STUDIES ON THE CHEMICAL SYNTHESIS OF NATURAL AND NOVEL OLIGORIBONUCLEOTIDES USING ALKYLSILYL PROTECTING GROUPS

A Thesis

by

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To my late grandfather,

STUDIES ON THE CHEMICAL SYNTHESIS OF NATURAL AND NOVEL OLIGORIBONUCLEOTIDES USING ALKYLSILYL PROTECTING GROUPS

by *Taifeng Wu*

ABSTRACT

The phenoxyacetyl group was investigated as a base protecting group in oligoribonucleotide synthesis using the phosphoramidite coupling procedure. The N-phenoxyacetyl protected ribonucleoside methylphosphoramidites were prepared and they were evaluated in the synthesis of a number of oligoribonucleotides, including a 75-unit-long molecule whose base sequence is related to the yeast formylmethionine initiator tRNA.

A side reaction leading to some cleavage of the assembled oligoribonucleotide chain was observed in oligoribonucleotide synthesis, following the standard procedure. The nature of this side reaction was identified and a procedure to eliminate it was developed.

An extensive study has been carried out to prove the fidelity of alkylsilyl groups as the 2'-hydroxyl protecting group in oligoribonucleotide synthesis. A series of natural dinucleotides were prepared. The corresponding dinucleotides with the unnatural 2'-5' phosphate linkage were also synthesized. The products from the synthesis as well as the intermediates during the synthesis were characterized by ^{1}H and ^{31}P NMR and HPLC. Unambiguous chemical evidence of the stability of the phosphate linkages in synthetic oligoribonucleotides was provided.

Several ribozymes and their substrates were chemically synthesized. A general procedure to prepare novel mixed DNA-RNA polymers was developed. The usefulness of this type of molecule in molecular biology has been demonstrated in the study of the mechanism of ribozyme catalysis.

ÉTUDES DE LA SYNTHÈSE CHIMIQUE D'OLIGORIBONUCLÉOTIDES NATURELS ET SYNTHÉTIQUES UTILISANT LES GROUPES PROTECTEURS ALKYLSILYLE

par *Taifeng Wu*

RÉSUMÉ

La groupe phénoxyacétyle a été étudié comme groupe protecteur des bases dans la synthèse d'oligoribonucléotides par la méthode de couplage des phosphoramidites. Les méthylphosphoramidites de ribonucléosides N-protégés par des groupes phénoxyacétyle ont été préparés et évalués dans la synthèse d'une série d'oligoribonucléotides, y compris une molécule longue de 75 unités dont la séquence des bases correspond à l'ARN de transfert de levure, initiateur de la formylméthionine.

Une réaction secondaire résultant en une rupture de la chaîne oligoribonucléotidique a été observée lors de la synthèse d'oligoribonucléotides, lorsque la méthode standard est appliquée. La nature de cette réaction secondaire a été élucidée et un moyen d'enrayer cette réaction a été développé.

Une étude approfondie a permis d'éprouver la fiabilité des groupes alkylsilyles comme groupes protecteurs de l'hydroxyle en 2' dans la synthèse d'oligoribonucléotides. Une série de dinucléotides naturels ont été préparés. Les dinucléotides correspondants avec un lien phosphate 2'-5' ont également été synthétisés. Les produits de synthèse et les intermédiaires ont été charactérisés au moyen de la RMN ^{1}H et ^{31}P ainsi que par CLHP. Une preuve non ambigue de la stabilité des liens phosphates dans les oligoribonucléotides synthétiques est présentée.

Plusieurs ribozymes et leurs substrats ont été synthétisés chimiquement. Une méthode générale pour la préparation de polymères hybrides ADN-ARN a été développée. L'utilité de ce type de molécule en biologie moléculaire a été démontrée dans l'étude du mécanisme de la catalyse ribozymique.

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Abbreviations

A	adenosine
Ac	acetyl
ASBV	avocado sunblotch viriod
BPB	bromophenol blue
Bz	benzoyl
C	cytidine
CBZ	benzyloxycarbonyl
CPG	controlled pore glass
Ctmp	1-[(2-chloro·4-methyl)phenyl]-4-methoxypiperidine-4-yl
DABCO	1,4-diazobicyclo[2,2,2]undecane
DBU	1,8-diazabicyclo[5,4,0]undec-7-ene
DCC	N,N,-dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMSO	dimethylsulfoxide
DMF	dimethylformamide
DMT	4,4'-dimethoxytrityl
DNA	deoxyribonucleic acids
EtOH	ethanol
Fmoc	9-fluorenymethoxycarbonyl
G	guanosine
HF	hydrogen fluoride
HPLC	high pressure liquid chromatography
IVS	intervening sequence
LCAACPG	long chain alkylamine controlled pore glass
Lev	levulinyl
M	molar
mCPBA	*meta*-chloroperbenzoic acid
mg	milligram
min.	minute
ml	milliliter
MeOAc	methoxyacetyl

MeOH	methanol
mmol	millimole
MMT	4'-monomethoxytrityl
m.p.	melting point
mRNA	messenger RNA
MTHP	4-methoxytetrahydropyranyl
nm	nanometer
NMR	nuclear magnetic resonance
N_pM	dinucleotide with 3'-5' phosphate linkage
NPM	dinucleotide with 2'-5' phosphate linkage
O.D. unit	optical density unit, the amount of material which will yield an absorbance of 1.0 @ 260 nm when dissolved in 1.0ml in a 1.0 cm cell
p-An	*p*-anisol
PhAc	phenylacetyl
PhOAc	phenoxyacetyl
Py	pyridine
Rf	relative mobility
RNA	ribonucleic acid
rRNA	ribosomal RNA
R.T.	room temperature
SDS	sodium dodecyl sulfate
Si	alkylsilyl
STobRV	satellite tobacco ringspot virus
T	thymidine
TBAF	tetrabutylammonium fluoride
TBDMS	*t*-butyldimethylsilyl
TBPA	*p*-*t*-butylphenoxyacetyl
TCA	trichloroacetic acid
TEAA	triethylammonium acetate
THP	tetrahydropyranyl
TIPS	triisopropylsilyl
TLC	thin layer chromatography

TMS	trimethylsilyl
TRIS	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
U	uridine
U.V.	ultraviolet
VLTSV	lucerne transient streak virusoid
XC	xylene cyanol

CHAPTER 1

INTRODUCTION

1.1. DNA as Store of Genetic Information

The discovery of the double helix structure of DNA by Watson and Crick[1] is one of the landmarks in the history of biology. It has had a profound influence on the way we think in biology and in the life sciences in general. The discovery not only unraveled various important structural aspects of this unique biomolecule, but has also thrown light on a number of fundamental biological processes such as replication, transcription, and translation. Since then an era of molecular biology has begun.

A DNA molecule is a long polymer of four deoxyribonucleotides; i.e., deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine, with 3'-5' phosphate linkages between them (Fig. 1). The genetic information of a gene is stored in the polymer and is represented by the specific sequence of the four nucleotides. In a cell, two complementary DNA polymers form a right handed double helix as first proposed by Watson and Crick. The two chains are held together by the specific interaction between the bases A and T, or G and C via intermolecular hydrogen bonding. (Fig. 2). Such specific interaction allows one DNA molecule to be able to recognize its complementary partner by forming the complementary stable base pairing. The recognition nature of DNA is the essence of its ability to replicate, which allows the living organism to inherit genetic information. This was realized by Watson and Crick right after their discovery of the double helix and later confirmed by Kornberg in his classic DNA semi-conservative replication experiment.[2]

It was later realized that the structure of a DNA molecule in a cell is not so uniform as it was hitherto perceived. X-ray crystallography, NMR spectroscopy, electronic microscopy and other physical methods have established that DNA can assume various

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1. J.D. Watson, F.H.C. Crick, *Nature*, **171**, 737 (1953).
2. A. Kornberg, *"DNA Replication"*, Freeman Co., San Francisco, 1980.

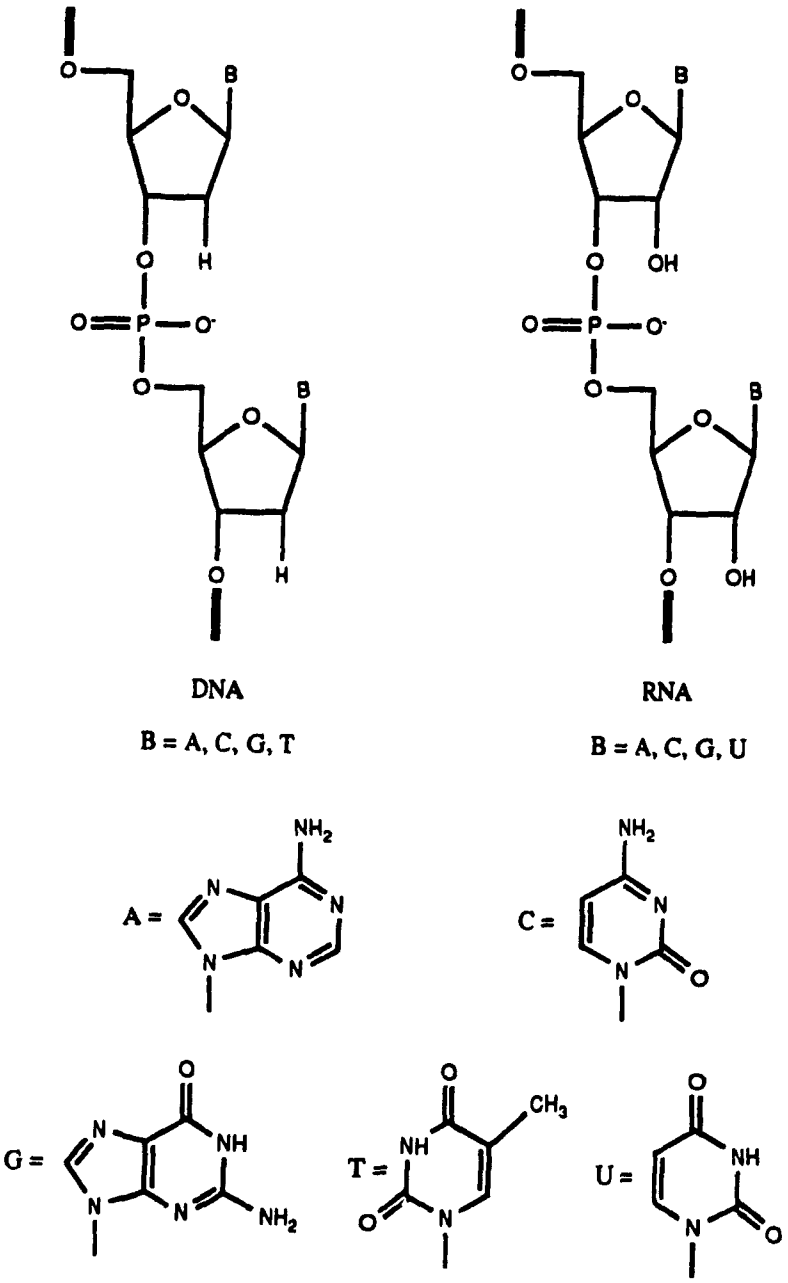

Figure 1. Structure of DNA and RNA

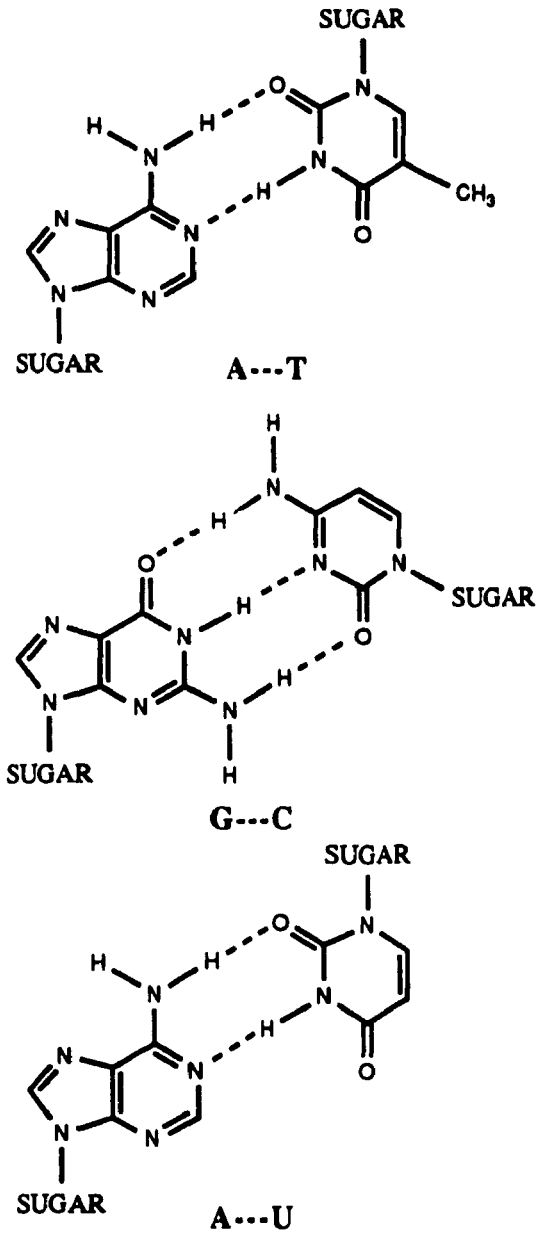

Figure 2. Hydrogen bondings in DNA (A-T, G-C) and in RNA (A-U, G-C).

forms: Z conformation,[3] bending,[4] and supercoiling.[5] DNA in a cell is in a dynamic process changing from one form to the other. It also interacts with other cellular components.

The genetic information of a given organism is stored in the DNA molecule in the form of the sequence of bases. The sequence of the DNA dictates the amino acid sequence of proteins which, in turn, determines the physical being, physiological state, and life style of an organism. Therefore the content of the DNA molecule lies at the beginning of the line in determining the feature of an organism. It also should be realized that the function of a sequence of DNA is much more than simply as a carrier of coding information for protein sequences. Some are involved in the regulation of the expression of genes;[6] some are the noncoding sequences called introns (*vide infra*). In fact in most eukaryotic organisms only a very small fraction of total bases of genome DNA have a coding function.

1.2 RNA in Cellular Gene Expression

An individual gene, which is essentially a segment of DNA out of the whole genomic DNA, will be expressed to give its corresponding product at a certain level and at a certain point during the life span of a cell. In a multicellular organism like a human being, the expression of a gene must also be tissue specific. The genetic information stored in a DNA sequence manifests itself by transcription into another type of nucleic acid: ribonucleic acid or RNA.

RNA is also a polymer of four nucleotides with 3'-5' phosphate linkages between them (Fig. 1). However, the nucleotide in an RNA polymer is a ribonucleotide instead of the deoxyribonucleotide found in DNA. From a chemical point of view, the structure of RNA is different from that of DNA in several aspects. Perhaps the most distinctive one is that the sugar moiety in RNA has an additional hydroxyl group at the 2'-position. It is largely the presence of this extra hydroxyl on the ribose that makes RNA so different from DNA in terms of stability, reactivity, and hence its biological activity. Another aspect is

3. (a) A. Rich, A. Nordheim, and A. H.-J. Wang, *Annu. Rev. Biochem.* **53**, 791 (1984). (b) A. H.-J. Wang, G.J. Quigley, F.J. Kolpak, J. L. Crawford, J.H. van Boom, G. van der Marcel, A. Rich, *Nature*, **282**, 680 (1979).
4. (a) H-S. Koo, H-M. Wu, D.M. Crothers, *Nature*, **320**, 501-506 (1986). (b). H-M. Wu, D.M., Crothers, *Nature*, **308**, 509-513 (1984).
5. J.C. Wang, *Annu. Rev. Biochem.*, **54**, 665 (1985)
6. J. Corden, B. Wasylyk, A. Buchwalder, P. Sassone-Corsi, C. Kedinger, P. Chambon, *Science*, **209**, 1406-1414 (1981).

base constitution. The thymidine in DNA is replaced by uridine in RNA and the base pairing becomes A and U, and G and C (Fig. 2). Also, there are a number of rare bases such as pseudouridine, ribothymidine, dihydrouridine, inosine, N-methylated guanosine and adenosine in RNA, in particular in the tRNAs.[7]

RNA has traditionally been given different names; i.e., messenger RNA (mRNA), transfer RNA (tRNA), and ribosome RNA (rRNA) purely based on their known biological functions. mRNA carries the information stored in DNA and it provides the template upon which the amino acids of a protein will be assembled. Thus mRNA is still typical of an informational genetic molecule like DNA since its function is to carry the genetic information. Two other types of ribonucleic acids, i.e., tRNA and rRNA, are also required for the biosynthesis of proteins. A tRNA will, with the help of amino acid synthetase, carry a specific cognate amino acid for the protein synthesis. The rRNA in a cell, present as a complex called ribosome with a group of riboproteins, will provide the machinery for the protein synthesis. tRNA and rRNA are very unique because they are still informational biopolymers, but they are also involved in assisting protein synthesis, an activity more like that of a protein.

That RNA plays an important role in the process of gene expression is well recognized. Unlike DNA, RNA has more versatile biological functions. DNA is relatively stable throughout the life cycle of a cell, while the messenger RNA is synthesized and degraded all the time. Synthesis of mature messenger RNA represents one of the major steps in the gene expression from DNA to protein and plays a critical role in the control of expression.[8] The operon and repressor system discovered by Jacob and Monod first illustrated the control of gene expression by means of RNA synthesis. Among the three types of RNA, messenger RNA is the most diversified, the most active and the most elusive one. In a eukarytic cell there are estimated to be about 10,000 to 20,000 proteins. Each of them has its own mRNA. They are constantly synthesized and destroyed during each life stage of a cell. Therefore the activity of messenger RNA is closely related to the cellular biological activity and it is the focus of current extensive investigation.

rRNA accounts for most of the RNA in the cell (about 80%). The base sequence of ribosomal RNA is highly conserved among the eukaryotic cells and also prokarytic cells.[9]

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7. S.M. Altman, "*Transfer RNA*", MIT Press, Cambridge, 1978.
8. J.E. Darnell, Jr., *Scientific American*, 68-78 (October, 1985).
9. "*Structure, Function and Genetics of Ribosome*", edited by B. Hardesty, G. Kramer, Springer, New York, 1986.

rRNA is unique because its active form is a constituent of a ribosome complex containing a group of riboproteins. It is this RNA containing complex that provides the site for protein synthesis. It was once widely believed that rRNA in the ribosome complex was merely a passive structural requirement. This notion has been challenged with the recent discovery of catalytic RNA and a number of other RNA-containing enzymes. It has been strongly suspected that the RNA component in the ribosome probably plays an active role in protein synthesis, most likely a catalytic one.[10]

A tRNA has about 70 bases and it appears to be the smallest RNA molecule present in a cell that has its own distinct biological function. All tRNAs have a significant number of modified bases in the molecule. Moreover the position of a modified base in a tRNA is highly conserved.[7] It is so far the best characterized molecule among the three RNAs in terms of the sequence arrangement, tertiary structure, and its interaction with proteins.[7] In the synthesis of an individual protein, messenger RNA molecules are different from each other but tRNAs and rRNAs are the same. Overall RNA acts as an active intermediate between DNA and proteins during the expression of an gene. It has also been found that RNA itself acts as the informational molecule in retroviruses.[11]

In the past few years, our understanding of cellular biological activities involving RNA has been expanded dramatically. This can be attributed to two facts. First of all, a more detailed picture of how rRNA, tRNA, and mRNA are made as well as the roles of the biosynthetic steps in the regulation of gene expression is emerging. The discovery of RNA splicing is one of them. Sharp *et al* and Chow *et al*[12] discovered that some genes coding for messenger RNA are interrupted by noncoding sequences, or introns. The intron sequence is transcribed together with the coding sequence (exon) and it is part of the preliminary RNA transcript sequence or pre-mRNA. The intron in the pre-RNA has to be removed via a unique process called RNA splicing (Fig. 3). The spliced intron RNA in mRNA splicing has the unique lariat structure (Fig. 4).[13] The key structural element of the lariat RNA is the branched unit where the two ribonucleotides are linked to a ribonucleotide,

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10. P.B. Moore, *Nature*, **331**, 223-227 (1988)
11. (a) D. Baltimore, *Nature*, **226**, 1209 (1970). (b) H. Temin, S. Mizutani, *ibid.*, **226**, 1211 (1970).
12. (a) S.M. Berget, C. Moore, P.A. Sharp, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 317 (1977). (b) L.T. Chow, K.E. Gelinas, T.R. Broker R.J. Roberts, *Cell*, **12**, 1 (1977).
13. (a) P.J. Grabowski, R.A.Padgett, P.A. Sharp, *Cell*, **37**, 415-427 (1984). (b) B. Raskin, A.R. Krainer, T. Maniatis, M.R. Green, *Cell*, **38**, 317-331 (1984). (c) R.A. Padgett, P.J. Grabowski, M.M. Konarska, S. Seiler, P.A. Sharp, *Annu. Rev. Biochem.* **55**, 1119-1150 (1986). (d) P.A. Sharp, *Science*, **235**, 766-771 (1987).

in most cases an adenosine ribonucleotide, by the 3'-5' and 2'-5' phosphate linkages as shown in Fig. 4. RNA splicing is particularly widespread in the synthesis of eukaryotic mRNA. In some cases, pre-mRNA transcripts can undergo an alternate splicing pathway.[14] In doing so several RNA templates coding for different proteins can be generated from the same preliminary transcript. It is widely believed that RNA splicing is another cellular post-transcription regulation step in the synthesis of mature messenger RNA for protein synthesis.

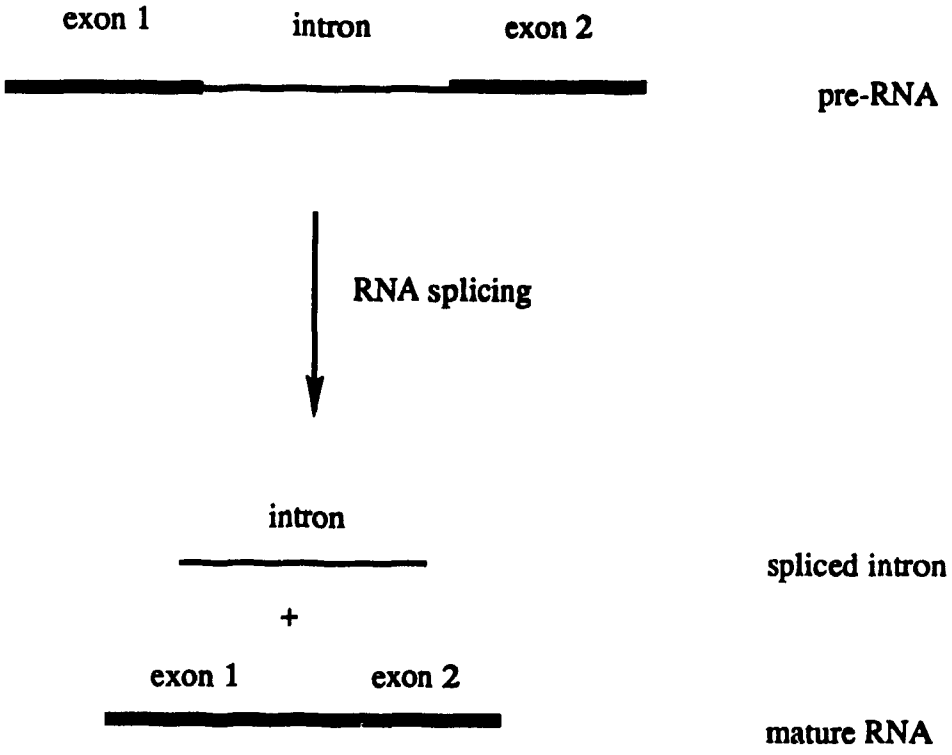

Figure 3. Pathway for removal of intron via RNA splicing

14. (a) J. Rogers, P. Early, C. Cantor, K. Calame, M. Bond, *Cell*, **20**, 313-319 (1980). (b) F.W. Alt, A.L.M. Bothwell, M. Knapp, E. Siden, E. Mather, *Cell*, **20**, 293-301 (1980).

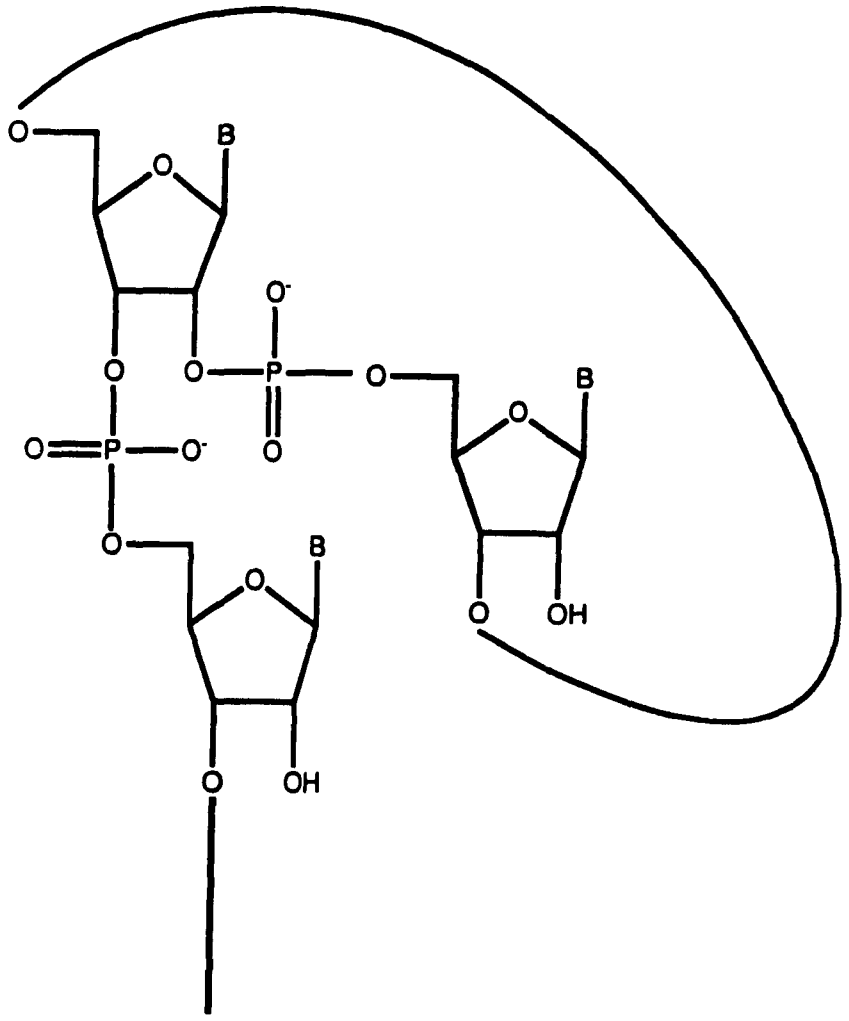

Figure 4. Structure of lariat RNA from mRNA splicing

Secondly, it has been realized that the role of RNA in the cellular gene expression is far more than those classic ones of mRNA, tRNA, and rRNA. For example, Soll *et al* have found that glutamate tRNA is required for the biosynthesis of chlorophyll in barley chloroplast, a very unusual role played by tRNA.[15] It has also been found that in some cases gene expression is solely controlled by RNA. These RNA molecules are synthesized with the sequence that is complementary to that of the mRNA to be translated.[16] Such RNA, or antisense RNA, binds to the mRNA by forming a double stranded duplex thereby inhibiting translation. The concept of antisense RNA regulation has been extended to the synthetic oligonucleotide analogues. For example, Miller *et al*[17] found that oligonucleoside methylphosphonates complementary to the messenger RNA can inhibit its translation. Zon *et al*[18] and Zamecnik *et al*[19] have shown that phosphorothioate analogues of oligonucleotides can also inhibit viral replication. RNA has also been found to assist the splicing of RNA itself. For example, in the splicing of eukaryotic mRNA, the splicing reaction is mediated by a cellular machinery called a splicesome.[20] One of the key elements of the splicesome is a group of small RNA molecules. The presence of RNA in the spliceosome is essential for the splicing reaction to take place.[21] Although the exact role of these small RNA molecules in the splicing reaction is not known yet, they nevertheless have demonstrated the very unusual biological activity of RNA. It has been further found that in the splicing of precursor RNA of the rRNA in *Tetrahymena*, the substance responsible for the process is simply an RNA molecule (*vide infra*).

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15. (a) A. Schon, G. Krupp, S. Gough, S. Berry-Lowe, C. G. Kannangara, D. Soll, *Nature*, **322**, 281-284 (1986). (b) A. Schon, C.G. Kannangara, S. Gough, D. Soll, *Ibid*, **331**, 187-190 (1988)
16. P.J. Green, O. Pines, M. Inouye, *Annu. Rev. Biochem*, **55**, 569-597 (1986).
17. (a) P.S. Miller, C.H. Agris, L. Aurelian, K.R. Blake, A. Murakami, M. P. Reddy, S.A. Spitz, P.O.P. Ts'o, *Biochemie*, **67**, 769-776 (1981). (b) C.H. Agris, K.R. Blake, P.S. Miller, M.P. Reddy, P.O.P. Ts'o, *Biochemistry*, **25**, 6268-6275 (1986).
18. M. Matsukura, K. Shinozuka, G. Zon, H. Mitsuya, M. Reitz, J.S. Cohen, S. Broder, *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 7706-7710 (1987).
19. S. Agrawal, J. Goodchild, M.P. Civeira, A.H. Thornton, P.S. Sarin, P.C. Zamecnik, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7079-7083 (1988).
20. (a) E. Abelson, J. Abelson, *Science*, **228**, 963 (1985). (b) P.J. Grabowski, S.R. Seiler, P.A. Sharp, *Cell*, **42**, 345 (1985).
21. (a) M.R. Lerner, J.A. Steitz, *Cell*, **25**, 298 (1981). (b) H. Busch, R. Reddy, L. Rothblum, Y.C. Choi, *Annu. Rev. Biochem.* **51**, 617 (1982).

1.3. Biological Catalysis by RNA

Among the many roles discovered for RNA, the discovery of catalytic RNAs, or ribozymes is probably the most dramatic event in the study of RNA in the past few years. The RNA catalysis was discovered by Cech.[22] He and his colleagues first found that the splicing of rRNA in *Tetrahymena* is a 'self-catalyzed' process.[23] They further showed that the excised intron in this case can undergo a series of spontaneous reactions involving ligation and cleavage of RNA molecules.[24] They finally concluded and were able to show that the intervening sequence of rRNA in *Tetrahymena* is an RNA enzyme or ribozyme which can catalyze the cleavage and joining of the specific ribonucleotide substrates.[22] In the splicing of pre-rRNA from *Tetrahymena*, the ribozyme and the substrate are in the same molecule, leading to a self-catalyzed system, or self-splicing. They further extended the RNA substrates into other exogenous RNAs.[22b, 22c] The intron RNA was shown to be like an RNA restriction enzyme, able to cleave a specific sequence.[22c] Inoue *et al* recently discovered a novel type of reaction catalyzed by the *Tetrahymena* RNA intron ribozyme.[25] In this case the ribozyme catalyzes the transesterification between dinucleotide substrates (Fig. 5).

$$\mathrm{GpX} + \mathrm{CpU} \xrightarrow{\text{IVS - RNA}} \mathrm{CpUpX} + \mathrm{G}$$

Figure 5. A transesterification reaction catalyzed by the ribozyme of Tetrahymena rRNA intron.

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22. (a) B.L. Bass, T.R. Cech, *Nature*, 308, 820-826 (1984). (b) A.J. Zaug, M.D. Been, T.R. Cech, *Nature*, 324, 429-433 (1986). (c) A.J. Zaug, T.R. Cech, *Science*, 231, 470-475. (d) T.R. Cech, B.L. Bass, *Annu. Rev. Biochem.*, 55, 599-629 (1986).
23. (a) T.R. Cech, A.J. Zaug, P.J. Grabowski, *Cell*, 27, 487-496 (1981). (b) K. Kruger, P.J. Grabowski, A.J. Zaug, J. Sands, D.E. Gottschling, T.R. Cech, *Cell*, 31, 147-157 (1982).
24. (a) A.J. Zaug, T.R. Cech, *Science*, 229, 1060-1064 (1985). (b) P.J. Grabowski, A.J. Zaug, T.R. Cech, *Cell*, 23, 467-476 (1981).
25. P.S. Kay, T. Inoue, *Nature*, 327, 343-346 (1987).

Since the first discovery, a number of other ribozymes have been characterized. For example, Altman discovered that the RNA component in ribonuclease P, a RNA containing enzyme responsible for the maturation of tRNA, can itself catalyze the processing of the precursor tRNA transcript.[26] In both the *Tetrahymena* intron ribozyme and ribozyme from the ribonucleases P, an RNA enzyme catalyzes the cleavage of substrate RNA to give the 5'-phosphate and 2',3'-hydroxyl in the product. The substrate is limited to RNA in all these RNA catalyzed reactions . However it has been shown in one case that RNA catalysis can be extended to a substrate other than RNA. Shvedova *et al* found that a 31-mer RNA with high content of modified bases catalyzes a reaction involving a carbohydrate as the substrate.[27]

1.4. `Hammer-Head' Ribozyme

One of the best characterized ribozymes involves those RNAs with a so called `hammer-head' structure. It has been found that certain plant viroids, virusoids, and linear satellite RNAs have very unusual replication and processing mechanisms. During the life cycle of these small infectious RNAs (ca. 400 nucleotide units), they are replicated in the multi-unit form, which is subsequently processed to give the single unit length molecule with 5'-hydroxyl and 2',3'-cyclic phosphate[28] in the product. Part of the processing reaction was found to be an autocatalytical process independent of any proteins. The auto-processing reaction has been demonstrated in the STobRV (satellite tobacco ringspot virus),[29] ASBV (avocado sunblotch viriod),[30] and VLTSV (lucerne transient streak virusoid).[31] Analysis of the nucleotide sequence of these autocatalytic RNAs suggests the conserved `hammer-head' structure present in these molecules (Fig. 6).[30, 31] This structure has been found to be essential for the autocatalytic activity. Symons[30,31] has shown that the mutated viroid RNA molecules containing the conserved `hammer-head' structure still have

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26. (a) R. Kole, M.F. Baer, B.C. Stark, S. Altman, *Cell*, **19**, 881-887 (1980). (b) C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace, S. Altman, *Cell*, **35**, 849-857 (1983). (c) C. Guerrier-Takada, S. Altman, *Science*, **223** (1984).
27. T.A. Shvedova, G.A. Korneeva, V.A. Otroshchenko, T.V. Venkstern, *Nucleic Acids Res.* **15**, 1745 (1987).
28. A.D. Branch, H.D. Robertson, E. Dickson, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6381-6386 (1981).
29. G.A. Prody, J.T. Bakos, J.M. Buzayan, I.R. Scheider, G. Bruening, *Science*, **231**, 1577-1580 (1986). (b) J.M. Buzayan, W.L. Gerlach, G. Bruening, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 8859-8862 (1986).
30. C.J. Hutchins, P.D. Rathjen, A.C. Foster, R.H. Symons, *Nucleic Acids Res.* **14**, 3627-3640 (1986).
31. A.C. Forster, R.H. Symons, *Cell*, **49**, 211-220 (1987).

the autocatalytic activity. On the other hand, deletion of the conserved nucleotides in the `hammer-head' structure destroys the autocatalytic activity.[31]

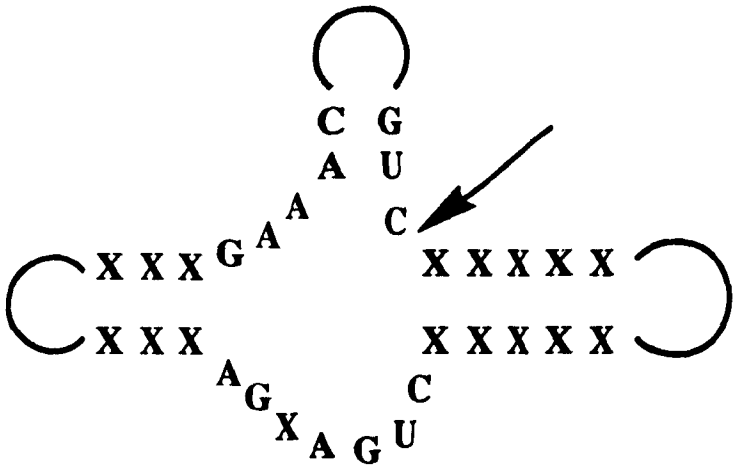

Figure 6. Conserved `hammer-head' structure and conserved bases of certain auto-processing viroids, virusoids and its cleaving sites

Using the *in vitro* transcription, Uhlenbeck prepared the two oligoribonucleotides that correspond to the `hammer-head' structure (Fig. 7).[32] In the presence of magnesium, the two small RNAs were found to be able to undergo the catalyticl reaction as in the case of the natural viroid RNA. As expected the site of cleavage is also the same, at the C16 position in the substrate. The cleavage product contains the 5'-hydroxyl and 2',3'-phosphate at the terminus. A 19-mer oligoribonucleotide participates in the reaction and also increases the cleavage of a specific phosphate bond, yet it is not consumed at the end of the reaction. The 19-units oligomer was shown to have all the properties characteristic of a catalyst.[32] The result from the reaction not only confirms the importance of the `hammer-head' structure to the catalytical activity, but it has also illustrated that the intramolecular `self-

32. O.C. Uhlenbeck, *Nature*, **328**, 586-600 (1987).

cleaving' reaction involving the viroid RNA is also working in the intermolecular fashion. Further, catalytic system involving these small oligoribonucleotides makes it possible to fully establish the role of the individual nucleotides in the reaction.

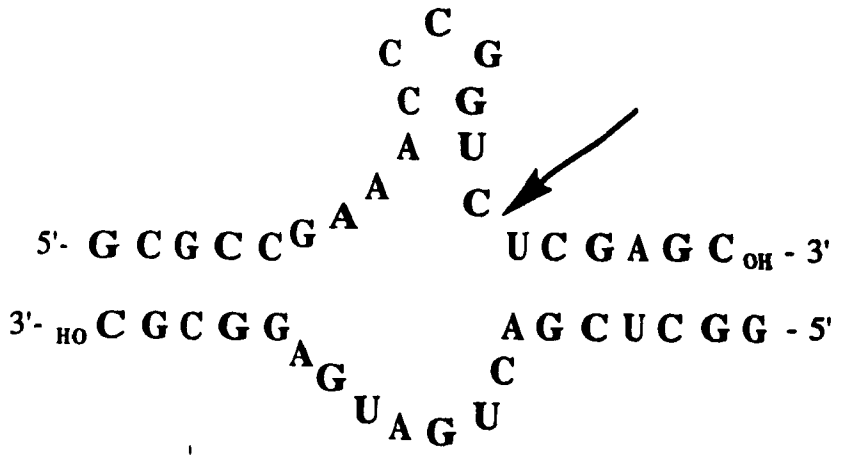

Figure 7. A catalytic oligoribonucleotide and its substrate

In the model proposed by Uhlenbeck, however, the substrate also contains some bases essential for the reaction. Gerlach *et al* has proposed a different model for the system(Fig. 8).[33] They have demonstrated that the ribozyme RNA in the model also cleaves the substrate RNA as predicted. In this model, most of the conserved bases that are required for the reaction have been incorporated in the ribozyme unit. The substrate in the model has the minimum amount of bases required for the reaction. The size of substrate is also not related to the ribozyme activity. Therefore the model separates the substrate unit from the catalytic unit and allows the full characterization of the ribozyme itself. They have further designed the sequence of the ribozyme such that it forms this structure with targeted

33. J. Haseloff, W.L. Gerlach, *Nature*, 334, 585-591 (1988).

substrate eukarytic mRNA. Under the conditions of ribozyme reaction, the ribozyme catalyzes cleavage of the substrate mRNA at the expected position.[33] This demonstrated, for the first time, the practical use of ribozyme in manipulating RNA.

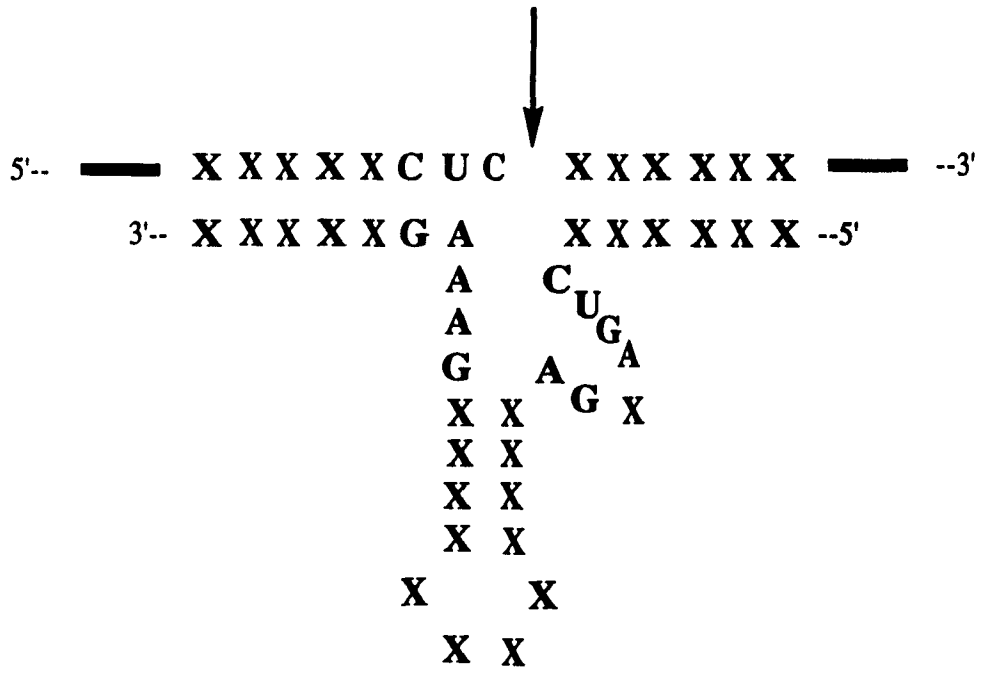

Figure 8. Another structural model of `hammer-head' ribozyme

A self-cleavage reaction similar to the viroid RNA has also been observed in the RNA transcript of satellite DNA from *Notophthalmus viridescens*.[34] Based on the secondary structure of this virus and the known `hammer-head' structure for the autocleavage plant viroid RNAs, Symons has been able to design a ribozyme with only 13 ribonucleotides (Fig. 9).[35] Recently, Wu *et al* showed that the human hepatitis virus, a single strand circular RNA, can also undergo the autocleavage reaction in the presence of magnesium.[36]

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34. L.M. Epstein, J.G. Gall, *Cell*, **48**, 535-543 (1987).
35. A.C. Jeffries, R.H. Symons, *Nucleic Acids Res.* **17**, 1371-1377 (1989).
36. H.N. Wu, Y.J. Lin, F.P. Lin, S. Makino, M.F. Chang, M.M.C. Lai, *Proc. Natl. Acad. Sci.*, **86**, 1831-1835 (1989).

