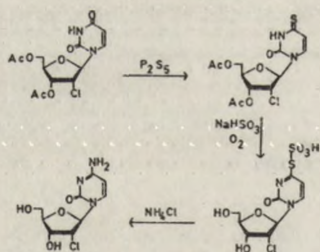
The way in which a substituent in the 2'-position of the ribose moiety influences structure, stability and function of polynucleotides is not well understood. One of best known examples of such an influence is the difference in thermal stability of polyribo- and polydeoxyribonucleotides[1]. In an attempt to understand this influence better a number of laboratories have synthesized a variety of polynucleotides with modifications in the 2'-position[2a-h]. A further drive for the synthesis of such compounds is the biological activity of certain double-stranded polyribonucleotides in conveying resistance against viral infection through the induction of interferon[3] as well as the observation that RNA-dependent DNA-polymerase which is associated with RNA tumor viruses[4] can be inhibited by single-stranded polymers[5]. Furthermore, it is conceivable that modified polynucleotides can be used as carriers of modified nucleotides into cells. Finally, nucleotides with a reactive group in the 2'-position might be useful as potential affinity labels for certain nucleotide dependent enzymes.

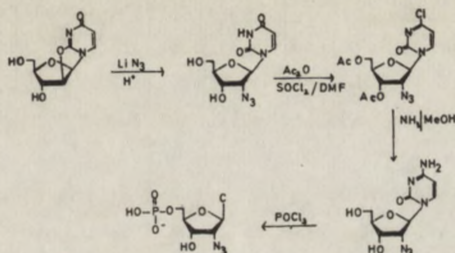
Our own efforts in this field have been restricted so far to modifications of the 2'-position in pyrimidine nucleosides[2g,h] because of the ease of synthesis of such derivatives. This is due to the facile formation of O^2,2'-cyclouridine[6]. The 5'-trityl derivative can easily be prepared from 5'-trityluridine by reaction with diphenylcarbonate in DMF at 140°C. On detritylation with HCl in dioxane one obtains the cyclouridine as the chloride which upon heating leads to 2'-chloro-2'-deoxyuridine. This is a convenient method of synthesis and has the additional advantage that at this stage ^{36}Cl can be introduced by addition of $Li^{36}Cl$ prior to heating.



The uridine derivatives are the best starting materials for the corresponding cytidine derivatives. A similar nucleophilic opening of a O^2,2'-cyclocytidine is not a favourable approach because of the easy reformation of the cyclonucleoside under the usual drastic reaction conditions. The transformation of 2'-chloro-2'-deoxyuridine was achieved by the bisulfite-oxygen reaction of Hayatsu on 2'-chloro-2'-deoxy-4-thiouridine[7].



The introduction of the azido group into the 2'-position of uridine can be brought about by reaction of O^2,2'-cyclouridine with LiN_3 as described by Verheyden et al.[8]. The transformation into the corresponding cytidine derivative can not be achieved as described for 2'-chloro-2'-deoxyuridine since on reaction with P_2S_5 to the 4-thiouridine derivative at least partial reduction of the azido group occurs. Instead, after acetylation the 3',4'-diacetyl-2'-azido-2'-deoxyuridine can be reacted with DMF-thionylchloride by a procedure of Zemlicka and Sorm[9] to the 4-chlorouridine derivative which on reaction with methanolic ammonia yields the desired 2'-azido-2'-deoxyuridine.



All these 2'-modified nucleosides can be phosphorylated in the 5'-position with $POCl_3$ in triethylphosphate, if desired by the use of $^{32}POCl_3$ to obtain labelled material. The synthesis of the 5'-di- and triphosphates can be performed by the conventional procedures by activation of the monophosphate with diphenylphosphorochloridate[1o] or carbonyldiimidazol[11] and subsequent reaction with phosphoric or pyrophosphoric acid.

Reduction of the 5'-diphosphates of 2'-azido-2'-deoxyuridine and 2'-azido-2'-deoxycytidine with hydrogen and Pd gives the corresponding 2'-amino-2'-deoxyuridine and -cytidine derivatives.

The 5'-diphosphates of these 2'-modified nucleosides are all substrates for polynucleotide phosphorylase from Micrococcus Luteus in the presence of Mn^{++}. This polymerisation is considerably slower than that of the natural substrates UDP and CDP. Analysis of the isolated polymers after degradation to the nucleosides with snake venom phosphodiesterase and alkaline phosphatase on a nucleoside analyzer shows that the polymers do not contain any nucleoside constituents other than the substrates indicating that no O^2,2'-cyclonucleoside or arabinonucleoside was polymerized or formed in the polymer.

Poly(2'-chloro-2'-deoxyuridylic acid) as well as poly(2'-chloro-2'-deoxycytidylic acid) can be annealed to their base complementary polymers, poly(rA) and poly(rI), respectively. The resulting double-stranded polymers have thermal stabilities comparable to those of the unmodified polymers. Poly(2'-azido-2'-deoxyuridylic acid)[2f] as well as poly(2'-azido-2'-deoxycytidylic acid) can also form double stranded polymers with poly(rA) and poly(rI), respectively,

with thermal stabilities similar to those of the unmodified polymers. However, it seems as if neither poly(2'-amino-2'-deoxyuridylic acid) nor poly(2'-amino-2'-deoxycytidylic acid) could form double stranded polymers with poly(rA) or poly(rI) which are stable under normal conditions.

As to the question in which way a substituent at the 2'-position alters the stability of a polymer, these results as well as others[2a-f] indicate that most substituents - actually all except for the amino group - seem not to destabilize the double helical structure of ribopolynucleotides, i.e. the thermal stabilities compare with those of double stranded polyribonucleotides and not with those of the polydeoxyribonucleotides. Before a more refined analysis has been carried out it appears that this stabilizing effect of a substituent in the 2'-position is mainly a steric effect as has been discussed by Crothers[11]. Experiments carried out by others mainly with 2'-O-methyl- and 2'-O-ethyl substituents indicate that an increase in size of the substituent also increases the thermal stability of the polymer in the absence of a complementary polynucleotide.

All the 2'-modified polynucleotides are considerably more stable than the unmodified polymers against enzymatic degradation by a variety of nucleases including human serum nucleases[12]. Since enzymatic breakdown decreases the concentration of polymers in cells, these resistant polymers are interesting for a number of biological applications. One of such possible applications seemed to be their use for the induction of interferon, a protein which confers resistance against viral infection to mammalian cells[3]. It is known that synthetic polynucleotides, in particular poly(rI)·poly(rC) can induce interferon in vivo and in vitro. Since a longer lifetime of such a polynucleotide in the serum might conceivably increase its activity we have tried together with other analogues the duplex poly(rI)·poly(rCcl) in cell cultures of human skin fibroblasts as well as in mice for its ability to induce interferon and resistance against viral infection[12,13]. These studies show that the modified polymer although very stable in the serum does not induce interferon indicating together with the work of others[14a-d] that the 2'-OH group is essential for this activity. These experiments revealed that the toxicity of polynucleotides seems to increase with its interferon inducing activity[12].