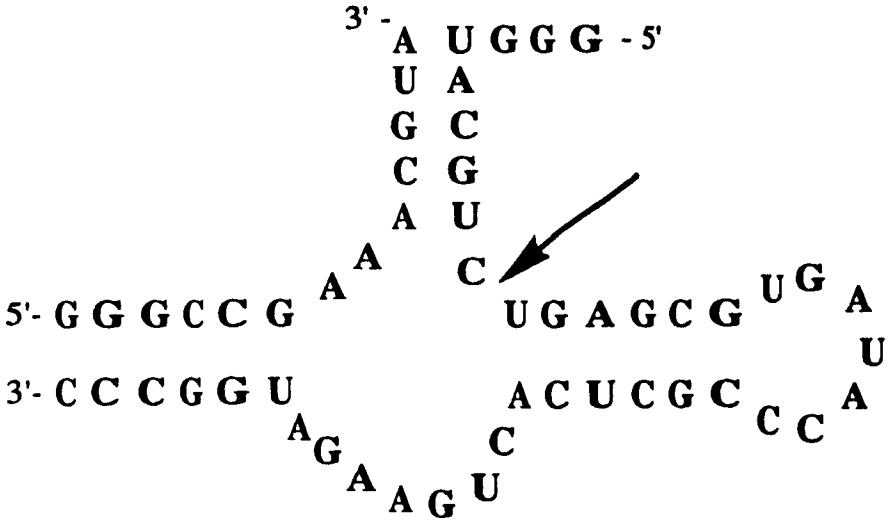

Figure 9. A 13-mer ribozyme system

1.5. Chemical Synthesis of RNA

The ability to make a specific sequence of DNA or RNA will be of great use in the understanding of various biological activities in the context of its sequence. While biologists developed enzymatic methods to make DNA and RNA, synthetic organic chemists have devoted much effort to find a convenient, efficient procedure to synthesize these two genetic molecules, in particular, RNA. Michelson and Todd described the first example of chemical synthesis of a dinucleotide in 1955.[37] Work in nucleic acid synthesis was, however, rather quiet until Khorana started an extensive investigation into the synthetic methodology for preparing DNA and RNA in the late 60's. He and his colleagues successfully demonstrated the use of synthetic RNA fragments in the deciphering of the genetic code.[38] Their effort also led them to the accomplishment of the total synthesis of

37. A.M. Michelson, A.R. Todd, *J. Chem. Soc.*, 2632 (1955).
38. D. Soll, E. Ohtsuka, D.S. Jones, R. Lohrmann, H. Hayatsu, S. Nishimura, H.G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **54**, 1378 (1965).

genes for yeast alanine tRNA,[39] and *E. Coli* tyrosine suppressor tRNA[40] in the 1970's. Itakura and his coworkers were also able to synthesize the genes coding for insulin,[41] and somatostatin.[42] Refinement in the methodology of DNA synthesis in combination with modern electronic technology has led to the development of the `gene machine'.[43] After the great effort by organic chemists for nearly two decades, synthetic DNA of 30-40 units long is now readily available on the commercial DNA synthesizers.[43,44] Not surprisingly, synthetic DNA has been found to be very useful in the understanding of the fundamental biological processes involving DNA. For example, the discovery of the novel Z conformation of the DNA duplex, which is suggested to be involved in gene expression, was made possible through the use of synthetic DNA molecules.[3b] Synthetic DNA is now widely used in gene cloning, site specific mutagenesis, structure/function study and it is one of the important tools for modern molecular biologists.

The development of the chemical synthesis of RNA was even slower than that of DNA. This is probably due to the fact that RNA synthesis is more difficult than DNA synthesis. The complication arises largely from the fact that there exists a core structure of a phosphate diester adjacent to a cis 2'-hydroxyl of a ribose in an RNA molecule. Unlike the phosphate diester in DNA, the phosphate in this unique core structure is chemically very sensitive. Under basic conditions, the interribonucleotide phosphate bond can be attacked by the 2'-hydroxyl group, leading to the cleavage of the nucleotide chain (Fig. 10). In aqueous acidic media, the interribonucleotide 3'-5' phosphate linkage can undergo isomerization to give internucleotide 2'-5' phosphate linkage in addition to cleavage, a process also assisted by the 2'-hydroxyl.[45] Further, RNA molecules are very susceptible to digestion by ribonucleases which are ubiquitous in the surrounding environment.

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39. K.L. Agarwal, H. Buchi, M.H. Caruthers, N. Gupta, H.G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, U.L. RajBhandary, J.H. van de Sande, V. Sgaramella, H. Weber, T. Yamada, *Nature*, 227, 27 (1970).
40. H.G. Khorana, K.L. Agarwal, P. Besmer, H. Buchi, M.H. Caruthers, P.J. Cashion, M. Fridkin, E. Jay, K. Kleppe, R. Kleppe, A. Kumar, P.C. Miller, K. Minamoto, A. Panet, U.L. RajBhandary, R. Ramamoorthy, T. Sekija, T. Takeya, J.H. van de Sande, *J. Biol. Chem.*, **251**, 565 and subsequent article (1976).
41. R. Crea, A. Kraszewski, T. Hirose, K. Itakura, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 5765-5769 (1978).
42. K. Itakura, T. Hirose, R. Crea, R.D. Riggs, H.L. Heyneker *et al*, *Science*, **198**, 1056-1063 (1977).
43. G. Alvarado-Urbina, G.M. Sathe, W.C. Liu, M.F. Gillen, P.D. Duck, R. Bender, K.K. Ogilvie, *Science*, 214, 270 (1981).
44. M.H. Caruthers, *Science*, **230**, 281-285 (1985).
45. D.M. Brown, D.I. Magrath, A.H. Neilson, A.R. Todd, *Nature*, **177**, 1124 (1956).

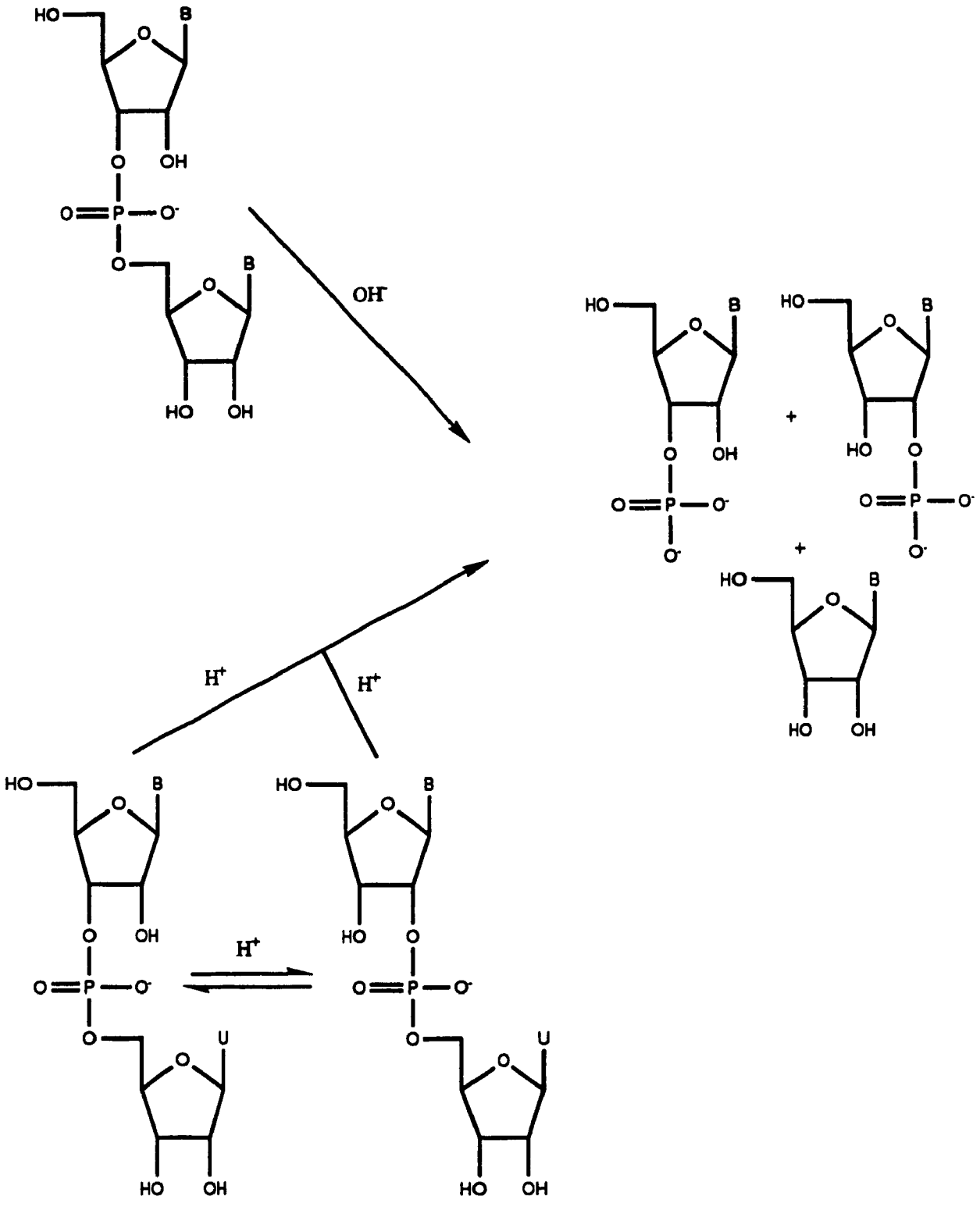
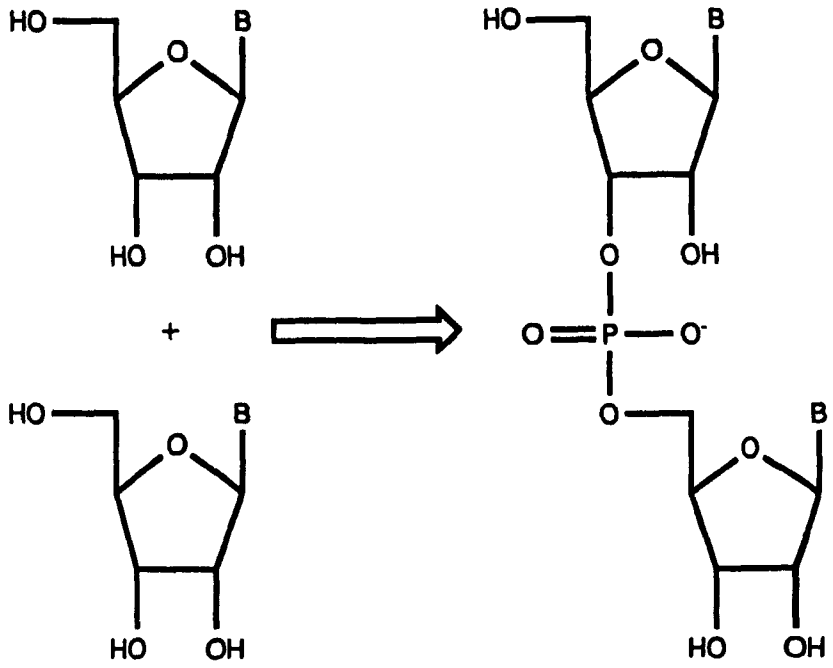


Figure 10. Acid and base catalyzed degradation of RNA

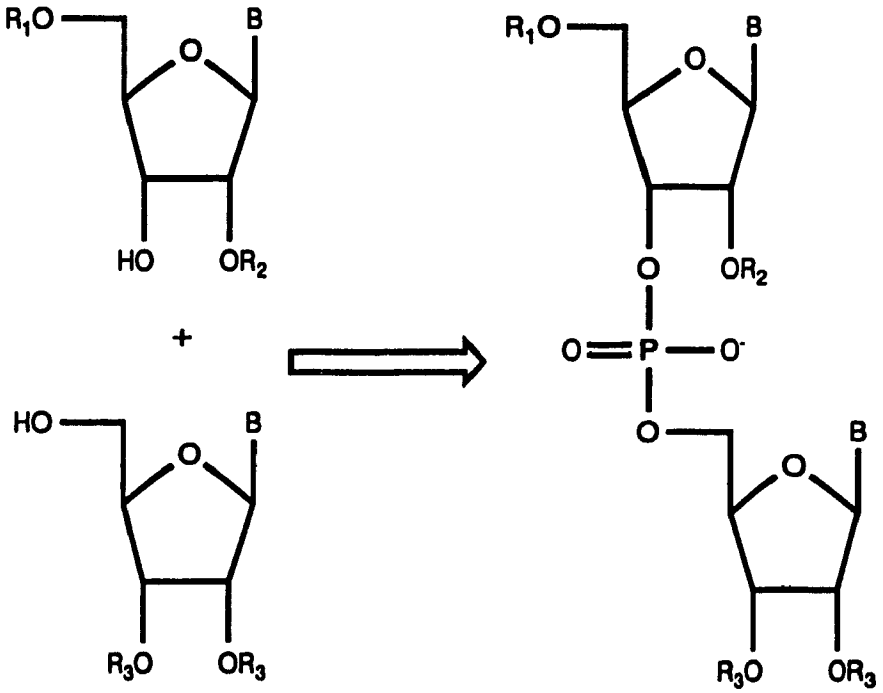
From the synthetic point of view, synthesis of RNA still shares some general strategies with that of DNA. The goal is to assemble the nucleotide chain from the individual ribonucleoside units (Scheme 1). One has to develop a set of compatible protecting groups and an efficient coupling procedure. One needs the protection of the 5'-hydroxyl and the amino group on the base. In the case of RNA there is an additional problem of 2'-hydroxyl protection. The suitably protected nucleosides then have to be coupled to give a 3'-5' phosphate bond. Therefore, historically, a number of strategies presently used in the RNA synthesis were first developed for DNA synthesis and later they were adapted to the RNA synthesis.

SCHEME 1



Two general approaches have been widely used for the nucleotide synthesis. They are solution synthesis and solid phase synthesis. Solution synthesis of an oligoribonucleotide resembles a classic organic synthesis where a phosphate is introduced at the 5'-hydroxyl of a 2',3'-protected nucleoside and the 3'-hydroxyl of another 5',2'-protected nucleoside (Scheme 2). The 5'-hydroxyl protecting group of 5',2'-protected dinucleotide is such that it can be removed without affecting the rest of the protecting groups thereby permitting the regeneration of the free 5'-hydroxyl for the extension of the nucleotide polymer. Thus a desired nucleotide can be assembled by adding one after another. The assembled chain was then deprotected to give the fully deprotected nucleotide. This approach was followed in the early stage of nucleotide synthesis to make all the triribonucleotides for deciphering the genetic code[38]. The synthesis is useful for the investigation of basic chemistry of nucleotide synthesis because during the synthesis, each intermediate can be isolated and characterized. Therefore one can find out the effectiveness of a coupling procedure or a protecting group in the synthesis by characterizing intermediates and products. The strategy can, at least theoretically, be used to prepare a desirable amount of nucleotides of any length.

SCHEME 2



Practically, however, solution synthesis is probably only suitable for the preparation of small size nucleotides. At the end of each assembly, one has to purify the product, remove the 5'-hydroxyl protecting group and then purify the 5'-hydroxyl free intermediate for the next extension.

The introduction of solid phase synthesis revolutionized the strategy in synthesizing macrobiomolecules such as peptides or polynucleotides. And it is surely one of the keys to the success for our achievement of automated procedures of preparing polypeptides and polynucleotides. In the solid phase synthesis, the first unit at the end of a polymer is attached on an inert solid surface. The next unit is then assembled onto the first molecule. The unreacted material is removed by the simple separation of liquid reagents from the solid support. The assembly of a polymer can also be performed on a machine. Thus soon after the invention of solid phase synthesis the automated peptide synthesizer was developed.

The solid phase synthesis was originally introduced by Merrifield for the synthesis of polypeptides.[46] The solid supports developed for the peptide synthesis were swellable polystyrene resins.[46] These were found to be unsuitable for nucleotide synthesis.[47] A non-swellable solid support like silica gel was introduced and it turned out to be very satisfactory for nucleotide synthesis.[48] Later, another version of a rigid solid support, controlled pore glass or CPG was introduced.[49] CPG became the most widely used solid support in the synthesis of oligodeoxyribonucleotides.[50] Ogilvie and Pon also used CPG in the synthesis of oligoribonucleotides and prepared a number of oligoribonucleotides up to 17-units long.[51] CPG, in particular, the long chain alkyl amino controlled pore glass (LCAACPG) has since been commonly used in the synthesis of oligoribonucleotides.

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46. (a) R.B. Merrifield, *Science*, **150**, 178 (1965). (b) R.B. Merrifield, *Angew. Chem. Int. Ed. Engl.* **24**, 799 (1985).
47. (a) R.L. Letsinger, V. Mahadevan, *J. Am. Chem. Soc.* **87**, 3526 (1965). (b). H. Hayatsu, H.G. Khorana, *ibid*, **89**, 3880 (1967). (c) F. Cramer, H. Koster, *Angew. Chem. Int. Ed. Engl.* 7, 473 (1968).
48. (a) M.D. Matteucci, M.H. Caruthers, *Tetrahedron Lett.* 21, 719-720 (1980). (b) K.K. Ogilvie, M.J. Nemer, *ibid*, 21, 4159 (1980).
49. (a) G.R. Gough, M.J. Brunden, P.T. Gilham, *Tetrahedron Lett.* 22, 4177-4180 (1981). (b) S.P. Adams, K.S. Kavka, E.J. Wykes, S.B. Holder, G.R. Galluppi, *J. Am. Chem. Soc.*, **105**, 661 (1983).
50. M.J. Gait, ed., "*Oligonucleotide Synthesis- A Practical Approach*", IRL Press, 1984.
51. R.T. Pon, K.K. Ogilvie, *Nucleosides & Nucleotides*, 3, 485 (1984).

In this approach, the ribonucleotide of 3'-end of the polymer to be synthesized is linked to the amino group on the solid support by succinate[51] (Fig. 11). The nucleotide chain is extended from the 5' position.

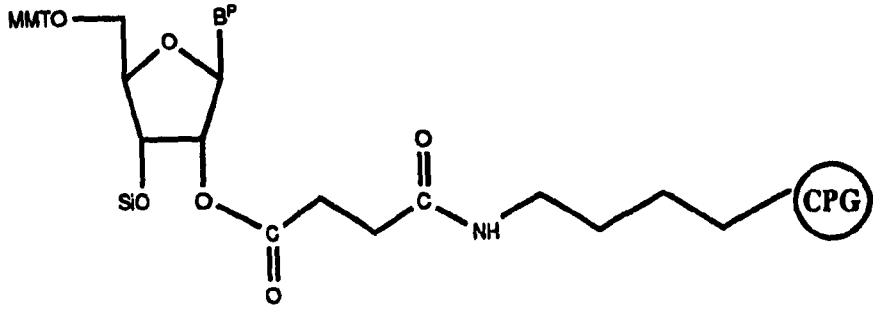
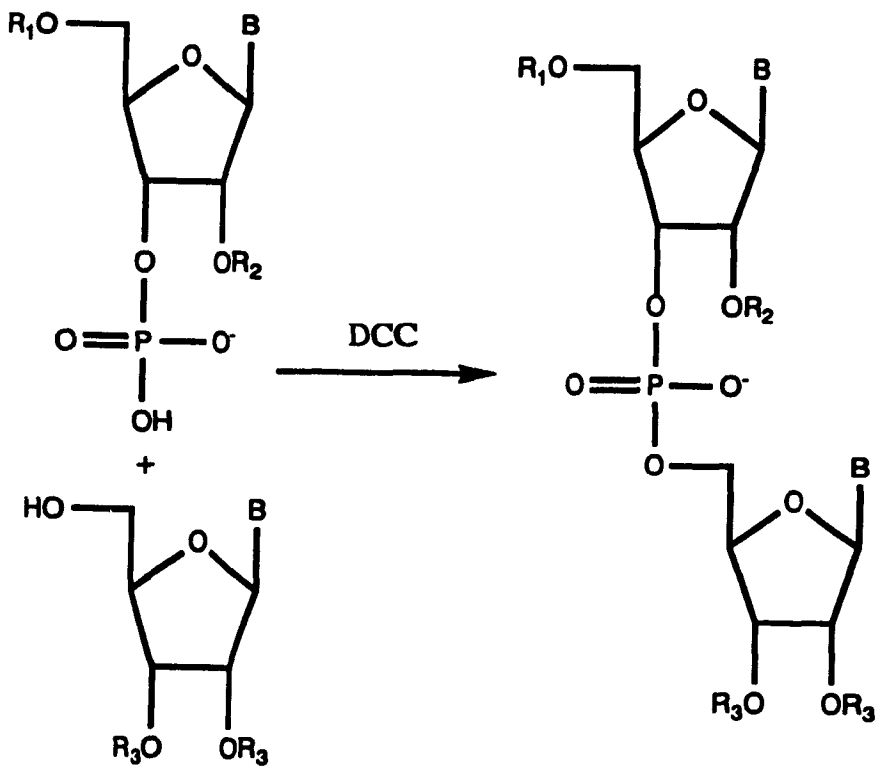


Figure 11. Controlled pore glass used in ribonucleotide synthesis

The success of solid phase synthesis relies heavily on the coupling yield for the formation of each internucleotide phosphate linkage during the chain assembly. Several methods have been developed in the search for the most efficient and convenient method of forming the phosphate linkage in the nucleotide synthesis. They are the phosphodiester, phosphotriester, phosphite triester, and H-phosphonate approaches. They were all first investigated in the synthesis of oligodeoxyribonucleotides and then introduced into the synthesis of oligoribonucleotides.

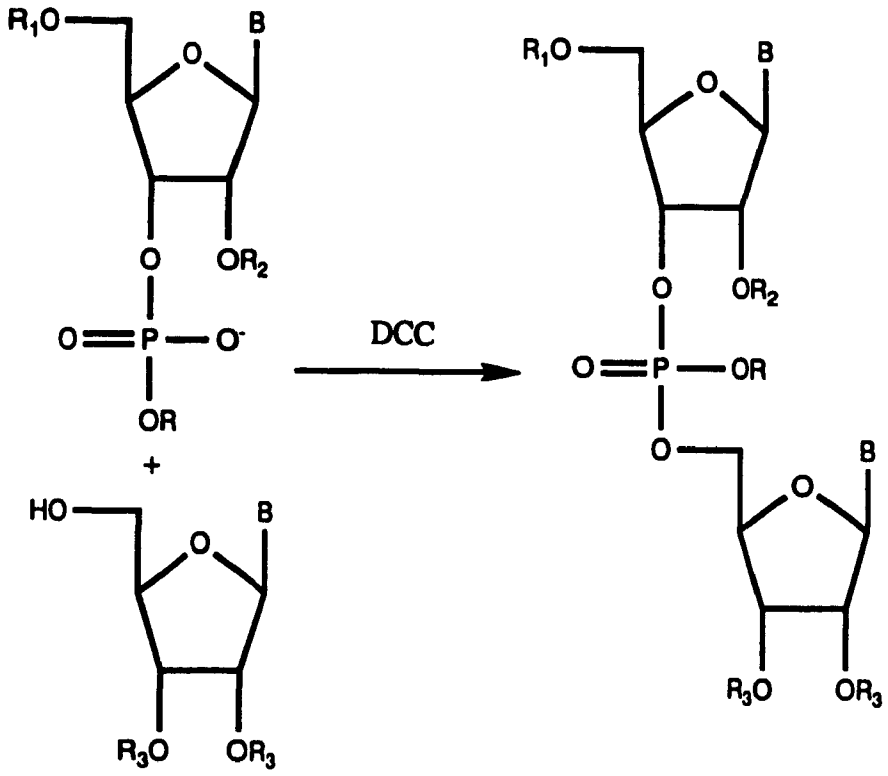
The phosphodiester method was introduced by Khorana[52] at a very early stage of ribonucleotide synthesis. In this approach (Scheme 3), a protected nucleoside 3'-phosphate was condensed with the 5'-hydroxyl of the next nucleoside in the presence of N,N,-dicyclohexylcarbodiimide (DCC). The unreactive nature of the phosphate monomer resulted in very low yields for the reaction. A strong condensing reagent such as aryl sulphonyl chloride was used to increase the coupling efficiency. The yield was still not good enough for the successful synthesis of long oligoribonucleotides on a solid support. Further, the existence of a charge on the phosphate in the product leads to side reactions in the next round of addition.

SCHEME 3



52. (a) M. Smith, D.H. Rammler, I.H. Goldberg, H.G. Khorana, *J. Am. Chem. Soc.*, 84, 430-440 (1962). (b) D.H. Rammler, Y. Lapidot, H.G. Khorana, *ibid.*, 85, 1989-1997 (1963).

SCHEME 4



As a result, a phosphotriester coupling procedure was introduced in the ribonucleotide synthesis. In this approach, the nucleoside 3'-phosphate was in the form of a diester (Scheme 4). After the condensation, a neutral phosphate triester was obtained as the product. Following this method, Neilson,[53] van Boom,[54] and Takaku[55] prepared several short oligoribonucleotides. However, the starting material in this approach, nucleoside 3'-phosphate diester, is ionic. It is difficult to prepare, therefore a modified triester was developed where the starting nucleoside 3'-phosphate is in the fully protected triester form, introduced by van Boom for the ribonucleotide synthesis.[56] Using this approach, van

53. (a) T. Neilson, *J. Chem. Soc. Chem. Comm.*, 1139 (1969). (b) E.S. Werstiuk, T. Neilson, *J. Am. Chem. Soc.* 96, 2295 (1974)
54. (a) J.H. van Boom, P.M.J. Burgers, G.R. Owen, C.B. Reese, R. Saffhill, *J. Chem. Soc. Chem. Comm.*, 869 (1971). (b) J.H. van Boom, P.M.J. Burgers, R. Crea, G. van der Marcel, G. Wille, *Nucleic Acids Res.* 4, 747 (1977).
55. H. Takaku, M. Yoshida, M. Kato, *Chem. Lett.* 811 (1979).
56. J.H. van Boom, P.M.J. Burgers, *Tetrahedron Lett.*, 4875 (1976).

Boom[57] as well as Ikehara[58] prepared a number of short oligoribonucleotides including a 20-mer. The modified triester method is, however, still not efficient enough for the automated solid phase synthesis. The coupling yield is still not very high.[59] Further, the preparation of a nucleoside monomer for nucleotide synthesis involves laborious work.

A novel approach to generate the internucleoside phosphate linkage in DNA synthesis was introduced by Letsinger in the 70's.[60] Ogilvie[61] as well as Daub[62] soon adapted the method to the synthesis of ribonucleotides. In this approach, the active synthon is a nucleoside 3'-chlorophosphite which is formed by the reaction between a properly protected nucleoside and dichlorophosphite (Scheme 5). This highly reactive nucleoside chlorophosphite is reacted with the 5'-hydroxyl of another nucleoside to give the phosphite triester intermediate, which was oxidized *in situ* to phosphate. Several ways to oxidize the phosphite to phosphate have been developed. One can use an aqueous oxidation by iodine/water/pyridine[60] or a nonaqueous *meta*-chloroperbenzoic acid (mCPBA) oxidation.[63] All the steps in the formation of dinucleotides are almost quantitative without any side reactions. Because of the high coupling yield of this reaction and because it is also free of side reactions, the reaction seemed to have great potential in the solid phase and solution synthesis of oligoribonucleotides. Indeed, following this coupling procedure, Ogilvie *et al* successfully synthesized a number of ribonucleotides on the solid support[48b, 51, 64] and in solution.[65]

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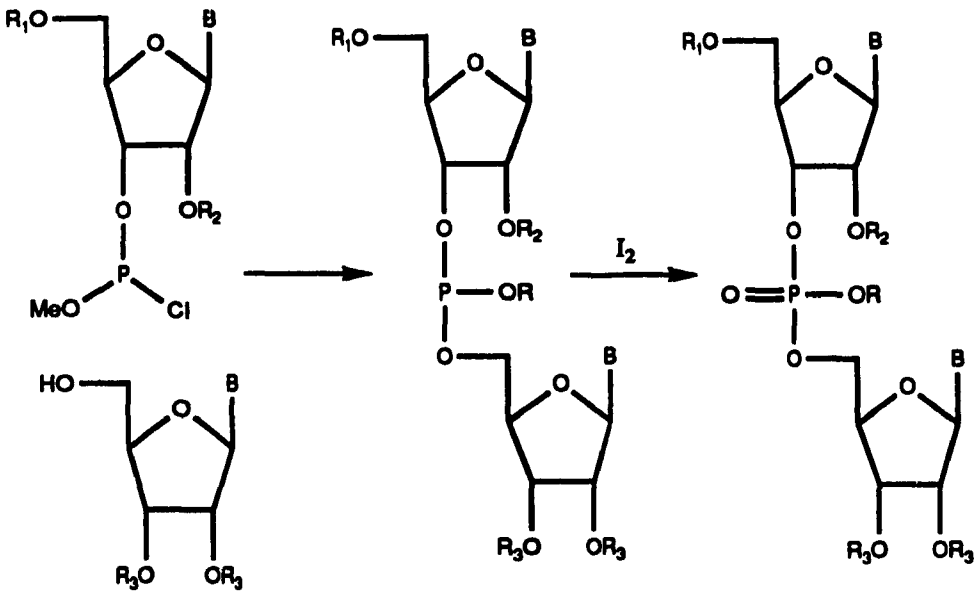
57. J.A.J. der Hartog, G. Wille, J.H. van Boom, *Biochemistry*, **21**, 1009 (1982).
58. E. Ohtsuka, K. Fujiyama, M. Ikehara, *Nucleic Acids Res.*, **9**, 3503 (1981).
59. K.K. Ogilvie, R.T. Pon, *Nucleic Acid Res.* 8, 2108 (1980).
60. R.L. Letsinger, W.S. Lunsford, *J. Am. Chem. Soc.*, **98**, 3655 (1976).
61. K.K. Ogilvie, N. Theriault, K.L. Sadana, *ibid*, **99**, 7741 (1977).
62. G.W. Daub, E.E. van Tamelen, *ibid*, **99**, 3526 (1977).
63. M.J. Nemer, K.K. Ogilvie, *Tetrahedron, Lett.*, **21**, 4149 (1980).
64. R.T. Pon, K.K. Ogilvie, *Tetrahedron Lett.* 25, 713 (1984)
65. (a) K.K. Ogilvie, N.Y. Theriault, *Can. J. Chem.* **57**, 3149 (1979). (b) K.K. Ogilvie, S.L. Beacauge, A.L. Schifman, N.Y. Theriault, K.L. Sadana, *ibid*, **56**, 2768 (1978). (c) K.K. Ogilvie, A.L. Schifman, C.L. Penney, *ibid*, **57**, 2230 (1979). (d) K.K. Ogilvie, N.Y. Theriault, *Tetrahedron Lett.* 2111 (1979). (e) K.K. Ogilvie, M.J. Nemer, *Can. J. Chem.*, **58**, 1389-1397 (1980).

SCHEME 5

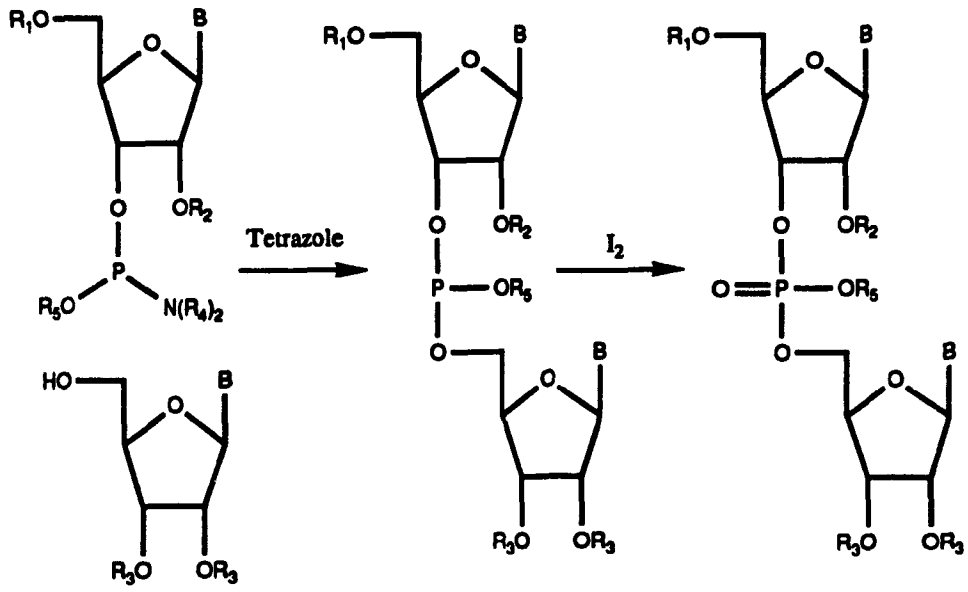


In order to overcome these drawbacks, the nucleoside phosphoramidite procedure was developed for nucleotide synthesis. The method was first developed by Caruthers[66] for DNA synthesis and it is now the most widely used technique in commercial DNA synthesizers. The phosphoramidite procedure was subsequently adapted by Ogilvie[67] to ribonucleotide synthesis. The key intermediate in this approach is the ribonucleoside 3'-phosphoramidite. It was prepared by a phosphorylation reaction between a properly

66. (a) S.L. Beaucage, M.H. Caruthers, *Tetrahedron Lett.* **22**, 1859 (1981). (b) L.J. McBride, M.H. Caruthers, *ibid*, **24**, 245 (1983).
67. (a) N. Usman, R.T. Pon, K.K. Ogilvie, *Tetrahedron Lett.* **26**, 4567-4570 (1985). (b) N. Usman, K.K. Ogilvie, M.Y. Jiang, R.J. Cedergren, *J. Am. Chem. Soc.*, **109**, 7845-7854 (1987).

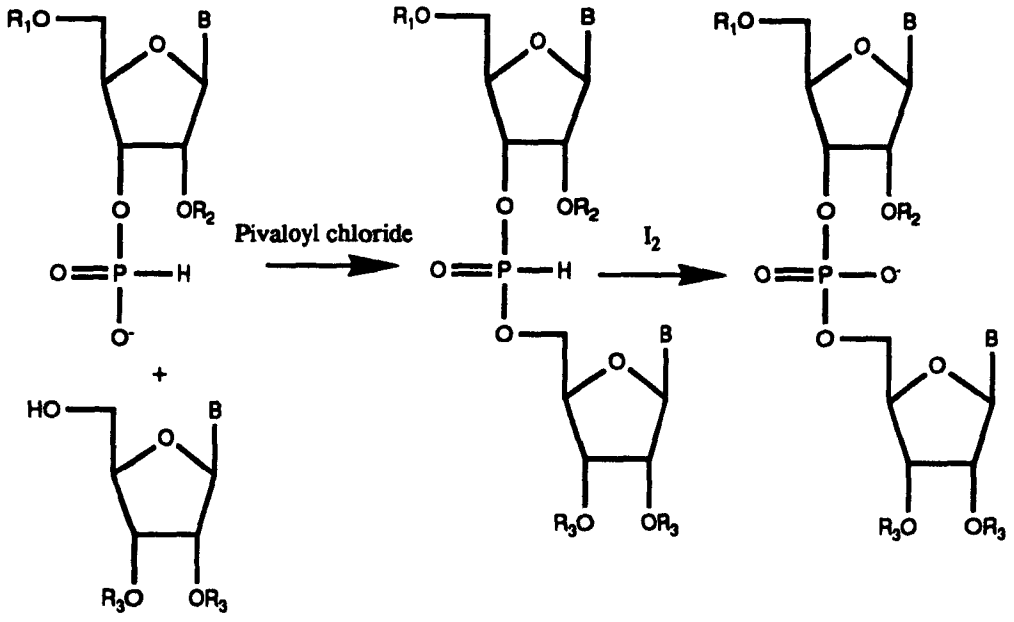
protected ribonucleoside with chloro(N,N,-diisopropylamino)methoxyphosphine in yields of 70-90%. During the synthesis, the nucleoside phosphoramidite is activated with a weak acid, tetrazole, to give the reactive nucleoside tetrazolide intermediate, which is then coupled with the 5'-hydroxyl of another nucleoside to form a dinucleoside phosphite. This intermediate can be oxidized *in situ* to the phosphate (Scheme 6). The coupling yield of this reaction is as high as the previous chlorophosphite method. Moreover, the synthon in this case is very stable toward air and moisture. The reaction is carried out at room temperature and the reaction time is generally completed in a few minutes. The procedures are readily automated. Several long oligoribonucleotides including a 43-mer were prepared using the phosphoramidite coupling procedure on the solid support.[67] Ogilvie *et al* also investigated the difference between diisopropylaminoamidites and morpholinoamidites in terms of their coupling efficiency. The diisopropylamino was found to give a higher coupling yield and therefore it is currently used in ribonucleotide synthesis.[67b]

SCHEME 6



Recently, another novel coupling reagent, nucleoside hydrogenphosphonate, was developed. The method was first used by Todd[68] in the preparation of diribonucleotides and reexplored by Froehler and Matteucci in the deoxyribonucleotide synthesis.[69] This coupling procedure was found to be very efficient and has been adapted to the automated DNA synthesis. Stawinski *et al* subsequently adapted the H-phosphonate procedure to oligoribonucleotide synthesis.[70] The key intermediate in this method is a nucleoside 3'-H-phosphonate (Scheme 7). The phosphonate can be activated by pivaloyl chloride, after which it can then be coupled with the next nucleoside to give the dinucleotide H-phosphonate intermediate. Oxidation with iodine/pyridine/water gives the dinucleotide phosphate. The advantage of this method is that there is no need to have the phosphate protecting group and therefore no need for the phosphate deprotection step at the

SCHEME 7



68. R.H. Hall, A.Todd, R.F. Webb, *J. Chem. Soc.*, 3291 (1957).
69. (a) B.C. Froehler, M.D. Matteucci, *Tetrahedron Lett.*, **27**, 469-472 (1986). (b) B.C. Froehler, P.G. Ng, M.D. Matteucci, *Nucleic Acids Res.* **14**, 5399-5407 (1986).
70. P.J. Garegg, I. Lindh, T. Regberg, J. Stawinski, R. Stromberg, C. Henrichson, *Tetrahedron Lett.*, **27**, 4055-4058 (1986). (b) J. Stawinski, R. Stromberg, M. Thelin, E. Westman, *Nucleic Acids Res.* **16**, 9285-9298 (1988).

deprotection stage. Also, theoxidation can be performed in the final step when the whole nucleotide chain has been assembled. However, the activation of H-phosphonate needs very reactive pivaloyl chloride and the condensation requires large excess of phosphorylating reagent. It could become an alternative to the widely used phosphoramidite coupling procedure if these problems can be solved.

No doubt, the most challenging problem in developing a method to synthesize oligoribonucleotides is to establish a set of compatible protecting groups[71,72] to protect the 5'-hydroxyl (R_1), the 2'-hydroxyl (R_2), and the amino of the base (R_3) (Fig. 12). In particular, one has to find a suitable 2'-hydroxyl protection group. A successful 2'-hydroxyl protecting group should allow the preparation of authentic oligoribonucleotides with correct 3'-5' phosphate linkages. Specifically, a suitable group has to be such that it can provide the isomeric pure 2'-protected 3'-phosphorylated nucleoside which dictates that only the 3'-position will be joined to the 5'-hydroxyl of the next nucleoside via a phosphate. This first requires that the protecting group can specifically protect the 2'-hydroxyl of a ribonucleoside and leave only the 3'-hydroxyl free for the phosphorylation. This is a difficult objective because when a protecting group is directly introduced to a free cis-diol system, a mixture of 2'-protected and 3'-protected isomers will form, which must be separated. If the introduction is not specific then the 2'-protected and 3'-protected isomers must be separated with relative ease. The next very important point is that the 2'-protecting group should be stable to allow the exclusive 3'-phosphorylation. The free 3'-hydroxyl in a 2'-protected nucleoside is extremely reactive to nucleophilic attack on the 2'-protecting group leading to its removal or isomerization. The 2'-protecting group must also stay intact in assembling nucleotide chains and in deblocking other protecting groups. In the end, it should be removed from the 2'-hydroxyl without leading to cleavage or isomerization of the desired 3'-5' phosphate linkage, which is again quite challenging since the 2'-protecting group-removed ribonucleotide core structure is very sensitive to extreme chemical conditions as mentioned previously (Fig. 10). Based on this last reqirement, either an acid labile or a base labile protecting group would be expected to lead to difficulties.

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71. C.B. Reese, *Tetrahedron*, 34, 3143-3179 (1978).
72. K.K. Ogilvie, M.J. Damha, N. Usman, R.T. Pon, *Pure & Appl. Chem.* 59, 325-330 (1987).

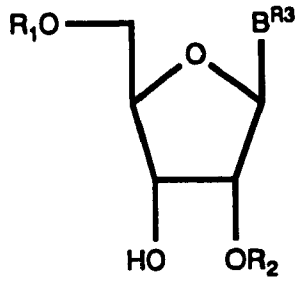

Figure 12. Protection of ribonucleosides

The development of a suitable 2'-protecting group was slow for some time because of such complications. Khorana introduced the acyl type; i.e. benzoyl or acetyl, (Fig. 13) as 2'-hydroxyl protecting group at the beginning of his work on ribonucleotide synthesis.[52] In combination with the phosphodiester coupling method, he and his colleagues prepared all 64 triribonucleotide codons. However, it was found to be very difficult to get the pure 2'-protected nucleoside. It has been also been shown that an acyl type group is easy to isomerize under the conditions of phosphorylation, contributing unnatural 2'-5' phosphate linkages to the contamination in the final synthetic product.[73] Kemple *et al* recently attempted to use an acyl protected 2'-hydroxyl in the phosphoramidite coupling procedure[74] but found that the final synthetic oligoribonucleotides contained about 5% unnatural 2'-5' phosphate linkages.

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73. (a) C.B. Reese, D.R. Trentham, *Tetrahedron Lett.*, 2467 (1965). (b) H.P.M. Fromageot, C.B. Reese, G.F. Stephenson, D.R. Trentham, *Ibid*, 4349 (1966). (c) B.E. Griffin, M. Jarman, C.B.Reese, J.E. Sulston, D.R. Trentham, *Biochemistry*, 5, 3638 (1966).
74. T. Kemple, F. Chow, W.I. Sundquist, T.J. Nardi, B. Paulson, S.M. Peterson, *Nucleic Acids Res.* 10, 6695-6714 (1982).

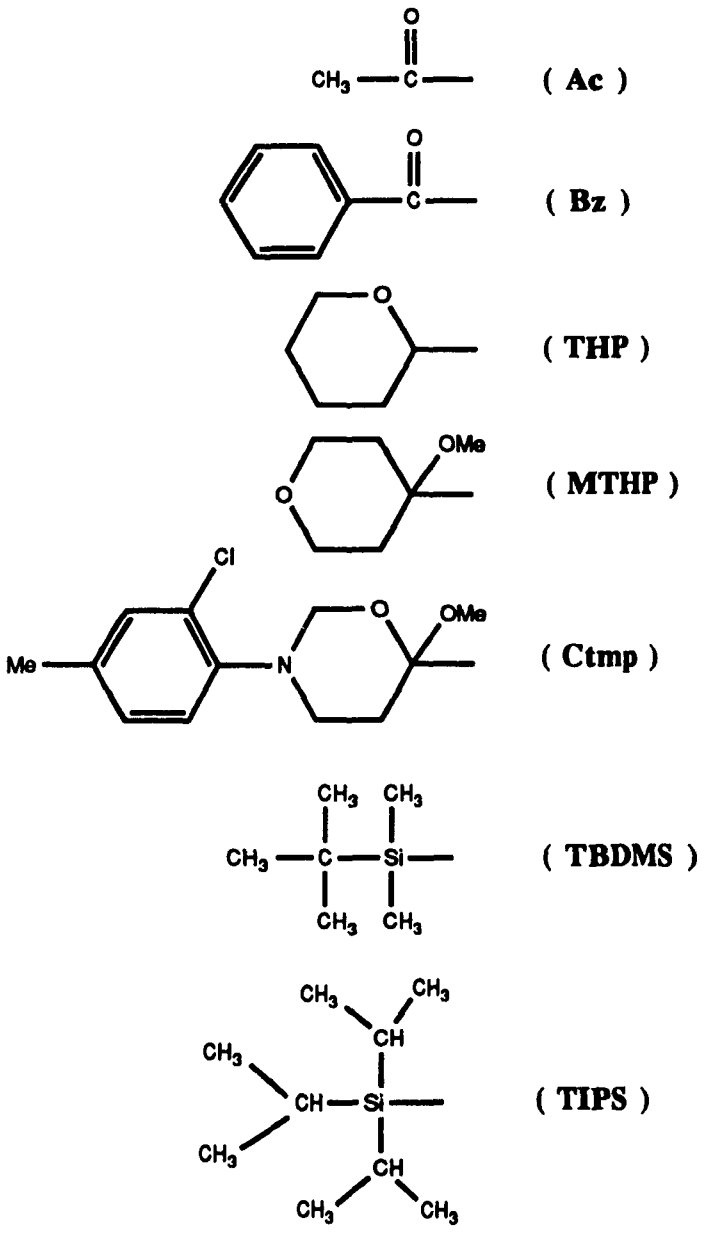

Figure 13. 2'-Protecting groups used in oligoribonucleotide synthesis

Another frequently used 2'-protecting group is an acid labile ketal type such as the tetrahydropyranyl (THP) group (Fig. 13), originally introduced by Reese.[75] THP has also been used by Caruthers to prepare several octamers on solid supports.[76] The original THP has the disadvantages that : 1) the introduction results in diastereomers of the nucleoside monomer;[73] 2) it is not compatible with the standard acid labile 5'-dimethoxytrityl protection used in nucleotide synthesis.[77] The first problem was solved by using achiral 4-methoxytetrahydropyranyl-4-yl (MTHP)[78] (Fig. 13). In a recent study, however, it was found that MTHP had the same difficulty as THP in terms of its stability toward the acidic treatment used to remove the 5'-trityl.[75] One solution to the problem is to design a 2'-protecting group that is stable under the conditions required to remove the 5'trityl group. In recent work, a 9-phenylxanthen-9-yl (Px) was used as a 5'-hydroxyl protecting group and 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (Ctmp) for 2'-protection (Fig. 13) in combination with the phosphoramidite coupling procedure to prepare a nonadecaribonucleotide.[79]

The other approach is to find a 5'-protecting group that can be removed under conditions without affecting MTHP. Indeed, recently, Ohtsuka *et al*[80] used the levulinyl which can be deblocked with hydrazine (Fig. 14) for the 5'-hydroxyl protection. This method suffers from the drawback that the yields during the synthesis can not be monitored since the levulinyl does not have an absorbance in the UV or visible region. Most recently, Gait introduced the 9-fluorenylmethoxycarbonyl (Fmoc), shown in Fig. 14, for 5'-protection which can be removed with 0.1 M DBU.[81]

In conclusion, the use of acid labile protecting groups for 2'-hydroxyl has a number of difficulties and they are still not satisfactorily solved.

In the early 70's, Ogilvie *et al* first introduced the alkylsilyl protecting groups, in particular, the *t*-butyldimethylsilyl (TBDMS) and triisopropylsilyl (TIPS), shown in Fig. 13,

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75. (a) B.E. Griffin, C.B. Reese, *Tetrahedron Lett.* 2925 (1964). (b) B.E. Griffin, M. Jarman, C.B. Reese, *Tetrahedron*, **24**, 639 (1968).
76. R. Kierzek, M.H. Caruthers, C.E. Longfellow, D. Swinton, D.H. Turner, S.M. Freier, *Biochemistry*, **25**, 7840-7846 (1986).
77. (a) C. Christodoulou, S. Agrawal, M.J. Gait, *Tetrahedron Lett.* **27**, 1521-1522 (1986). (b) C. Reese, P.A. Skone, *Nucleic Acids Res.* **13**, 5215 (1985).
78. C.B. Reese, R. Saffhill, J.E. Sulston, *J. Am. Chem. Soc.* **89**, 3366 (1967).
79. T.S. Rao, C.B. Reese, H.T. Serafinowska, H. Takaku, G. Zappia, *Tetrathedron Lett.*, **28**, 4897-4900 (1987).
80. S. Iwai, E. Ohtsuka, *Nucleic Acids Res.* **16**, 9443-9456 (1988).
81. C. Lehmann, Y-Z. Xu, C. Chritotoulou, Z-K. Tan, M.J. Gait, *Nucleic Acids Res.*, **17**, 2379-2391 (1989).

to protect the 2'-hydroxyl of ribonucleosides in oligoribonucleotide synthesis.[61,82] In addition to their stability under a number of conditions, one of the most remarkable features of alkylsilyl groups is that they can be removed by fluoride ion under neutral conditions.[83] The final removal of silyl groups under such conditions provides a mild environment avoiding any undesired degradation or isomerization of the deprotected nucleotide. The silyl groups have been used in ribonucleotide synthesis following the phosphate triester coupling procedure.[59] Silyl groups have been most extensively investigated in the phosphite triester coupling procedure. Both solution[65] and solid phase[48b,51,84] using silyl groups as 2'-protecting groups and the phosphite condensation procedure have been described.

In the approach by Ogilvie *et al* using 2'-silyl group protection, one of the key intermediates is the N-benzoylated 5'-monomethoxytrityl 2'-silylated ribonucleoside[65] (**2a-d**, Scheme 8). This was prepared by the silylation reaction of N-protected 5'-tritylated nucleoside (**1a-d**) with a silyl chloride. The reaction gives a mixture of the desired 2'-silylated nucleoside and its isomer: 3'-silylated nucleoside. However, the undesired 3'-silylated isomer can be removed by simple silica gel column chromatography. Ogilvie *et al* further developed selective silylation conditions. They found that if the reaction is carried out in the presence of silver nitrate, pyridine, and *t*-butyldimethylsilyl chloride, the 2'-hydroxyl will be selectively silylated, allowing the isolation of 2'-silylated nucleoside (**2a, 2b, 2d**), the desired isomer for ribonucleotide synthesis in higher yield.[85,86] On the other hand, the use of 1,4-diazabicyclo[2,2,2]undecane (DABCO) as catalyst gave more than 80% 3'-silylated nucleoside.[87] In order to facilitate the separation of the two isomers, triisopropylsilyl was used for the protection of guanosine. The silylation was carried out in DMF in the presence of imidazole.[67b]

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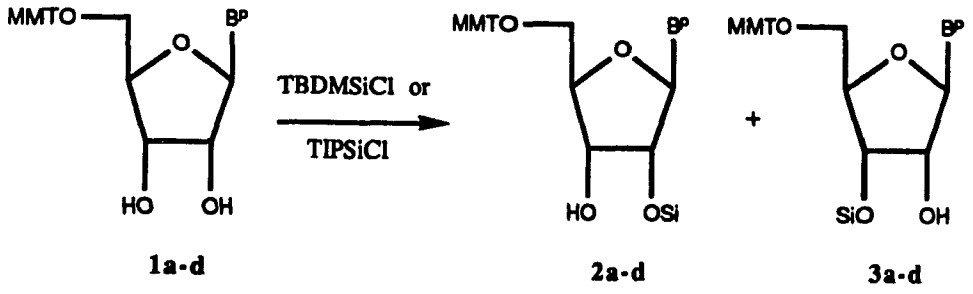
82. (a) K.K. Ogilvie, K.L. Sadana, E.A. Thompson, M.A. Quilliam, J.B. Westmore, Tetrahedron Lett., 33, 2861-2863 (1974).
83. E.J. Corey, A. Venkateswarlu, *J. Am. Chem. Soc.*, **94**, 6190-6191 (1972).
84. R.T. Pon, K.K. Ogilvie, *Tetrahedron Lett.* **25**, 713 (1984).
85. H.H. Hakimelahi, Z.A. Proba, K.K. Ogilvie, *Can. J. Chem.* **60**, 1106-1113 (1982.
86. H.H. Hakimelahi, Z.A. Proba, K.K. Ogilvie, *Tetrahedron Lett.*, **22**, 5243-5246 (1981).
87. H.H. Hakimelahi, Z.A. Proba, K.K. Ogilvie, *Ibid*, 22, 4775-4778 (1981).

SCHEME 8



1a-d 2a-d 3a-d

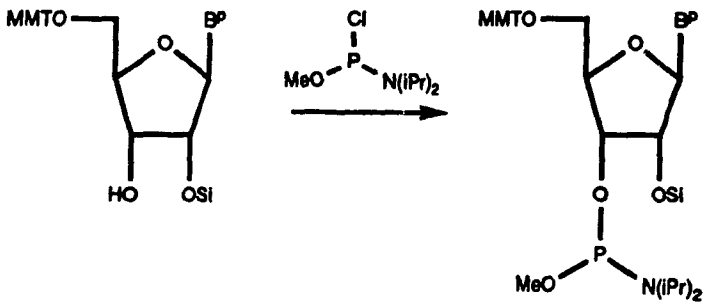
1.a. B = N^6-benzoyl-5'-monomethoxytrityladenosine
b. B = N^4-benzoyl-5'-monomethoxytritylcytidine
c. B = N^2-benzoyl-5'-monomethoxytritylguanosine
d. B = 5'-monomethoxytrityluridine

2,3. a,b,d, Si = t-butyldimethylsilyl (TBDMS)
2c,3c, Si = triisopropylsilyl (TIPS)

The silyl group as a 2'-protecting group has been recently elaborated in the phosphoramidite coupling procedure in the solid phase synthesis of oligoribonucleotides.[67] The key intermediates are N-benzoylated 5'-monomethoxytrityl 2'-silylated nucleoside 3'-phosphoramidites (4a-d) prepared by the phosphorylation of 2a-d with chlorophosphite (Scheme 9). Benzoyl is used for the amino protection and monomethoxyltrityl is used for 5'-hydroxyl protection (*vide infra*). The procedure for preparing these amidites was straightforward and convenient for facile large-scale preparation. The phosphoramidites are stable and easy to handle. When the synthesis is carried out on a commercial synthesizer, the overall process of preparing ribonucleotides is as convenient as the automated procedure developed for DNA.[44] The scope of the technique has been demonstrated in the synthesis of a number of oligoribonucleotides including a 43-mer corresponding to the 3'-half of the formylmethionine tRNA[67b] from *E. Coli.* Using this method, Ogilvie *et al* have further synthesized this whole tRNA which is 77-units long[88] The result has fully demonstrated the practicality of using a silyl group as a 2'-hydroxyl protecting group in oligoribonucleotide synthesis.

88. K.K. Ogilvie, N. Usman, K. Nicoghosian, R.J. Cedergren, *Proc. Natl. Acad. Sci.*, **85**, 5764-5768 (1988).

SCHEME 9



2a-d

2.a, B^P = N^6-benzoyladenine
b, B^P = N^4-benzoylcytosine
c, B^P = N^2-benzoylguanine
d, B^P = uracil

4a-d

There are also several other functional groups in ribonucleosides that need to be protected. They are the 5'-hydroxyl, the phosphate, and the amino group in the case of adenosine, guanosine, cytidine.

The protection of the 5'-hydroxyl is very straightforward. Virtually all the methods of ribonucleotide synthesis use the monomethoxytrityl (MMT) or dimethoxytrityl (DMT) groups, introduced by Khorana[52a] for 5'-hydroxyl protection (Fig. 14). The monomethoxytrityl group is removed with mild acid (usually 5% trichloroactetic acid in methylene chloride) at the end of each assembly. Because its cation UV absorbance (UVmax. 475nm for MMT and 506nm for DMT), it can be used in the solid phase

nucleotide synthesis to monitor the yield of each coupling.[89] Because depurination by acid for RNA is less severe than that for DNA[71], MMT is often used in the RNA synthesis[67]. The MMT group used for as 5'-hydroxyl protection was also found to be compatible with the 2'-silyl protecting group[65, 67].

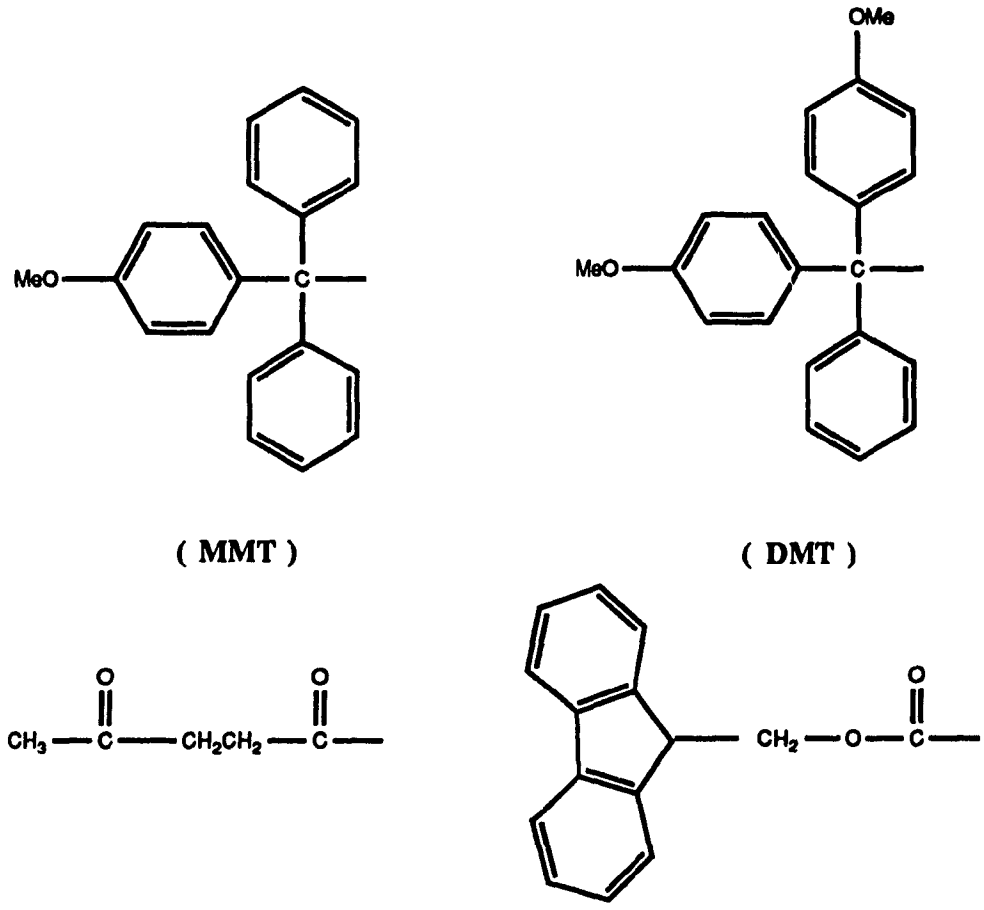


Figure 14. 5'-Protecting groups used in oligoribonucleotide synthesis

89. E.F. Fisher, M.H. Caruthers, *Nucleic Acids Res.* 11, 1589 (1983).

The phosphate protecting group is not required in the H-phosphonate method because the intermediate after each assembly is still H-phosphonate.[70] These phosphonate linkages are oxidized to the phosphate diester in one step at the end of assembly (Scheme 7). However, in both phosphate diester or triester and phosphite triester, one needs to have a phosphate protecting group. The most frequently used phosphate protecting group is methyl or cyanoethyl. Methyl as phosphate protecting group was used by Letsinger in deoxyribonucleotide synthesis[90] and by Ogilvie in oligoribonucleotide synthesis.[67, 91] In the deprotection stage, the methyl group can be removed with thiophenol. Cyanoethyl was first used by Letsinger and Ogilvie in the deoxynucleotide synthesis[92] by the triester method. Later, Koster described the combination of cyanoethyl as phosphate protecting group with the phosphoramidite coupling method.[93] The cyanoethyl protecting group has been extensively investigated in the triester method[94] and also in the phosphite method[95] for ribonucleotide synthesis. The advantage of using this is that the group can be removed by concentrated ammonia which is also used to remove the base protecting group (*vide infra*). Thus one can avoid the use of noxious thiophenol in the deprotection. However, it was observed in the phosphoramidite approach to oligoribonucleotide synthesis that the cyanoethyl phosphoramidite is less reactive than the methylphosphoramidite.[67] Higher coupling yields were obtained with methyl amidite.

The need for amino protection is due to the fact that it was found that the amino group was phosphorylated when using some of the procedures for the phosphorylation of the 3'-hydroxyl group.[96] The other reason for protecting the amino is that the nucleoside derivatives without the amino protecting group have poor solubility. This can lead to difficulties in the preparation of suitable protected nucleosides.[97,98] There are a number of protecting groups available for the amino group (Fig. 15). Khorana *et al* used an acyl type group to protect all three bases. This has since been followed in most phosphate triester, phosphite triester and phosphonate methods. In the deoxyribonucleotide synthesis, the most

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90. R.L. Letsinger, V. Mahedevan, *J. Am. Chem. Soc.* **87**, 3526 (1965).
91. D.J.H. Smith, K.K. Ogilvie, M.F. Gillen, *Tetrahedron Lett.*, **21**, 861-864 (1980).
92. R.L. Letsinger, K.K. Ogilvie, *J. Am. Chem. Soc.*, **89**, 4801 (1967).
93. N.D. Sinha, J. Biernat, H.Koster, *Tetrahedron Lett.*, **24**, 5843 (1983).
94. M.J. Damha, Ph.D. Thesis, McGill University, Montreal, Canada (1987).
95. K.K. Ogilvie, N.Y. Theriault, J.M. Seifert, R.T. Pon, M.J. Nemer, *Can. J. Chem.*, **58**, 2686 (1980).
96. H. Schaller, G. Weimann, B. Lerch, H.G. Khorana, *J. Am. Chem. Soc.* **85**, 3821 (1963).
97. R. Arentzen, C.B. Reese, *J. Chem. Soc. Perkin I*, 445 (1977).
98. J.B. Chattopadhyaya, C.B. Reese, *J. Chem. Soc. Chem. Comm.*, 639 (1978).

frequently used protecting groups are isobutyryl for guanosine,[99] benzoyl for adenosine and cytidine.[96] They are removed in the end with aqueous ammonium hydroxide at 55°C for 18 h. A number of others have been proposed to balance the base lability of the protecting group on the base. For example, Khorana also used *p*-anisoyl (*p*-An)for cytidine[100] and acetyl for guanosine.[101] Others have also proposed *p*-*t*-butylphenoxyacetyl (TBPA),[98] phenylacetyl (PhAc),[102] benzyloxycarbonyl (CBZ)[103] as base protecting groups (Fig. 15). Koster[104] carried out a quantitative study on the base lability of various base protecting groups used in the deoxyribonucleotide synthesis. However, most of them have not been extensively investigated and are not commonly used in nucleotide synthesis.

A detailed study on the use of base labile phenoxyacetyl (PhOAc) and methoxylacetyl (MeOAc) protecting group (Fig. 15) in DNA synthesis was recently carried out by Teoule and coworkers.[105] They introduced the phenoxyacetyl for adenosine and guanosine, and isobutyryl for cytidine. Using this combination of protecting groups, the final deprotection conditions were 4 h in ammonium hydroxide at room temperature as compared with the previous 18 h at 55°C.

A novel amino protecting group was recently introduced by Caruthers *et al*[106] in oligodeoxyribonucleotide synthesis. In this case, they used amidine (Fig. 15) for the amine protection in order to alleviate the degree of depurination. The deprotection conditions for this protecting group is still similar to the benzoyl deprotection; i.e., 16h, 60°C.

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99. H. Buchi, H.G. Khorana, *J. Mol. Biology*, 72, 251 (1972).
100. H.G. Khorana, *Pure & Appl. Chem.*, 17, 349 (1968).
101. R. Lohrmann, D. Soll, H. Hayatsu, E. Ohtsuka, H.G. Khorana, *J. Am. Chem. Soc.* 88, 819 (1966).
102. B. Watkins, H. Rapoport, *J. Org. Chem.*, 47, 4471-4477 (1982).
103. C.B. Reese, P.A. Skone, *J. Chem. Soc. Perkin Trans. I*, 1263-1271 (1984).
104. H. Koster, K. Kulikowski, T. Liese, W. Heikins, V. Kohli, *Tetrahedron*, 37, 363-369 (1981).
105. J.C. Schulhof, D. Molko, R. Teoule, *Nucleic Acids Res.*, **15**, 397-415.
106. L.J. McBride, R.Kierzek, S.L. Beaucage, M.H. Caruthers, *J. Am. Chem. Soc.*, **108**, 2040-2048 (1986).

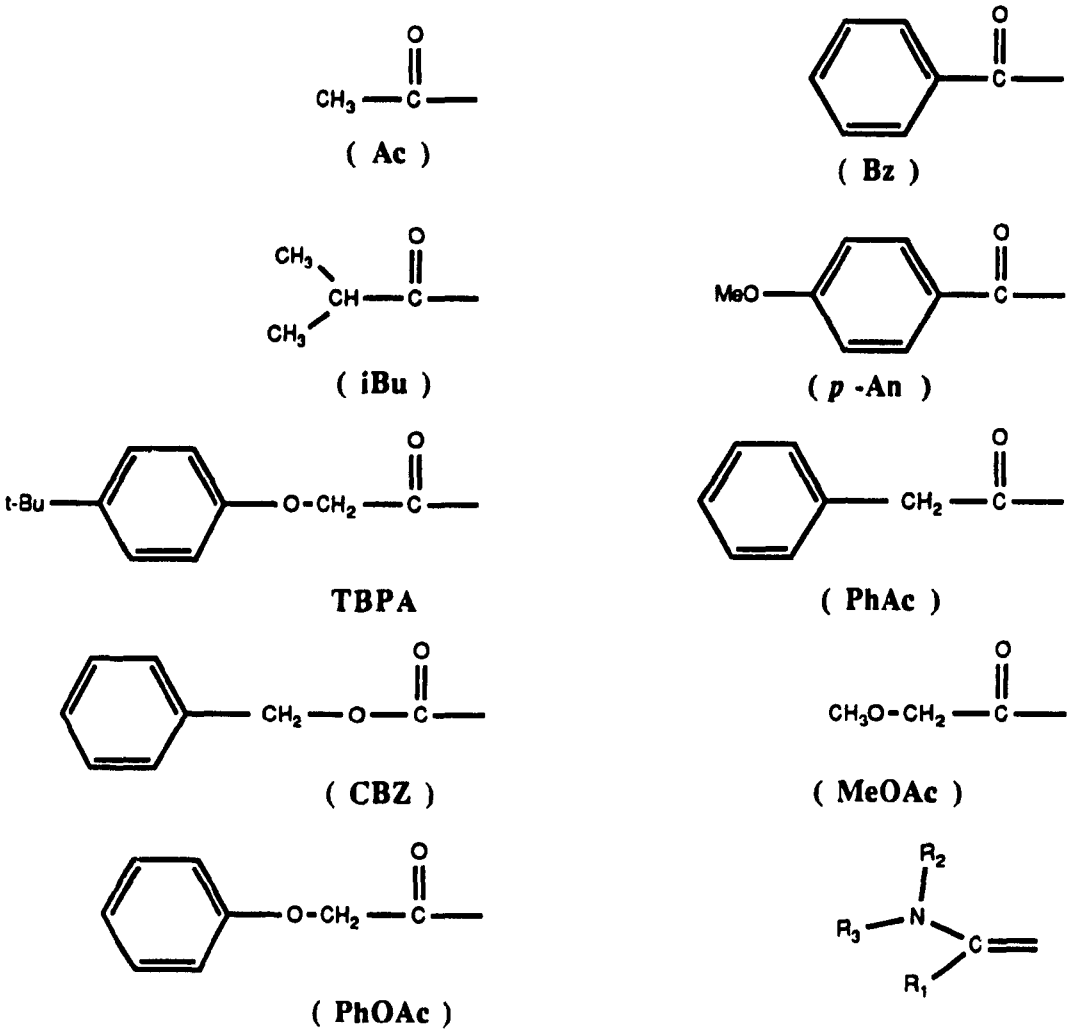

Figure 15. Base protecting groups

In an effort to develop a reliable method to prepare nucleotides with a high degree of chemical homogeneity, attentions were also directed to some side reactions which occured during the synthesis. For example, it was found that a side reaction occurs at the O-6 position of the guanosine nucleoside in both DNA[107,108] and RNA[109] synthesis. Therefore, it has been suggested, that the O-6 of guanosine should be protected in order to prepare the nucleotide with high fidelity.[110] The *p*-nitrophenylethyl group was used for O-6 protection of guanosine. However, other alternatives to prevent such a side reaction, other than using a protecting group, have been proposed[109,111].

1.6. Concluding Remarks.

RNA plays a very important role in cellular gene expression. The various RNAs include the classic types such as tRNA, rRNA, mRNA as well as those novel ones such as intron RNA and ribozyme RNA, that were recently discovered. The development of a reliable and convenient chemical method to synthesize RNA is useful for the study of these novel reactions involving RNA and the further understanding of the various biological aspects of this genetic molecule. Despite its more challenging nature, chemical synthesis of RNA has nevertheless made great progress. The current most successful RNA synthesis uses the methodology developed in this laboratory. The key to the success to this approach is the use of alkylsilyl as the 2'-hydroxyl protecting group and the phosphoramidite coupling procedure. Other protecting groups used in this approach include methyl for the phosphate, benzoyl for the amino, and monomethoxytrityl for the 5'-hydroxyl.

This thesis will deal with some aspects of the chemical synthesis of oligoribonucleotides following the approach above and will also illustrate the use of this synthetic method in the mechanistic study of RNA catalysis.

Part of the thesis will investigate the use of a new more base labile phenoxyacetyl group as the base protecting group in ribonucleotide synthesis. During the work on the synthesis of oligoribonucleotides following the standard approach, a side reaction was discovered which was found to result in an abnormal amount of default short sequences in

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107. R.T. Pon, M.J. Damha, K.K. Ogilvie, *Tetrahedron Lett.*, **26**, 2525-2528.
108. M.H. Caruthers, McBride, L.J., L.P. Bracco, J.W. Dubendorff, *Nucleosides & Nucleosides*, **4**, 95-105 (1985).
109. R.T. Pon, N. Usman, M.J. Damha, K.K. Ogilvie, *Nucleic Acids Res.*, **14**, 6453-6479 (1986).
110. R.T. Pon, M.J. Damha, K.K. Ogilvie, *Nucleic Acids Res.*, **13**, 6447-6470 (1985).
111. J.S. Eadie, D.S. Davidson, *Nucleic Acids Res.*, **15**, 8333-8349.

the synthetic product. This side reaction has been characterized and its source identified. The solution to the problem is provided.

The key to the success of this approach is the use of an alkylsilyl group as the 2'-protecting group. An extensive model study has been carried out to prove the fidelity of 2'-silyl protection in ribonucleotide synthesis.

Although a number of ribozyme systems have been discovered and the catalytic activity of the ribozyme has been extensively studied, the mechanism of the reaction is still poorly understood. The fact that the ribozyme is only 20-30 units in length facilitates the study of the relationship between its structure and activity. With the advent of a convenient chemical method to synthesize RNA, this problem can be approached from the chemical side. Part of this thesis will also demonstrate the chemical synthesis of ribozymes and novel types of ribozyme as well as the use of this synthetic technique in the study of the mechanism of ribozyme reactions.

CHAPTER 2

PHENOXYACETYL AS A BASE PROTECTING GROUP IN THE SOLID PHASE SYNTHESIS OF OLIGORIBONUCLEOTIDES

2.1. Introduction

The protection of purine and pyrimidine bases with a benzoyl group in nucleotide synthesis dates back to the early work by Khorana.[96] This basic method has been followed ever since in most nucleotide syntheses including the current oligoribonucleotide synthesis by the phosphoramidite procedure.[67] The final removal of the benzoyl group involves the treatment of the assembled nucleotide chain in ethanol/ ammonium hydroxide (1/3) at 55°C for 18 h.[67] This step obviously becomes a time consuming step in the deprotection of oligoribonucleotides. Further, recent study has shown that such strong basic conditions resulted, to a small extent, in cleavage of the assembled oligoribonucleotide chains (see Chapter 3). It becomes an objective of the thesis work to introduce a more labile base protecting group in order to prevent such a side reaction. The introduction of a milder deprotection condition would also allow us to incorporate certain base labile modified nucleotides such as 5,6-dihydrouridine in the synthetic products. It would therefore be possible to study the roles of the rare bases present in tRNA.

This chapter will investigate phenoxyacetyl group, which was used by Teoule *et al*[105] for the protection of adenine and guanine in oligodeoxyribonucleotide synthesis, as a more base-labile protecting group in oligoribonucleotide synthesis. The N-phenoxyacetylated guanosine, adenosine, and cytidine methylphosphoramidites were prepared. They have been evaluated in the synthesis of several oligoribonucleotides including a 76-mer RNA corresponding to the sequence of yeast formylmethionine initiator tRNA. After the completion of the work,[112] Teoule *et al*[113] also described a similar result.

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112. T. Wu, K.K. Ogilvie, R.T. Pon, *Tetrahedron Lett.*, **29**, 4249-4252 (1988).
113. C. Chaix, D. Molko, R. Teoule, *Tetrahedron Lett.*, **30**, 71-74 (1989).

2.2. Preparation of N-Phenoxyacetylated Adenosine, Guanosine, and Cytidine (5a-c)

In introducing the N-protecting group, the most frequently used method was the transient protection procedure originally developed by Jones[114] and introduced by McLaughlin *et al* [115] in the preparation of the N-benzoylated adenosine, guanosine, and cytidine. In this procedure, the hydroxyl groups of a ribonucleoside are first temporarily protected with trimethylsilyl groups. The acyl group is then introduced. The trimethylsilyl group was finally removed at the end of acylation. The reaction can be done in one flask with the use of a minimum amount of acylating agent. This is different from the full acylation method used by Teoule *et al*[105] in preparing the N-phenoxyacetylated deoxyadenosine and deoxyguanosine, where the ribonucleosides were directly acylated and the acyl group on a hydroxyl group was selectively removed after the acylation. It was felt that the transient procedure would also work in introducing the more base labile N-phenoxyacetyl group. But it is obvious that care has to be taken when the trimethylsilyl is removed with ammonium hydroxide since the N-phenoxyacetyl is much more labile than benzoyl.

The procedure to prepare the N-phenoxyacetylated ribonucleosides (**5a-c**) is shown in Scheme 10. In preparing N^6-phenoxyacetyl adenosine (**5a**), the nucleoside was first silylated with excess trimethylsilyl chloride in pyridine solution. Phenoxyacetic anhydride (3 eq.), prepared from phenoxyacetyl chloride and phenoxyacetic acid sodium salt[116] was used to introduce the phenoxyacetyl group. On considering the base labile nature of phenoxyacetyl, hydrogen fluoride/pyridine complex[117] (1M) instead of ammonium hydroxide, was added to the reaction mixture to remove trimethylsilyl groups. The reaction mixture was worked up and the crude product was purified by recrystallization from absolute ethanol to give pure **5a**. The yield of the reaction (65%) is comparable to that obtained with the full acylation method as reported by Teoule[105], but much less amount of the expensive phenoxyacetic anhydride was consumed in this case.

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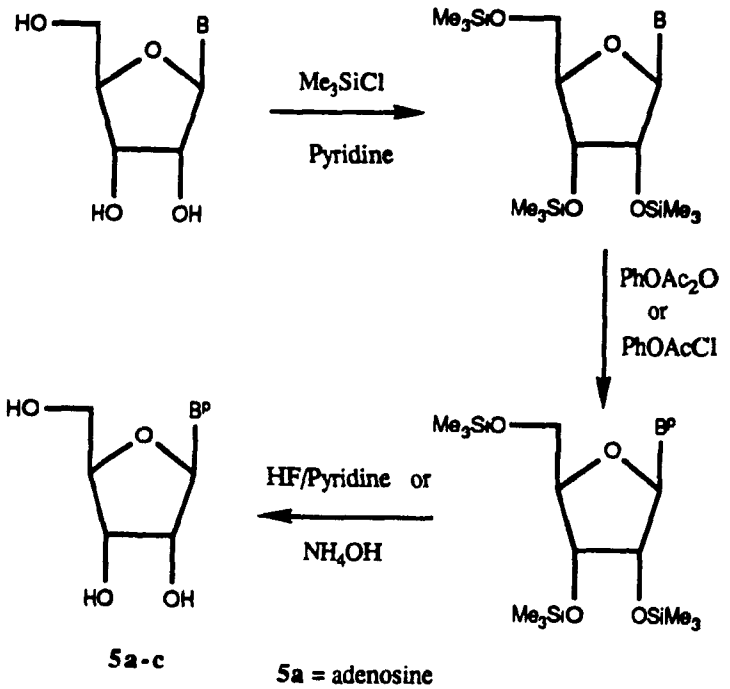
114. G.S. Ti, B.L. Gaffney, R.A. Jones, *J. Am. Chem. Soc.*, **104**, 1316-1319 (1982).
115. L.W. McLaughlin, N. Piel, T. Hellmann, *Synthesis*, 322 (1985).
116. R.K. Smalley, H. Suschitzky, *J. Chem. Soc.* 755 (1964).
117. X. Gao, B.L. Gaffney, S. Hadden, R.A. Jones, *J. Org. Chem.* **51**, 755 (1986).

In preparing N^2-phenoxyacetylguanosine (**5c**) it was found that the ratio of anhydride over nucleoside is crucial to the success of the reaction. The use of a large excess of anhydride (3 eq.) as in the case of adenosine caused extensive side reactions during the acylation of guanosine. The TLC of the product, after workup, indicated a very complicated product composition. The reaction worked when a slight excess of acylation reagent (1.2 eq.) was used and the reaction was left longer (20 h). It was also found that either phenoxyacetic anhydride or simply the phenoxyacetic chloride could be used as acylating agent with comparable efficiency. The commercially available chloride can be used, thereby avoiding the preparation of the anhydride. Also, the hydrogen fluoride/pyridine complex, which was used in the case of adenosine, was found to be ineffective to remove the trimethylsilyl groups on the hydroxyl groups in the case of guanosine. TLC of the crude mixture after treatment with HF/Py showed the presence of mostly very nonpolar silylated derivatives. The trimethylsilyl on the hydroxyl of guanosine appears to be more stable than that on adenosine. This agrees with the result from Mclaughlin[4] who found that the 5',2',3'-trimethylsilylated N^2-benzoylated guanosine was so stable that it can in fact be isolated. It was then decided to try to use ammonium hydroxide to remove the trimethylsilyl groups. It is obvious that the conditions to treat with ammonium hydroxide are very critical. It has to be such that will deprotect the trimethylsilyl without substantially removing the phenoxyacetyl on the base. Finally, it was found that treatment of the reaction mixture with a small amount of ammonium hydroxide (10ml in a 50 mmol scale reaction) for 10-15 min. at 0°C served the purpose. **5c** crystalized from the aqueous solution during the work-up (49%).

N^4-phenoxyacetyl cytidine (**5b**) was prepared in 70% yield in a similar manner as that for guanosine, using phenoxyacetyl chloride as the acylating reagent (1.2 eq) and a small amount of ammonium hydroxide to remove the trimethylsilyl in the end.

These N-phenoxyacetylated nucleoside derivatives were verified by ^1H NMR. They have a very distinctive methylene group (CH_2) signal from the phenoxyacetyl group in proton NMR. In addition, UV absorbances of these derivatives are different from either the benzoylated or unprotected nucleosides. The change of UV absorbance of these products compared with that of unprotected nucleosides is additional evidence that the bases are acylated.

SCHEME 10



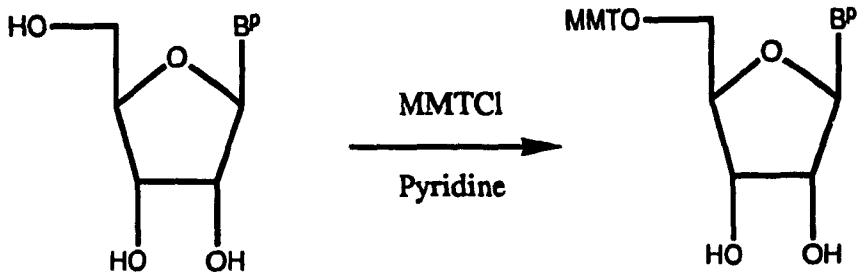
5a-c

5a = adenosine
5b = cytidine
5c = guanosine

2.3. Preparation of N-Phenoxyacetylated Nucleoside Methylphosphoramidites (9a-c)

Preparation of fully protected nucleoside phosphoramidites involves 5'-protection, 2'-protection, and finally 3'-phosphorylation. MMT has been mostly used in oligoribonucleotide synthesis as a 5'-protecting group[65,67]. The tritylation was completed following the same procedures[65] as those used for the N-benzoylated derivatives to give N-phenoxyacetylated 5'-tritylated nucleosides (**6a-c**, Scheme 11). The yields (55%-70%) of these N-phenoxyacetylated derivatives are comparable to those of the N-benzoylated derivatives.

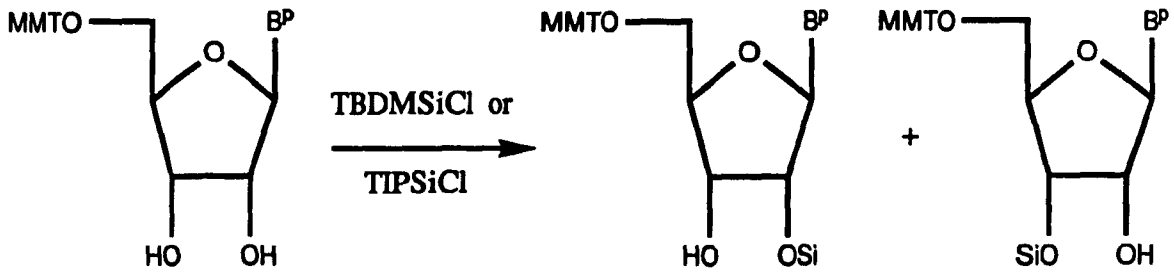
SCHEME 11



5a-c **6a-c**

The silylation of **6a-c** is outlined in Scheme 12. TBDMS was employed for **6a** and **6b**. The reaction involved the addition of *t*-butyldimethylsilyl chloride to a stirred THF solution of nucleosides **6a**, **6b** in the presence of silver nitrate, and pyridine. After the silylation was completed, the reaction was worked up to give a mixture of the 2'-silylated and 3'-silylated isomers with the former having higher percentage (TLC). These two isomers were separated using the routine silica gel column chromatography to give pure **7a** and **7b**.