There is another biological application conceivable for poly(2'-chloro-2'-deoxycytidylic acid). Aracytidine is an inhibitor for DNA synthesis in mammalian cells[15] and has been used to treat leukaemia. In the cell arabinocytidine can

be phosphorylated to the monophosphate by kinases and further to the triphosphate which acts as an inhibitor of DNA-polymerase. However, these tumors can develop resistance against aracytidine presumably by altering the nucleoside kinase so that this nucleoside cannot be phosphorylated. To circumvent this problem and since nucleoside triphosphates cannot penetrate the cell wall the application of the nucleotide in form of the 3',5'-cyclophosphate has been tried although with limited success since this compound gets hydrolyzed rapidly in the serum first to the 5'-phosphate and further to the nucleoside leaving the problem where it was[16]. Poly(2'-chloro-2'-deoxycytidylic acid) might offer an alternative. We know it is stable in the serum but on the other hand on standing at 37° for 50 hrs at pH 8.9 or pH 7.5 concomitated with a slow breakdown of the polymer an increase of up to 15 % of aracytidinephosphate can be detected[17]. This suggests that this polymer can possibly be used as a carrier for arabinocytidine-phosphate provided it can penetrate the cells. This has not been established as yet but it is known from other work that synthetic polynucleotides can enter mammalian cells[18a-c] and it is possible that poly(2'-chloro-2'-deoxycytidylic acid) is no exception. The biological evaluation has still to be carried out.

There is one more possible biological application for the 2'-modified polynucleotides. RNA tumor viruses contain a RNA-directed DNA-polymerase which catalyses the synthesis of a DNA copy of the resident or endogenous RNA[4]. The synthesis of this DNA copy is considered to be an obligatory step in infection and cell transformation by these viruses. Single stranded polynucleotides inhibit this viral DNA-polymerase[5] and some inhibit virus replication in tissue culture[19a,b]. In search for polynucleotides which are resistant against nucleolytic degradation and which exhibit selective inhibition of the viral DNA-polymerase in comparison with bacterial and mammalian DNA-polymerases, a number of 2'-modified polynucleotides have been tested[20a,b]. The results show that such polymers exhibit the desired properties <u>in vitro</u>. It is conceivable that they might be useful as analytical tools for the detection of such viral DNA-polymerases and thus for the presence of such tumor viruses.

Finally, in a long-standing cooperation with Prof. Reichards laboratory in Stockholm we have tested the interaction of some of the 2'-modified cytidinediphosphates with the E.coli ribonucleotide reductase, the enzyme which reduces the ribonucleoside diphosphates to deoxyribonucleoside diphosphates. These, of course, are the precursors for the corresponding triphosphates, the substrates

for DNA-polymerases. This enzyme is highly regulated by nucleoside triphosphates and is responsible for the pool of deoxyribonucleoside triphosphates in the cell and thus represents one way of regulation of DNA-synthesis. The enzyme consists of two proteins, B_1 and B_2, which in turn are made up by probably two identical subunits each[21]. The effectors bind to B_1[22]. One of the problems still awaiting an answer is the question as to what effect positive or negative effectors have on the binding of substrate. In an effort to produce competitive inhibitors for this enzyme with which one could tackle this problem we tested 2'-chloro-, 2'-azido- and 2'-amino-2'-deoxycytidine diphosphate. It turned out that only 2'-amino-2'-deoxycytidine diphosphate is a reversible competitive inhibitor which will be useful for the studies indicated. The two other compounds, however, inhibit the enzyme irreversibly in a specific manner, i.e. only the di- and not the monophosphate inhibits and this only in the presence of both proteins, B_1 and B_2. Moreover, the inhibition can be controlled by effectors and by the presence of substrate. These criteria suggest that this irreversible inhibition occurs probably on the substrate binding site. The mechanism by which these compound inactivate the enzyme is still under investigation[23].

In summary it can be said that 2'-modified nucleosides and in particular 2'-modified nucleotides could have a wide application in a number of biochemical and biological systems.

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STUDIES ON Y BASE AND RELATED COMPOUNDS

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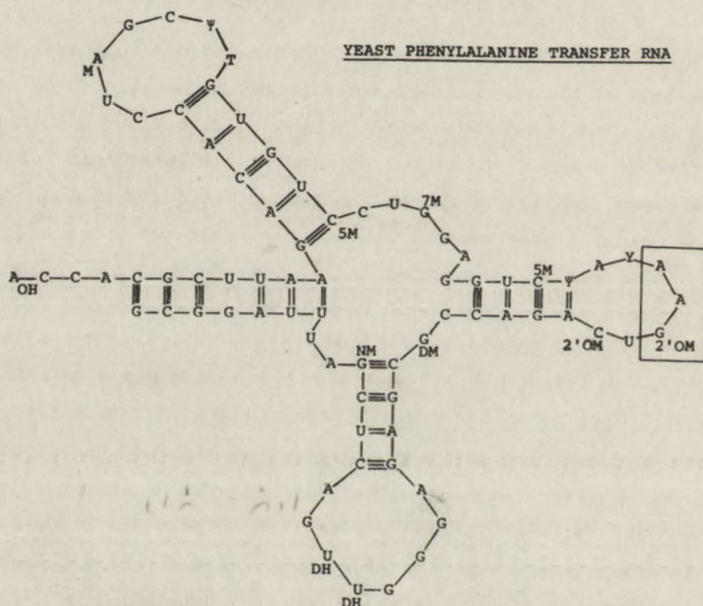
Transfer RNA (tRNA) differs from most other RNA and DNA in that a substantial percentage of the nucleosides are modified. The structures of about forty of these modified nucleosides have been determined and there are at least five of unknown structure reported in the literature. Although there have been many theories as to the function of modified nucleosides, very little has actually been proven. Of all the positions where modified bases occur in tRNA, two are of special interest since they are the only ones known to contain several different hypermodified nucleosides (nucleosides containing a carbon chain larger than a methyl) and they are located in positions which would likely affect ribosome binding and maybe amino acylation. These two positions are at the 5'-end of the anticodon (wobble base) and adjacent to the 3'-end of the anticodon. It has been shown that in some cases alteration of the modified bases at either of these two positions will affect ribosome binding and amino acylation activity. Nishimura and coworkers have thoroughly demonstrated that the presence of certain modified nucleosides in these two positions is related to the codon for that tRNA in _E. coli_.[1)]

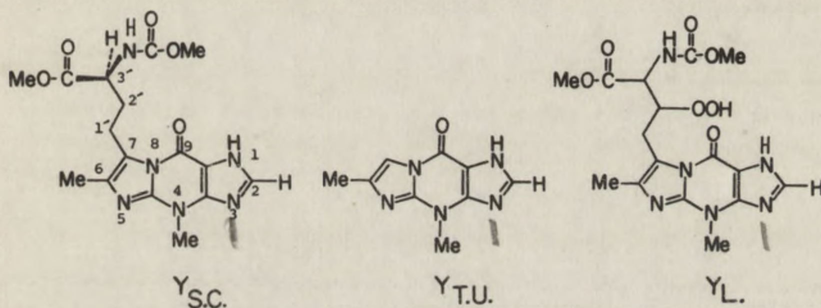
Of all the known modified bases our interest lies especially with the "Y" bases. The presence of the Y nucleoside was first noticed in the determination of the primary sequence for yeast $tRNA^{Phe}$.[2,3)] At that time it was discovered that Y compounds are less stable than most naturally occurring nucleosides and that they possess greater fluorescence than other nucleosides occurring in tRNA. Prior to the structure elucidation of a Y base, 1-methylinosine, 6-(Δ^2-isopentyl)adenosine, 2-methylthio-6-(Δ^2-isopentyl)adenosine and N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine were known to occur in the position adjacent to the 3'-end of the anticodon.

Figure 1



Through studies on model compounds the structure of Y base from Saccharomyces cerviseae (Y_{SC}) was shown by Nakanishi and coworkers to have the unique fused tricyclic system along with a complex side chain.[4] Using this compound as a basis, Kasai and coworkers proved that Y base from Torulopsis utilis (Y_{TU}) had the same ring system but lacked the side chain.[5] Later Nakanishi and coworkers indicated that Y base from mammalian liver (Y_L) and probably wheat germ not only contained the imidazo[1,2-a]purine ring system and a side chain similar to Y_{SC}, but also contained a hydroperoxide, a rare functional group in natural compounds.[6] The various Y bases have distinctive mobility upon silica gel or cellulose thin layer chromatography as demonstrated in Figure 3.

Figure 2



Structure of the fluorescent bases from phenylalanine tRNA of Saccharomyces cereviseae ($Y_{S.C.}$), Torulopsis utilis ($Y_{T.U.}$) and of mammalian liver (Y_L).

Since the structure elucidation has been published, it will not be covered in this paper, except to indicate that the structure determination involved comparison of the physical properties of Y base with many model compounds. The final structure proof of a compound is usually considered to be its unambiguous synthesis; i.e., a compound with the same physical properties as the isolated sample is synthesized by reactions whose outcome could be predicted. As a definitive structure proof, the published synthesis of Y_{SC} has a major handicap in that the formation of the third ring by the reaction of 3-methylguanine with the appropriate α-bromoketone goes in such low yield (~2%)[7] that the possibility exists that the product results from an undetectable contaminate in the starting materials.

Figure 3

t-RNA^{Phe} source	R_f[6]		R_f[7]	
Saccharomyces cervisiae[1]		.48		.84
Torulopsis utilis[2]	major and minor bands		.63	
Wheat germ[3]			.67	
Beef liver[4]	.34	.48 (?)	.67	
Rat liver[5]	.35	.48		
Chicken liver	.35			
Calf liver	.34	.50 (?)		

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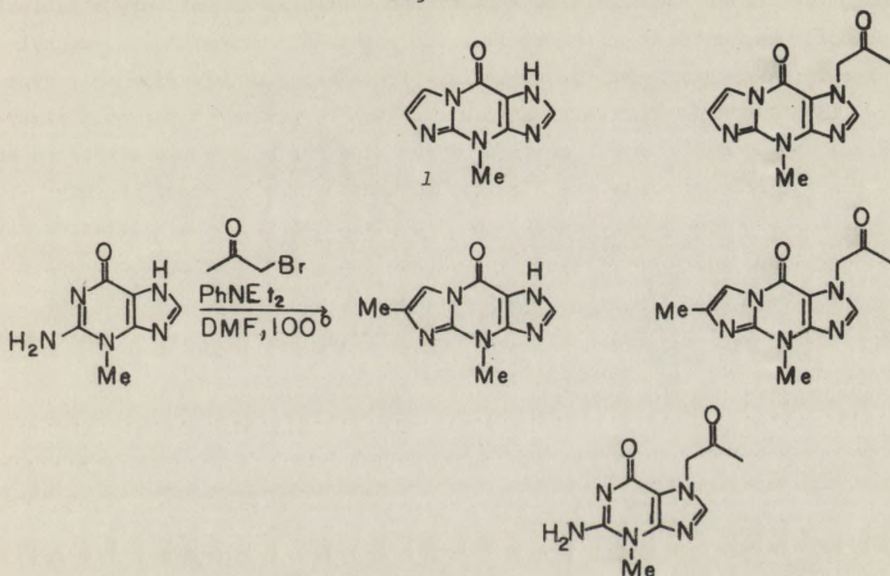
Because of this concern about the synthesis of Y and because of a desire to obtain more Y_{SC} for studies on its physical properties, we investigated alternate syntheses of Y bases. Before discussing the new synthesis it would be useful to show how the problems encountered in the old synthesis led to our improved route. Even in the simplest compound, Y_{TU}, this method of synthesis was poor. Causing excess bromoacetone to react with 3-methylguanine at 60-100° in DMF, DMA, DMSO, etc., with various bases $PhNEt_2$, Et_3N and $NaHCO_3$ gave Y_{TU} in 25% along with the 6-desmethyl-Y_{TU} (numbering system is illustrated in Figure 2), and the 1-alkylated derivatives of both compounds. Only by using 1 N aq NaOH could we avoid the formation of the desmethyl-Y_{TU}, *1*, but these conditions are clearly not applicable to the synthesis of Y_{SC}.

Figure 4



In going on to the synthesis of the model compound 7-butyl-Y_{TU}, the problems increase for not only the desired compound (5% yield) and the desmethyl-Y_{TU} are formed, but also the 6-(2-butenyl)-6-desmethyl-7-methyl-Y_{TU}, *2*, and the 1-alkylated derivatives of these compounds are formed. The separation of all of these compounds is very tedious. It should be noted that most of the unwanted products are formed from the decomposition products of the α-bromoketone. It is likely that the desmethyl-Y_{TU} comes from the α,α'-dibromoketone (formed by disproportionation of the α-bromoketone) by first a cyclization reaction involving the primary bromide and the ketone, followed by a cleavage reaction. The oxidation of the α-bromoketone to an α,β-diketone, especially in DMSO, results in the formation of the compound with the unsaturated side chain in the 6-position of the Y base.

In the case of Y_{SC}, using a three-fold excess of the proper α-bromoketone, whose synthesis will be covered later, gave the desired Y base in 2% yield plus other compounds analogous to those formed in the model compounds.[7)]

Although we obtained the desired Y base, the synthesis is not very efficient. The problem encountered in the cyclization reaction is two-fold: 3-methylguanine is mainly insoluble in solvents which are compatible with this type of reaction and thus the reaction requires high temperatures for solubility reasons, and secondly the 7-position of the base can be readily alkylated and thus some of the reactants are diverted to nonuseful compounds. A very reasonable solution was to protect the 7-position of the 3-methylguanine with a benzyl group, which would greatly increase the solubility of the guanine and would leave only the 1- and N^2-positions for reaction; hopefully the protecting group could be removed by mild hydrogenation conditions that would not effect the olefinic bond of the added ring.