SCHEME 12



6a-c **7a-c** **8a-c**

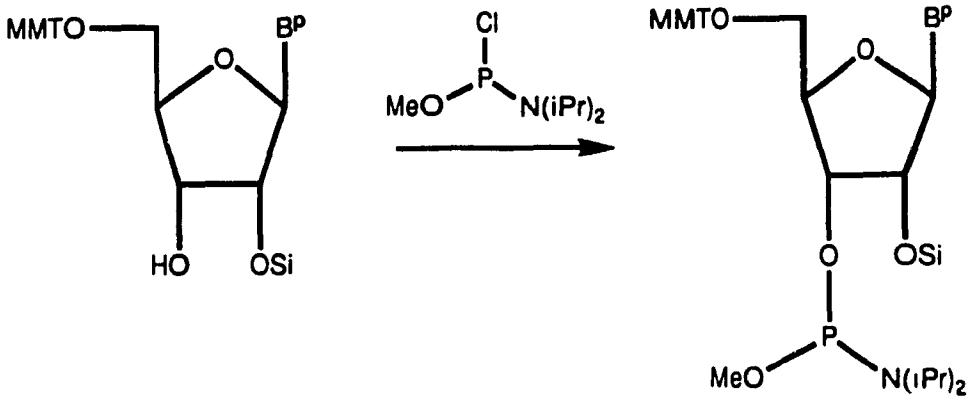
7a,b, 8a,b, Si = *t*-butyldimethylsilyl (TBDMS)

7c, 8c, Si = triisopropylsilyl (TIPS)

In the case of guanosine nucleoside **6c**, the silylation was carried out in DMF in the presence of imidazole and triisopropylsilyl chloride was used since it has been shown that the use of triisopropylsilyl protection facilitates the separation of the two isomers. The reaction took longer to complete (R.T., overnight). The separation between new N-protected 2'-silylated and 3'-silylated isomers has been greatly improved relative to N-benzoylated derivative with the introduction of the phenoxyacetyl group. The two isomers can be separated on a silica gel column with a routine solvent system (20% ethyl acetate in methylene chloride). This allows for the isolation of the desired isomer, the 2'-silylated guanosine (**7c**), much easily and less tediously than was the case with the analogous N^2-benzoylated 2'-silylated guanosine.

The phosphorylation of these new protected nucleoside derivatives was carried out using chloro(N,N,-diisopropylamino)methoxyphosphine as the phosphorylating reagent[67] (Scheme 13). Compared with the other phosphorylating reagents such as bis-diisopropylaminophosphine[118,119] which has been used in preparing deoxyribonucleoside phosphoramidites, the chlorophosphine is much more reactive. This is probably desirable in preparing the ribonucleoside amidites since the 3'-hydroxyl in a 2'-protected ribonucleoside is much less reactive than that in a deoxyribonucleoside due to the steric hindrance.[71] This effect was further observed in the phosphorylation of a guanosine derivative which was protected with the triisopropylsilyl group (7c). It was found that a catalytic amount of DMAP is also needed in order to speed up the phosphorylation. Even with that, the reaction was longer (16 h) than for adenosine and cytidine which generally finishes in 4 h. All the new amidites (**9a-c**) are characterized by ^{1}H and ^{31}P NMR. ^{31}P NMR of each amidite shows two peaks corresponding to a pair of diastereomers.

SCHEME 13



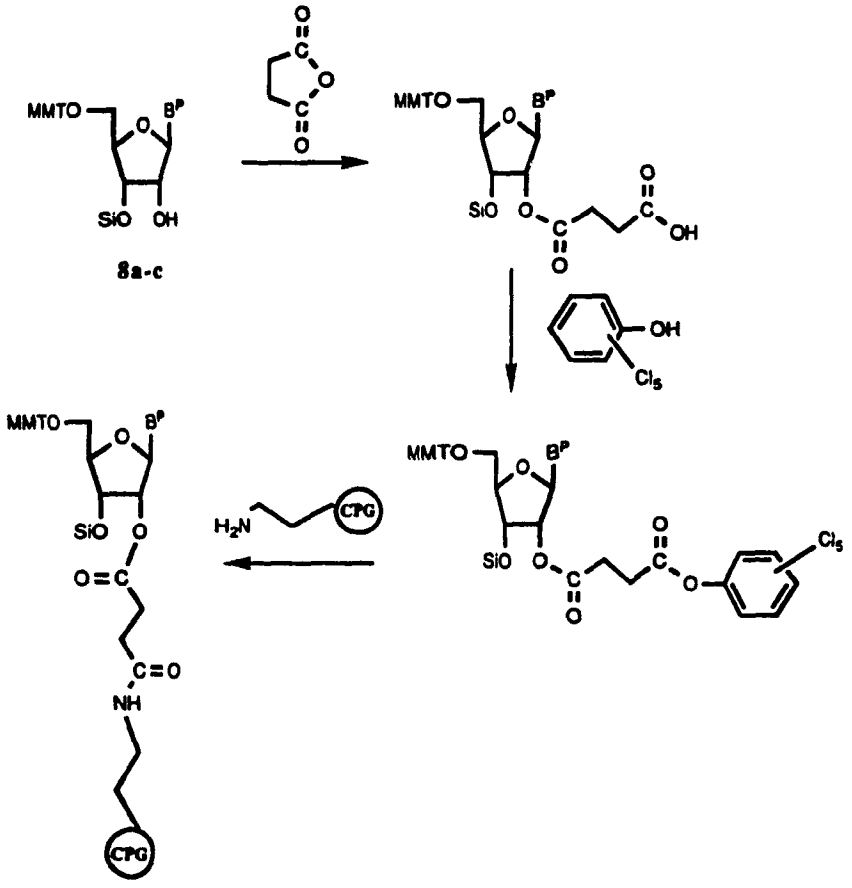
7a-c **9a-c**

118. S.L. Beaucage, *Tetrahedron Lett.*, **25**, 375 (1983).
119. A.D. Barone, J.Y. Tang, M.H. Caruthers, *Nucleic Acids Res.*, **12**, 4051 (1984).

2.4. Synthesis of Oligoribonucleotides

In order to carry out solid phase syntheses with these N-phenoxyacetyl amidites, a CPG that is derivatized with these new N-protected nucleosides was prepared following the previously described procedure.[120] (Scheme 14). Compounds **8a-c**, which are not used in ribonucleotide synthesis, are used for the derivatization of the CPG. They are succinylated with succinic anhydride, then activated with pentachlorophenol in the presence of DCC to give the pentachlorophenyl ester. This activated intermediate was coupled with the amino groups on the LCAACPG to give the solid support needed for nucleotide synthesis. The procedure gives the solid support with loadings of 25-30 umol per gram of CPG.

SCHEME 14



120. N. Usman, Ph.D. Thesis, McGill University, Montreal, 1986.

The phenoxyacetylated phosphoramidites were evaluated in the solid phase synthesis of oligoribonucleotides. However, difficulty was observed with the cytidine derivative **9b** since this compound was poorly soluble in acetonitrile. The compound tended to crystalize out of acetonitrile solutions after standing for a few minutes. This problem precluded further use of **9b** in the standard RNA synthesis. However, it must be realized that there is a marked difference among the lability for benzoyl on adenosine, guanosine, and cytidine. While the benzoyl on adenosine or guanosine needs elevated temperature to be completely deblocked (Chapter 3), the benzoyl group on cytidine can be removed at room temperature in ammonium hydroxide. The half life of debenzoylation of benzoylated cytidine in ammonium hydroxide at room temperature is about 3 h. On the other hand, the half lives to remove the phenoxyacetyl on adenosine and guanosine are only 10 and 15 min. respectively (see Chapter 3). The combination of benzoyl for cytidine and phenoxyacetyl for adenosine and guanosine will still allow the deacylation to be carried out in ammonium hydroxide at room temperature.

A 13-mer oligoribonucleotide (CACUUGACUAGCC) was then prepared on a solid support using N-phenoxyacetylated adenosine and guanosine phosphoramidites, N-benzoylated cytidine amidite (**4b**), and uridine amidite (**4d**). The later two were prepared following the procedures previously described.[67] The nucleotide chain was assembled on an automated synthesizer (Applied System 381B) using the synthesis cycle shown in Table 1 with an average coupling yield of 97.7%.

Table 1. Automated RNA synthesis cycle

Step	Reagent	Time (sec.)
1	Detritylation (5% TCA/DCM)	120
2	Washing (acetonitrile)	120
3	Coupling (0.11 M amidite + 0.5 M Tetrazole, 1/1)	7
4	Wait	60
5	Coupling (0.11 M amidite + 0.5 M Tetrazole, 1/1)	3
6	Wait	240
7	Capping (0.25 M Ac_2O/0.25 M DMAP/0.125 M Collidine in THF)	90
8	Oxidation (0.1 M I_2 in THF/Pyridine/H_2O, 7/2/1)	30
9	Washing	105

The deprotection of ribonucleotides was done following the standard DNA manual deprotection procedures provided on the machine[121]. The methyl phosphate protecting group was first removed with thiophenoxide. This was done on the automated machine by using its manual control operation. Thiophenoxide solution was delivered to the column where the solid support was packed. This was left standing for 1 h and then the support was extensively washed with methanol. This was followed by cleaving the nucleotide from solid support with ammonium hydroxide (30 min.) and then debenzoylation in the same reagent at 55°C for 18 h. However, when CPG from the synthesis was unpacked and left in ethanolic ammonium hydroxide for half an hour, the ammonium hydroxide solution obtained contains only 35 O.D. material, much less than one expected from the coupling yields. Some product must still have remained on the solid support. Indeed, when the above CPG was treated in ethanolic ammonium hydroxide for another 12 h., UV of this solution showed an additional 20 O.D. product. The solution from the 30 min. ammonium hydroxide treatment was left at room temperature for 12 h to further deblock the amino protecting groups. It was then combined with the solution from the sample from the subsequent 12 h treatment. The result suggests that the time to cleave the assembled ribonucleotide chain from solid support should be considerably longer than 30 min. which is routinely used in the deprotection of deoxyribonucleotide. The slow cleavage may probably be due again to the steric hindrance of the silyl group next to the succinate linkage.

The 2'-silyl groups were finally removed with 1M TBAF (tetra-n-butylammonium fluoride).[67] The crude desilylation mixture was desalted on a G-25 Sephadex column to give the crude product. HPLC of this crude product (Fig. 16a) indicates the presence of one major peak corresponding to the desired product.

121. User Bulletin No. 13 (Revised), Model 380/381 DNA Synthesizer (1987) Applied Biosystem, Inc., Foster City, CA.

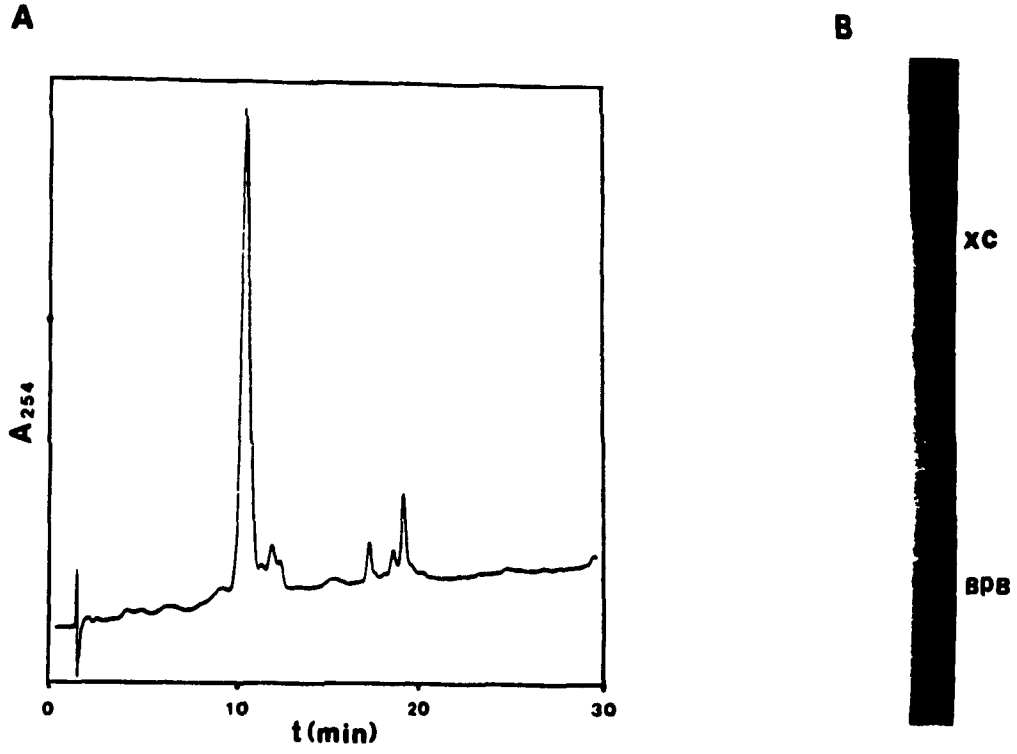


Figure 16. (A) HPLC of the crude oligoribonucleotide CACUUGACUAGCC from the synthesis. HPLC conditions: column, AQUAPORE RP-300 (4.6x100mm); solvent, 7%-20% CH_3CN in TEAA (0.1 M, PH 7); flow, 1ml/min. (B) Polyacryamide gel electrophoresis (20%, 8M urea) of the purified sample.

In order to characterize the synthetic product, the crude product was purified by preparative gel electrophoresis. The final pure, homogeneous material is shown in Figure 16B. The chemical homogeneity was established by the enzymatic total digestion of the pure product followed by the HPLC analysis of the digested mixture.[122] The pure product was incubated with snake venom phosphodiesterase/alkaline phosphodiesterase to give the individual nucleosides. HPLC of digested material showed the four peaks corresponding to the four nucleosides with the expected ratio of 3:5:2:3 (A:C:G:U). No extra peaks were observed. This suggests that there is no base modification in the purified product. The product was also analyzed with spleen phosphodiesterase which is specific to 3'-5' phosphate linkage. There were no unexpected peaks present, suggesting the exclusive 3'-5' phosphate internucleotide linkage.

122. R.T. Pon, Ph.D. Thesis, McGill University, Montreal, 1985.

2.5. Chemical Synthesis of an Analogue of Yeast Formylmethionine Initiator tRNA

Transfer RNAs are the smallest RNAs present in living cells that have their own distinct biological functions. With the current efficiency in oligoribonucleotide synthesis, the total synthesis of this size of molecule becomes a possibility. In fact the chemical total synthesis of a tRNA was recently accomplished, for the first time, in this laboratory. The 77-units RNA related to the sequence of formylmethionine initiator tRNA of *E.Coli* was made in a stepwise fashion.[88] The synthetic analogue was shown to have 11% of the activity of the natural one.

In order to further investigate the scope of the new base protecting group, we decided to make another transfer RNA, a 75-unit-long RNA corresponding to the yeast formyl methionine initiator tRNA (Fig. 17). All the modified bases in the molecule are replaced with their parent ribonucleotides.

Using the reagents described above, the 75-unit nucleotide chain was assembled on the commercial automated synthesizer. The synthetic cycle is the same as the one used before except the detritylation time was increased from 120 to 150 sec. after the 57th base. The average coupling yield is 96% and the overall yield is 31% as measured by the trityl assay.

The assembled molecule was then deprotected as before except that methanolic ammonia (12 h, R.T.) was used to remove the N-acyl groups and cleave the nucleotide form the solid support since these conditions were found to be better than the aqueous ethanolic ammonia (Chapter 3). Most of the product was stored with the 2'-position protected with a silyl group and at -20°C because of the sensitive nature of the fully deprotected ribonucleotides. Only part of this sample (c.a. 30 O.D.) was further treated with TBAF to remove the 2'-silyl protecting group and desalted to give fully deprotected nucleotides. The pure synthetic molecule was prepared by purifying the crude product on preparative polyacryamide gel electrophoresis and is shown in Figure 18.

The molecule was fully characterized by a number of biochemical techniques (work carried out in Dr. Cedergren's lab at Université de Montréal). The total digestion of the purified sample indicates the presence of four nucleotides. Its nucleotide sequence was established using enzyme sequencing. The analogue was found to have 34% of the amino acid acceptance activity of the natural tRNA.

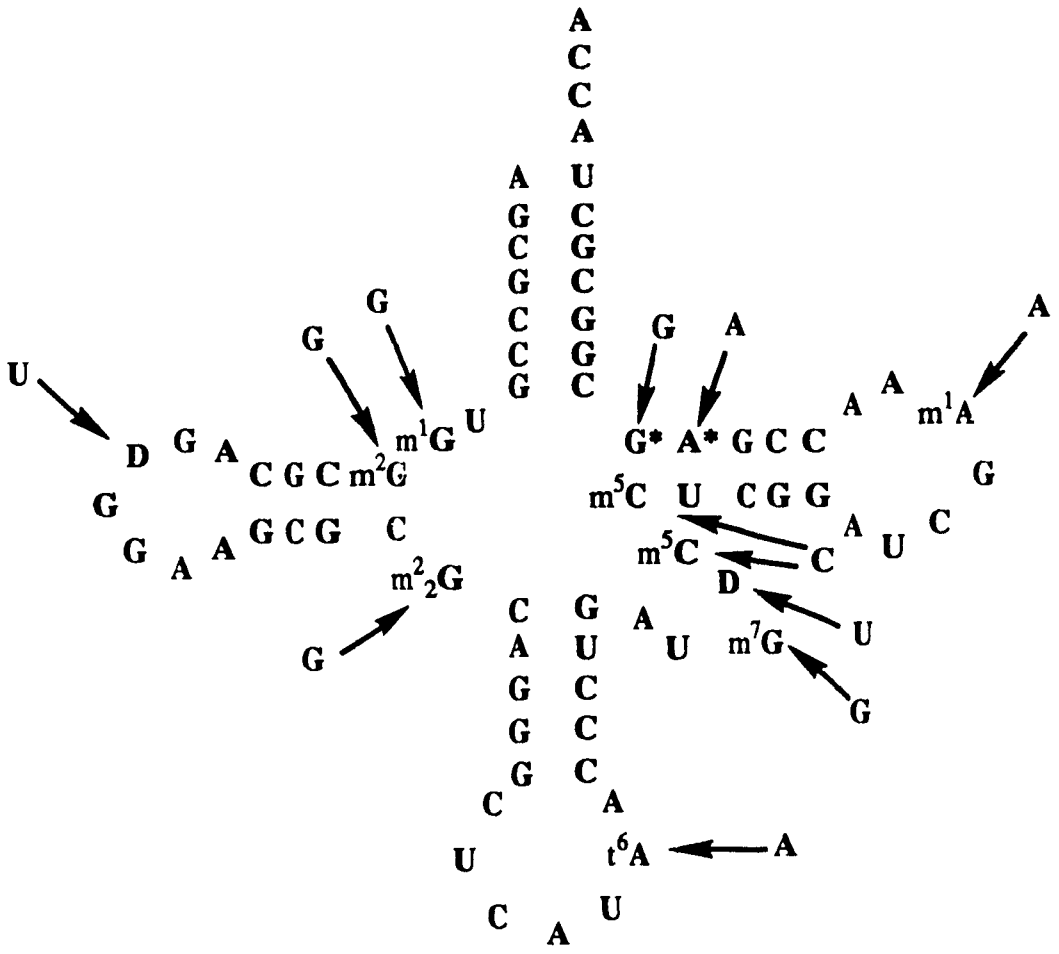


Figure 17. Nucleotide sequence of yeast formylmethionine initiator tRNA.

Figure 18. Polyacryamide gel electrophoresis (20%, 8M urea) of the purified 75-mer analogue of yeast formymethionine tRNA

2.6. Conclusion

The phenoxyacetyl group has been investigated as a more base labile amino protecting group in oligoribonucleotide synthesis. N^4-phenoxyacetyl protected cytidine methylphosphoramidite was found to be inadequate for the present standard RNA synthesis system since it is not sufficiently soluble in acetonitrile. The combination of benzoyl for cytidine and phenoxyacetyl for adenosine and guanosine, as base protection, allows the deacylation step to be carried out at room temperature. The phenoxyacetyl group has been found to be stable under the conditions of preparing ribonucleotides yet it can be removed under mild basic conditions. The use of this new base labile protecting group was demonstrated in the chemical synthesis of a 76-mer RNA related to the yeast formyl methionine initiator tRNA.

The introduction of mild base conditions in the deprotection will be useful in preparing the oligoribonucleotides containing the base-labile modified or rare nucleosides. This further extends the capacity of the standard synthetic technique. The fact that the synthetic tRNA analogue without modified bases in the molecule still has substantial activity suggests the importance of the overall structure of a tRNA molecule. With the availability of a synthetic technique, it is possible to study the relationship between its sequence and biological activity.

CHAPTER 3

PREVENTION OF CHAIN CLEAVAGE IN THE CHEMICAL SYNTHESIS OF 2'-SILYLATED OLIGORIBONUCLEOTIDES

3.1. Introduction

Chemical synthesis of long sequences of RNA has become a practical reality. The scope of the technique has been established by the chemical synthesis of several large size RNA molecules including the half and the whole molecule of a 77-unit-long initiator tRNA of *Escherichia coli* [67b,88] and also a 76-unit-long initiator tRNA of yeast (Chapter 2). This chemical synthesis represented the end result of nearly twenty years of development in which a key element leading to the successful synthesis of long RNA sequences was the use of alkylsilyl (principally *t*-butyldimethylsilyl and triisopropylsilyl) protecting groups on the 2'-position[65,67]. The silyl protecting groups have been used in combination with triester, chlorophosphite and phosphoramidite coupling procedures. Others have recently reported successful RNA syntheses using the same alkylsilyl groups in the H-phosphonate coupling procedure.[70]

As a set of compatible protecting groups and effective nucleotide coupling procedures were developed, the major obstacles to RNA synthesis were overcome. It becomes possible to focus on increased efficiency in experimental protocols. We try to optimize the conditions both in the assembly and the deprotection to allow the synthesis of oligoribonucleotides in higher efficiency. One problem that became apparent was the cleavage of synthetic chains during deprotection steps. On careful investigation it was apparent that the severe aqueous ammonia treatment used in the removal of N-acyl protecting groups was also causing the removal of some silyl groups leading to chain cleavage.

In this chapter we describe the cleavage problem arising from the standard deprotection procedures and the use of methanolic ammonia and N-phenoxyacetyl groups as a means to prevent the problem. Since the completion of our study,[123] Stawinski and co-workers [70b] have reported similar observations using the H-phosphonate method.

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123. T. Wu, K.K. Ogilvie, R.T. Pon, *Nucleic Acids Res.*, 17, 3501-3517 (1989).

3.2. Cleavage of Nucleotide Chains under the Standard Deacylation Conditions

RNA is chemically much less stable than DNA due to the presence of the 2'-hydroxyl. The synthesis of oligoribonucleotides has always been more difficult than the synthesis of oligodeoxyribonucleotides because of the need for the 2'-protection. In DNA synthesis, relatively severe conditions (30% NH_4OH, 55°C, 16-24 h) are used to remove the N-protecting group since this is the last step of the synthesis and DNA is stable under such conditions. Natural RNA will be completely hydrolyzed under such conditions. In our oligoribonucleotide synthesis, the deacylation was carried out with the 2'-positions protected with silyl groups. Also, we used more moderate conditions (NH_4OH/EtOH, 3/1, 55°C, 16h) to deprotect the N-protecting groups. These conditions have been confirmed to be superior to the standard conditions used in the synthesis of oligodeoxyribonucleotide synthesis by an independent investigation.[70b] However, we found that some cleavage of nucleotide chains occurs even under these conditions.

In order to investigate this situation we chose to study oligouridylates. The uridine nucleoside does not require an amino protecting group. Thus the assembled homopolymers of uridine do not possess exocyclic amino protecting groups and thus there is no need to apply a deacylation procedure during the deprotection stage. Thus it is possible to compare the stability of the internucleotide linkage to various conditions of ammonia hydrolysis. A pentadacameric homopolymer of uridine was assembled by using uridine methylphosphoramidite with an average coupling yield of 97% as measured by the trityl assay. The protected nucleotide from the synthesis was treated with thiophenoxide (thiophenol/dioxane/triethylamine, 1/2/2) to remove the methyl group from the phosphate linkages. The nucleotide was cleaved from the solid support by treatment in concentrated ammonium hydroxide (1h) at room temperature. One half of this product was subjected to the desilylation conditions without any further treatment and the other half was exposed to concentrated ammonium hydroxide/ethanol for 18h at 55°C and was then desilylated. The final crude products from these two trials were analyzed by polyacryamide gel electrophoresis (Fig. 19A) and HPLC (Fig. 19B & C).

The sample which did not receive strong ammonium hydroxide-ethanol treatment contained the full-length product predominantly, as expected from the trityl coupling yields (lane a). The sample which was prepared following the standard deacylation conditions; i.e., ammonium hydroxide-ethanol, 18h, 55°C, contained (lane b) noticeably higher amounts of short chain fragments. These can be seen as a ladder of short bands in the gel, or as multiple eluting peaks with shorter retention times on HPLC. The increased chain cleavage in the ammonium hydroxide treated sample is the immediate result of silyl group hydrolysis, since RNA linkages with exposed 2'-hydroxyl groups are readily cleaved under alkaline conditions (Fig. 10). Clearly, however, the desired product is by far the major component even with the severe ammonium hydroxide treatment. Given the 14 phosphate linkages in this chain, the degree of cleavage at any single internucleotide bond is quite small. This was in agreement with the study by Stawinski et. al. [77] on a uridine dinucleotide, which found that the amount of chain cleavage occurring under these conditions was barely detectable.

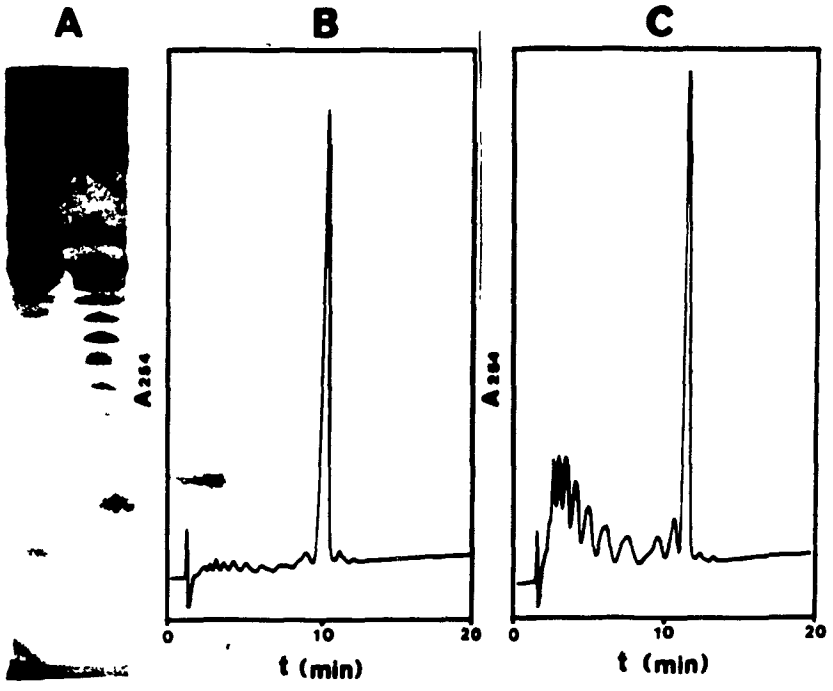


Figure 19. **A.** Polyacryamide gel electrophoresis (20%, 8M urea) of unpurified U_{15}. Lane a: sample without 3/1 NH_4OH/EtOH; Lane b: sample after treatment with NH_4OH/EtOH, 3/1 (55°C, 18 h). **B.** HPLC profiles of sample from lane a. **C.** HPLC profile of sample from lane b. HPLC conditions: column, RP300 (4.6x100mm); solvent, linear gradient (0-30min.) of 7-20% acetonitrile in 0.1 M TEAA (PH7); flow, 1ml/min.

Additional oligoribonucleotides, C_{15}, A_{14}, G_{15}, and $(AUG)_8$, were also prepared following the standard procedure using N-benzoylated nucleoside phosphoramidites. Average coupling yields of 96-97% were obtained for all sequences except A14 which had average coupling yields of 94%. The presence of the benzoyl protecting groups in these sequences made hydrolysis in 3/1 ammonium hydroxide-ethanol (18h, 55°C) a mandatory step of the deprotection sequence. Polyacryamide gel electrophoresis of the crude product in each of these three preparations indicates the presence of a few fast moving bands corresponding to default sequences in addition to the desired major product (Figure 20). The least amount of cleavage was observed with the G_{15} sequence since the triisopropylsilyl group used on guanosine was less susceptible to hydrolysis than the *t*-butyldimethylsilyl group used on the other nucleosides.

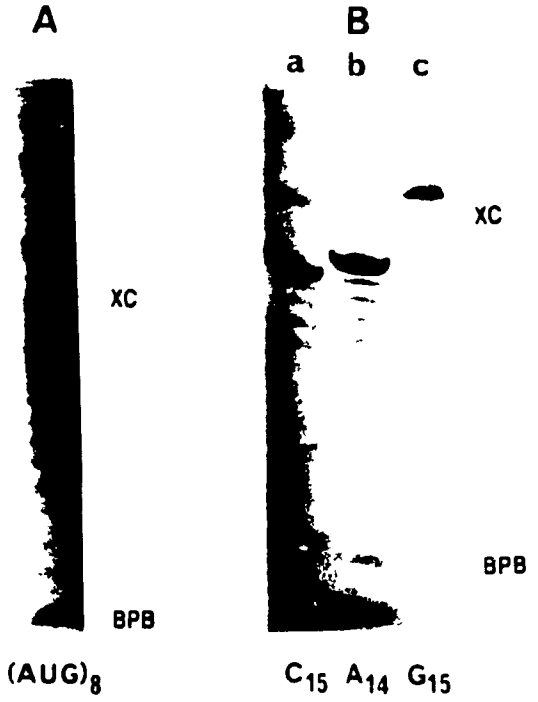
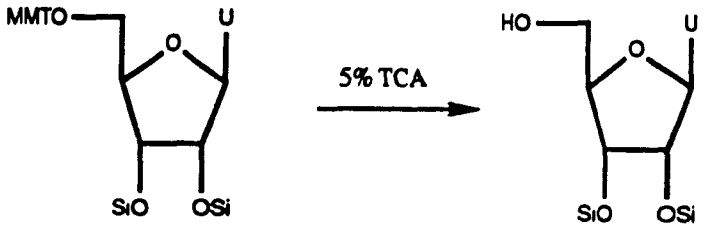


Figure 20. 20% Polyacryamide/8M urea gel electrophoresis of oligonucleotides prepared using standard procedure (NH4OH/EtOH, 3/1, 55°C, 18 h). **A**: $(AUG)_8$. **B**: lane **a**, A_{14}; lane **b**, C_{15}; lane **c**, G_{15}.

3.3. Stability of *t*-Butyldimethylsilyl Group under Various Deacylation Conditions in Nucleotide Synthesis

In order for chain cleavage to occur during ammonium hydroxide treatment some silyl groups must be removed under these conditions. This could happen by the direct attack of hydroxide on the 2'-silyl group. We investigated the stability of the *t*-butyldimethylsilyl group at the secondary hydroxyl positions using 2',3'-di-*t*-butyldimethylsilyluridine (**12**) under various conditions that have been used in removing acyl protecting groups. **12** was prepared by detritylation of 5'-tritylated 2',3'-bis-t-butyldimethylsilyl uridine (**11**, Scheme 15). We first tested the compound in ammonium hydroxide-ethanol (3/1) at 55°C for 18 h, the standard conditions that are used to remove the benzoyl protecting group. Compound **12** was incubated in the solution and it was then analyzed on reverse phase HPLC. HPLC of the sample from this treatment indicated the appearance of three new peaks corresponding to the three products from the silyl hydrolysis: 2'-*t*-butyldimethylsilyluridine, 3'-*t*-butyldimethylsilyluridine, and uridine (Table 2). The identity of these peaks were verified by comparison to authentic standards, prepared by detritylation of 5'-monomethoxytrityl-2'-*t*-butyldimethylsilyluridine (**2d**) and 5'-monomethoxytrityl-3'-*t*-butyldimethylsilyluridine (**3d**). These are the product formed by the attack of hydroxide on the silyl group (Scheme 16). Uridine could result from the further loss of the silyl from the 2'-silylated or 3'-silylated derivative.

SCHEME 15



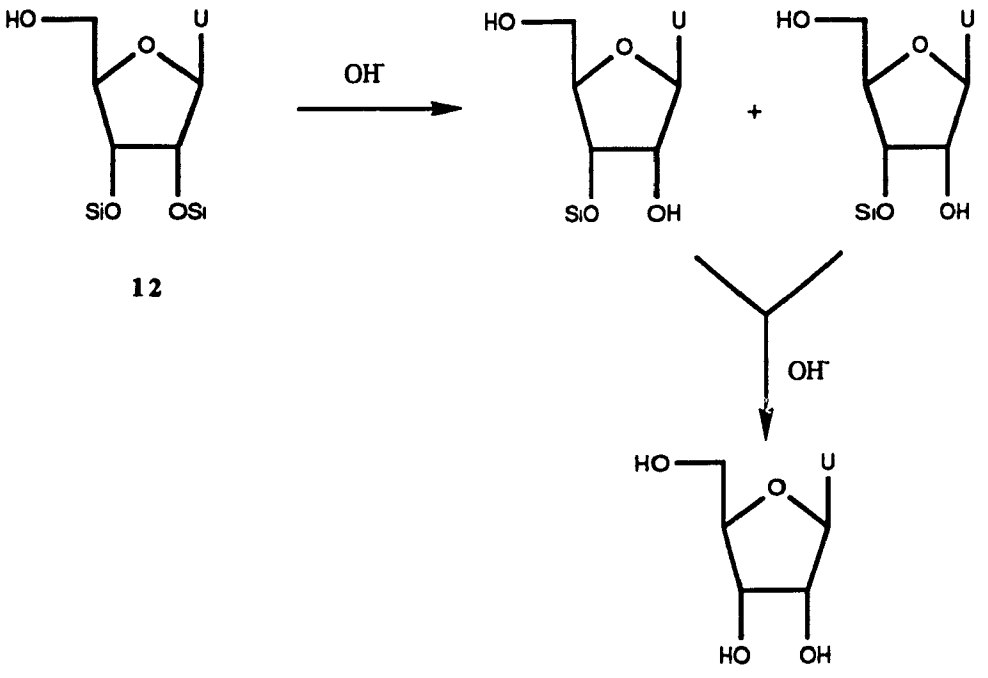
11 **12**

Table 2. Data on the hydrolysis of *t*-butyldimethylsilyl groups from 2',3'-di-*t*-butyldimethylsilyl uridine in 3:1 ammonium hydroxide-ethanol and in anhydrous methanolic ammonia, as determined by HPLC analysis on a 4.6x250 mm Whatman C8 column (60/40 MeCN:H_2O, 1ml/min.)

Hydrolysis Products	Hydrolysis Conditions		
	(NH_4OH-EtOH, 3/1)		(NH_3/MeOH)
	55°C, 18 h	R.T., 12 h	R.T., 12 h
2'-*t*-butyldimethylsilyluridine	0.7%	0.3%	0%
3'-*t*-butyldimethylsilyluridine	0.7%	0.3%	0%
uridine	7.6%	0.7%	0%
2',3'-*t*-butyldimethylsilyluridine	91.0%	98.7%	100%

The test was repeated on 2',3'-disilylated uridine using the much milder condition of concentrated ammonium hydroxide-ethanol (3/1) for 12 h at room temperature, the conditions that are employed when phenoxyacetyl is used for adenosine and guanosine and benzoyl is used for cytidine (see Chapter 2). The result is shown in the Table 2. The hydrolysis was still detected under the conditions but it had been greatly reduced.

SCHEME 16



12

It has been observed that the free 5'-hydroxyl can participate in the cleavage of the phosphate triester, leading to the cleavage of the phosphate bond in nucleotide synthesis[71]. Thus the chain cleavage could also arise from initial 5'-deprotection followed by ammonium hydroxide treatment. In order to clarify such a possibility, we carried out another model study. A uridine nonadecamer was prepared on solid support on an automated synthesizer. At the end of chain assembly, half of the solid support was detritylated and deprotected following the standard procedures including the ammonium hydroxide treatment step (18 h, 55°C). The other half of the solid support was not detritylated, thus the 5'-hydroxyl of the nucleotide is blocked. This trityl-on sequence was deprotected as follows. After the methyl phosphate protection was removed with thiophenoxide, the 5'-tritylated sequence on the solid support was treated with ammonium hydroxide/ethanol (3/1) for 18h, 55°C. After that the 5'-dimethoxytrityl was removed with 80% acetic acid (15 min.). The sample was then desilylated and desalted to give the crude product. The samples from these two different preparations were analyzed by gel electrophoresis (Fig. 21). The samples from the same synthesis but different deprotection sequences give virtually the same result. Both of them contain an abnormal amount of default sequences. The 5'-hydroxyl does not participate in the hydrolysis of the silyl group.

Figure 21. Polyacryamide gel electrophoresis (24%, 8M urea) of U_9 prepared using different procedures. lane 1, sample prepared using standard procedure. Lane 2, sample prepared with deacylation being carried out with trityl on the 5'-hydroxyl group.

To further verify the above result, 5'-tritylated 2',3'-(bis)-*t*-butyldimethylsilylated uridine (**11**) was incubated under the standard conditions (ammonium hydroxide/ethanol, 3/1, 18 h, 55°C). HPLC indicated that there was still the formation of 5'-tritylated uridine (**1d**), 5'-tritylated 2'-silylated uridine (**2d**) and 5'-tritylated 3'-silylated uridine (**3d**). The silyl group is still hydrolyzed under these strong conditions when the 5'-hydroxyl is protected.

The result clearly demonstrates that the presence of strong aqueous hydroxide is detrimental to the silyl groups in the deacylation step during nucleotide deprotection. Methanolic ammonia has also been used to remove acyl protecting groups, a procedure introduced by Khorana and previously used in our solution synthesis of oligoribonucleotides.[65a,65c] We therefore subjected 2',3'-disilylated uridine to saturated ammonia in methanol (prepared at 0°C) for 12h at room temperature. HPLC analysis, in contrast to the previous examples, showed no new peaks corresponding to uridine or monosilylated uridine (Table 2). These results demonstrate that the silyl group is stable under methanolic ammonia conditions.

The above studies suggest that the use of N-protecting groups that can be removed under mild conditions could completely eliminate the loss of alkylsilyl protecting groups, and the resulting chain cleavage.

In order to confirm the effectiveness of various deacylation conditions to remove amino protecting groups, the half life of the deacylation reaction for N-benzoylated and N-phenoxyacetylated nucleosides was measured.

The N-protected nucleoside was incubated in methanolic ammonia for different periods of time at room temperature. The reaction was monitored by HPLC analysis of the mixture on a reverse phase column. The half life of the reaction was obtained by plotting the percentage of starting material against the time of the reaction. The half life of deacylation for N-protected derivatives under various conditions is shown in Table 3. For comparison, the half-time of the reaction in ammonium hydroxide was also measured (by Dr. R.T. Pon at University of Calgary).

Table 3. Half-time for N-Deacylation of Various Nucleosides in Aqueous or Methanolic Ammonia at Room Temperature.

Nucleoside	28-30% NH_4OH[a]	Saturated NH_3/MeOH[b]
N^6-Benzoyladenosine	11h	1h
N^6-Phenoxyacetyladenosine	7min.	<1min.
N^4-Benzoylcytidine	3h	30min.
N^4-Phenoxyacetylcytidine	2min.	<1min.
N^2-Benzoylguanosine	10h	8-10h
N^2-Phenoxyacetylguanosine	8min.	<1 min.

a - Determined by UV monitoring
b - Determined by HPLC analysis on an Aquapore RP300 (4.6x100mm) column using 15-25% MeOH in H_2O gradient over 30 min., 1 ml/min.

The data shows that the benzoyl group is inadequate for the protection of guanosine if methanolic ammonia is used for the deacylation. Also, since N-phenoxyacetylated cytidine methylphosphoramidite (9c) is not soluble in acetonitrile, it can not be used in the present standard RNA synthesis procedure. The fact that the half life for the removal of the benzoyl on cytidine (30min.) and adenosine (1 h) in methanolic ammonia suggests that the benzoyl remains adequate for these two nucleosides under these conditions. The combination of benzoyl for cytidine, benzoyl or phenoxyacetyl for adenosine and phenoxyacetyl for guanosine allows the use of methanolic ammonia as the deacylation condition. Clearly, treatment with saturated ammonia in methanol (for less than 8h) should be sufficient for the deacylation of oligoribonucleotides using this combination of protecting groups. Under such condition the silyl group used to protect the 2'-hydroxyl should not be affected and chain cleavage would be eliminated.

3.4. Synthesis of Oligoribonucleotides Using Improved Deacylation Conditions

The effectiveness of this new combination of protecting groups and the improved deprotection conditions was demonstrated through the synthesis of several oligoribonucleotides, A10, C10, G10, and CACUUGACUAGCC. Benzoylated cytidine, either benzoylated or phenoxyacetylated adenosine and phenoxyacetylated guanosine nucleoside methylphosphoramidites were used. The chain was assembled on an automated synthesizer using our standard protocol, with an average coupling efficiency of 96-98%.