Figure 5

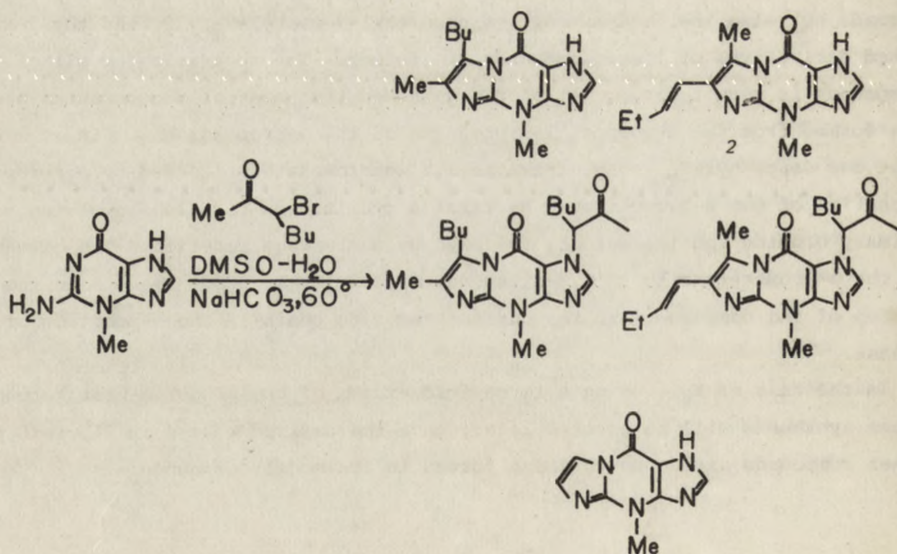
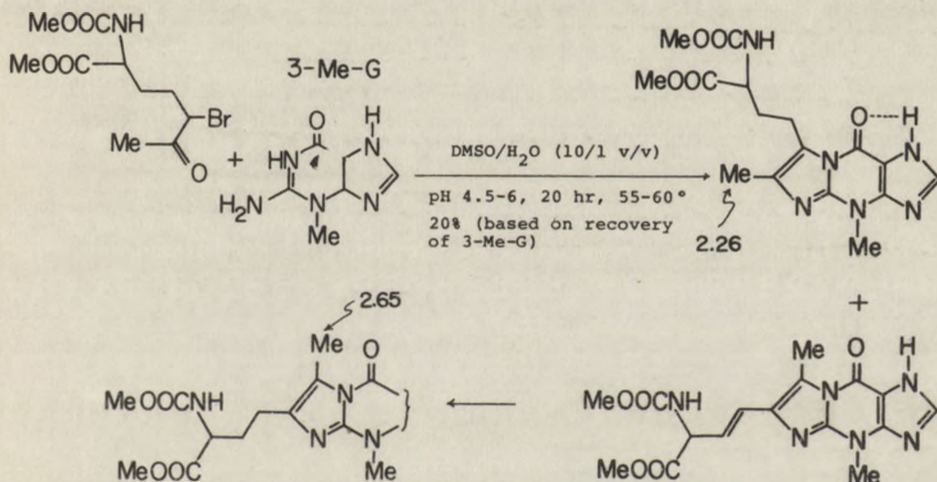


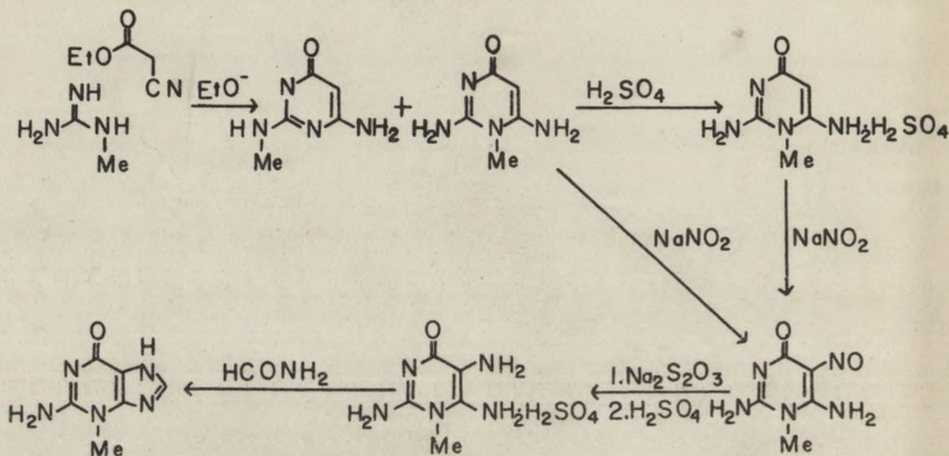
Figure 6



A short digression is in order to explain the preparation of the 3-methylguanine that we used. By several modifications of the literature preparation of 1-methyl-4-oxo-2,5,6-triminopyrimidine·sulfate from methylguanidine[8] and by following the literature procedure for the cyclization to 3-methylguanine,[9] we improved the yield of 3-methylguanine from 3 to 40% overall yield (figure 7).

Adding a protecting group to the 7-position of 3-methylguanine proved more troublesome than expected. The anion of 3-methylguanine failed to react with either benzyl chloride or benzyl chloromethyl ether even at 80° in DMF. However if the 2-amino group was transformed to a palmitamido group, the desired alkylation would take place at the 7-position. Since the overall yield from 3-methylguanine to the 7-benzyl derivative was 50% and to the 7-benzyloxymethyl derivative was 46%, we desired a better method of protection.

Figure 7

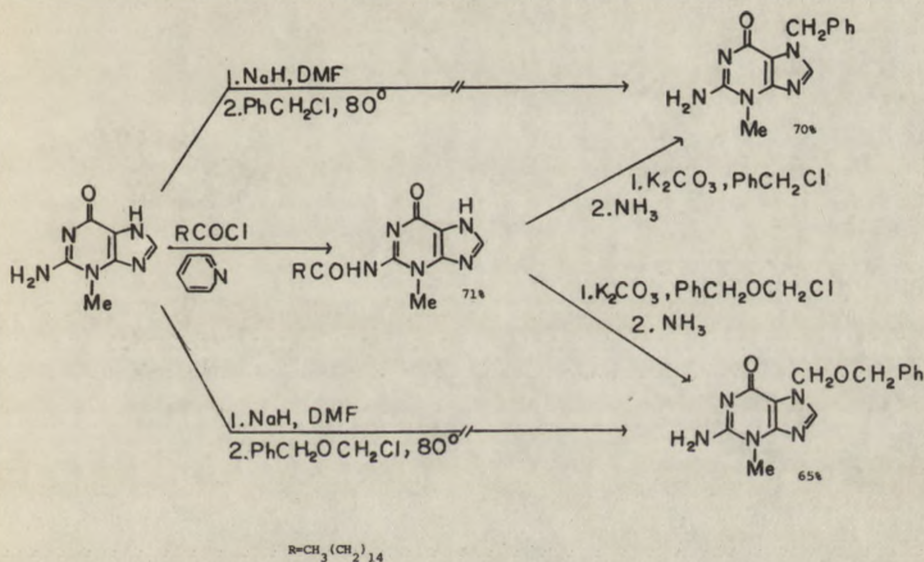


Roth, Smith, Hultquist, JACS, 73, 2864 (1951)
Towsend, Robins, JACS, 84, 3008 (1962)

Unlike the chloride, benzyl bromide reacted readily with the anion of 3-methylguanine in high yield; thus this method of adding the protecting group was used. Because of some trouble in synthesizing the benzyl bromomethyl ether and in handling such an unstable lacrimator, and because we found conditions under which the benzyl group could be removed easily, we terminated our investigation on the benzyloxymethyl compound.

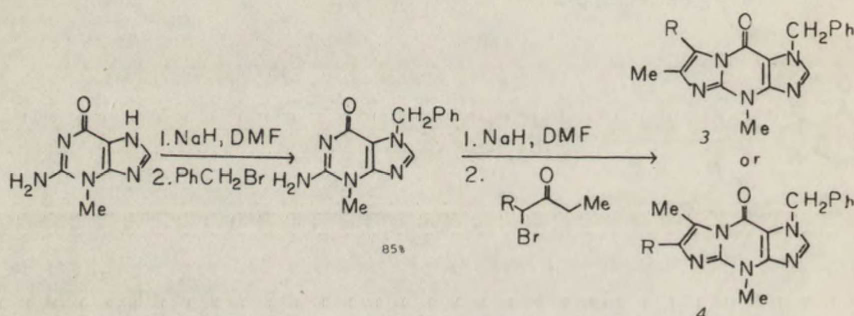
Now that we have the 3-methylguanine protected at the 7-position, we turned our attention to the alkylation-condensation reaction which will form the third ring. Since the reaction is carried out under basic conditions, the first step, alkylation with a bromide would be expected to occur at the 1-position of guanine by comparison with a 7-alkylguanine. Although this comparison seems reasonable, it was necessary to verify this point since it determines the orientation of the two alkyl groups on the third ring, *3* or *4*. It should be noted that the nmr spectroscopy supports the predicted reaction pathway if one assumes that the anisotripic effect of the carbonyl will deshield

Figure 8



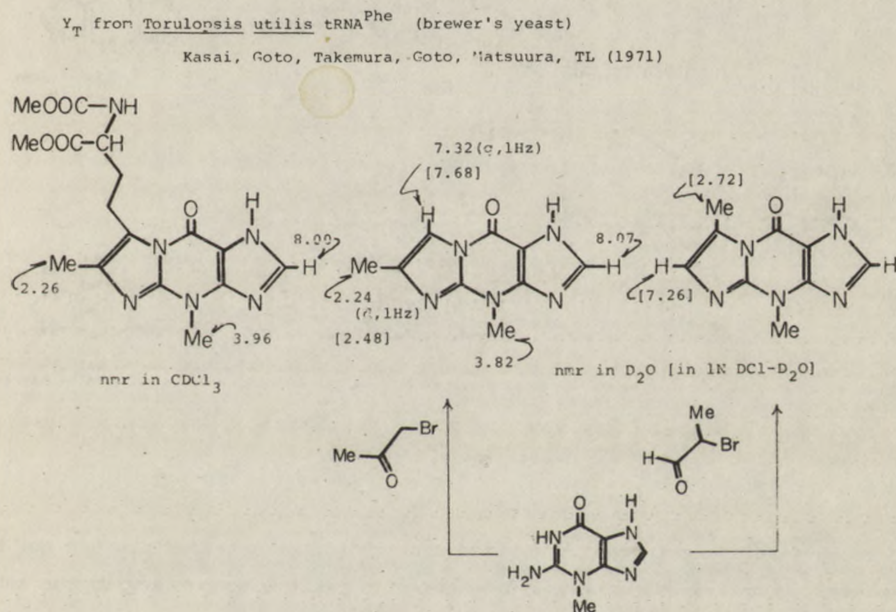
the proton at 7-position or on a methyl attached at the 7-position more than at the 6-position, as illustrated in Figure 10. Our method for verifying the position of alkylation was to substitute methyl iodide for the α-bromoketone. The product (89% yield) of this reaction was compatible with that expected for 7-benzyl-1,3-dimethylguanine. Although this compound was stable to strong aqueous acid conditions, it was readily hydrolyzed in 3 N KOH to the known 7-benzyl-1,3-dimethylxanthine in 75% yield, which in turn was hydrogenated to give a compound with the same physical properties as an authentic sample of theophylline (1,3-dimethylxanthine). This proves that the alkylation occurs as predicted and the assigned orientation of the side chains of Y bases is correct.

Figure 9



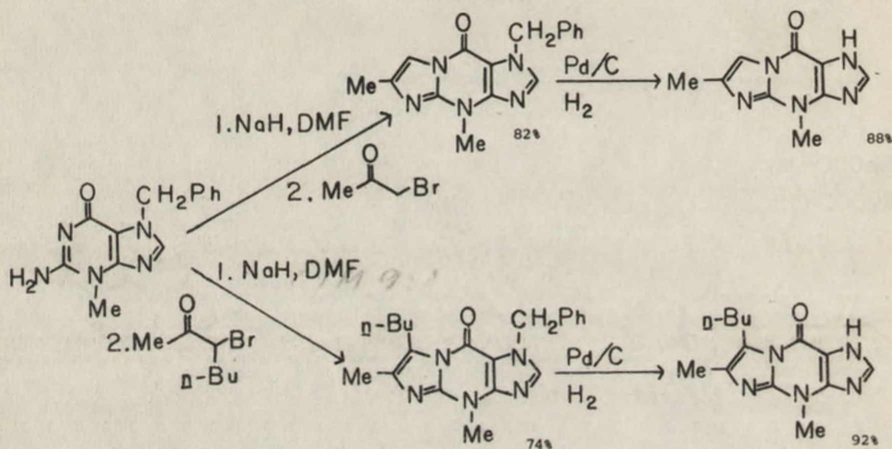
Progressing to the cyclization reaction, we found that the anion of 7-benzyl-3-methylguanine reacted cleanly with one equivalent of bromoacetone to give the nicely crystalline 1-benzyl-Y_{TU}. All that remained now was the removal of the benzyl protecting group, but this turned out to be a harder problem than expected. After trying many conditions which either caused no reaction, the formation of many fluorescent products or the loss of fluoresence entirely, it was found that reduction occurred rapidly in i-propanol-acetic acid with a trace of mineral acid, 10% Pd/C and 1 atm of hydrogen. In the model compound 7-butyl-Y_{TU} both the cyclization and deprotection steps occurred smoothly and in good yield using the same conditions as with Y_{TU} as illustrated in Figure 11.