In order to compare the use of methanolic ammonia with the previous standard conditions (ammonium hydroxide, 55°C, 18 h), after thiophenoxide treatment, each polymer was cleaved from the support using methanolic ammonia at room temperature for 1h. Each

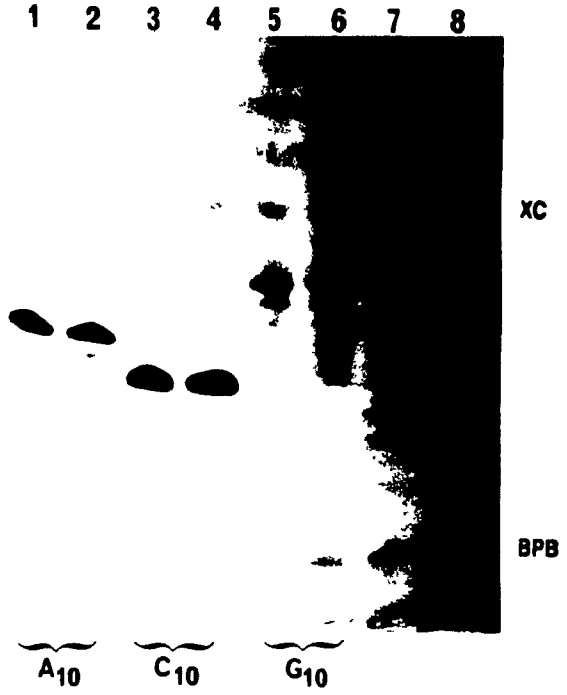


Figure 22. Polyacryamide gel electrophoresis (24%, 8M urea) of crude oligoribonucleotides prepared using either anhydrous methanolic ammonia (8 h, room temperature, lanes **1, 3, 5,** and **8**) or 3/1 ethanol/NH_4OH (18 h, 55°C, lanes **2, 4, 6,** and **7**). Lanes **1** and **2**: A_{10}; Lanes **3** and **4**: C_{10}; Lanes **5** and **6**: G_{10}; Lanes **7** and **b8**: CACUUGACUAGCC..

sample was divided into two parts, one of which was treated with methanolic ammonia at ambient temperature for 8h while the other was treated with ammonium hydroxide at 55°C for 18h. Following desilylation and desalting the crude products were characterized on polyacrylamide gel electrophoresis.

The results, shown in Fig. 22, indicate a substantially cleaner product from methanolic ammonia treated samples (lanes 1,3,5,8) compared to those from ammonium hydroxide treatments (lanes 2,4,6,7). The samples that were prepared using methanolic ammonia as the deacylation condition show up as a single well defined band without any appreciable amount of default sequences.

3.5. Conclusion

It is clear from this study that the standard ammonium hydroxide treatment of 2'-silylated oligoribonucleotide chains, one of the intermediates in the oligoribonucleotide synthesis, leads to the cleavage of the molecule, resulting in the formation of short default sequences in the final product. The cleavage is found to be brought about by the hydrolysis of a small percentage of the 2'-*t*-butyldimethylsilyl protecting groups.

The silyl group on the 2'-hydroxyl is hydrolyzed in the aqueous basic solution containing the hydroxide ion. At elevated temperature (55°C), the hydrolysis is most severe in concentrated ammonium hydroxide but substantially reduced in ammonium hydroxide/ethanol (3/1). Hydrolysis is even further decreased in ammonium hydroxide/ethanol (3/1) at room temperature. Finally, the silyl group is stable in the anhydrous methanolic ammonia.

The problem of chain cleavage can be greatly reduced by using methanolic ammonia deacylation conditions made possible by using the easily removed phenoxyacetyl group for the protection of amino groups on guanosine. The N-benzoyl group remains adequate for the protection of cytidine and adenosine. The deacylation can be satisfactorily carried out in methanolic ammonia under such combination of base protecting groups without causing any detectable chain cleavage. Oligoribonucleotides thus can be prepared in higher yield using these new procedures.

CHAPTER 4

CHEMICAL SYNTHESIS OF NATURAL AND NOVEL RIBOZYMES

4.1. Introduction

The discovery of catalytic RNAs, or ribozymes, is one of the most surprising observations in molecular biology in the past few years[22,124]. The ribozyme with the so called "hammer-head" structure is perhaps the best characterized[32,33]. The small size of "hammer-head" ribozyme RNA makes it an attractive model for the study of its structure/activity relationship. Although the activity of the ribozyme containing the `hammer-head' structure has been extensively studied, the mechanism of the reaction remains poorly understood. The answers to several questions are required in order to unravel the novel catalytic reaction: what is the role of the conserved bases? Where is the active site of the ribozyme? How does the catalysis proceed? The ability to prepare the ribozyme would enable us to address the roles of these structural elements in its catalytic activity. The size of the ribozyme has made its total chemical synthesis a practical possibility. Part of this chapter will describe the chemical synthesis of two ribozymes and their substrate RNA molecules.

One of the attractive features of chemical synthesis of RNA is that it is flexible. We explored the RNA synthetic method and describe herein a general procedure for the preparation of mixed RNA-DNA hetero polymers in which some ribonucleoside units of the natural sequences have been replaced by deoxyribonucleosides. The method allows the substitution of specific ribonucleotides with corresponding deoxyribonucleotides in a ribonucleotide sequence or *vice versa*. Several such rationally modified ribozymes, only available through the synthetic chemical method, have been used to probe the mechanism of RNA catalysis. The study provides a deep insight into the mechanism of ribozyme catalysis.

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124. F.H. Westheimer, *Nature*, **319**, 534-536 (1986).

4.2. Chemical Synthesis of Ribozymes

Ribozymes have been previously prepared using enzymatic synthesis. The ribozymes described by Uhlenbeck (Figure 7), Symons (Figure 9), and Gerlach (Figure 8) were all prepared using the *in vitro* T_7 RNA polymerase[125]. Chemical synthesis would be an another alternative to prepare RNA and other related molecules for the structure/function study. To demonstrate the chemical method, we targeted the ribozyme system shown in Figure 23. The 35-unit ribozyme and its 14-unit substrate were designed according to the model proposed by Gerlach[33] (Figure 23).

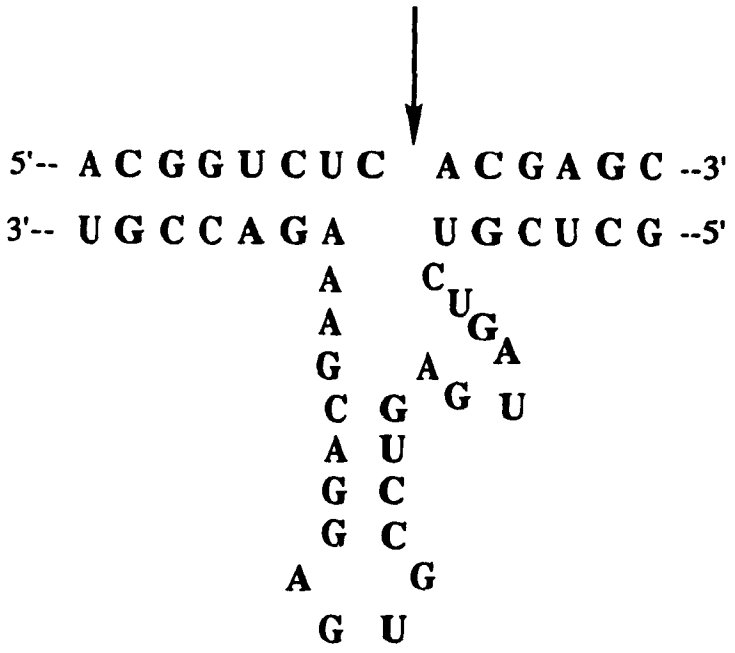

Figure 23. Ribozyme RNA derived from the model proposed by Gerlach[33]

125. J.F. Milligan, D.R. Groebe, G.W. Witherell, O.C. Uhlenbeck, *Nucleic Acids Res.*, 15, 8783-8798 (1987).

The synthesis followed the procedure described in previous chapters. The nucleotide chain was assembled using N-phenoxyacetyl protected adenosine and guanosine, N-benzoyl protected cytidine, and uridine methylphosphoramidites (average coupling yield 97%, overall yield 32%). In the deprotection, saturated methanolic ammonia, instead of ethanolic ammonia hydroxide, was used to eliminate the undesired chain cleavage reaction (see Chapter 3). The crude product was obtained after desilylation and desalting. The gel electrophoresis (Figure 24) shows that the crude product contains very few short sequences, in agreement with the high yields of the synthesis. The molecule was characterized by the RNA sequencing method (Figure 25). The 14-mer substrate was prepared in the same manner. The product is shown in Figure 24 (14C) and its sequencing gel is shown in Figure 26 (14C).

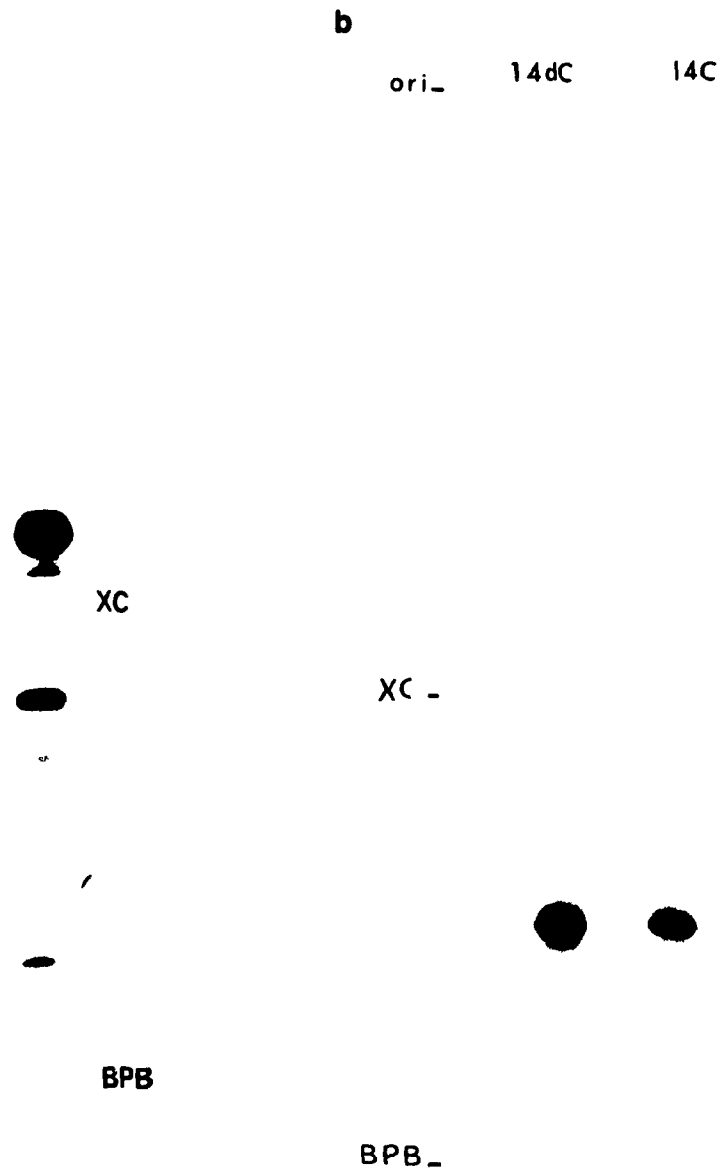

Figure 24. (a) Polyacryamide gel electrophoresis (20%, 8M urea) of crude sample of 35-mer ribozyme from the synthesis. (b) Polyacryamide gel electrophoresis (20%, 7M urea) of a 14-mer oligoribonucleotide (14C) and a 14-mer mixed oligonucleotide (14dC).

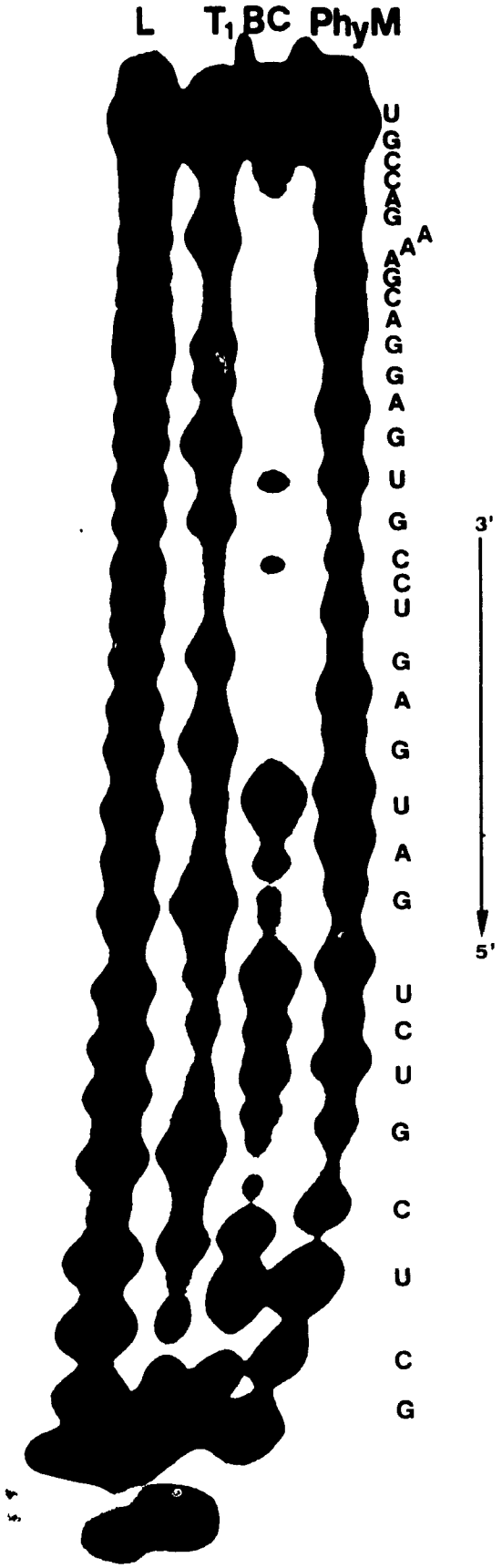

Figure 25. Sequencing gel (15%, 7M) of 35-mer ribozyme. L, ladder; T_1, specific for G; BC, specific for C and U; Phy M, Specific for A and U

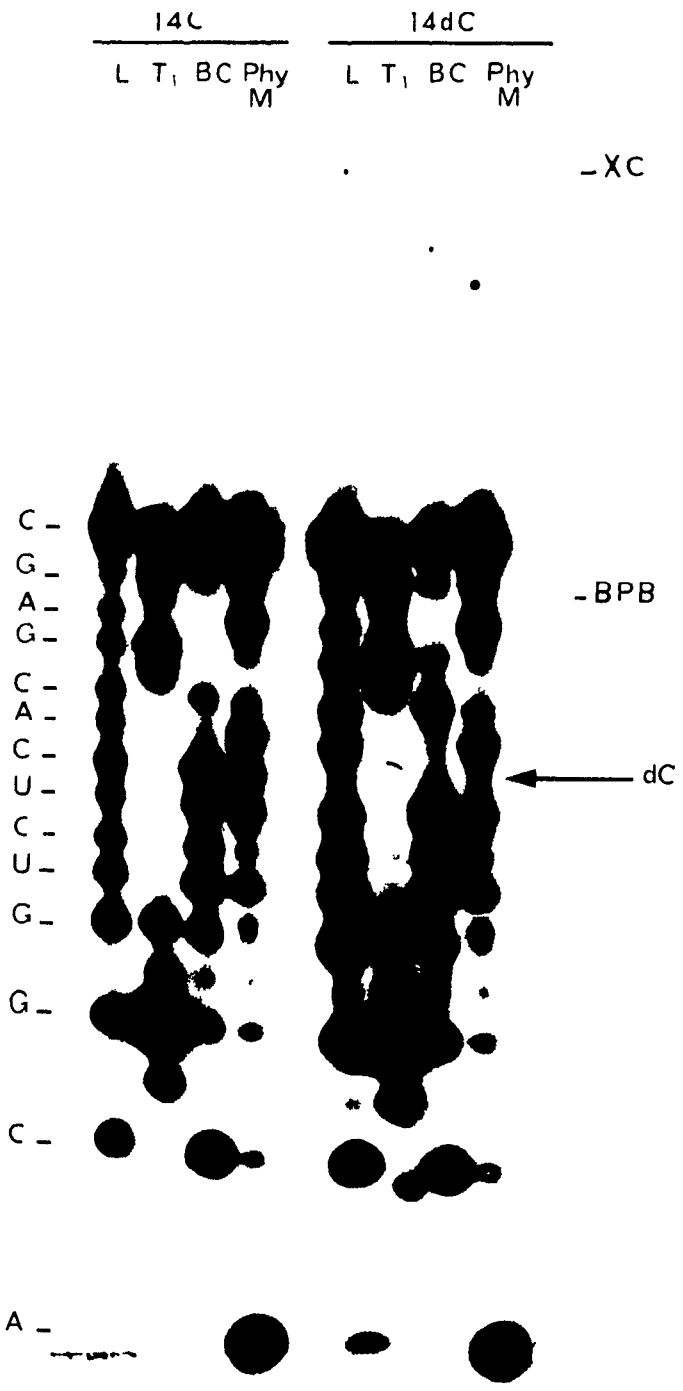

Figure 26. Sequencing gel (15%, 7M) of 14-mer oligoribonucleotide (**14C**) and 14-mer mixed deoxy-ribonucleotide (**14dC**). Enzymes are same as those in Figure 25.

The activity of these synthetic molecules was also tested. When the two molecules were incubated under the conditions described previously[33], the 14-mer substrate was cleaved to give an octamer with the 2',3'-cyclic phosphate at the 3'-end and a hexamer with 5'-hydroxyl at the 5'-end. The reaction is virtually quantitative. (Figure 27, lane 3)

Figure 27. Cleavage reactions by ribozyme monitored by polyacryamide gel electrophoresis (20%, 8M urea). Lane 1, control sample; Lane 2, ribozyme and 14dC substrate; Lane 3, ribozyme and its natural RNA (14C) substrate.

The 19-mer ribozyme (GCGCCGAAACCCGGUCUCGAGC) and its substrate, a 24-mer oligoribonucleotide (GGCUCGACUGAUGAGGCGC), described by Uhlenbeck (Figure 7, Chapter 1) were also prepared. The average synthetic yields for these two molecules were 97% and 98.4% respectively. The two oligoribonucleotides were characterized in the same manner as above. The yield of cleavage of the substrate by ribozyme was found to be 80%, close to that observed by Uhlenbeck using enzymatically prepared molecules.

The results from these two examples shows that synthetic RNAs are biologically as active as the one made using other biochemical methods. This, in addition, suggested the possibility of applying the synthetic technique in the study of the ribozyme reaction itself.

4.3. Chemical Synthesis of the Mixed DNA-RNA Polymer

The automated solid phase synthesis of RNA that we have developed is compatible, in terms of equipment, with DNA synthesis using phosphoramidite chemistry. Thus the synthesis of RNA sequences can be performed on any commercial DNA synthesizer designed for phosphoramidite chemistry. In the automated RNA synthesis using a synthesizer, ribonucleoside phosphoramidites are used in place of deoxyribonucleoside phosphoramidites during the chain assembly. Other reagents are the same as those used in DNA synthesis. An RNA synthesis cycle involves the coupling of an activated ribonucleoside phosphoramidite to the free 5'-hydroxyl of the next nucleoside on the growing chain, oxidation of the phosphite to phosphate, capping to eliminate the unreacted 5'-hydroxyl, and detritylation to generate a free 5'-hydroxyl ready for the next cycle. These steps are in the same order as those in a DNA synthetic cycle. But the time involved is longer for detritylation and coupling steps in RNA synthesis. The former is because the monomethoxytrityl is often used instead of dimethoxytrityl for 5'-hydroxyl protection in the oligoribonucleotide synthesis. The later is considering that the activation of ribonucleoside phosphoramidite with tetrazole appears to be slower than that of deoxyribonucleoside phosphoramidite[121]. The deprotection of RNA sequences requires a desilylation step to remove alkylsilyl groups from the 2'-hydroxyl.

The similarity of the two procedures allows the chemical synthesis of a specific nucleotide polymer with deoxyribonucleosides and ribonucleosides being interspersed on an automated synthesizer. The structure of such a molecule is shown in Fig. 28. The Okazaki fragment in which a fragment of DNA linked with an RNA fragment, is a similar molecule. While van Boom[126] described the solution synthesis of an Okazaki fragment, the method we describe herein allows the solid phase synthesis of a specific nucleotide polymer in which the four deoxyribonucleotides and the four ribonucleotides are interspersed.

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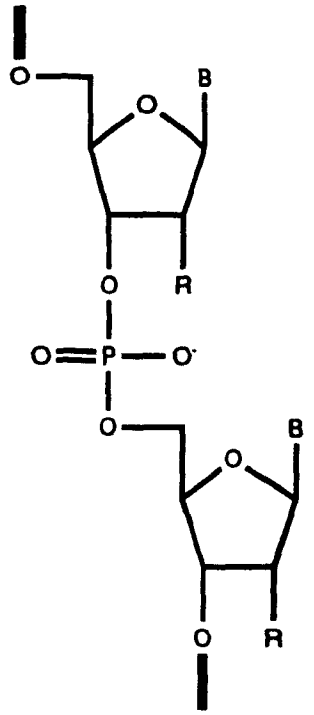
126. E.de Vroom, H.C.P.F. Roelen, C.P. Saris, T.N.W. Budding, G.A. van der Marel, J.H. van Boom, *Nucleic Acids Res.*, **16**, 2987-3003 (1988).



DNA, R = H; B = Adenine, Cytosine, Guanine, Thymine
RNA, R = OH; B = Adenine, Cytosine, Guanine, Uracil
DNA-RNA Hybrid, R = H or OH;
B = Adenine, Cytosine, Guanine,
Thymine, Uracil

Figure 28. Chemical structure of DNA, RNA, and mixed DNA-RNA polymers

To make the mixed DNA and RNA polymers a standard RNA synthesis was set up and deoxyribonucleotide unit were added through the extra base ports, or vice versa. Most commercial DNA synthesizers have the capacity for the addition of extra bases. Deprotection of the assembled mixed nucleotide sequence involves the same steps as for the regular ribonucleotide synthesis, i.e., the removal of the phosphate protecting groups, N-protecting groups, and finally the silyl protecting groups from the ribonucleotide units in the chain.

To illustrate the procedure, a 14-mer ribonucleotide (ACGGUCU*dC*ACGAGC), a substrate of the ribozyme in Figure 23, with a deoxycytidine inserted at the 8th position (**14dC**) was prepared. N-protected 5'-O-monomethoxytrityl-2'-O-alkylsilylribonucleoside methylphosphoramidites, and the commercially available N-protected-5'-dimethoxytrityl deoxyribonucleotide cyanoethylphosphoramidites were used as assembling units. The assembly was performed on an automated DNA synthesizer. The standard RNA synthetic cycle (Table 1) was used for adding ribonucleotide units while the DNA synthesis cycle[122] was used when deoxyribonucleotide units were added. This was easily achieved by using the full programming flexibility for synthetic cycles on the commercial synthesizer available to us. The average coupling yield was calculated by U.V. quantitation of the released trityl cation (476nm for MMT and 504nm for DMT) following the addition of each nucleotide unit (average coupling yield 97%).

The assembled chain was deprotected as follows. First, the phosphate protecting group was removed by thiophenoxide (thiophenol/dioxane/triethylamine, 1/2/2). Treatment of the nucleotide chain with saturated methanolic ammonia for 16h resulted in the cleavage from the solid support and removal of the phenoxyacetyl group on the guanosine and adenosine as well as the benzoyl group on cytidine and deoxyadenosine. The product was then treated with tetra-n-butylammonium fluoride (1M in the THF) for 12h to remove silyl groups from the ribonucleotide units in the chain. The reaction mixture was desalted on Sephadex G25 to give the crude product from the synthesis.

The HPLC profile of the crude products is shown in Figure 29. The mixed oligoribonucleotide was found to have a longer retention time (retention time, 13.5 min.) on HPLC than the corresponding all ribonucleotide sequence (retention time, 11.5 min.) prepared before. Gel electrophoresis of the sequence shows it has virtually the same mobility as the all ribo-sequence (Figure 24).

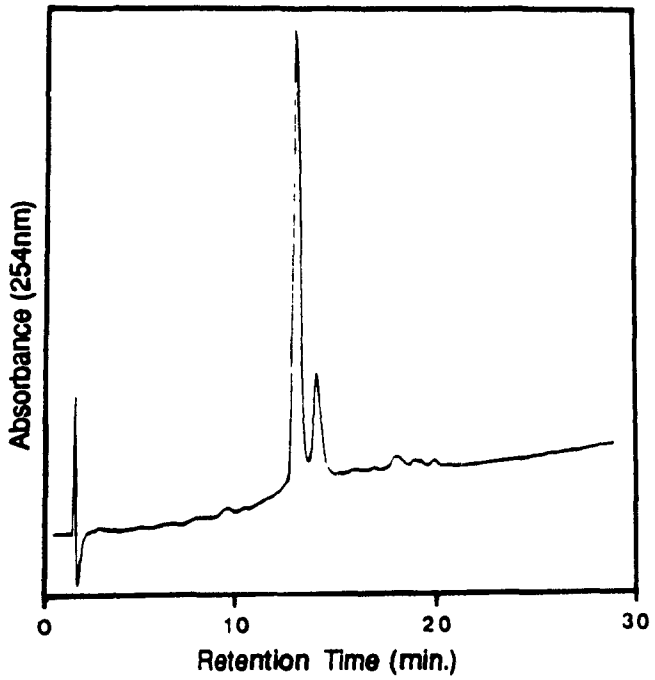


Figure 27. HPLC of **14dC** nucleotide

Full characterization of the mixed oligomer was carried out together with the corresponding ribonucleotide. These two purified oligomers were analyzed by the rapid RNA gel-sequencing technique. The two 5'-labeled oligomers were treated with ribonucleases under conditions of partial digestion. The gels establish the sequence of the polyribonucleotide portion of the oligomer. The DNA segment in the sequence will not be digested by ribonucleases. On the gel, the deoxyribonucleoside positions showed as blank regions when compared to the natural RNA sequence (Figure 26). Comparison of the two gels confirms the lack of a band corresponding to the deoxycytidine position in the mixed 14-mer, whereas the cytidine is prominent in the all RNA 14-mer.

Following the same procedure, a 35-unit oligonucleotide containing 8 deoxynucleotides (8-deoxy) and a 35-unit oligonucleotide containing 5 deoxynucleotides (5-deoxy), shown in Figure 31, were prepared and their sequences established. Both of them are related to the sequence of the ribozyme shown in Figure 23.

4.4. 2'-Hydroxyl Group at the Cleavage Site in the Catalysis

The 5'-hydroxyl and the 2',3'-cyclic phosphate products of the ribozyme reaction suggest the direct involvement of the 2'-hydroxyl of the ribonucleoside at the cleavage site (C_8, Figure 23). This is therefore similar to RNA cleavage by base hydrolysis and protein ribonucleases[127]. In these cases, phosphodiester bond hydrolysis is initiated by the nucleophilic attack of the adjacent 2'-hydroxyl. The ribozyme catalysis is probably more like the lead catalyzed cleavage of tRNA[128]. It has been shown in this case that lead was bound by RNA in such a position that it activates the 2'-hydroxyl group at the cleavage site. The activated 2'-hydroxyl in turn attacks the phosphate to give the 5'-hydroxyl and 2',3'-cyclic phosphate termini in the product. In the catalytic cleavage by ribozymes, it has also been shown that magnesium is required for the ribozyme reaction[32]. It seems to be quite possible that magnesium has a role in activating the 2'-hydroxyl. In any event, the presence of an adjacent 2'-hydroxyl would be essential for the cleavage reaction.

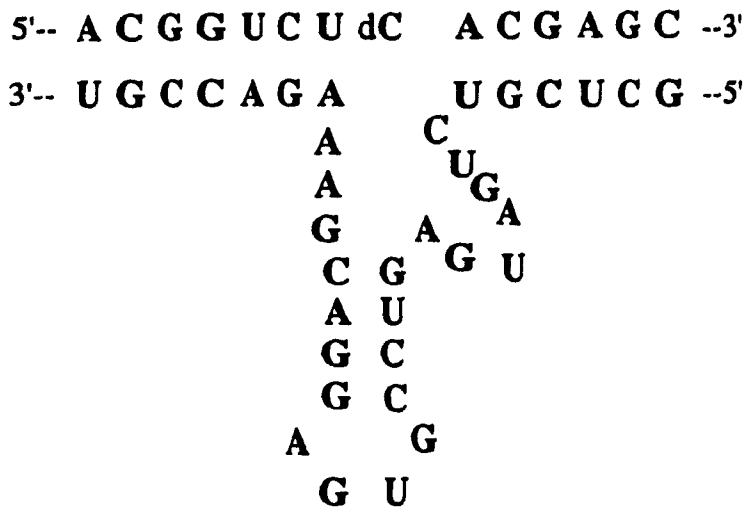


Figure 30. Ribozyme and its analogue of the natural substrate

127. R. J. Cedergren, B.F. Lang, D. Gravel, *FEBS Lett.*, **226**, 63-66 (1987).
128. R.S. Brown, J.C. Dewan, A. Klug, *Biochemistry*, **24**, 4785-4801 (1985).

To test the proposal, the substrate oligonucleotide with deoxycytidine at the 8th position, (**14dc**) prepared previously (Figure 30), was incubated under the conditions required for the reaction. While the natural all ribonucleotide substrate was almost quantitatively cleaved, the dC_8 analogue showed no cleavage at all (Figure 27, lane 2). The result unambiguously shows that the 2'-hydroxyl group on the C at position 8 is required for the reaction.

4.5. Mixed DNA-RNA Polymers as Novel Types of Ribozymes

In order to further characterize some aspects of the structural element of ribozyme itself, we prepared hybrid ribozyme sequences and subjected them to this technique. Although the cleavage reaction requires an adjacent 2'-hydroxyl at the cleavage site in the substrate, no 2'-hydroxyl would seem to be necessary in the ribozyme. Since it is not practical to test the 2'-OH requirement of each residue in the ribozyme, the 8 consensus nucleotides (Figure 8, Chapter 1) in the non-duplex region were chosen. We first designed two 35-mer ribozymes having 8-deoxy nucleotide and 5-deoxy nucleotide substitution respectively. The sequences are shown in Figure 31.

The two hybrid ribozymes were incubated with the natural 14-mer RNA substrate under the standard conditions for ribozyme reaction. The 8-deoxy analogue was shown to have an activity of 15% of the natural one and the 5-deoxy analogue has 50% of the natural one (Figure 32).

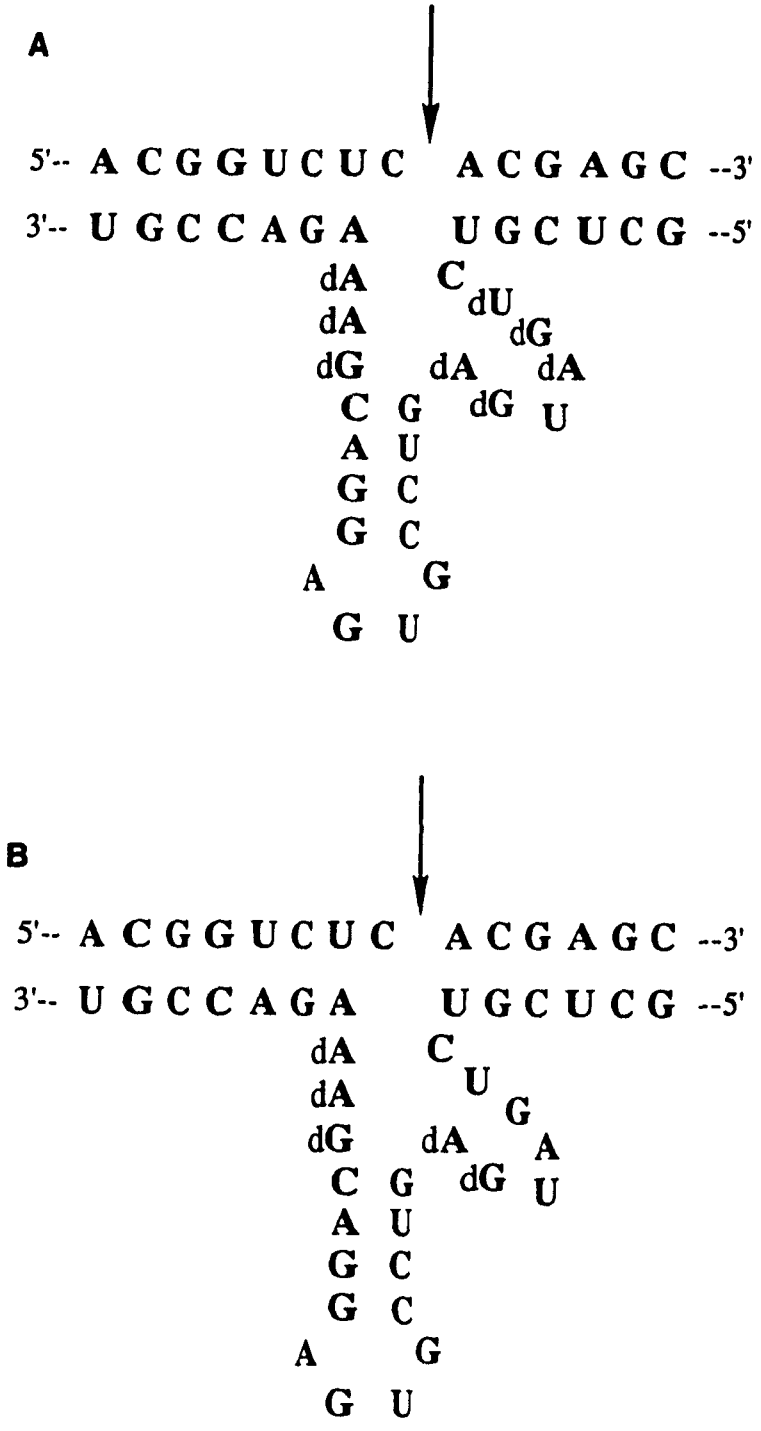


Figure 31. Structure model of 8-deoxy (A) and 5-deoxy (B) ribozymes.

Figure 32. Catalytical activity of mixed DNA-RNA ribozymes. Lane 1, 8-deoxy ribozyme; Lane 2, 5-deoxy ribozyme, Lane 3, all RNA ribozyme. Polyacryamide gel, 20%/8M urea.

The fact that the 8-deoxy ribozyme has such low activity compared with the all RNA ribozyme suggests that the 2'-hydroxyl group plays an important role in catalysis. In the ribozyme model by Gerlach (Figure 23), the bases in the duplex region and those in the loop region have quite different roles in the catalytic activity[33]. The former are involved in the classic Watson-Crick base pairing to form a duplex structure. Any of these base pairs can be replaced with another one without affecting the ribozyme activity. On the other hand, the bases in the loop are not involved in base pairing and they are highly conserved. Substitution of any of these bases has been shown to effect or destroy the catalysis[129], indicating that each of these bases is essential for the ribozyme activity. It has been shown in a tRNA that those bases in the single strand loops are involved in the tertiary interaction

129. M. Koizumi, S. Iwai, E. Ohtsuka, *FEBS Lett.*, 228, 228-230 (1988).

to generate the active L-shaped tertiary structure[130]. It is highly possible that these bases play the same role as those in a tRNA. A metal cation has been shown to be essential for the catalytic reaction. In the well studied metal cation catalyzed tRNA hydrolysis[5], the metal was found to bind to the ribonucleoside units in the molecule. These binding sites hold the divalent metal cation in a position that allows the metal cation to increase the nucleophilicity of the 2'-hydroxyl of the cleavage site. It seems to be also possible that 2'-hydroxyls of some of these bases are directly involved in the binding of magnesium.

On the other hand, the 5-deoxy ribozyme was shown to have 50% of the activity of the all RNA ribozyme. Comparison of this 5-deoxyribozyme with 8-deoxyribozyme allows one to identify influential 2'-hydroxyls. The significant increase in activity seen in the 5-deoxy suggests that at least one of the three hydroxyls in position 8, 9, and 10 is important to the ribozyme activity.

To define the specific hydroxyl essential for the ribozyme, one could prepare a series of molecules in which the U, G, and A in position 8, 9, and 10 are selectively replaced by their deoxy analogues. This project would be an extension of the present work.

4.6. Conclusion

As the first step toward the study of ribozymes using the synthetic RNA technique, several ribozymes have been chemically synthesized. The fact that the synthetic ribozymes have a comparable biological activity with the one prepared with standard biochemical methods has further demonstrated the fidelity of the synthetic methodology. In addition, chemical synthesis is straightforward and has wide flexibility compared to the biochemical method. The flexibility of the chemical method has been illustrated in the synthesis of rationally modified ribozymes for mechanistic study.

A general procedure to prepare the mixed DNA-RNA polymers has been described. This provides a highly specific approach to selective mutation of natural DNA or RNA in the study of structure and function. This technique is likely to be useful in mutagenesis and cloning in the future.

The potential of using novel DNA-RNA hybrids in molecular biology has been amply demonstrated in this study of RNA catalysis. We have made the DNA-RNA polymers whose base sequence correspond to a ribozyme and also its substrate. By studying

130. A. Rich, *Acc. Chem. Res.* **10**, 388-396 (1977).

the activity of the mutated substrate, we have provided, for the first time, evidence of the direct involvement of 2'-hydroxyl of the nucleoside at the cleaving site in the substrate. The modified ribozyme was found to have a much lower activity than the unmodified one. The result suggests that not only the 2'-hydroxyl group at the cleavage site in the substrate is essential but also some of the 2'-hydroxyl groups of the consensus bases in the ribozyme are important for the ribozyme activity. This study has provided insight into the mechanism of catalysis by "hammer-head" ribozymes. Future work will be directed at identifying the specific hydroxyl essential for the catalysis.

CHAPTER 5

PROOF OF THE FIDELITY OF ALKYLSILYL GROUPS IN OLIGORIBONUCLEOTIDE SYNTHESIS

5.1. Introduction

The use of alkylsilyl groups for 2'-protection is the key to our development of a procedure for oligoribonucleotide synthesis. We have demonstrated the use of alkylsilyl groups as the 2'-hydroxyl protecting group in the phosphoramidite coupling method in the solid phase synthesis of a number of oligoribonucleotides.[67] Synthetic molecules were found to have the same distinctive biological activity known for the natural RNAs. For example the synthetic analogue of a tRNA retains some amino acid acceptance activity.[88] Recently, several ribozymes have been synthesized. These small catalytical RNAs do not have a rare base in their molecules and they can be totally synthesized by the standard RNA synthesis technique. The synthetic ribozyme was shown to have an activity comparable to the one made by biochemical methods (see Chapter 4). The synthetic method has been successfully used in the mechanistic study of the ribozyme reactions. Enzyme sequencing of synthetic RNA molecules indicated the correct nucleotide sequences. All these biochemical characterizations have clearly shown the fidelity of the synthetic product.

Despite these results there have been some concerns in literature about the stability of *t*-butyldimethylsilyl as a 2'-protecting group in the oligoribonucleotide synthesis and the phosphate linkage in the synthetic product.[131,132] As a result, this study was undertaken to prove that silyl groups are effective as the 2'-hydroxyl protecting group in the phosphoramidite approach in the synthesis of oligoribonucleotides. We provide NMR and HPLC data establishing the phosphate linkage of the intermediates during the synthesis and in the final product from the synthesis.

To examine the synthetic intermediate at each stage of a synthesis, solution synthesis of a series of 3'-5' dinucleotides, A_pU, C_pU, G_pU, U_pU, has been carried out using 2'-silylated nucleoside 3'-O-phosphoramidites. Dinucleotides with an unusual 2'-5' phosphate

131. C.B. Reese, *Nucleosides & Nucleotides* 4, 117-127 (1985).
132. C.B. Reese, *Ibid* 6, 121-129 (1987).

linkage, ApU, CpU, GpU, UpU, were also synthesized using 3'-silylated nucleoside 2'-O-phosphoramidites for comparison. The intermediates in the synthesis of these two series of compounds were analyzed by ^{1}H NMR, ^{31}P NMR, and HPLC and then compared. A uridine 3'-5' dinucleotide was prepared on a solid support. This sample was compared with the one prepared via the solution procedure and with a standard commercial sample.

5.2. Isomeric Purity of N-Acylated 5'-Tritylated 2'-Silylated Nucleosides (2a,b,d,7c)

The preparation of pure 2'-hydroxyl protected nucleosides is the first key step in order to prepare the ribonucleotide with the correct phosphate linkage, since it will determine the hydroxyl that will be phosphorylated and thereafter the position of the phosphate linkage. Of equal importance is that the procedure has to be straightforward to allow the large scale preparation of protected nucleoside monomers. The preparation of 2'-silylated nucleosides involves a one step reaction and a one step purification.[65,72] The purification to separate the two isomers is performed on a routine silica gel column with a common solvent system. Following the standard procedure[65,67b], N-protected 5'-tritylated nucleosides (**1a,b,d,6c**, Chapter 1&2) were silylated. The crude product from the reaction is a mixture of the 2'-silylated and 3'-silylated isomers for each nucleoside. The two isomers were separated by silica gel column chromatography using a previously described solvent system (ref. 65 and Chapter 2). TLC of the purified isomer, either 2'-silylated (**2a,b,d,7c**) or 3'-silylated (**3a,b,d,8c**), in Solvent D shows one single spot (Table 4).

2a,b,d,7c from the purification were analyzed by ^1H NMR spectroscopy and the spectra were compared with those of **3a,b,d,8c**. The ^1H NMR chemical shifts data are shown in Table 4. To illustrate the spectral interpretation of the region of ribose and silyl protons , the proton spectra of 2'-silylated (**2d**) and 3'-silylated (**3d**) uridine, together with the spectrum for an artificial mixture of the two isomers (c.a. 90% **3d**, 10% **2d**) are presented (Fig. 33). **2d** and **3d** can be distinguished by their different proton chemical shifts. In particular, the chemical shifts of the anomeric proton for each isomer are distinctive. The two isomers are also easily distinguishable by their different chemical shifts of silyl protons. While an artificial mixture of the two isomers shows the presence of signals for both isomers as expected, either sample **2d** or sample **3d** shows only its corresponding signals, suggesting that the purified **2d**, the desirable isomer for nucleotide synthesis, is free of its isomer **3d**. The same results were observed for guanosine (**7c**, **8c**), cytidine (**2b**, **3b**), and adenosine (**2a**, **3a**).

The 2'-silylated isomer also has a different ^{29}Si chemical shift from its 3'-silylated isomer.[133]

133. S. Boisvert, Ph.D. Thesis, McGill University, Montreal, 1986.

Table 4. ^{1}H NMR Data[a] and Rf Values[b] of 2'-Silylated Ribonucleosides (2a,b,d,7c) and 3'-Silylated Ribonucleosides (3a,b,d,8c).

Compd	H1' (J)	H2 (5)	H 8(6)	OCH_2CO	OCH_3	*t*-Bu	MeSi	SiMe	Rf
2a	6.08 (d, 5.4)	8.23	8.71	4.84	3.78	0.82	-0.03	-0.17	0.60
3a	6.05 (d, 5.0)	8.25	8.77	4.85	3.77	0.88	0.08	-0.01	0.36
2b	5.94 (s)	8.48	7.88		3.82	0.93	0.32	0.20	0.56
3b	6.06 (d, 2.6)	8.42	7.87		3.81	0.81	0.02	-0.10	0.14
7c	5.94 (d, 5.8)		7.93	4.57	3.76	0.99	0.92	0.90	0.52
8c	5.92 (d, 4.3)		7.95	4.61	3.77	0.97	0.97	0.97	0.24
2d	5.92 (d, 3.1)	5.25	7.92		3.80	0.92	0.17	0.15	0.70
3d	5.93 (d, 4.1)	5.35	7.50		3.79	0.84	0.05	-0.06	0.46

[a] Signals are referenced to $CDCl_3$. [b]TLC solvent: Solvent D.

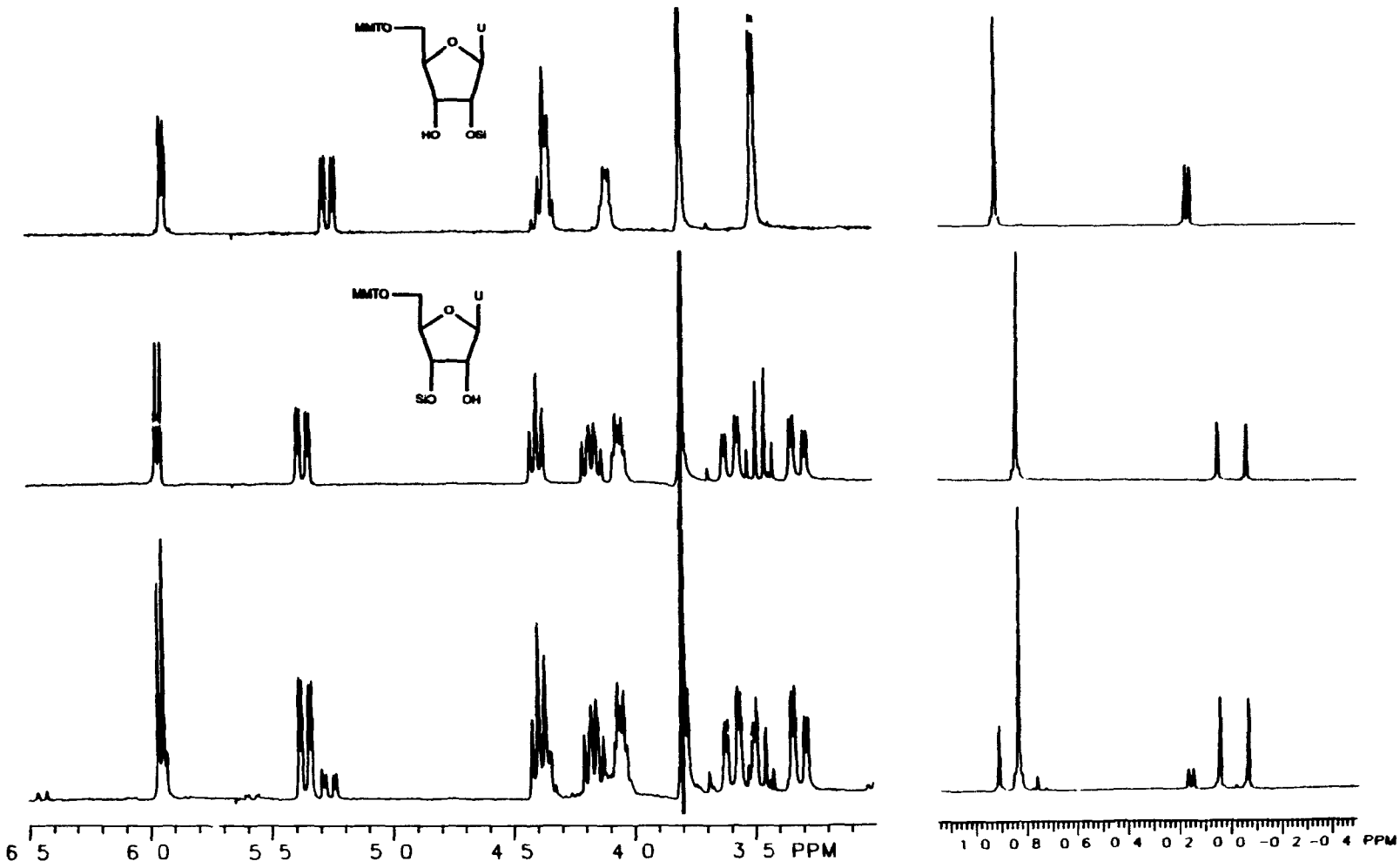


Figure 33. ^{1}H NMR spectra of 2'-silylated uridine (2d), 3'-silylated uridine (3d), and an artificial mixture of the two.

5.3. Isomeric Purity of 2'-Silylated Nucleoside 3'-O-Phosphoramidites (4a,b,d,9c)

The isomeric purity of the 2'-protected, 3'-phosphorylated nucleoside is crucial in the synthesis of oligoribonucleotides since this will determine that only 3'-5' phosphate linkages will be introduced in the coupling step. A 2'-hydroxyl protecting group in the 2'-protected nucleoside should therefore be stable under the conditions required to phosphorylate the 3'-hydroxyl group.

The 2'-silyl group in compounds **2a,b,d,7c** is unique because its stability differs from the case where a *t*-butyldimethylsilyl is on an ordinary secondary hydroxyl, for example, the silyl group on the 3'-hydroxyl of deoxyribonucleoside. The presence of the adjacent cis 3'-hydroxyl group makes the 2'-silyl group susceptible to isomerization under certain conditions to give a mixture of 2'-silylated and 3'-silylated nucleoside. The stability of the silyl groups in **2a,b,d,7c** under various conditions has been extensively discussed previously.[134] The 2'-silyl group was found to isomerize in the neutral protic solvent such as methanol, ethanol.[135] Others have also found that it isomerizes in pyridine/water, wet pyridine or DMF.[136] On the other hand, HPLC analysis of the 2'-silylated nucleoside exposed to various solvents has clearly shown that the 2'-silyl group is stable in a dry aprotic solvent such as chloroform, or DMF.[23] The stability of the 2'-silyl group in nucleotide synthesis was also recently confirmed by Stawinski[77] in preparing the 2'-silylated nucleoside H-phosphonates for ribonucleotide synthesis.

4a,b,d,9c were prepared from **2a,b,d,7c** as previously described (ref 67 and Chapter 2). The isomeric 3'-silylated nucleoside 2'-phosphoramidites for the four nucleosides were prepared for comparison. The isomeric purity of the 3'-amidites was established by comparing them with the corresponding 2'-amidites. 2'-Amidites were also used later to prepare the 2'-5' phosphate linked dinucleotides. The preparation of these 2'-amidites (**13a-d**) is similar to that of the 3'-amidite (Scheme 17). The key intermediates are 3'-silylated nucleosides (**3a,b,d,8c**). To illustrate the procedure, a dry THF solution of **3a** was added to a dry THF solution containing a slight excess of chloro(diisopropylamino)methoxyphosphine in the presence of diisopropylethylamine and a catalytical amount of DMAP at room temperature. The phosphorylation is generally completed in 4-6 h with **8c** being longer (12

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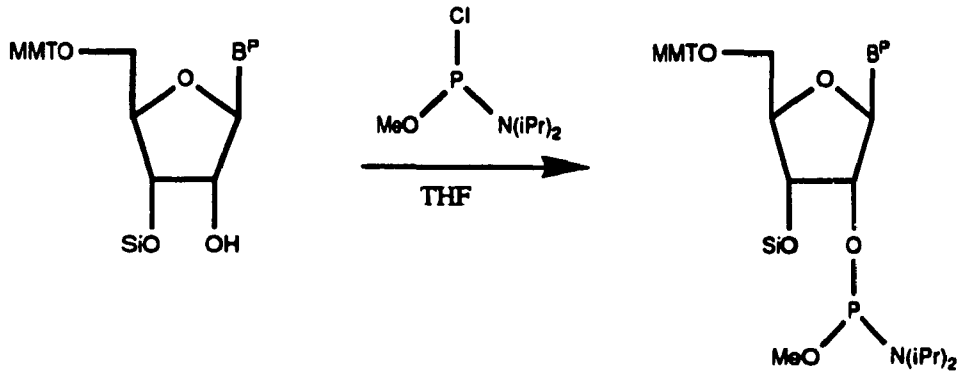
134. K.K. Ogilvie, *Nucleosides, Nucleotides and Their Biological Applications* Academic: New York, 209-256 (1983)
135. K.K. Ogilvie, D.W. Entwistle, *Carbohydrate Res.* **89**, 203-210 (1981).
136. S.S. Jones, C.B. Reese, *J. Chem. Soc. Perkin Trans.* **1**, 2762 (1979).

h). After the workup of the reaction, the crude product was purified by silica gel column chromatography. **13a-d** were prepared in yields of 70-80%.

SCHEME 17



3a,b,d,8c **13a-d**

^1H and ^{31}P NMR data for **4a,b,d,9c** and **13a-d** are shown in Table 5. Examination of the data in Table 5 suggests several interesting features. ^1H NMR indicates that the ribose protons and silyl protons of a 3'-amidite have different chemical shifts from those of its corresponding 2'-isomer. In particular, the anomeric proton and the silyl protons in each amidite are distinctive. The two isomers are easily distinguishable by their different anomeric and silyl proton chemical shifts. In addition, the anomeric proton of the 2'-amidite is shifted downfield compared to that of the corresponding 3'-amidite. This is probably related to the inductive effect of the 2'-phosphoramidite on the anomeric position. ^{31}P NMR of an amidite is much simpler and easier to diagnose. ^{31}P NMR of a 3'-amidite , like that of a 2'-amidite, shows two peaks corresponding to a pair of diastereomers. There is a substantial difference between the ^{31}P chemical shifts of a 3'-amidite and its corresponding 2'-amidite.

Table 5. ^{1}H and ^{31}P NMR Data of 2'-O-Silylated Nucleoside 3'-O-Phosphoramidites (**4a,b,d,9c**) and 3'-O-Silylated Nucleoside 3'-O-Phosphoramidites (**13a-d**)

Compd	^{1}H NMR chemical shifts									^{31}P
	H1' ($J_{1'-2'}$)	H2(5)	H6(8)	OCH_2CO	$POCH_3(J_{P-H})$	OCH_3 (MMT)	t-Bu	MeSi	SiMe	
4a	6.10 (d, 6.1)	8.23	8.71		3.21 (d, 13.1)	3.77	0.76	-0.01	-0.20	149.8
	6.03 (d, 6.0)	8.23	8.67		4.42 (d, 13.2)	3.77	0.75	-0.03	-0.24	151.7
13a	6.22 (d, 5.4)	8.21	8.73		3.30 (d, 13.1)	3.78	0.87	0.12	0.04	150.8
	6.17 (d, 5.5)	8.20	8.70		3.00 (d, 13.0)	3.77	0.85	0.08	0.03	150.3
4b	5.92 (d, 1.2)	7.84	7.84		3.23 (d, 13.1)	3.82	0.91	0.26	0.16	150.1
	5.84 (s)	7.84	7.84		3.33 (d, 13.1)	3.82	0.90	0.25	0.14	149.1
13b	6.26 (d, 3.5)	7.87	7.87		3.40 (d, 13.2)	3.82	0.77	0.03	-0.06	151.2
	6.08 (d, 1.4)	7.87	7.87		3.33 (d, 13.1)	3.81	0.74	0.02	-0.11	150.2
9c	6.03 (d, 6.0)		7.96	4.58	3.40 (d, 13.1)	3.77	0.97	0.97	0.97	152.3
	5.98 (d, 5.4)		7.94	4.53	3.14 (d, 13.0)	3.77	0.97	0.97	0.97	150.4
13c	6.11 (d, 6.4)		7.93	4.62	3.29 (d, 13.1)	3.77	1.09	1.06	1.02	152.2
	6.05 (t)		7.93	4.60	3.07 (d, 13.1)	3.77	1.09	1.06	1.02	150.9
4d	5.94 (d, 3.7)	5.16	8.07		3.40 (d, 13.1)	3.79	0.90	0.13	0.13	150.4
	5.83 (d, 3.1)	5.12	8.04		3.25 (d, 13.1)	3.79	0.88	0.13	0.11	150.3
13d	6.16 (d, 5.4)	5.34	8.01		3.34 (d, 13.0)	3.79	0.81	0.07	-0.01	151.1
	6.03 (t)	5.26	7.82		3.27 (d, 13.0)	3.79	0.79	0.05	-0.04	150.2
4e[a]	6.08 (d, 6.0)	8.24	8.69	4.83	3.20 (d, 13.1)	3.77	0.75	-0.02	-0.20	151.9
	6.02 (d, 5.9)	8.19	8.65	4.83	3.41 (d, 13.0)	3.77	0.74	-0.04	-0.25	150.1
13e[a]	6.20 (d, 5.6)	8.43	8.70	4.84	2.97 (d, 13.0)	3.78	0.87	0.11	0.03	151.2
	6.15 (d, 5.7)	8.20	8.67	4.83	3.29 (d, 13.0)	3.77	0.85	0.08	0.02	151.1

[a] N-Phenoxyacetyl protected adenosine methylphosphoramidites.

^{1}H NMR (Fig. 34) and ^{31}P NMR (Fig. 35) of uridine 2'-amidite (**13d**) and 3'-amidite (**4d**) are presented. In addition to the above features, the proton spectra indicate that no silyl and anomeric proton signals corresponding to 2'-amidite were detected in the spectrum of 3'-amidite and *vice versa*. ^{31}P NMR of an artificial mixture of **13d** and **4d**, prepared by mixing the two pure amidites, shows the expected signals corresponding to the two isomers, that of **13d** indicates that there exist only signals of its own. ^{1}H and ^{31}P NMR of phosphoramidites for adenosine, cytidine and guanosine have the same results. Within the detection range of NMR, the 3'-amidite is clearly free of the isomeric contamination. The preparation of the isomerically pure 3'-amidite further proves that the 2'-silyl group is stable under the phosphorylation conditions.

The preparation of pure 2'-silylated 3'-phosphorylated nucleosides and the successful removal of the 2'-protecting group in the end (*vide infra*) are two crucial steps in the synthesis of oligoribonucleotides. The 3'-phosphorylated nucleoside will dictate that only the 3'-hydroxyl will be linked to the 5'-hydroxyl in the condensation.

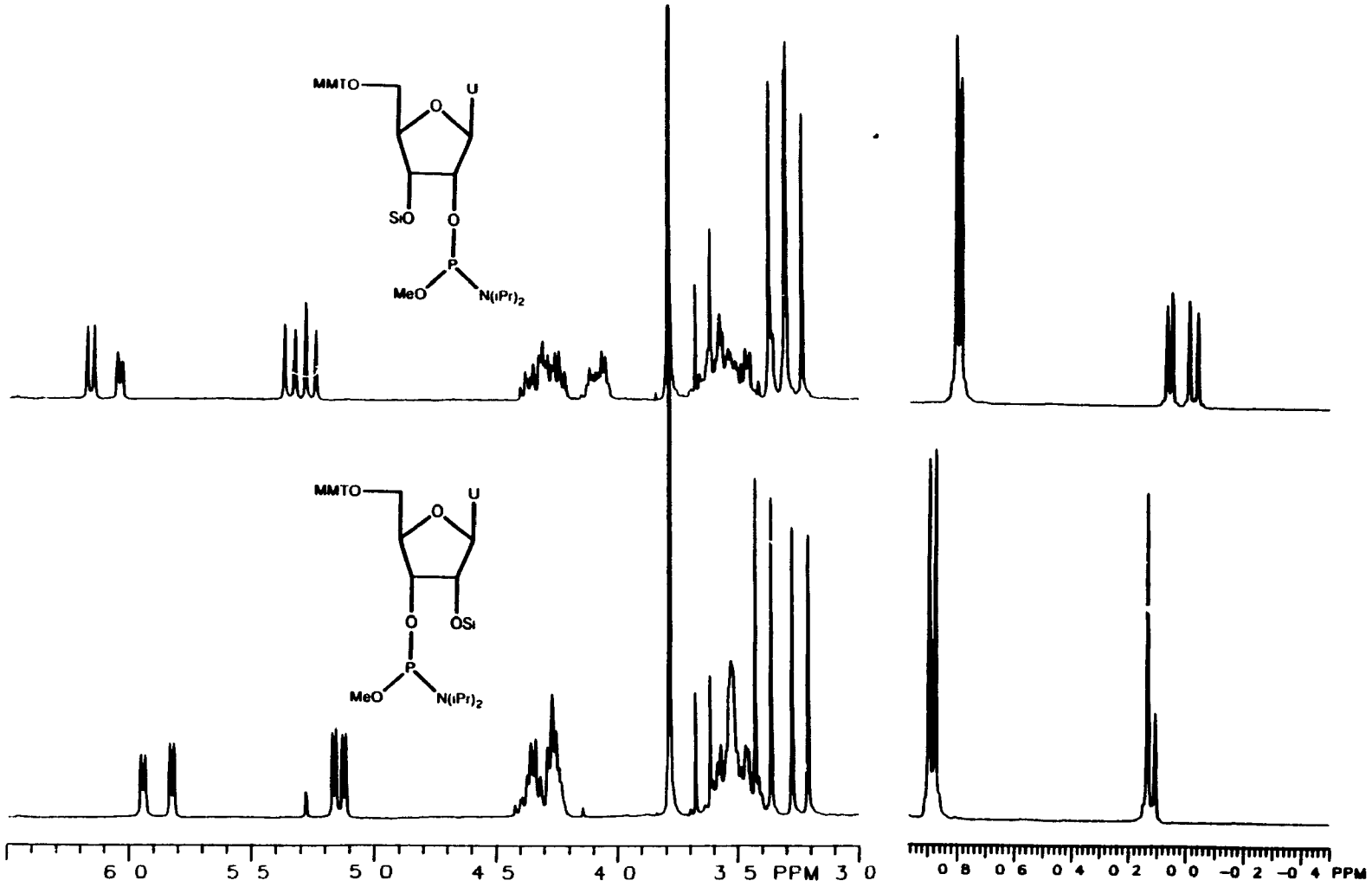


Figure 34. ^{1}H NMR spectra of the uridine nucleoside 3'-methylphosphoramidite (4d) and 2'-methylphosphoramidite (13d)

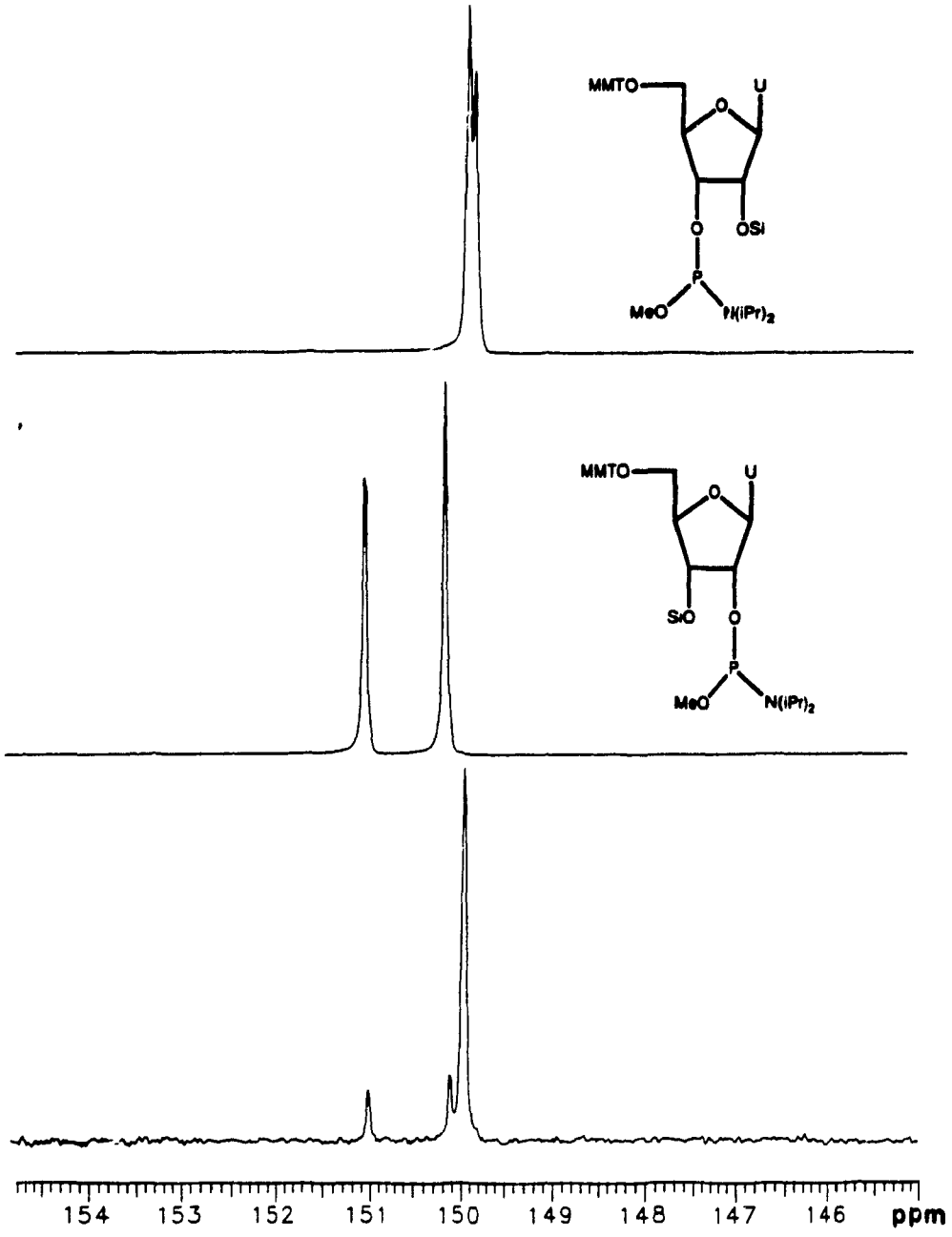


Figure 35. ^{31}P NMR of the uridine nucleoside 3'-amidite, 2'-amidite, and the artificial mixture of the two.

5.4. Preparation of Protected 3'-5' Dinucleotides (16a-d)

Dinucleotides A_pU, C_pU, G_pU, and U_pU (3'-5') were prepared to demonstrate the use of nucleoside 3'-amidites in the synthesis of oligoribonucleotides. The isomeric 2'-5' linked nucleotides, ApU, CpU, GpU, and UpU were also prepared using nucleoside 2'-amidites for comparison.

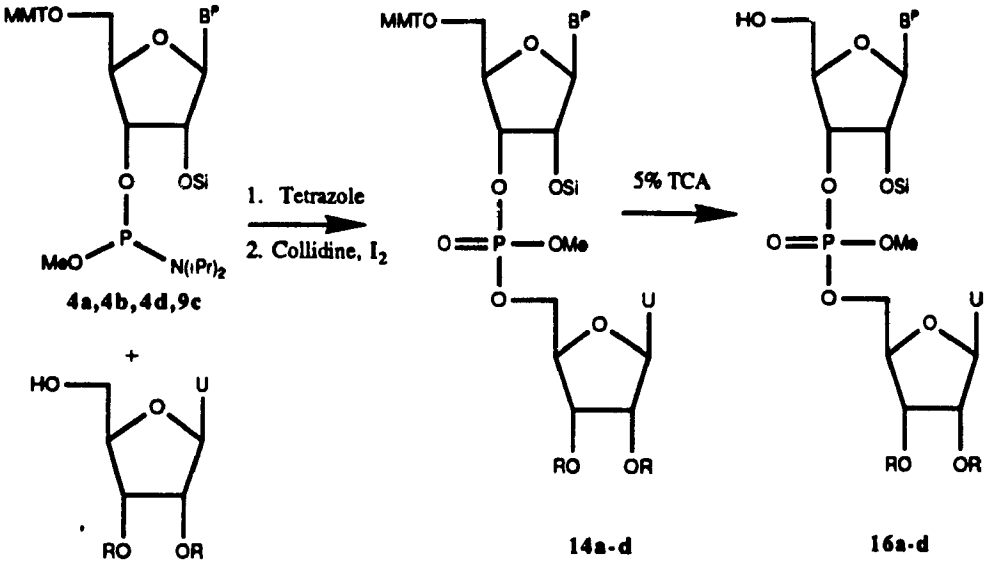
The synthesis of 3'-5' linked dinucleotides involves similar steps to those used in the solid phase synthesis, i.e., assembling the chain to obtain the protected dinucleotide; deprotecting other protecting groups to isolate the 2'-silylated nucleotide; and finally removing the 2'-silyl group to give the fully deprotected ribonucleotide

The synthesis started by the condensation between the 3'-amidites (**4a,b,d,9c**) and 2',3'-protected uridine to give the fully protected 3'-5' dinucleotides **14a-d** (Scheme 18, A). Thus each 3'-phosphoramidte (**4a,b,d,9c**, 1.2 to 1.3 eq.), 2',3'-acylated (for **4a**, **4b**, and **9c**) or 2',3'-silylated uridine (for **4d**), and tetrazole (4 equivalents relative to the phosphoramidite) were mixed in a dry vial. The reaction was initiated by adding THF to the system. Oxidation by collidine and an aqueous iodine solution gave the fully protected dinucleotides (**14a-d**). Purification on silica gel column gave pure **14a-d**.

The fully protected dinucleotides containing 2'-5' pnosphate linkage (**15a-d**) were prepared following essentially the same procedure but using **13a-d** (Scheme 18, B).

SCHEME 18

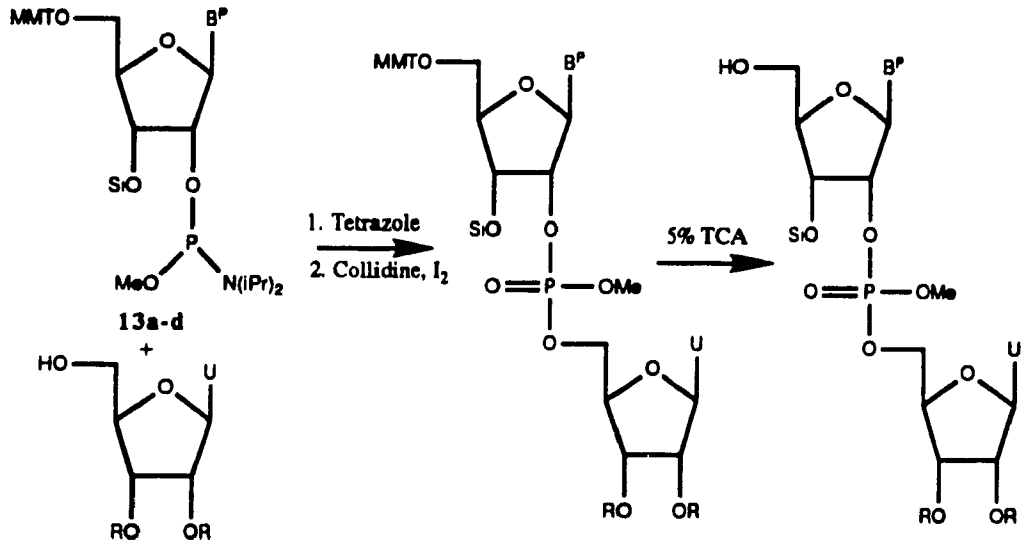
A



14a-d **16a-d**

14a-c, 16a-c: R = acetyl; **14d, 16d:** R = Si

B



15a-d **17a-d**

15a-c, 17a-c: R = acetyl; **15d, 17d:** R = *t*-butyldimethylsilyl

^{31}P and ^{1}H NMR data of **14a-d** and **15a-d** are shown in Table 6. Each dinucleotide has the two signals corresponding to a pair of diastereomers in ^{31}P NMR. The 3'-5' dinucleotide has different phosphorus chemical shifts from the corresponding 2'-5' linked dinucleotide. The proton chemical shifts of the silyl group are diagnostic in distinguishing the two isomeric nucleotides. 2'-silyl protons of the purine nucleoside in a 3'-5' linked dinucleotide (**14a, 14c**) have chemical shifts at a higher field than 3'-silyl protons of the purine nucleoside in a 2'-5' linked nucleotide (**15a, 15c**). For the pyrimidine nucleotides (**14b,d** vs **15b,d**) the shifts are reversed.

These features are illustrated by ^{1}H NMR (Fig. 36) and ^{31}P NMR (Fig. 37) for compounds **14a** and **15a**. Comparison of the spectra for the two isomers further indicates that neither of them has the signals from its isomer.

14a-d and **15a-d** were then detritylated to form **16a-d** and **17a-d** respectively (Scheme 18). **14a-d** and **15a-d** were treated with a large excess of 5% trichloroacetic acid in CH_2Cl_2 for half an hour at room temperature. Excess acid was neutralized by aqueous sodium bicarbonate solution. The product was purified either by silica gel chromatography or simply by precipitation in ether.

16a-d and **17a-d** were also characterized by ^{1}H NMR and ^{31}P NMR (Table 7). Similarly, the ^{31}P chemical shifts and the proton chemical shifts of the silyl group of each dinucleotide are diagnostic in differentiating the isomeric nucleotides. The 2'-silyl protons in the 3'-5' dinucleotide appear at higher field compared to the 3'-silyl protons in the 2'-5' dinucleotides. Examination of data in Table 6 and 7 indicates that the removal of the 5'-trityl group has the opposite consequence on the chemical shifts of 2'-silyl protons in 3'-5' dinucleotides and on those of 3'-silyl protons in 2'-5' dinucleotides. The chemical shifts of 2'-silyl protons in the detritylated 3'-5' dinucleotide are at a higher field compared to those of corresponding tritylated dinucleotide. On the other hand, the chemical shifts of 3'-silyl protons in detritylated 2'-5' dinucleotide are at a lower field compared to the corresponding tritylated ones. This is probably due to the fact that the 5'-trityl has a shielding effect on the 3'-silyl group in the 2'-5' dinucleotide (**15a-d**) while this 5'-trityl has a deshielding effect on the 2'-silyl group in the 3'-5' dinucleotide because of their relative position to each other.

Table 6. ^{1}H NMR and ^{31}P NMR Data of Fully Protected Dinucleotides (**14a-d, 15a-d**)

	^{1}H NMR Chemical Shifts (ppm)													^{31}P
	X			U			POMe (J_{P-H})	OMe (MMT)	OAc	OAc	t-Bu	MeSi	SiMe	
	H1'	H2(5)	H8(6)	H1'	H6	H5								
14a	6.04- 6.18	8.23 8.28	8.67 8.63	6.04- 6.18	8.01 8.04	5.76 5.78	3.82 (d, 11.4) 3.76 (d, 11.4)	3.78 3.78	2.14 2.12	2.07 2.07	0.72 0.69	-0.06 -0.08	-0.28 -0.29	0.1 -0.5
15b	6.28- 6.32	8.26 8.25	8.72 8.70	5.95- 6.40	8.04 8.03	5.75- 5.71	3.76 (d, 11.4) 3.58 (d, 11.4)	3.77 3.76	2.07 2.07	2.02 2.03	0.86 0.85	0.12 0.11	0.03 0.03	0.0 -0.3
14b	5.92- 5.99	7.18 7.18	8.20 8.20	5.92 5.99	8.20 8.20	5.62- 5.70	3.73 (d, 11.3) 3.60 (d, 11.2)	3.82 3.81	2.10 2.09	2.07 2.07	0.90 0.89	0.22 0.23	0.13 0.13	0.1 0.2
15b	6.14- 6.20	7.62 7.79	8.43- 8.56	6.14- 6.20	7.86 7.86	5.73- 5.82	3.84 (d, 11.4) 3.89 (d, 11.4)	3.83 3.82	2.10 2.09	2.05 2.03	0.75 0.73	0.07 0.05	-0.11 -0.09	0.3 -0.6
14c	6.11 (d) 6.16 (d)		7.93 7.93	5.85- 5.91	7.52 7.60	5.85- 5.91	3.80 (d, 11.4) 3.63 (d, 11.3)	3.76 3.75	2.11 2.10	2.07 2.07	0.97 0.97	0.88 0.88	0.86 0.86	1.4 0.4
15c	6.15 (d) 5.85		7.91 7.91	5.80- 5.89	7.20- 7.40	5.66 5.70	3.55 (d, 11.3) 3.70 (d, 11.4)	3.77 3.75	2.09 2.08	2.07 2.07	1.02 1.02	1.00 1.00	0.99 0.99	0.4 0.2
14d	5.94- 6.00	5.65- 5.70	7.81 7.95	5.94- 6.00	7.50- 7.50	5.65- 5.70	3.62 (d, 11.3) 3.65 (d, 12.3)	3.79 3.65			0.87- 0.89		0.05- 0.13	0.6 0.3
15d	6.09- 6.13	5.75- 5.80	7.68 7.68	6.09- 6.13	8.00 8.00	5.75 5.80	3.81 (d, 11.4) 3.79 (d, 11.3)	3.80 3.80			0.77- 0.89		-0.03 0.07	0.8 0.3

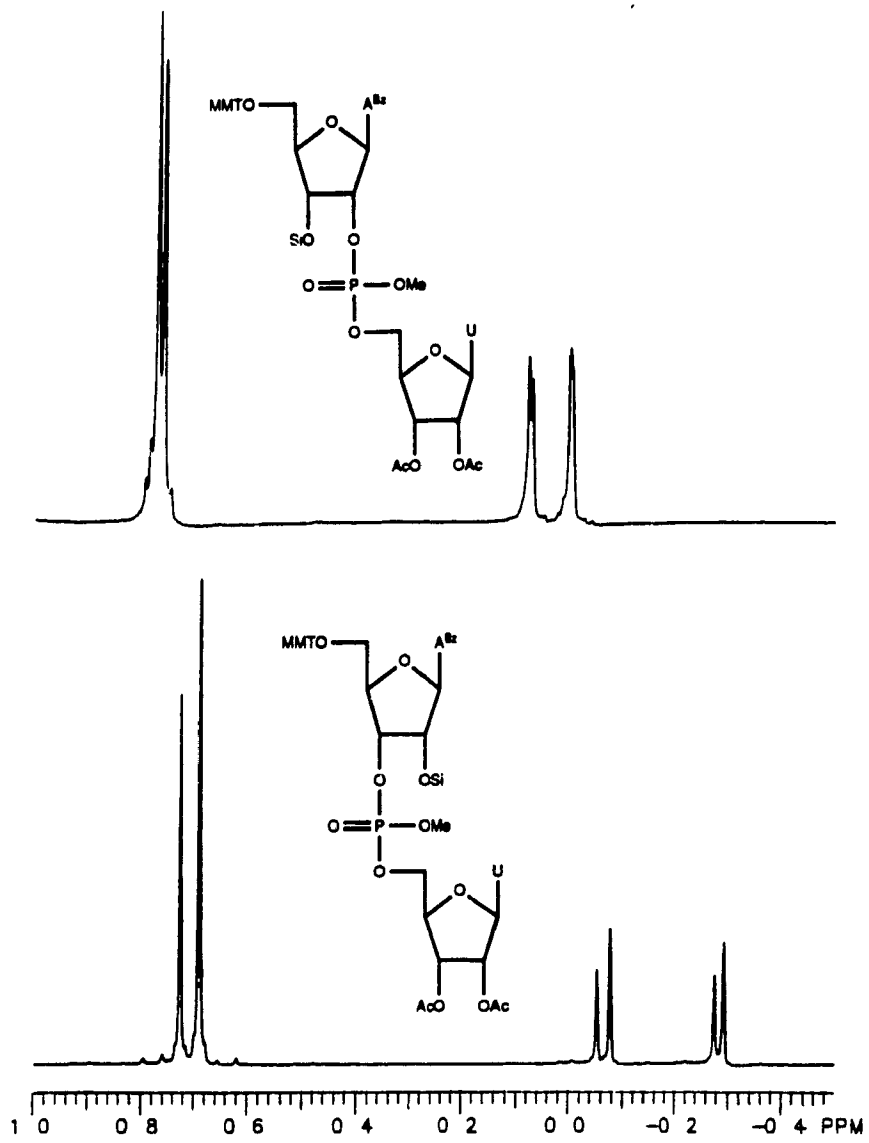


Figure 36. ^{1}H NMR of **14a** and **15a** in the region of *t*-butyldimethylsilyl group.

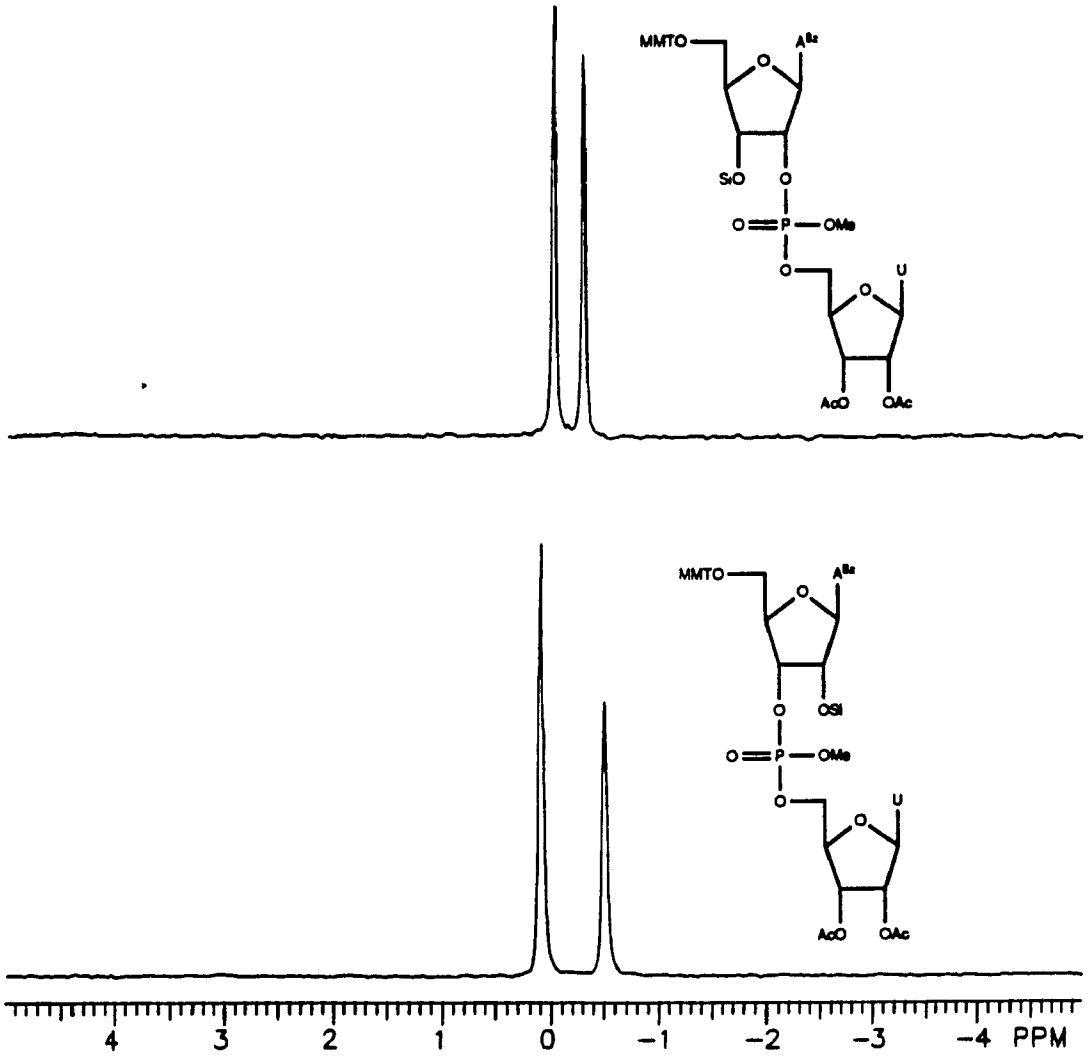


Figure 37. ^{31}P NMR of **14a** and **15a**

Table 7. ^{1}H NMR and ^{31}P NMR Data of Detritylated Dinucleotides (**16a-d, 17a-d**)

	^{1}H NMR Chemical Shifts												^{31}P
	X			U			POMe (J_{P-H})	OAc	OAc	t-Bu	MeSi	SiMe	
	H1'	H2(5)	H8(6)	H1'	H6	H5							
16a	6.00-	8.35	8.79	6.00-	8.02	5.99	3.86 (d, 11.4)	2.15	2.05	0.67	-0.21	-0.4	0.4
	6.18	8.20	8.79	6.18	5.95	8.02	3.86 (d, 11.4)	2.13	2.05	-0.69	-0.18	-0.45	-0.9
17a	6.03-	8.17	8.76	5.73-	8.09	5.92	3.60 (d, 11.4)	2.06	2.02	0.93	0.15	0.15	-0.3
	6.16	8.22	8.74	5.78	8.07	5.89	3.60 (d, 11.4)	2.02	1.98	0.93	0.14	0.14	-0.7
16b	5.79-	7.49	8.31	6.03-	7.89	5.68	3.81 (d, 11.3)	2.12	2.07	0.89	0.16	0.08	1.2
	5.77	7.49	8.43	5.72	7.89	5.69	3.81 (d, 11.4)	2.09	2.03	0.87	0.11	0.08	0.1
17b	5.98-	7.67	8.23	5.98-	7.89	5.78	3.90 (d, 11.3)	2.08	2.03	0.90	0.13	0.11	-0.3
	6.11	7.67	8.32	6.11	7.89	5.78	3.78 (d, 11.4)	2.06	2.00	0.89	0.11	0.10	-0.4
16c	5.75-		8.00	5.75-	7.32-	5.75-	3.86 (d, 11.4)	2.13	2.09	0.84-			0.3
	5.99		8.00	5.99	7.48	5.79	3.86 (d, 11.4)	2.13	2.08	0.89			0.1
17c	5.96-		7.80	5.11-	7.45	5.61-	3.65 (d, 11.4)	2.12	2.07	1.09-			0.3
	6.02		7.80	5.84	7.97	5.84	3.52 (d, 11.3)	2.09	0.08	1.11			0.3
16d	5.63-	5.52-	7.58	5.63-	7.88	5.52-	3.81 (d, 11.4)			0.87-	0.05-		1.7
	5.75	5.59	7.58	5.75	7.88	5.75	3.81 (d, 11.4)			0.88	0.12		1.6
17d	5.83-	5.64-	7.65	5.83-	7.75	6.64-	3.79 (d, 11.3)			0.86-	0.04		0.6
	5.99	5.87	7.65	5.99	7.75	5.87	3.77 (d, 11.3)			0.91	0.16		0.0

^{1}H NMR of silyl protons and ^{31}P NMR for **16a** and **17a** are shown in Figure 38 and 39. Both of them suggest that the sample is isomerically pure as no signal belonging to its isomer was detected.