Figure 10



For the synthesis of Y_{SC}, the appropriate side chain is required. The published synthesis is the addition of a homoserine equivalent to a three-carbon chain containing an activating group, which is then removed to give the appropriate seven-carbon chain.[7)] Using a literature procedure to go from α-bromo-γ-butyrolactone to methyl 2-amino-4-iodobutyrate·hydrochloride, the later compound is converted into the desired α-bromoketone 5. Alternative routes to this compound are under investigation since the previous synthesis is lengthy and only of moderate yield. In this case, the sodium hydride-DMF cyclization reaction went in ~20% yield, which is a ten fold increase over the old synthesis, but is lower than in the simpler Y compounds.

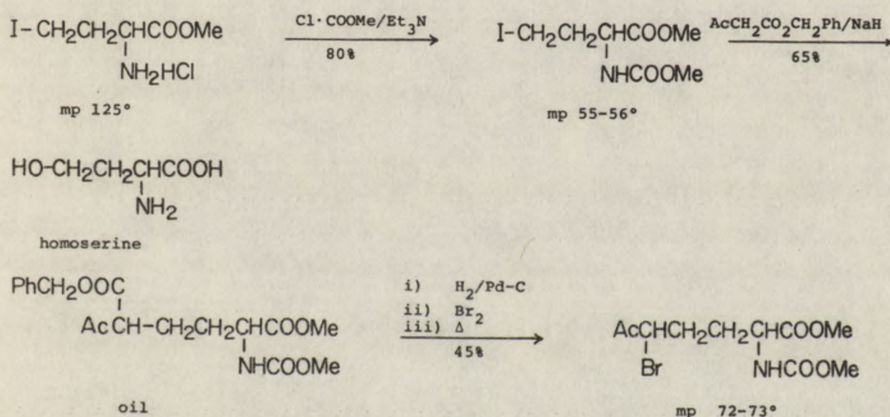
Figure 11



Due to an insufficient quantity of the side chain the intermediate has not been completely purified but it was hydrogenated to yield a compound with the same physical properties as the previously synthesized Y_{SC}. Further investigation into this method for the synthesis of Y bases is being carried out.

A completely different route for the synthesis of Y compounds was also investigated. The condensation of benzyl 3-mesyloxy-4-oxopentanoic acid with 3-methylguanine gives a Y compound with an activated methylene attached to the 7-position. Attempts to generate an anion at this position, followed by reaction with a serine derivative and removal of the activating group to give Y_{SC}, failed to give any of the desired product. As of yet this reaction sequence has not been tried with 7-benzyl-3-methylguanine. A similar sequence with a triphenylphosphinyl in place of the carbobenzoxy group was tried, but it failed in the same manner.

Figure 12

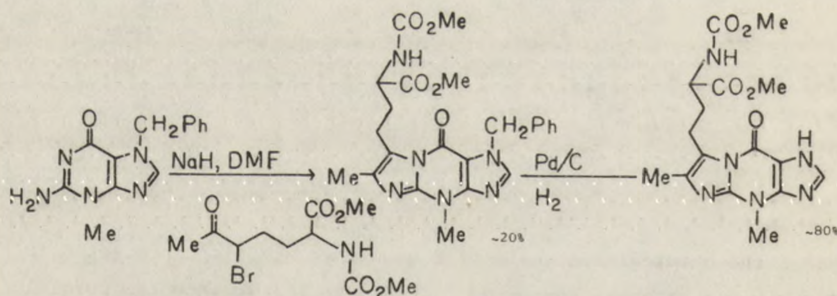


5

Besides the chemical synthesis of Y bases, we are also interested in the biosynthesis of these compounds. In Figure 15, we show two possible biosynthetic pathways for Y_{SC}. The originally proposed components for the formation of the third ring and the side chain were a glutamic acid[4)] derivative and an acetic acid. Another route involves the use of a homoserine and a pyruvic acid (or a reduced form of it). The second scheme is considered more likely since the formation of Y_{TU} would require only a single modification reaction, while the first method requires both a two-carbon and a one-carbon unit. The second scheme also allows the consideration of Y_{SC} as just a further modification of Y_{TU}. A probable source of the homoserine residue is S-adenosylmethionine, which has recently been

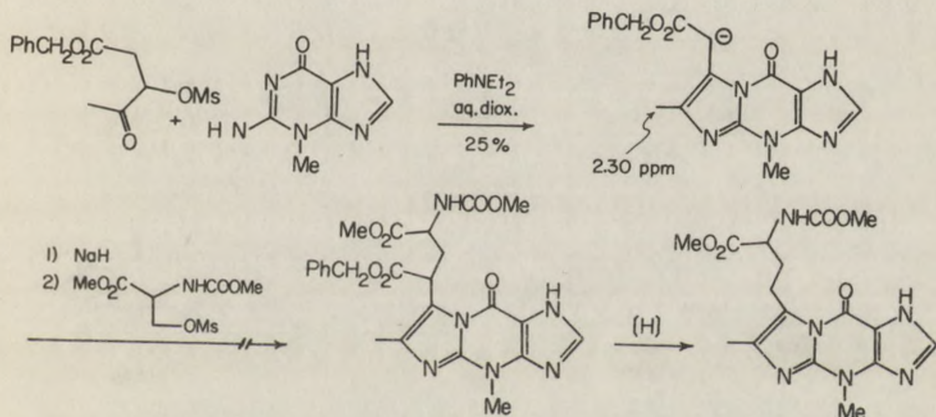
shown to be the source of the side chain for the modified nucleoside 3-(3-amino-3-carboxypropyl) uridine.[11,12] It is likely that the 4-methyl, the ester methyl and the carbamate methyl also come from S-adenosylmethionine.

Figure 13



To be sure that guanosine is the source of the purine portion of Y, an incorporation study using labelled guanine was carried out. The isolated Y base from a guanine-requiring yeast mutant, which was grown in a medium

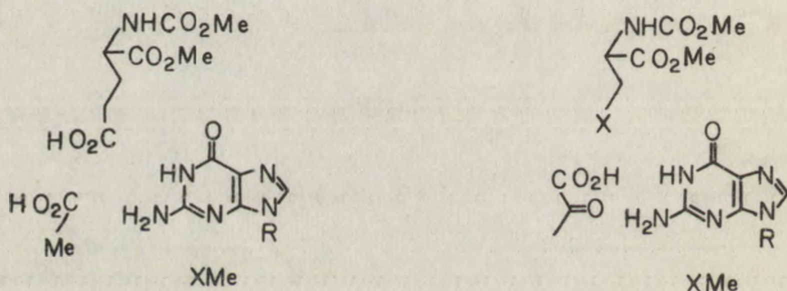
Figure 14



containing [8-^{14}C]-guanine, had 80% of the specific activity of the isolated GMP from the same tRNA.[13,14] No incorporation of glutamic acid above the random level was observed when a glutamic acid-requiring yeast mutant was grown in a medium containing labelled glutamic acid; thus the originally proposed biosynthesis is less likely to be correct. The study of the biosynthesis of Y base is just in its early stages since once the sources of the various portions of the molecule are known, the order of their introduction will have to be determined before the biosynthetic pathway has been elucidated.

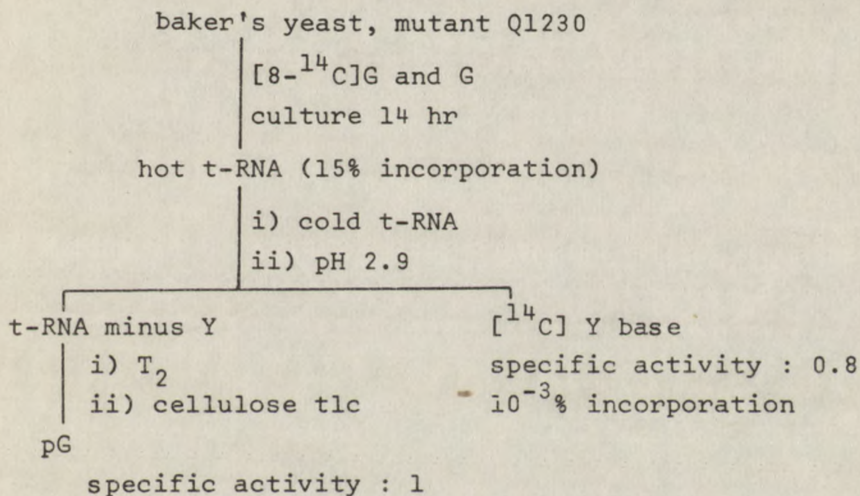
Figure 15

Possible Biosynthetic Pathways



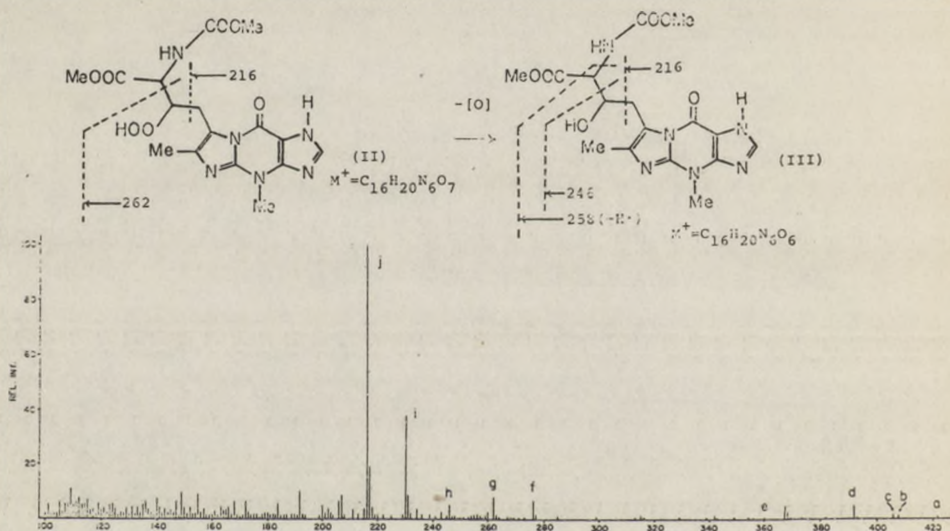
The Y base about which the least is known is also apparently the most widely distributed (beef, calf, chicken and rat liver and probably wheat germ). Since we had only a limited amount (ca. 5 μg) of this compound, we are very pleased to have found that the plant Lupinus luteus (Papilionacae) contains Y_L also.[14] By isolating a larger sample (ca. 30 μg), we could obtain much better physical data on this interesting compound. By performing a specific color test, ferrous thiocyanate solution, the presence of a hydroperoxide group was verified. The bar graph of the mass spectrum of the natural Y_L on a MS-9 at 210° and 70eV is given in Figure 17. The mass spectrum, along with the color test, demonstrates the presence of the hydroperoxide group in Y_L.

Figure 16



The FT nmr spectrum on this sample showed four methyl absorption peaks at 2.20, 3.71, 3.76 and 3.94 δ as compared with 2.26, 3.68, 3.71 and 3.96 δ. Since there were some contaminants from the silica gel plates in the sample, the other protons of the base could not be detected. The presence of the hydroperoxide alters the long wavelength cd spectrum, but not the uv spectrum of the Y compounds, indicating the difference between Y_{SC} and Y_{TU} is the presence of an additional chiral center in Y_{TU} adjacent to that already present in Y_{SC}

Figure 17



In summary, the complexity of the Y bases, which inhibited the structure elucidation of the compounds, has led to many very interesting avenues of research on these molecules. Now that we have an efficient synthesis of simpler molecules, we are applying these methods to the more complex molecules. Since only minute quantities are available from natural sources, synthetic samples are necessary for studying in more detail the physical properties of these molecules, such as the +14 peaks observed in the mass spectra, the increased ε values of the uv spectra in MeOH over those in H_2O and not least, the crystal structure of the Y_{SC}.

Acknowledgements: Some of this research was carried out by S. Blobstein, R. Crouch, I. Doerr, A. Feinberg, H.J. Li under Professor Nakanishi's constructive leadership.

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III. Communications presented by posters

Complete list of communications

1. NEW KEY INTERMEDIATE FOR OLIGORIBONUCLEOTIDE SYNTHESIS

E.Biała, P.Dembek, K.Grześkowiak, A.Kraszewski, J.Stawiński, M.Wiewiórowski,
Adam Mickiewicz University, Institute of Chemistry, Department of Organic Chemistry, Poznań, Poland.
Polish Academy of Sciences, Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.

2. CHEMICAL SYNTHESIS OF ALKALI-STABLE DIRIBONUCLEOSIDE MONOPHOSPHATE: 2'- O - METHYL-CYTIDYLYL /3'- 5'/ CYTIDINE

W.Markiewicz, M.Wiewiórowski,
Polish Academy of Science, Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.

3. ISOLATION AND CHARACTERIZATION OF PEROXY - Y BASE FROM PHENYLALANINE TRANSFER RIBONUCLEIC ACID OF THE PLANT Lupinus luteus

A.M.Feinberg, K.Nakanishi, J.Barciszewski, A.J.Rafalski, H.Augustyniak, M.Wiewiórowski,
Columbia University, Department of Chemistry, New York, N.Y., U.S.A.
Polish Academy of Sciences, Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.

4. EXPERIMENTS ON UREIDONUCLEOSIDES

R.W.Adamiak, M.Wiewiórowski,
Polish Academy of Sciences Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.

5. CRYSTAL AND MOLECULAR STRUCTURE OF RUBIDIUM SALT OF N-/PURIN-6YLCARBAMOYL/-L-THREONINE TETRAHYDRATE, THE HYPERMODIFIED BASE OF SOME tRNAs

D.A.Adamiak, Z.Kosturkiewicz, T.L.Blundell, I.J.Tickle,
Adam Mickiewicz University, Institute of Chemistry, Department of Crystallography, Poznań, Poland.
University of Sussex, Biochemistry Department, Brighton, England.