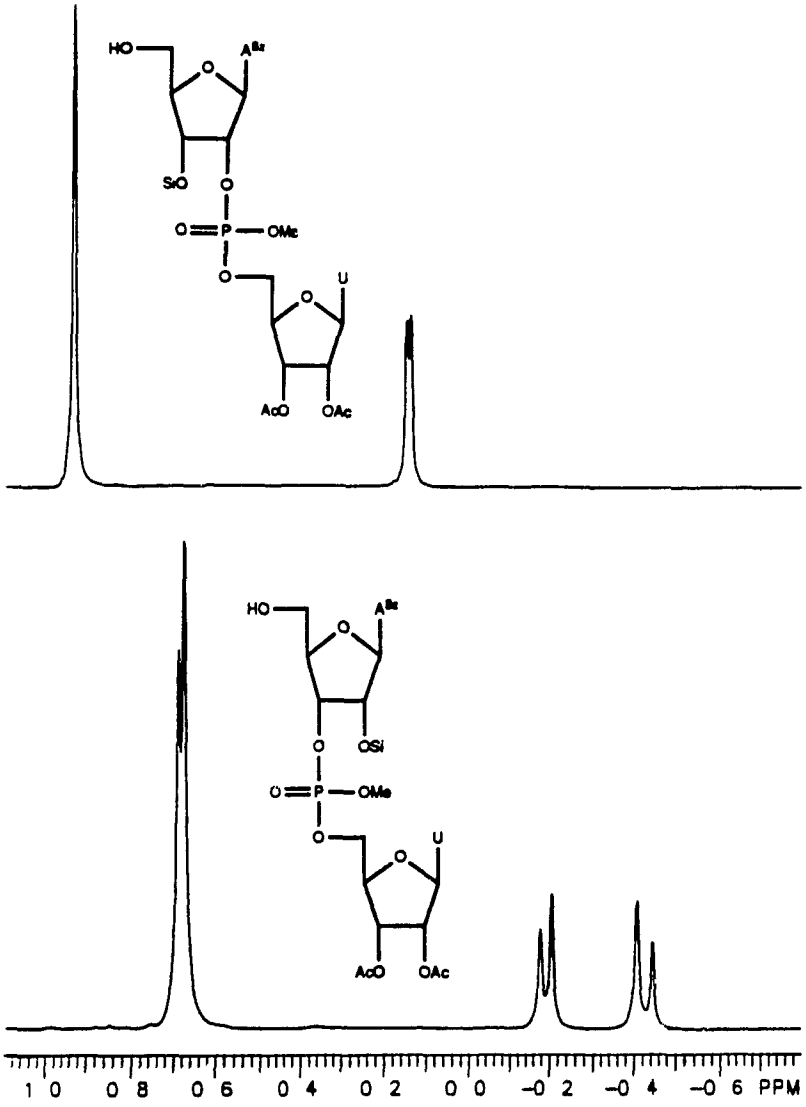


Figure 38. ^{1}H NMR of **16a** and **17a** in the region of *t*-butyldimethylsilyl group.

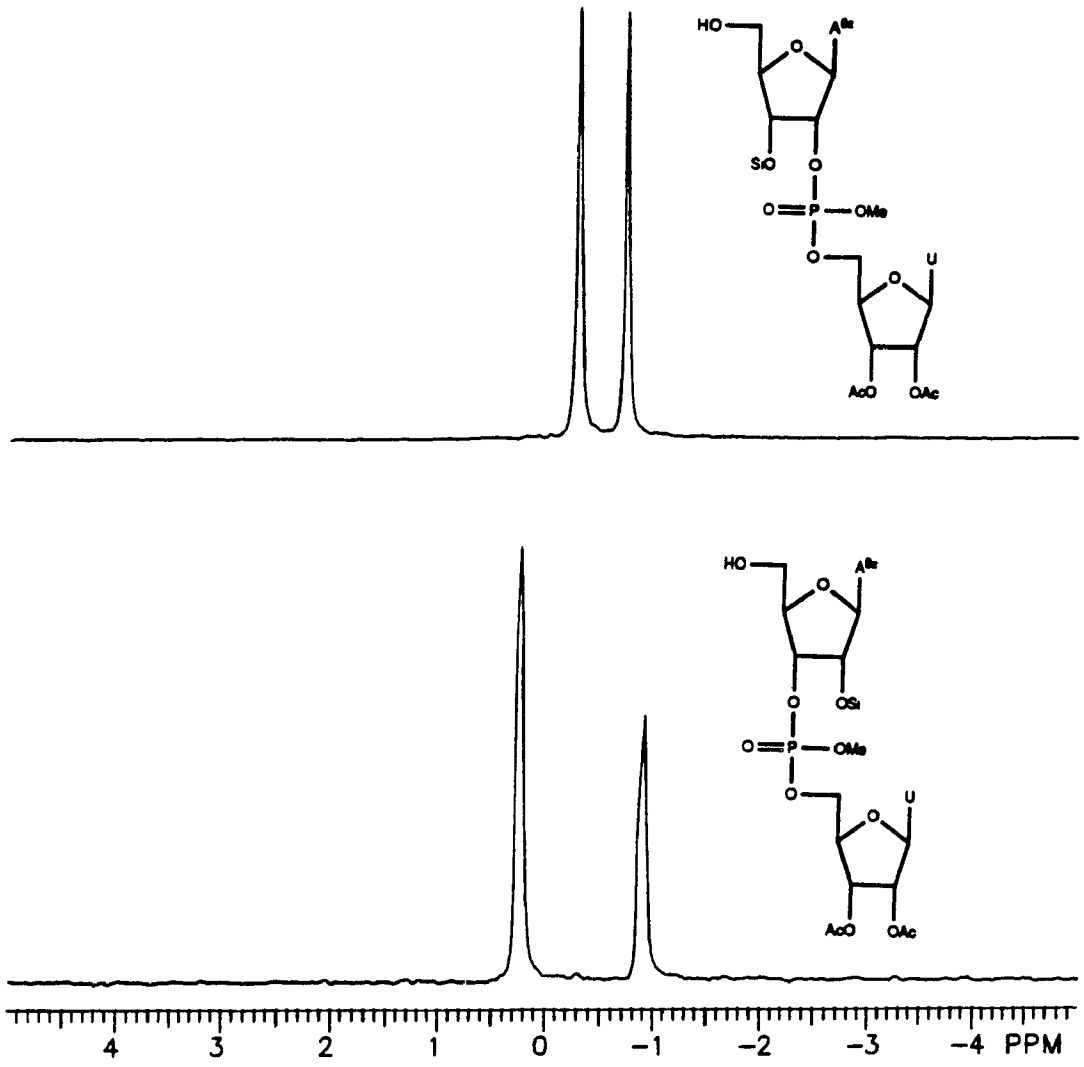


Figure 39. ^{31}P NMR of **16a** and **17a**.

The above results establish that the nucleotide chain assembled using 2'-silylated nucleoside 3'-O-phosphoramidites has the correct structure.

5.5. Isomeric Purity of 2'-O-Silylated Dinucleotides (20a-d)

A 2'-protecting group should also allow the successful removal of other protecting groups. We next analyzed the intermediates in the deprotection of the assembled nucleotides. The first step is to remove the methyl phosphate protecting group. Thus compounds **16a-d** and **17a-d**, typically 10-50mg, were treated with excess thiophenoxide in an Eppendorf tube to give N-protected 2'-silylated 3'-5' dinucleotides (**18a-d**) and 2'-5' dinucleotides (**19a-d**) respectively (Scheme 19, **A** and **B**). TLC of the reaction mixture in solvent B indicated that the starting material was converted to a very polar compound. The excess thiophenol was removed by precipitation of crude product in hexane.

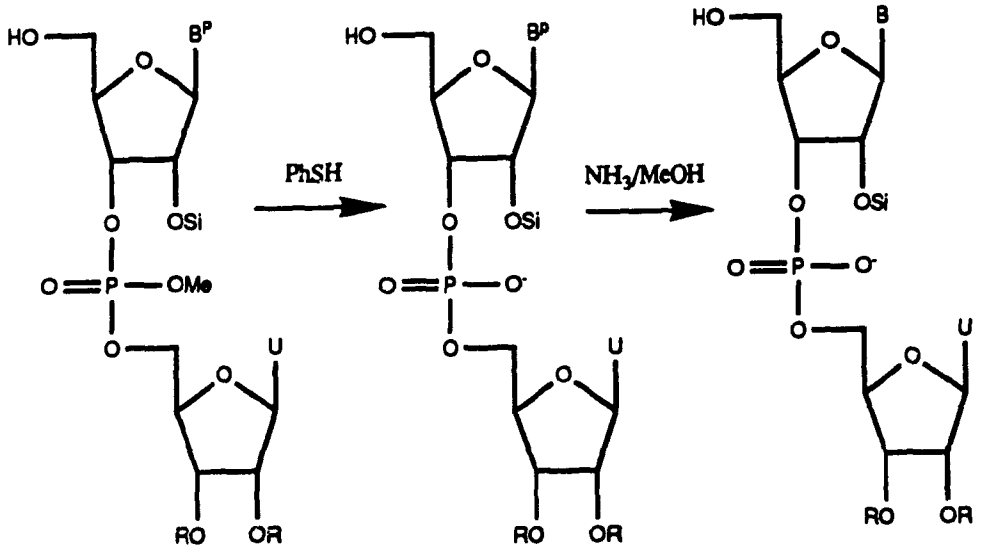
In contrast to all the ^{31}P spectra of previous triester intermediates, ^{31}P NMR spectra of methyl deprotected dinucleotides all showed only one peak (Table 8). ^{31}P chemical shift of the 3'-5' dinucleotide is downfield compared that of the corresponding 2'-5' dinucleotide. No signal belonging to the 2'-5' dinucleotide was observed in the ^{31}P of corresponding the 3'-5' dinucleotide. These results are exemplified in the ^{31}P NMR spectra of **18a** and **19a** shown in Figure 40.

Compounds **18a-d** and **19a-d** were also analyzed by HPLC on a reverse phase C_8 column. For each isomeric pair, an artificial mixture of the two was prepared by mixing the two individual samples. This was analyzed on HPLC to find the HPLC conditions under which the two isomers can be cleanly separated. Then the individual isomer was analyzed. The result is shown in Table 8. All the isomeric pairs of dinucleotides (**18a-d** vs **19a-d**) were separated on the Whatman C_8 reverse phase column (4.6x250mm) using acetonitrile in triethylammonium acetate buffer (0.1 M, pH7).

HPLC profiles for **18a** and **19a** are presented in Figure 41 as an example. The artificial mixture of the two samples shows the presence of two peaks corresponding to each isomer on HPLC. The individual sample of either **18a** or **19a** only shows one major elution peak and no peak corresponding to its isomer is detected. The same result was obtained for all the other isomeric pairs. HPLC data further confirms the NMR result that **18a-d** does not have any isomeric contamination.

SCHEME 19

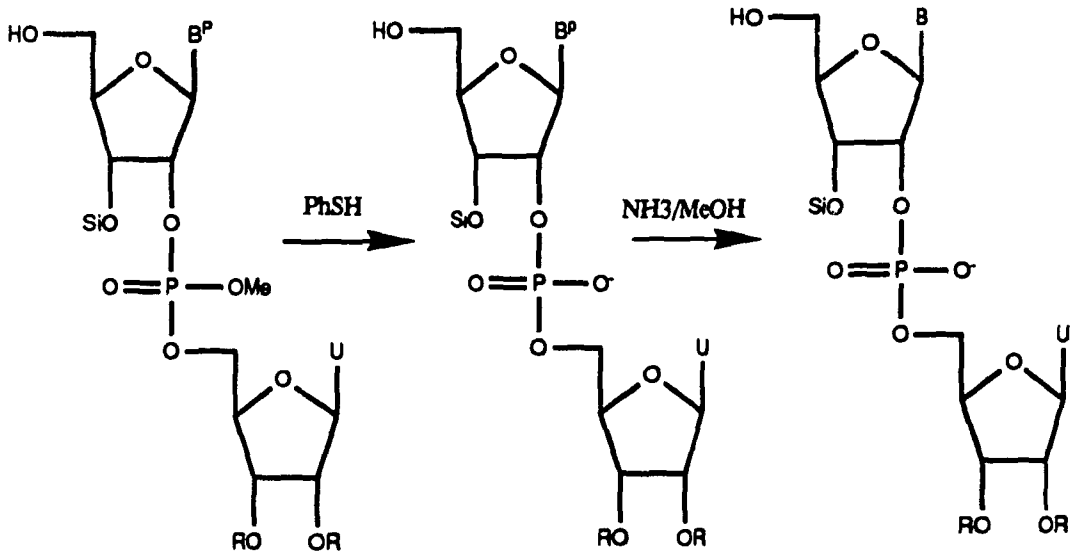
A



16a-d **18a-d** **20a-d**

20a-c, R = H; 20d, R = Si

B



17a-d **19a-d** **21a-d**

21a-c, R = H; 21d, R = Si

Table 8. ^{31}P Chemical Shifts and HPLC Retention Times of N-Acylated 2'-O-Silylated Dinucleotides (**18a-d, 19a-d**)[a]

Entry	^{31}P Chemical Shifts (ppm)	Retention Time (min.)	HPLC Solvent (% CH_3CN in TEAA)
18a	-0.3	18.2	33% (isocratic)
19a	-1.4	19.6	33% (isocratic)
18b	0.3	11.4	30% (isocratic)
19b	-0.9	9.8	30% (isocratic)
18c	0.1	8.0	38% (isocratic)
19c	-1.0	12.4	38% (isocratic)
18d	0.7	19.8	45-57% (linear gradient)
19d	-0.7	18.8	45-57% (linear gradient)

[a] ^{31}P Chemical shifts are reported as downfield positive to the external 85% H_3PO_4. HPLC conditions were described in experimental.

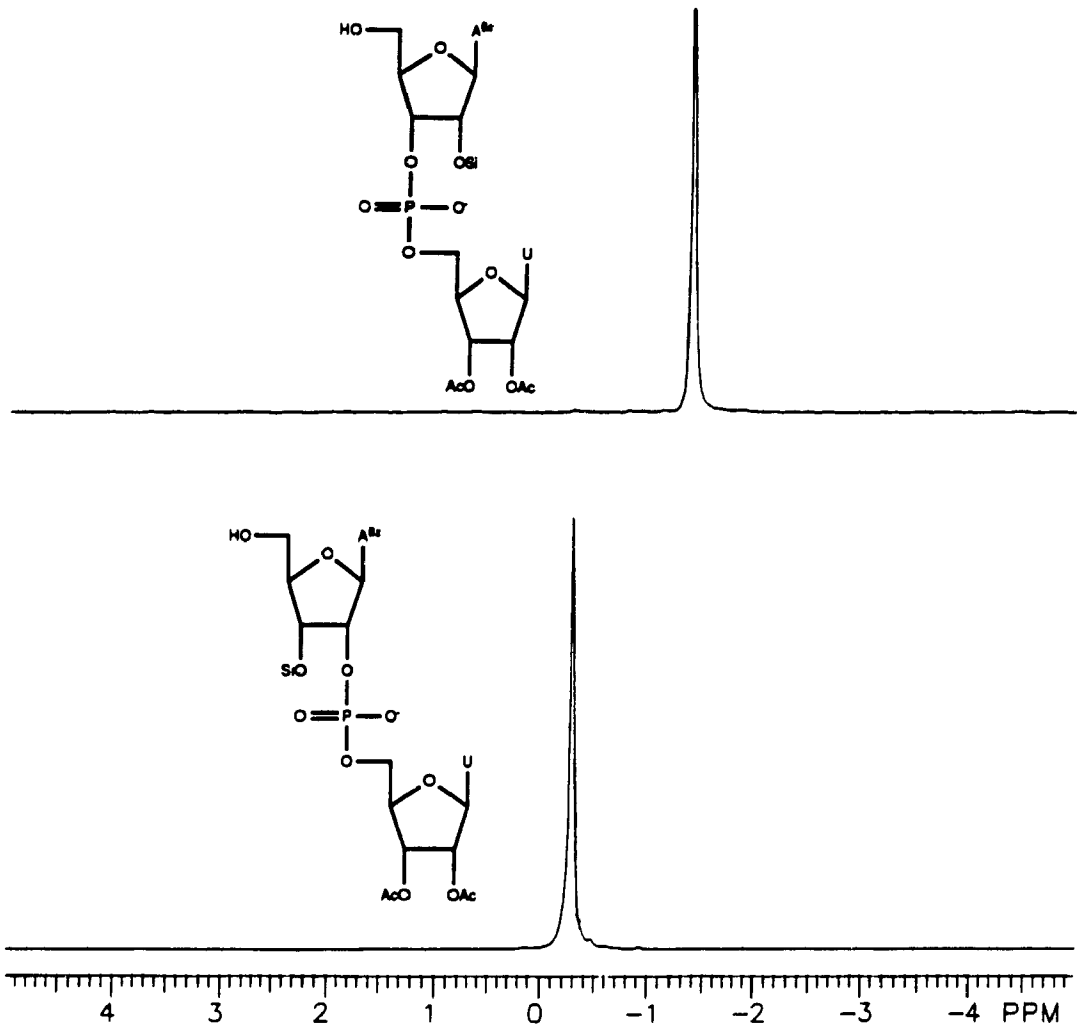


Figure 40. ^{31}P NMR of **18a** and **19a**

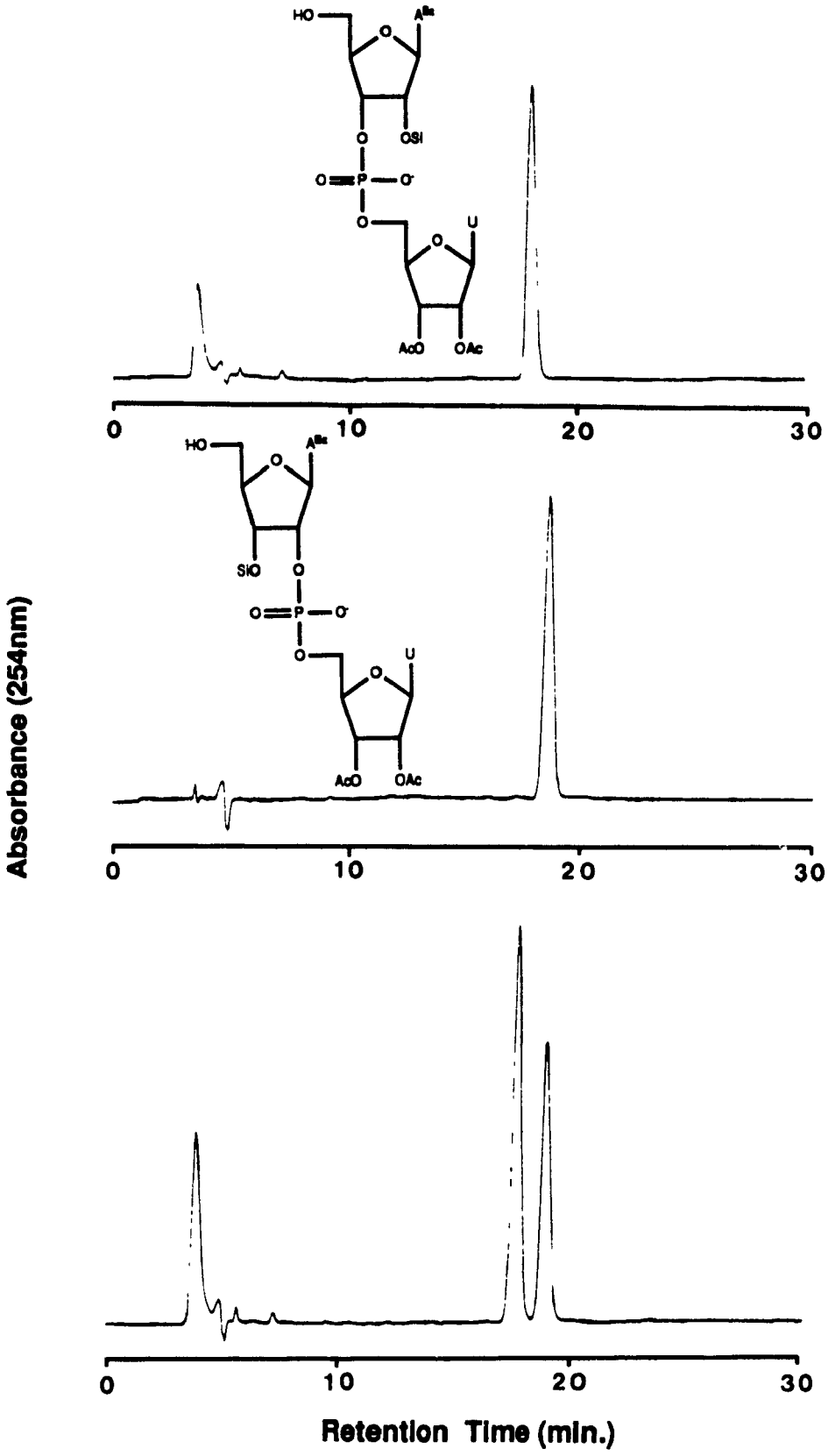


Figure 41. HPLC profiles of **18a**, **19a**, and an artificial mixture of **18a** and **19a**

18a-c and **19a-c** were next deacylated to give the 2'-silylated 3'-5' linked (**20a-c**) and 3'-silylated 2'-5' linked (**21a-c**) dinucleotides respectively (Scheme 19). This also removed the terminal 2',3'-acetyl groups. This step is not necessary for **18d** and **19d** since no acyl protecting group is used. Benzoyl on cytidine and adenosine, phenoxyacetyl on guanosine were removed using methanolic ammonia (Chapter 3). The crude sample from the treatment was directly analyzed on HPLC on a C_8 reverse phase column using isocratic acetonitrile in triethylamine as the solvent. The results are shown in Table 9. The 2'-silylated 3'-5' dinucleotide and its corresponding 3'-silylated 2'-5' dinucleotide have different retention times. Each of the samples from the deprotection shows one major peak on the HPLC profile. None of them have been found to indicate the presence of any impurity belonging to its isomer. The HPLC profiles for **20a**, **21a** and an artificial mixture of the two are presented in Fig 42 as an example.

Cyanoethyl is one of the other phosphate protecting groups used in the ribonucleotide synthesis[93,94]. The use of cyanoethyl will greatly simplify the deprotection procedure since ammonia treatment removes both the cyanoethyl phosphate protection and the N-acyl protection. Based on this study the 2'-silyl group should also be stable if the conditions to remove cyanoethyl phosphate protection were used.

This part of the study has established that the 3'-5' phosphate linkages on the assembled nucleotide chain remained intact in deblocking the phosphate protecting groups and the N-protecting groups. The silyl group is stable during the deprotection stage of nucleotide synthesis and it stays on the 2'-position throughout the synthesis and deprotection. The 2'-O-silylated oligoribonucleotide, one of the key intermediates in the chemical synthesis of oligoribonucleotides, contains the correct 3'-5' phosphate linkages.

Table 9. HPLC Retention Times of 2'-O-Silylated Dinucleotides (**20a-d, 21a-d**)[a]

Entry	Retention Time (min.)	HPLC Solvent (% CH3CN in TEAA)
20a	13.7	17% (isocratic)
21a	35.5	17% (isocratic)
20b	10.6	20% (isocratic)
21b	16.7	20% (isocratic)
20c	6.5	26% (isocratic)
21c	15.3	26% (isocratic)
20d	19.8	45-57% (linear gradient)
21d	18.8	45-57% (linear gradient)

[a] HPLC conditions were described in experimental section.

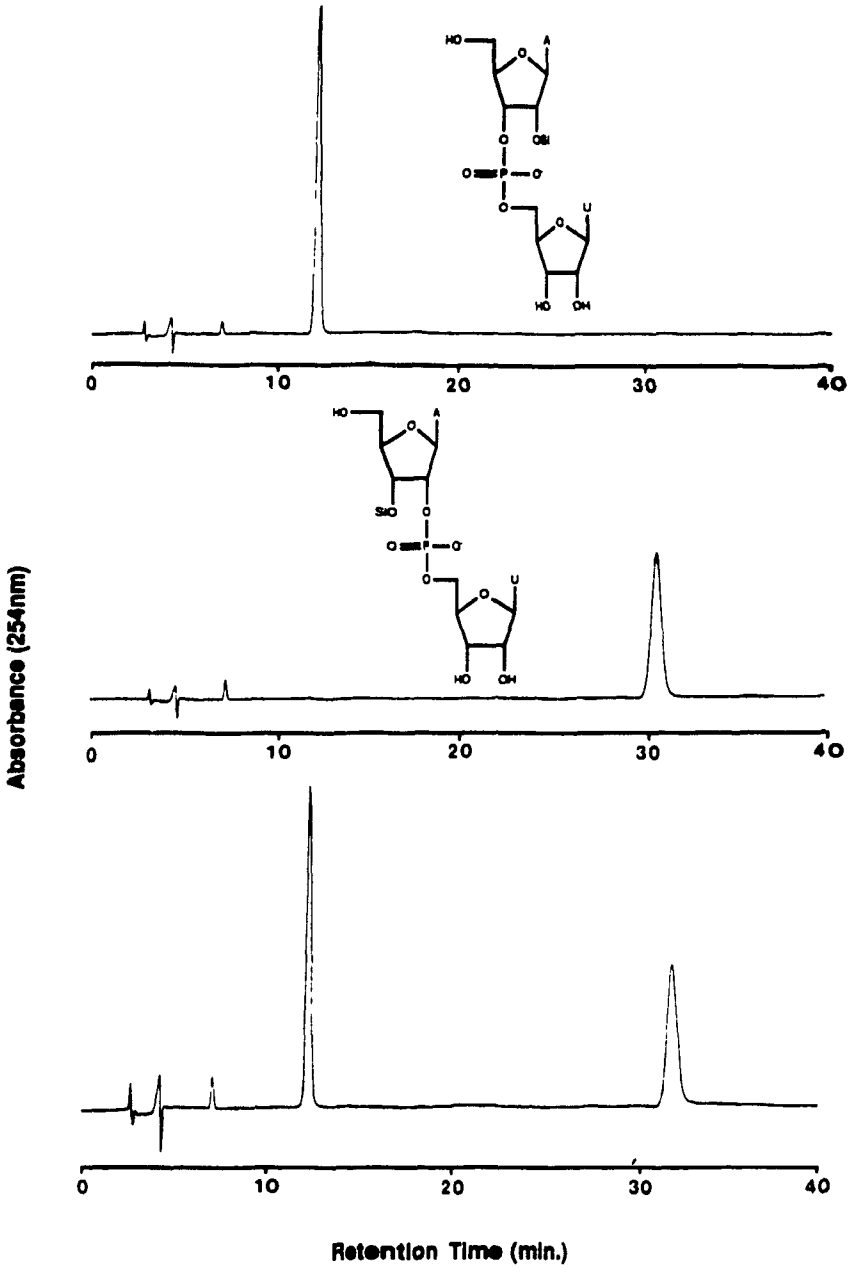


Figure 42. HPLC profiles of **20a**, **21a**, and an artificial mixture of the two samples

5.6. Removal of 2'-Silyl Protecting Groups

Deprotecting the 2'-protecting group is always the last and also perhaps the most critical step in the synthesis of oligoribonucleotides. The natural ribonucleotide unit released during the deprotection of 2'-position is so sensitive to the chemical conditions that the few protecting groups that meet all of the previous criteria fail miserably at this very last step due to the severity of the deprotection conditions. For example, in addition to the possible isomerization during the phosphorylation, another drawback of using acyl as 2'-protecting group is that the final removal of acyl groups under basic conditions may cause cleavage of the assembled nucleotide chain[73]. Ketal type groups such as THP were found to also meet the requirement with difficulty since the acidic conditions required to remove the ketal sometimes seems to lead to the more troublesome isomerization in addition to the chain cleavage.[77]

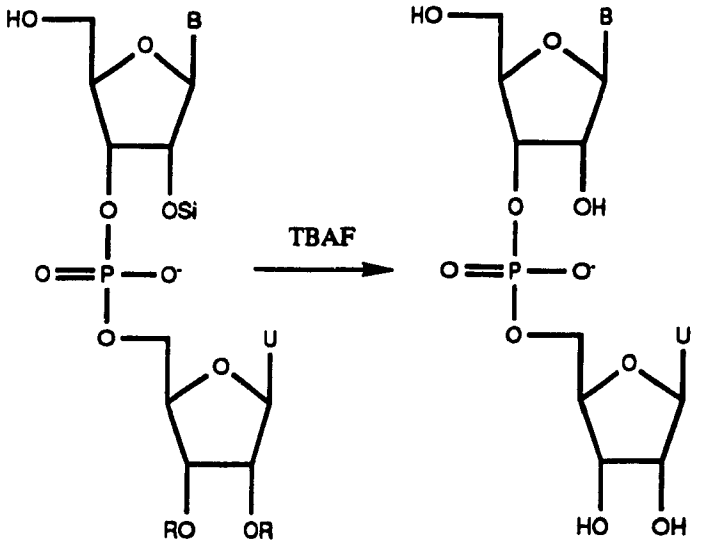
The *t*-butyldimethylsilyl or triisopropysilyl group can be easily removed by the fluoride ion under neutral conditions.[137] A THF solution of TBAF (1M) was used in our deprotection.[65,67] Thus dinucleotides **20a-d** and **21a-d** were treated with TBAF for 10-12 h to give the fully deprotected dinucleotides **22a-d** and **23a-d** respectively (Scheme 20). In the case of **20d** and **21d**, this also removes the terminal 2',3'-silyl protection. The crude reaction mixture from the treatment was analyzed without purification on a Whatman C8 reverse phase column. The HPLC result is shown in Table 10.

The natural 3'-5' phosphate linked dinucleotide was cleanly separated from the corresponding 2'-5' dinucleotide on a reverse phase C_8 column under the conditions shown in the Table. The 2'-5' phosphate linked isomer has a shorter retention time than its corresponding 3'-5' dinucleotide on a reverse phase column. This seems to suggest that the phosphate in the 3'-5' nucleotide is more `shielded' than that in the 2'-5' nucleotide.

137. E.J. Corey, B.B. Snider, *J. Am. Chem. Soc.*, 94, 2549 (1972).

SCHEME 20

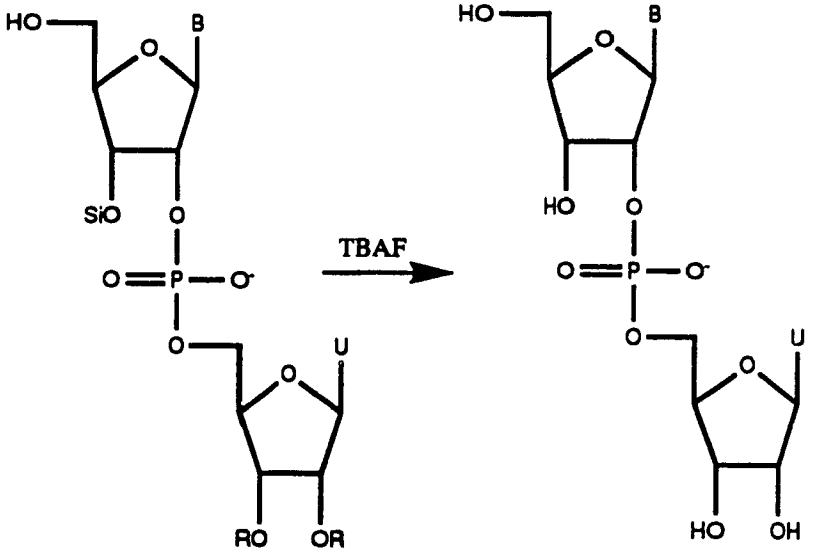
A



20a-d

22a-d

B



21a-d

23a-d

Table 10. HPLC Retention Times of Fully Deprotected Dinucleotides (**22a-d, 23a-d**) [a]

Entry [b]	Retention Time (min.)	Solvent (% CH3CN in TEAA)
22a	21.6	6% (isocratic)
23a	17.0	6% (isocratic)
22b	11.6	4% (isocratic)
23b	8.9	4% (isocratic)
22c	12.0	3% (isocratic)
23c	7.5	3% (isocratic)
22d	12.5	5% (isocratic)
23d	10.4	5% (isocratic)

[a] HPLC conditions were described in experimental sections.

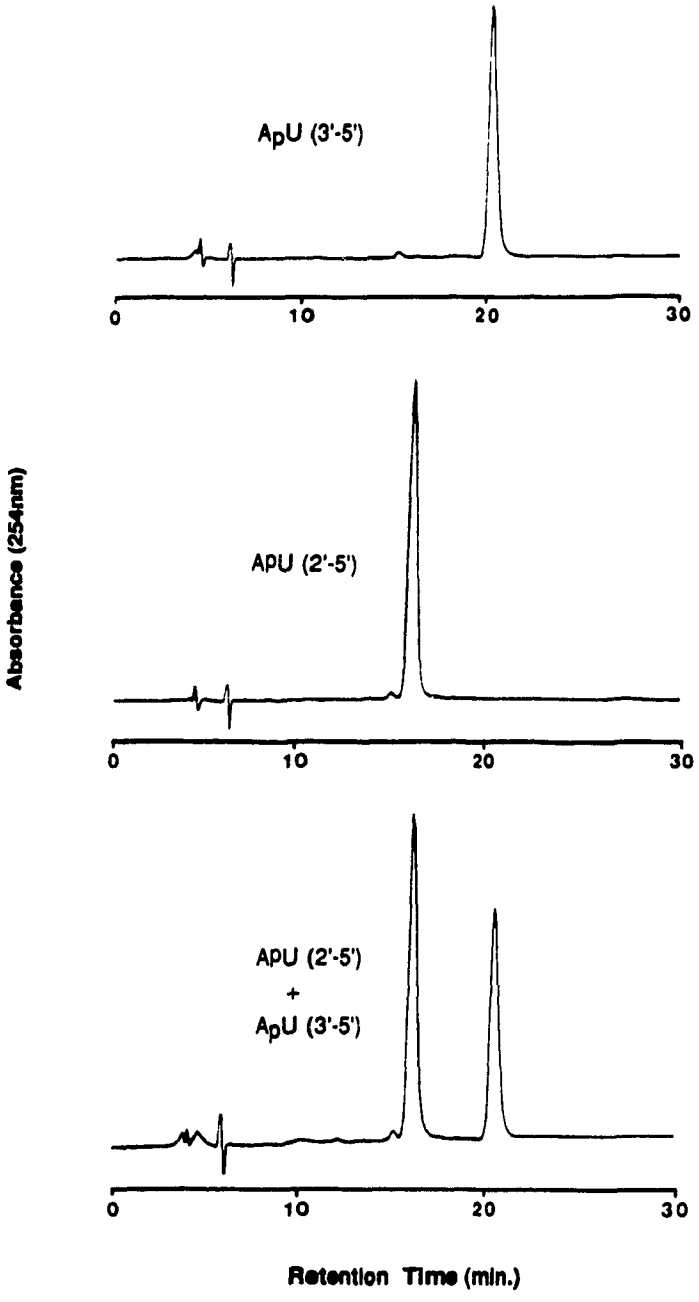


Figure 43. HPLC profiles of the fully deprotected dinucleotides **22a**, **23a**, and an artificial mixture of the two.

The HPLC profiles of **22a** and **23a** are shown in Fig. 43 as an example. The HPLC profile of the mixture of the two compounds, prepared by mixing the two samples, shows the clean separation of the two isomers. A sample of **22a** has one peak on HPLC. No trace of a peak corresponding to **23a** was detected in the synthetic sample of **22a**. The same result was obtained for **22b-d**. Compound **22d** (U_pU) is also compared with a sample from Sigma (Fig. 44). The synthetic sample is superimposable on the commercial sample. HPLC data proves that the phosphate linkage remains intact in the removal of 2'-silyl groups and the synthetic oligoribonucleotides have the correct phosphate linkage.

5.7. Solid Phase Synthesis of a Uridine Dinucleotide

The solid phase synthesis of oligoribonucleotides using nucleoside 3'-phosphoramidite essentially follows the same steps as the solution synthesis. Solid phase synthesis replaces all the time consuming silica gel column purification steps by the simple washing steps to remove the undesirable reagents in the solution from the solid support. An extra capping step is needed in solid phase synthesis to deactivate the unreacted 5'-hydroxyl at the end of each assembling stage. The procedures for deprotection are the same as those in solution synthesis. The reaction conditions in the automated solid phase synthesis were optimized and a high synthetic yield can be achieved (95%). A uridine 3'-5' dinucleotide was assembled using the uridine 3'-amidite and deprotected using the same deprotection conditions. HPLC profile of this sample is shown in Fig. 44. The crude product from the synthesis appears as one dominant peak. Like the one prepared from the solution synthesis (**22d**), no 2'-5' phosphate linked uridine dinucleotide was detected in this sample. The synthetic samples from solid phase and solution are superimposable on the commercial sample from Sigma.

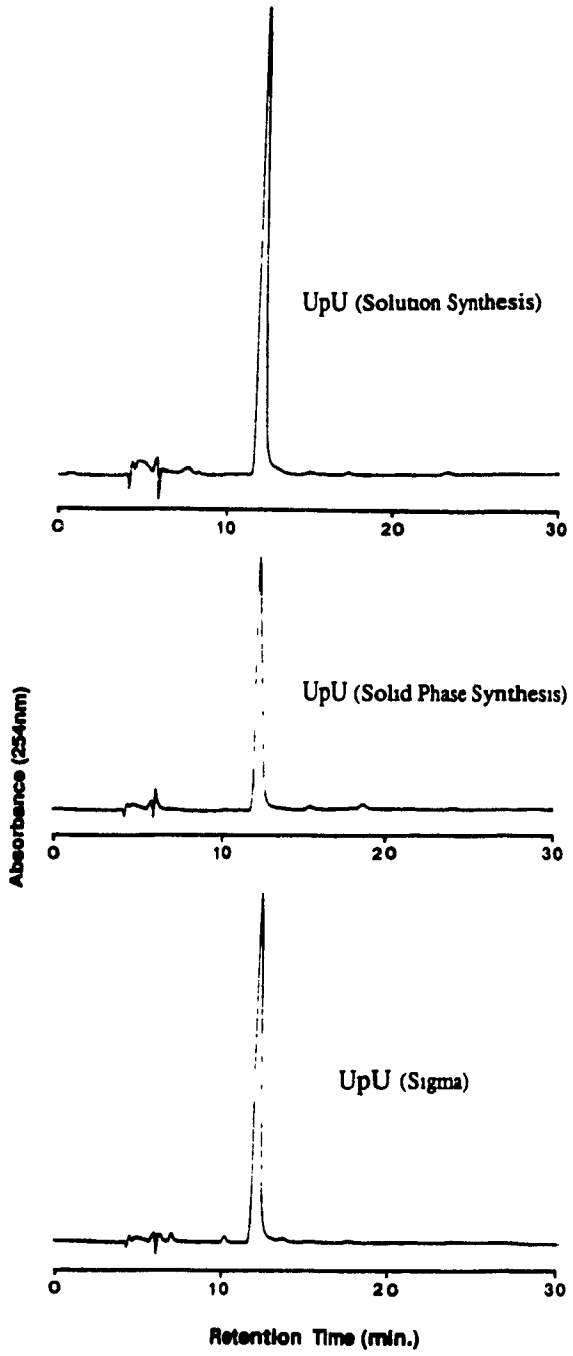


Figure 44. HPLC profiles of several samples of a uridine dinucleotides

5.8. Conclusion

The study based on the preparation of a series of dinucleotides in solution has provided rigorous proof that oligoribonucleotide synthesis using *t*-butyldimethylsilyl or triisopropylsilyl as the 2'-protecting group in combination with the phosphoramidite coupling procedure is reliable. The 2'-silylated nucleoside 3'-O-phosphoramidite, the key reagent for ribonucleotide synthesis, is prepared free of the isomeric 3'-silylated nucleoside 3'-O-phosphoramidite. The 2'-silyl group is stable under the chemical conditions used in the preparation of the nucleoside phosphoramidites. The nucleotide intermediate at each stage of the synthesis has the correct phosphate linkage. The removal of the 2'-silyl group from the assembled nucleotide also does not cause any undesired reaction. The synthetic oligoribonucleotide, both from the solution synthesis and solid phase synthesis, does not have any contamination with unnatural 2'-5' linkages. The present model study, together with previous biochemical results, fully establishes the fidelity of alkylsilyl groups as 2'-hydroxyl protecting groups in the chemical synthesis of RNA sequences.