6. MODIFIED NUCLEOSIDES DERIVED FROM INOSINE

B.Golankiewicz, M.Mazur, K.Golankiewicz,
Polish Academy of Sciences, Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.
Adam Mickiewicz University, Institute of Chemistry, Department of Organic Chemistry, Poznań, Poland.

7.SYNTHESIS AND BIOCHEMICAL PROPERTIES OF PURINE - α -NUCLEOSIDES AND α -NUCLEOTIDES

H.Rokos,

Constance University, Department of Chemistry, G.F.R.

8.THYMINE NUCLEOSIDES OF 2-DEOXY-D - AND L-THREO PENTAFURANOSE

K.Miersch, H.Venner,

Academy of Sciences of G.D.R. Research Center of Molecular Biology and Medicine, Central Institute of Microbiology and Experimental Therapeutics, Jena, G.D.R.

9.DIMETHYL SULPHOXIDE OXIDATIONS. A NEW SELECTIVE CONVERSION OF THE THIOCARBONYL-CONTAINING NUCLEIC ACID COMPONENTS INTO THEIR OXYGEN ANALOGUES

M.Mikołajczyk, J.Łuczak,

Polish Academy of Sciences, Center of Molecular and Macromolecular Research, Łódź, Poland.

10.MASS SPECTRA OF THE THIO ANALOGUES OF PYRIMIDINE BASES

K.Golankiewicz, E.Wyrzykiewicz,

Adam Mickiewicz University, Institute of Chemistry, Department of Organic Chemistry, Poznań, Poland.

11.SYNTHESIS OF ALKYLATED DERIVATIVES OF ISOGUANINE

Z.Kaźmierczuk

Warsaw University, Department of Biophysics, Warsaw, Poland.

12.STUDIES ON ALKYLATION OF CYTOSINE RESIDUE IN 1-SUBSTITUTED CYTOSINES

J.Kuśmierek,

Polish Academy of Sciences. Institute of Biochemistry and Biophysics, Warsaw, Poland.

13.PHYSICOCHEMICAL STUDIES ON CONFORMATION OF DIKETOPYRIMIDINE NUCLEOSIDES

A.Rabczenko, I.Kułakowska, K.Zakrzewski, K.Bolewska,

Polish Academy of Sciences, Institute of Biochemistry and Biophysics, Warsaw, Poland.

14.FLUORESCENCE AND DIELECTRIC STUDIES ON CONFORMATION OF AMINO GROUP IN AMINO-PYRIMIDINES

J.Smagowicz, I.Kułakowska, K.L.Wierzchowski,

Polish Academy of Sciences, Institute of Biochemistry and Biophysics Warsaw, Poland.

15.STACKING EQUILIBRIA IN AQUEOUS SOLUTIONS OF DIKETOPYRIMIDINES

E.Plesiewicz, E.Stępień, K.L.Wierzchowski,

Polish Academy of Sciences, Institute of Biochemistry and Biophysics, Warsaw, Poland.

16.FLUORESCENCE STUDIES OF STACKING EQUILIBRIA IN MODEL AMINOPURINES

A.Bierzyński, K.L.Wierzchowski

Polish Academy of Sciences, Institute of Biochemistry and Biophysics, Warsaw, Poland.

17.ROLE OF CONFORMATION ON FRACTIONATION OF NUCLEOSIDES AND O'- ALKALI - NUCLEOSIDES

J.Giziewicz,
Polish Academy of Sciences, Institute of Biochemistry and Biophysics, Warsaw, Poland.

18.INFLUENCE OF METAL IONS ON ELECTRONIC STRUCTURE OF NUCLEIC ACIDS FRAGMENTS

B.Jeżowska-Trzebiatowska, A.Antonow,
Wrocław University, Institute of Chemistry, Wrocław, Poland.

19.INTERACTION OF RARE EARTH CATIONS WITH POLYNUCLEOTIDES

K.Golankiewicz, A.Rajchel,
Adam Mickiewicz University, Institute of Chemistry, Department of Organic Chemistry, Poznań, Poland.

20.MUNG BEAN NUCLEASE: SPECIFITY AND MODE OF ACTION ON SYNTHETIC ESTERS OF 3'-NUCLEOTIDES

R.Kole,
Polish Academy of Sciences, Institute of Biochemistry and Biophysics, Warsaw, Poland.

21.SOME MINOR NUCLEOSIDES IN tRNA FROM Lupinus luteus

H.Augustyniak, K.Gulewicz,
Polish Academy of Sciences, Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.

22.TWO - DIMENSIONAL CHROMATOGRAPHY OF HYDROLYSATES OF Lupinus luteus tRNA

K.Szyfter, W.Daniluk, A.Joachimiak,
Polish Academy of Sciences, Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.
Adam Mickiewicz University, Institute of Chemistry, Department of Organic Chemistry, Poznań, Poland.

23.DETERMINATION OF NUCLEOTIDE COMPOSITION IN AMMONIA HYDROLYSATES OF tRNA,

K.Gulewicz, H.Augustyniak, K.Szyfter,
Polish Academy of Sciences, Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.

24.APPLICATION OF RESORCINE RESIN FOR ISOLATION OF NUCLEIC ACID COMPONENTS

K.Szyfter, J.Chełkowski,
Polish Academy of Sciences, Institute of Organic Chemistry, Department of Stereochemistry of Natural Products, Poznań, Poland.
Agriculture Academy, Department of Food Biochemistry, Poznań, Poland.

25.DIRECT PHOSPHORYLATION OF UNBLOCKED DIRIBONUCLEOSIDE MONOPHOSPHATE

K.Golankiewicz, J.Antkowiak,
Adam Mickiewicz University, Institute of Chemistry, Department of Organic Chemistry, Poznań, Poland.

26.SYNTHESIS AND PHOTOCHEMISTRY OF NUCLEIC ACID ANALOGUES. PART I.

K.Golankiewicz, F.Kaźmierczak, J.Langer,

Adam Mickiewicz University, Institute of Chemistry, Department of Organic Chemistry, Poznań, Poland.

27.SYNTHESIS AND PHOTOCHEMISTRY OF NUCLEIC ACID ANALOGUES. PART II.

K.Golankiewicz, F.Kaźmierczak, J.Langer,

Adam Mickiewicz University, Institute of Chemistry, Department of Organic Chemistry, Poznań, Poland.

28.PMR STUDY OF THE MOLECULAR CONFORMATION OF THIOURIDINES IN SOLUTION

J.Bujarski, S.Paszyc,

Adam Mickiewicz University, Institute of Chemistry, Department of Photochemistry, Poznań, Poland.

29.tRNA NUCLEOTIDYL TRANSFERASE IN Lupinus luteus SEEDS

M.Pietrzak, H.Cudny,

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