CHAPTER 6

EXPERIMENTAL

6.1. Materials and Methods

6.1.1. Reagents and chemicals

Nucleosides were obtained from either Sigma Chemical Co. (St. Louis, Mo.) or Boehringer Mannheim Canada (Dorval, P.Q.). Long chain controlled pore glass for solid phase synthesis (LCAA-CPG, pore diameter 500A°, particle size, 125-177u) was from Pierce Chemical Co (Rockford, IL). All other chemicals were reagent grade from commercial sources and used as they were unless specified otherwise.

Pyridine, N,N,-dimethylformamide (DMF), diisopropylethylamine, dioxane, and collidine were distilled over calcium hydride. THF used for silylation, phosphorylation, and condensation was dried over molecular sieve (12 h) before it was refluxed over sodium and benzophenone to generate a distinct purple solution. It was freshly distilled before use. Acetonitrile used for ribonucleotide synthesis was refluxed over calcium hydride under argon for 12 h and was distilled prior to use. Hexane, ethyl acetate, and triethylamine used for preparative silica gel column chromatographic solvents were distilled. Methylene chloride, methanol, ethyl ether, were used as reagent grade from commercial sources. Reagent grade silver nitrate, N,N,-dimethylaminopyridine (DMAP) were used. *t*-Butyldimethylsilyl chloride, triisopropylsilyl chloride, chloro(N,N,-diisopropylamino)methoxyphosphine were purchased from Aldrich and used as they were. Tetrazole was obtained from Aldrich and kept over P_2O_5 in a desiccator to keep dry. Standard 3'-5' diuridine nucleotide was obtained from Sigma. Ribonucleases were purchased from Boehringer Mannheim Canada (Dorval, P.Q.). The double distilled water used in the deprotection was autoclaved and contained 0.001% sodium azide. Deoxyribonucleoside cyanoethylphosphoramidites were obtained from Pharmacia. Deuterated NMR solvents were obtained from Merck Sharp and Dohme (Montreal, P.Q.)

All the standard reagents for nucleotide synthesis were prepared in this lab. Tetrazole solution (0.5 M) was obtained by dissolving the appropriate amount of tetrazole in acetonitrile. The detritylation solutions (5% Cl_3CCOOH in CH_2Cl_2) were prepared by dissolving trichloroacetic acid (25g, Aldrich) in methylene chloride (500ml, dried over 4 A molecular sieve). The 0.1 M I_2 oxidation reagent was prepared by dissolving solid I_2 (10.2g) in water (134ml) and THF (266ml). 0.5 M Ac_2O in THF (solution A) and 0.5 M DMAP in collidine (0.25 M) in THF (solution B) were used as capping solutions. Dioxane, triethylamine, and thiophenol (Aldrich) were mixed in the ratio of 2/2/1 (volume) to give the thiophenoxide solution for methyl phosphate deprotection. Methanolic ammonia was prepared by bubbling ammonia in ice cold methanol (20ml) for 15 to 20 min. and was used immediately. Tetra-n-butylammonium fluoride was obtained as 1M solution in THF (Aldrich) and used as is.

CH_3CN for HPLC was obtained from Caledon Laboratory (HPLC grade). HPLC methanol was obtained as spectral grade and filtered through 4.5um filter paper before use. Double distilled water was used for HPLC. HPLC buffer for analyzing oligoribonucleotides (triethylammonium acetate, 0.1 M, pH 7) was prepared by diluting the stock solution of triethylammonium acetate (1M, 100ml) with double distilled water to 1L. The pH of the solution was adjusted with glacial acetic acid or triethylamine to 7 on a Corning 125 pH meter. The solution was filtered through Millipore 0.45um filter paper before use. Stock triethylammonium acetate (1M) was prepared by slowly adding triethylamine (139ml, distilled) to a magnetically stirred, ice cold aqueous solution (500ml) of glacial acetic acid (57ml). The resulting solution was diluted to 1L as stock (1M).

The phosphorylation and condensation reactions were carried out in Hypovials (Pierce). The vials were dried in the oven (120°C, 12h) and cooled in an argon atomsphere. The equipment used in the deprotection of ribonucleotides was autoclaved or treated with a diethylpyrocarbonate solution to avoid nuclease contamination. The glassware used in the deprotection of nucleotides is silanized and then autoclaved before use.

All other nucleoside intermediates were dried on the vacuum line (4mm Hg) at room temperature for >12h. The aqueous nucleotide solution was lyophilized in a Speed-Vac concentrator (Savant Instruments).

6.1.2. Equipment and instrumentation

^1H NMR spectra were recorded on a Varian XL-200 spectrometer. $CDCl_3$ was used as the solvent unless specified otherwise. Proton chemical shifts were reported with reference to internal residual solvent signal at 7.26 ppm ($CHCl_3$). ^{31}P NMR was performed a on Varian XL-300 in $CDCl_3$. Phosphor signals were referenced to external 85% phosphoric acid (downfield positive). UV spectra were recorded on HP 8451A spectrometer. Melting points were taken on a Fisher-Johns melting point apparatus and reported uncorrected.

The automated synthesizer was generously provided by Applied Biosystem (Model 380B). The synthesizer provides the standard DNA synthesis cycle. The RNA synthesis used the standard synthesis procedure[67]. A typical synthesis uses about 30 mg CPG derivatized with the appropriate nucleoside. The solid support was packed in a Teflon plastic column (Applied Biosystem). The instrument was interfaced with a fraction collector (FRAC 100, Pharmacia) which collects the detritylated solution for the calculation of each coupling yield. Only the average coupling yields and overall yields for the entire synthesis were reported. Conditions for the removal of methyl protecting groups was previously described[67]. The silyl protecting groups were removed by dissolving the lyophilized 2'-silylated material in 1 M TBAF and the solutions were left at room temperature for 16 h. The sample was finally desalted on a size exclusion column.

6.1.3. Chromatography

Thin layer chromatography (TLC) were performed on Merck Kieselgel 60 F_{254} analytical sheets. TLC plates were developed in 5% methanol in methylene chloride (solvent A), 10% methanol in methylene chloride (solvent B), 20% ethyl ether in methylene chloride (solvent C), or 50% ethyl ether in methylene chloride (Solvent D) and visualized under a single wavelength (254 nm) UV source. Flash preparative silica gel column chromatography was performed on columns packed with E. M. Kieselgel 60 (230-400 mesh) silica gel (20g per gram of sample).

Size exclusion chromatography was performed on a column (60 x 0.9 cm, Pharmacia) packed with Sephadex G25F. The Sephadex was autoclaved in water before the

use. The column was eluted with autoclaved water and monitored with a Pharmacia UV-1 UV monitor at 254 nm.

Polyacryamide gel electrophoresis was performed in tris(hydroxylmethyl)aminomethane (TRIS) buffer and was run at 400-600 V. The gels were visualized and photographed by UV shadowing over a fluorescent TLC plate. For preparative gels, the desired band was sliced out and extracted by incubation in buffer (0.5 M NH_4OAc/1mM EDTA/0.1% SDS/1mM $MgCl_2$) for 18 h at room temperature. The supernatants were then desalted on the size exclusion column. Nucleotides were stored at -20°C.

HPLC analysis was carried out on a Spectra Physics 8000 chromatographic system equipped with a single wave length UV detector (254nm). The system has both isocratic and gradient mode. The instrument operates at 22°C with a flow of 1ml/min. A Whatman Partisil 5 C-8 (4.6x250 mm), or AQUAPORE RP-300 (4.6x100 mm), or Whatman ODS-2 Partisil 10 (4.6x250 mm) column was used. Typically, about 0.2mg of nucleoside derivative was dissolved in 1 ml of the eluting solvent. For oligoribonucleotides the sample (1 O.D.) was dissolved in sterile water (100ul). 10ul of the solution was injected for each run.

6.2. Synthetic Methods - Chapter 2

6.2.1. Preparation of N^6-phenoxyacetyladenosine (5a)

To a suspension of adenosine (50mmol, 13.35g) in dry pyridine (300ml), trimethylsilyl chloride (375mmol, 47.3ml) was introduced followed, after 2 h, by the addition of phenoxyacetic anhydride (150mmol, 42.9g). The reaction was stopped after another 2 h by addition of water (50ml). 1M HF/pyridine solution (13) (200ml) was added 5min later. It was stirred for another 25min. The mixture was then poured into methylene chloride (800ml). The organic phase was washed with saturated brine solution (3x500ml), and then dried over sodium sulfate. The solvents were evaporated to give a yellow gum. Crystalization of the residue from hot ethanol gave 11.0g (55%) of **5a**. Some product remained in the mother liquor which was concentrated to a brown gum and purified by flush column chromatography using a gradient of methanol in methylene chloride (5%-7%) as

eluant, giving another 2.5g, bringing the total yield to 65%. Rf=0.53 in 20% methanol in methylene chloride; m.p. 132-134°C; U.V. (H_2O), max (nm), 274, 210; ^1H NMR (DMSO-d_6, TMS as internal reference): 10.98 (s, 1, NH); 8.75 (s, 1, H8); 8.70 (s, 1, H2); 6.03 (d, 1, J=6Hz, H1'); 4.63 (m, 1, H2'); 4.19 (m, 1, H3'); 4.00 (m, 1, H4'); 3.64 (m, 2, H5'& H5"); 5.04 (s, 2, $PhOCH_2CO$); 3.58 (s, 3, $-OCH_3$); 6.97 (m, 3, ArH);7.32 (m, 2H, ArH). Anal. Calcd. for $C_{18}H_{19}N_5O_6$: C, 53.86; H, 4.74; N, 17.46. Found: C, 53.55; H, 4.78; N, 17.23.

6.2.2. Preparation of N^6-phenoxyacetyl-5'-monomethoxytrityladenosine (6a)

5a (11g, 27.4mmol) was dissolved in dry pyridine (200ml) and monomethoxytrityl chloride (33mmol, 10.12g,) was added. After stirring at ambient temperature (4 h), TLC showed incomplete reaction and more monomethoxytrityl chloride (2g) was added. After stirring for another 10 h methanol (50 ml) was added and the mixture was poured into methylene chloride (600ml). The solution was washed with saturated sodium bicarbonate aqueous solution (2x500ml), and dried with sodium sulfate. The solvents were removed and the residue was coevaporated with toluene (2x200ml) to remove residue pyridine. The crude mixture was purified by flush silica gel column chromatography using a gradient of methanol in methylene chloride (0%-5%) to give 12 g pure product (65%). Rf=0.36 in solvent B; m.p. 102-105°C; UV (95% ethanol), max. (nm), 278, 234; ^1H NMR ($CDCl_3$): 9.49 (s, 1, NH), 8.75 (s, 1, H8), 8.27 (s, 1, H2), 6.74-7.35 (m, 19, aryl), 6.23 (d, 1, J=0.6Hz, H1'), 4.85 (m, 1, H2'); 4.44 (m, 2, H3'&H4'); 3.30 (q, 1, H5'); 3.45 (q, 1, H5"); 4.84 (s, 2, $PhOCH_2CO$), 3.77 (s, 3, $-OCH_3$); Anal. Calcd. for $C_{38}H_{35}N_5O_7 \cdot 1CH_3OH$: C, 66.38; H, 5.53, N, 9.93. Found: C, 66.66; H, 5.81; N, 9.57.

6.2.3. Preparation of N^6-phenoxyacetyl-5'-monomethoxytrityl-2'-t-butyldimethylsilyladenosine(7a)

To a solution of **6a** (11mmol, 7.6g) in anhydrous THF (30 ml) were added silver nitrate (15mmol, 2.5g) and dry pyridine (45mmol, 1.3ml). Once the silver nitrate was dissolved, t-butyldimethylsilyl chloride (1.4 equiv., 15.4 mmol, 2.31g) was added. TLC (in solvent C) showed complete reaction after 4h. The solution was filtered off into a brine solution (400ml) and extracted with methylene chloride (2x400ml). The combined organic

solutions were dried with sodium sulfate, concentrated and coevaporated with toluene (2x200ml) to remove the residual pyridine. The 2'-silylated product was separated from the 3'-isomer by the flush column chromatography method using 40% ethyl acetate/hexane to give 5.5g pure **7a** (62%). Rf=0.35 in 50% ethyl acetate in hexane; m.p. 80-83°C; UV (95% ethanol), max. (nm), 268, 222; ^{1}H NMR ($CDCl_3$): 8.71 (s, 1, H8), 8.23 (s, 1, H2), 6.80-7.50 (m, 19, ArH), 6.08 (d, 1, J=5.4Hz, H1'), 5.00 (t, 1, H2'); 4.35 (q, 1, H3'); 4.28 (m, 1, H4'); 3.55 (q, 1, H5'); 3.38 (q, 1, H5"); 4.84 (s, 2, $PhOCH_2CO$), 3.78 (s, 3, $-OCH_3$), 0.82 (s, 9, $SiC(CH_3)_3$), -0.03&-0.17 (s, 6, $Si(CH_3)_2$). Anal. Calcd. for $C_{44}H_{49}N_5O_7Si$: C, 67.07; H, 6.27; N, 8.89. Found: C, 67.22; H, 6.19; N, 8.96.

6.2.4. Preparation of N^6-phenoxyacetyl-5'-monomethoxytrityl-2'-t-butyldimethylsilyladenosine-3'-N,N,-diisopropylmethylphosphoramidite (9a)

A solution of **7a** (3.14g, 4mmol) in THF (6ml) was slowly added, via a syringe, to a stirred THF solution (9ml) of dimethylaminopyridine (0.1g, 0.8mmol), diisopropylethylamine (2.8ml, 16mmol), and chloro(N,N-diisopropylamino)methoxyphosphine (1ml, 5.2mmol) in an argon purged vial. A precipitate was formed after a few minutes and the mixture was stirred at room temperature (3 h) until TLC indicated complete reaction. The mixture was added to a ethyl acetate solution (150 ml, previously washed with aqueous sodium bicarbonate solution), washed with brine solution (2x200ml), and dried with sodium sulfate. The crude mixture was purified by flush column chromatography with solvent methylene chloride/hexane/triethylamine, 50/47/3 to give 3.2g (**9a**) (83%). Rf=0.48&0.32 (two diastereomers) in 20% ethyl acetate in methylene chloride; m.p. 72-75°C; UV (95% ethanol), max (nm), 274, 234; ^{31}P NMR ($CDCl_3$): 151.9, 150.1 ppm.

6.2.5. Preparation of N^4-phenoxyacetylcytidine (5b)

Cytidine (50 mmol, 12.2g) was co-evaporated with anhydrous pyridine (3x) and suspended in pyridine (300 ml). Trimethylsilyl chloride (400 mmol, 51 ml) was added and the mixture stirred (1 h). Phenoxyacetyl chloride (62,5 mmol, 8.6 ml) was added via syringe and an orange color appeared. The reaction was stirred at room temperature (2.5 h) before the addition of first ice-water (150 ml) followed after 20 min. by 30% ammonium hydroxide

(2ml). The solution was concentrated to remove pyridine, redissolved in water (700 ml) and extracted with methylene chloride. **5b** was precipitated from the aqueous layer to give 12.1g. This was used without further purification. Rf=0.28 in solvent B; UV, max. (nm), 303, 277, 248; Anal. Calcd. for $C_{17}H_{19}N_3O_7$: C, 54.11; H, 5.07; N, 11.14. Found: C, 54.43; H, 5.21; N, 10.63.

6.2.6. Preparation of N^4-phenoxyacetyl-5'-monomethoxytritylcytidine (6b)

Monomethoxytrityl chloride (13.25g, 1.2eq.) was added to a solution of **5b** (obtained without purification) in pyridine (13.5 g, 36 mmol). After stirring 4 h, methanol (50 ml) was added and the solution was concentrated to a brown gum. This was redissolved in CH_2Cl_2 (600 ml), washed with brine, dried over sodium sulfate and coevaporated with toluene (2x) to remove pyridine. The mixture was then purified by flash chromatography using a 0-4% $MeOH/CH_2Cl_2$ gradient to give 14 g pure **6b** (60%). Rf=0.2 in solvent A; m.p., 115-117°C; UV. (95% ethanol), max. (nm), 304, 238, 208; ^1H NMR ($CDCl_3$): 8.13 (d, 1, H6, J=7.3 Hz), 6.75-7.33 (m, 19, ArH), 6.91 (d, 1, H1', J=1.1 Hz), 4.56 (s, 2, CH_2O), 4.37 (m, 3, H2', H3', H4'), 3.37 (s, 3, $-OCH_3$), 3.28-3.36 (m, 2, H5', H5"). Anal. Calcd. for $C_{37}H_{35}O_8N_3$: C, 68.40; H, 5.43; N, 6.47. Found: C, 68.09; H, 5.42; N, 6.51.

6.2.7. Preparation of N^4-phenoxyacetyl-5'-monomethoxytrityl-2'-t-butyldimethylsilylcytidine (7b)

Silver nitrate (4.4 g, 1.2 eq.), pyridine (7 ml, 4 eq.) and **6b** (14 g, 21.6 mmol) were dissolved in anhydrous THF (250ml) and then t-butyldimethysilyl chloride (4.1 g, 1.2 eq.) was added. After 4 h, TLC indicated incomplete reaction and more silver nitrate (0.37 g, 0.1 eq.) and t-butyldimethylsilyl chloride (0.34 g, 0.1 eq.) were added. After stirring 2 h, the mixture was filtered into 5% aqueous $NaHCO_3$ (400 ml). This was extracted with CH_2Cl_2 (2x300 ml) and the combined organic extracts were dried with sodium sulfate. The solution was coevaporated with toluene (2x200 ml) to remove pyridine and then purified by flash chromatography using a 5-10% Et_2O/CH_2Cl_2 gradient to yield 8g pure **7b** (49%). Rf=0.69 in solvent C; m.p., 107-109°C; UV (95% ethanol), max. (nm), 306, 238, 206; ^1H NMR ($CDCl_3$): 8.43 (d, 1, H6, J=7.4Hz), 6.76-7.35 (m, 19, ArH), 5.82 (s, 1, H1'), 4.50 (s, 2, $-CH_2O$), 4.20 (m, 2, H2', H3'), 4.02 (m, 1, H4'), 3.48 (m, 2, H5', H5"), 3.73 (s, 3, $-OCH_3$),

0.84 (s, 9, $Si(CH_3)_3$), 0.23 (s, 3, $-SiCH_3$), 0.10 (s, 3, CH_3Si-). Anal. Calcd. for $C_{43}H_{49}O_8N_3Si$: C, 67.69; H, 6.47; N, 5.50. Found: C, 67.43, H, 6.42; N, 5.54.

6.2.8. Preparation of N^4-phenoxyacetyl-5'-monomethoxytrityl-2'-t-butyldimethylsilylcytidine-3'-N,N,-diisopropylmethylphosphoramidite (9b)

A solution of **7b** (3.82 g) in THF (9 ml) was added dropwise via syringe to a solution of DMAP (0.12 g, 0.2 eq.), diisopropylethylamine (3.5 ml, 4eq.) and chloro(N,N-diisopropylamino)methoxyphosphine (1.26 ml, 1.3 eq.) in THF (12 ml). After stirring 6 h at room temperature, the reaction was added to ethyl acetate (200 ml), washed with 5% aqueous $NaHCO_3$ and dried over sodium sulfate. The material was purified by flash chromatography using hexane/CH_2Cl_2/triethylamine (50/48/2). The fractions containing the product were combined, concentrated under vacuum and coevaporated with absolute ethanol to yield **9b** (4.0 g, 86.5%). Rf=0.2 in hexane/CH_2Cl_2/triethylamine (50/48/2); m.p., 100-102°C; U.V. (95% ethanol), max. (nm), 306, 238, 214; ^{31}P NMR: 150.1, 148.7 ppm.

6.2.9. Preparation of N^2-phenoxyacetylguanosine (5c)

Guanosine (14.2g, 50mmol) was coevaporated with dry pyridine (2x200ml) and then suspended in pyridine (300ml). Trimethylsilyl chloride (31.5ml, 5eq.) was transferred into the solution via syringe, followed 2h later by the addition of phenoxyacetyl chloride or phenoxyacetic anhydride (1.2eq.). It was stirred for another 4h, and then cooled down with an ice bath. Water (50ml) was added, followed by the concentrated ammonium hydroxide (50ml) 15 minutes later. The slurry was vigorously stirred for another 10 minutes. The solution was evaporated on a vacuum pump to the volume of about 200ml and then poured into water (1.7L). **5c** was crystalized out of water upon extraction of water layer with methylene chloride (300ml) to yield 10.3g (49%). Rf=0.37 in 20% methanol in methylene chloride; m.p. 168-170°C; UV (water): max. (nm) 264; ^1H NMR (DMSO-d_6, TMS as reference): 8.29 (s, 1, H8); 7.29-7.37 (t, 2, Ar); 7.00-7.03 (m, 3, Ar); 5.83 (d, 1, H1', J=6Hz); 4.87 (s, 2, $PhOCH_2CO$); 4.45 (t, 1, H2'), 4.14 (t, 1, H3'); 3.93 (m, 1, H4'); 3.58 (m, 2, H5',H5''). Anal. Calcd. for $C_{18}H_{19}N_5O_7 \cdot 3/4\ H_2O$: C, 50.17; H, 4.64; N, 16.26. Found: C, 50.09; H, 4.76; N, 15.81.

6.2.10. Preparation of N^2-phenoxyacetyl-5'-monomethoxytritylguanosine (6c)

5c (16g, 38mmol) was coevaporated with dry pyridine (300ml) and then dissolved in the pyridine (200ml). Monomethoxytrityl chloride (14.2g, 1.2eq.) was added and after 6h the reaction was complete. The reaction was quenched with methanol (50ml). The solution was concentrated in vacuo to the volume of 100ml and added to methylene chloride (500ml). The organic phase was washed with brine (2x300ml), dried over sodium sulfate. The crude product was purified by silica gel column chromatography using a 0-5% methanol in methylene chloride gradient. 6c. (19g, 72%). Rf=0.23 in solvent B; m.p. 141-143°C; UV (95% ethanol): max. (nm), 278, 256, 236; ^{1}H NMR (DMSO-d_6, TMS as reference): 8.09 (s, 1, H8); 7.19-7.38 (m, 14, ArH); 6.82-7.00 (m, 5, ArH); 5.85 (d, 1, H1', J=4.5Hz); 4.81 (s, 2, $PhOCH_2CO$); 4.51 (t, 1, H2'); 4.19 (m, 1, H3'); 4.05 (m, 1, H4'); 3.72 (s, 3, $-OCH_3$); 3.21 (m, 2, H5', H5''). Anal. Calcd. for $C_{38}H_{35}O_8N_5 \cdot 1/2H_2O$: C, 65.31; H, 5.02; N, 10.20. Found: C, 65.52; H, 5.14; N, 9.99.

6.2.11. Preparation of N^2-phenoxyacetyl-5'-monomethoxytrityl-2'-triisopropylsilyguanosine (7c)

6c (11g, 16mmol) and imidazole (4.4g, 4eq.) were dissolved in dry DMF (50ml). Triisopropylsilyl chloride (6.85ml, 2eq.) was added and the solution was stirred at room temperature. The solution was poured into methylene chloride (300ml), washed with brine (2x200ml), dried with sodium sulfate, and concentrated in vacuo to give the crude product as a gum. The 2'-silylated product was separated from its 3'-isomer by flash silica gel column chromatography using 20% ethyl acetate in methylene chloride to give 6g of 7c (44.5%). Rf=0.6 in 40% ethyl acetate in methylene chloride; m.p. 103-105°C; UV (95% ethanol): max. (nm) 276, 254, 230, 214; ^{1}H NMR ($CDCl_3$): 7.93 (s, 1, H8); 5.94 (d, 1, H1', J=5.8); 4.89 (q, 1, H2'); 4.29 (m, 1, H3'); 4.24 (m, 1, H4'); 3.48 (q, 1, H5'); 3.33 (q, 1, H5''); 4.57 (s, 2, $PhOCH_2CO$); 3.76 (s, 3, $-OCH_3$); 0.99, 0.92, 0.90 (s, 18, iPr_3). Anal. Cacld. for $C_{47}H_{55}O_8N_5Si$: C, 66.71; H, 6.56; N, 8.28. Found: C, 66.56; H, 6.58; N, 8.22.

6.2.12. Preparation of N^2-phenoxyacetyl-5'-monomethoxytrityl-2'-triisopropylsilylguanosine -3'-N,N-diisopropylmethylphosphoramidite (9c)

To a stirring solution of DMAP (0.04g, 0.2eq.), diisopropylethylamine (1.6ml, 9eq.) and chloro(N,N-diisopropylamino)methoxyphosphine (0.61ml, 3eq.) in THF (2ml), a THF (1.5ml) solution of **7c** (.84g, 1mmol) was added. The reaction was stirred at room temperature overnight. The solution was added to ethyl acetate (100ml, prewashed with brine), washed with brine (3x100ml), and dried with anhydrous sodium sulfate. The solution was concentrated and purified by flash silica gel column chromatography using ethyl acetate/methylene chloride/triethylamine (20/75/5). (**9c**) (0.9g, 90%). Rf=0.62 in 50% ethyl acetate in methylene chloride; m.p. 93-95°C; UV (95% ethanol): max. (nm), 278, 256, 208; ^{31}P NMR ($CDCl_3$): 152.3, 150.4 ppm.

6.2.13. Synthesis of the analogue of yeast formylmethionine initiator tRNA

The synthesis was carried out as previously described[67b] except that N-phenoxyacetylated adenosine and guanosine phosphoramidites were used. The average coupling yields were 97% and overall yield of the synthesis was 31.4%. The synthesis was `aborted' at the end of assembly in order to do the deprotection manually. The column containing the solid support was left on the machine and a thiophenoxide solution was delivered to the column using the manual control mode on the machine. After 1h, the column was washed with methanol (5x), the column was taken off the machine and the column was unpacked. The solid support was transferred to a plastic tube (5ml) to which methanolic ammonia (5ml) was added. The tube was capped with a rubber septum and sealed with tape. After standing for 16 h at room temperature, a needle was inserted to release the presure inside the tube before it was exposed to the air. The supernatants were decanted and quantitated (ca. 300 O.D). The solvent was removed by blowing argon over the surface and it was then dried on Speed-Vac. Part of the sample (40 O.D.) was treated with TBAF (0.5ml) in an Eppendorf tube for 12 h. The desilylation was quenched by addition of water (0.5 ml). The mixture was applied on a Sephadex G-25 coulumn eluted with water to give the final crude product (15. O.D.). This was further 5'-end labeled with ^{32}P and purified on a preparative gel. The purified product was characterized by standard

procedures and its amino acid acceptance activity was assayed (34%) as compared with the natural one.

6.3. Synthetic Methods - Chapter 3

6.3.1. Studies on the pentadecameric homopolymer of uridine

After chain assembly (97% coupling yield) on the automated synthesizer (trityl off), the column was removed and filled with thiophenoxide (thiophenol/triethylamine/dioxane, 1/2/2, 0.5ml). After 45 min. at ambient temperature, the column was extensively washed with ethanol (95%, 5x1ml). Concentrated ammonium hydroxide/ethanol (3/1, 1ml) was introduced into the column via syringe. The ammonium hydroxide solution was pushed into a vial 15 min. later. This procedure was repeated three more times. Half of this solution (2ml) was frozen and then dried on the Speed-Vac. The residue was finally treated with TBAF (1M, 0.5ml, 12h) and then applied on a Sephadex G25 column (6 O.D. units) eluted with water. The fractions containing the product were lyophilized and dissolved in water to a concentration of 1.0 O.D./5ul. 1.0 O.D. of this solution was analyzed by polyacryamide gel electrophoresis. For HPLC analysis, Sample (1 O.D.) was diluted to 100 ul and 10ul was injected each time. The remaining NH_4OH-ethanol solution was diluted with more 3:1 NH_4OH-ethanol (3ml). The vial was sealed tightly and left in an oil bath at 55°C for 18h. Solvents were removed on a Speed-Vac. The residue was then desilylated and desalted to give 8.5 O.D. product.

6.3.2. Studies on the hydrolysis of *t*-butyldimethylsilyl group in 2',3'-O-bis-*t*-butyldimethylsilyluridine (12)

Compound **12** (ca. 2.5mg) was dissolved in concentrated ammonium hydroxide (4ml, 28% ammonium hydroxide/95% ethanol, 3/1) in a sealed vial and left in the oil bath at 55°C for 18h. The solution was then frozen before it was left on the Speed-Vac. The residue was dissolved in acetonitrile (200ul). An aliquot solution (20ul) was further diluted with a solution of acetonitrile/water (1.5ml, 50/50) to have the final sample for HPLC analysis.

HPLC conditions: column, Whatman C8 (4.6x250); solvent, isocratic 60% acetonitrile in water (F=1ml/min). This procedure was repeated under various deacylation conditions listed in Table 2.

6.3.3. Half-time of ammonialysis of N-acylated nucleosides in methanolic ammonia (N^4-benzoylcytidine as an example)

N^4-benzoylcytidine (ca. 1mg) was weighed into a open top screw cap vial with a teflon septum (Fisher), to which a solution of saturated ammonia in methanol (prepared at 0^0C, about 4ml) was added. The vial was sealed tightly and left at room temperature. At certain time intervals, part of the solution (0.5ml) was taken out with a syringe. 80% acetic acid (0.5ml) was added to it. The solution was lyophilized in the Speed-Vac. The residue was coevaporated with water (1ml) once and then dissolved in water (1ml) for HPLC analysis. HPLC conditions: column, AQUAPORE RP300 (4.6x100, Applied Biosystem); solvent, gradient 15-25% methanol in water over 30 minutes (F=1ml/min). The percentage area of N^4-benzoyl cytidine from the HPLC, after the correction for its extinction coefficient, was plotted against the reaction time to give the half-life of the reaction (30min). This was repeated for all the other N-protected derivatives in Table 3.

6.4. Synthetic Methods - Chapter 4

6.4.1. Synthesis of ribozyme RNAs and substrates

The synthesis is illustrated by the preparation of a 35-mer ribozyme shown in Figure 23. The nucleotide was assembled using methylphosphoramidites following the standard RNA synthesis cycle (Table 1) with the average coupling yield of 97% and overall yield of 32% as determined by the trityl assay. The solid support was treated with thiophenoxide and then methanolic ammonia as previously described to give 140 O.D. material. The sample was kept at -20°C. Part of this (ca. 60 O.D.) was desilylated and desalted to give the final fully deprotected product (35 O.D.).

6.4.2. Preparation of the mixed DNA-RNA polymers

The synthesis is illustrated by the preparation of the 8-deoxy ribozyme (Figure 31). The deoxynucleoside phosphoramidites (0.1M in acetonitrile) were added through the "rare base" ports on the synthesizer. The ribonucleoside phosphoramidites were prepared as a 0.11M solution in acetonitrile. CPG (30mg, derivatized with uridine, loading 30umol/mg) was packed in a Teflon column. All other reagents were standard RNA synthesis reagents. The synthesizer was set up such that ribonucleotides were assembled using the RNA synthesis cycle (Table 1) and the deoxyribonucleotides were assembled using the DNA synthesis cycle (provided on 380B DNA Synthesizer by Applied Biosystem). The detritylated solutions were collected and quantitated. Average coupling yields:96%; overall yield: 25%.

The deprotection was as follows. Demethylation was carried out on the machine by using the manual control mode and delivering thiophenoxide (thiophenol/triethylamine/dioxane, 1/2/2, 0.5 ml) to the column containing the solid support. After standing for 1h, the column was extensively washed with methanol. The CPG was taken out of the column and transferred to a 5ml plastic tube. Methanolic ammonia (4ml) was introduced into the tube. The tube was capped with a rubber septum (Aldrich) and tightly sealed with tape. The solution was allowed to stand at ambient temperature for 16h. A needle was then inserted to release the pressure in the tube before the solution was exposed to air. The methanol solution was decanted and the CPG was washed with methanol (3x1ml). The methanol solutions were combined and quantitated (160 O.D.). The solvents were removed by blowing argon over the surface of the solution. The residue was redissolved in water (1ml). Part of it (0.25ml, the rest was stored at -20°C.) was treated with TBAF (0.5ml) for 12h at room temperature. The reaction was quenched with water (0.5ml) and applied to a Sephadex G25 column (60x0.9cm) eluted with water. The fractions containing the product were combined and lyophilized to give the crude product (25 O.D.).

6.5. Synthetic Methods--Chapter 5

6.5.1. Preparation of N^6-benzoyl-5'-O-(monomethoxytrityl)-3'-O-(*t*-butyldimethylsilyl)adenosine 2'-N,N-diisopropylmethylphosphoramidite. (13a)

A THF solution (2ml) of **3a** (1.2g, prepared as previously described[65b]) was added to a stirring THF solution (3ml) of diisopropylethylamine (1.1ml, 4 equiv.), chloro(N,N,-diisopropylamino)methoxyphosphine (0.4ml, 1.2 equiv.), and DMAP (39mg). After stirring for 3 h, the mixture was poured into ethyl acetate (100ml, prewashed with brine), washed with brine (2x100ml), dried over sodium sulfate. The solvents were removed under reduced presure. The crude product was purified by flush chromatography. Chromatographic solvent: Et_3N/hexane/EtOAc (3/65/35). Yield: 76% (1.1g). m.p.: 90-92°C; U.V. (95% EtOH, max., nm): 213, 234, 283; Rf: 0.65 in solvent C.

The N-phenoxyacetyl protected amidite was prepared following the similar procedure. A dry THF (2ml) solution of **8a** (1.0g) was slowly added to a stirring dry THF (3ml) solution of diisopropylethylamine (0.8ml, 4 equiv.), DMAP (50mg, 0.2 equiv.), chloro(N,N,-diisopropylamino)methoxyphosphine (0.3ml, 1.4 equiv.) in a Hypervial. A precipitate formed in 10 mins. After it was stirred for 3h, TLC of reaction mixture indicated complete reaction. The reaction was worked up as **13a**. Chromatagraphic solvent: Et_3N/hexane/CH_2Cl_2 (3/47/50) to give pure **13e** as a white solid in 83% yield (1.0g). m.p.: 60-65°C; U.V. (95% EtOH, max., nm): 216, 274; Rf: 0.63, 0.58 in solvent C.

6.5.2. Preparation of N^4-benzoyl-5'-O-(monomethoxytrityl)-3'-O-(*t*-butyldimethylsilyl)cytidine-2'-N,N-diisopropylmethylphosphoramidite. (13b)

To a rubber septum capped Hypervial containing a stirring dry THF (2ml) solution of chloro(N,N-diisopropylamino)methoxyphosphine (0.34ml, 1.3 equiv.), diisopropylethylamine (0.95ml, 4 equiv.), and DMAP (33mg), a THF (3ml) solution of **3b** (1.36g) was dropwisely added via a syringe. A precipitate formed instantly upon addition. After the solution was stirred at room temperature for 3 h, the reaction mixture was worked up and purified as **13a**. Chromatographic solvent: hexane/EtOAc/NEt_3, 70/30/3. Yield: 74% (0.9g). m.p.: 87-90°C. U.V. (95% EtOH, max., nm): 213, 234, 264, 308; Rf: 0.42, 0.23 in solvent C.

6.5.3. Preparation of N^2-phenoxyacetyl-5'-O-(monomethoxytrityl)-3'-O-(triisopropylsilyl)guanosine-2'-N,N-diisopropylmethylphosphoramidite. (13c)

To a stirring THF (3ml) solution of chloro(N,N,-diisopropylamino)methoxyphosphine (0.61ml, 3equiv.), diisopropylethylamine (1.6ml, 9 equiv.), and catalytic amount of DMAP (40mg), a dry THF (2ml) solution of **8c** (0.88g) was added via a syringe. A precipitate appeared after stirring for 5 min.. The reaction was stirred at room temperature for another 12 h and then worked up as **13a**. The crude product was purified on flush silica gel column eluted with CH_2Cl_2/EtOAc/Et_3N (65/30/5) to give pure **13c** in 77% yield (0.81g). m.p.: 79-81°C; U.V. (95% EtOH, max., nm): 210, 258, 378; Rf: 0.35, 0.26 in solvent C.

6.5.4. Preparation of 5'-O-(monomethoxytrityl)-3'-O-(t-butyldimethylsilyl)uridine-2'-N,N-diisopropylmethylphosphoramidite. (13d).

To a stirring dry THF solution (3ml) of chloro(diisopropylamino)methoxyphosphine (1.39 ml, 1.3 equiv.), diisopropylethylamine (1.39 ml), and catalytic amount of DMAP (50mg), a dry THF solution (4ml) of **3d** (1.26g) was added dropwise. It was stirred for 3h at room temperature and then worked up as **13a**. The crude product was purified on flush silica gel column eluted with CH_2Cl_2/hexane/NEt_3 (50/48/2). The fractions containing the product were evaporated to give **13d** as a white solid in 88% yield (1.4g). m.p.: 81-83°C; U.V. (95% EtOH, max., nm): 234, 264; Rf: 0.53 in solvent C.

6.5.5. Synthesis of protected dinucleotides (14a-d, 15a-d).

a). **14a**: 2',3'-Bis-O-(acetyl)uridine (131mg, prepared by detritylation of 5'-O-monomethoxytrityl-2',3'-bis-O-(acetyl)uridine), **4a** (440mg, 1.2 equiv.), and tetrazole (134mg, 4 equiv. to **4a**) were transferred to a dry Hypervial. Dry THF (3ml) was introduced into the vial. After the reaction was stirred at ambient temperature for 3h, collidine (250ul) was added to the solution, followed by the dropwise addition a 0.1 M aqueous iodine solution (7/3, water/THF). The addition of iodine solution stopped until a dark brown color solution persisted. The solution was stirred for another 10 min. and then transferred to CH_2Cl_2 (50ml). The organic solution was extracted with saturated sodium chloride solution (50ml) containing 5% sodium bisulfite (5ml), dried with sodium sulfate, and then evaporated under reduced pressure. The crude product was purified on a flush silica gel

column eluted with 4% MeOH in CH_2Cl_2 to give 0.34g of pure **14a** (73%). m.p.: 118-121°C; U.V. (95% EtOH, max., nm): 210, 278; Rf: 0.24 in solvent A.

b). **15a**: Same as **14a**. Scale: **13a**, 440mg; 2',3'-O-bis-(acetyl)uridine, 131mg; tetrazole, 134mg; and THF (3ml). The reaction was worked up as **14a**. The crude product was loaded on flash silica gel column eluted with CH_2Cl_2/EtOAc (50/50), followed by 4% methanol in CH_2Cl_2. **14b** was obtained in 71% yield (330mg). m.p.: 111-112°C; U.V. (95% EtOH, max, nm): 210, 278; Rf: 0.33 in solvent A.

c). **14b**: Same as for **14a**. Scale: **4b**, 323mg; 2',3'-O-bis-(acetyl)uridine, 98mg; tetrazole, 101mg; THF (2.5ml). After the reaction was worked up as **14a**, the crude product was purified by flash chromatography using the elution of 50% EtOAc in CH_2Cl_2, followed by 4% methanol in CH_2Cl_2 to afford 210mg of **15a** (63%). m.p.: 119-122°C; U.V. (95% EtOH, max, nm): 208, 263, 306; Rf: 0.31 in solvent A.

d) **15b**: Same as for **14a**. Scale: **13b**, 323mg; 2'3'-O-bis-(acetyl)uridine, 98mg; tetrazole, 101mg; THF (2.5ml). The reaction mixture was worked up and purified as for **14a**. Chromatographic solvent (for flash silica gel column): 5% methanol in CH_2Cl_2. **15b** was obtained in 60% yield (200mg). m.p.: 123-125°C; U.V. (95% EtOH, max., nm): 206, 263, 306; Rf: 0.26 in solvent A.

e) **14c**: Same as **14a**. Scale: **7c**, 361mg; 2',3'-O-bis-(acetyl)uridine, 98mg; tetrazole, 100mg; THF, 2ml. The reaction was worked up and purified same as **14a**. Chromatographic solvent (flash silica gel column): 3% methanol in CH_2Cl_2. **14c** was obtained in 56% yield (210mg). m.p.: 119-121°C; U.V. (95% EtOH, max., nm): 210, 263; Rf: 0.29 in solvent A

f) **15c**: **8c** (361mg), 2',3'-O-bis-(acetyl)uridine (98mg) tetrazole (100mg) were dissolved in THF (2ml) in a dry Hypervial. The reaction was stirred for 6 h at room temperature and then worked up as **14a**. The crude product was loaded on silica gel column eluted with 3%, 4% methanol in CH_2Cl_2. **15c** was obtained in 54% yield (200mg). m.p.: 115-117°C; U.V. (95% EtOH, max., nm): 210, 263; Rf: 0.26 in Solvent A.

g) **14d**: **4d** (1.1g), 2',3'-O-bis-(*t*-butyldimethylsilyl)uridine (0.48g), tetrazole (0.4g) were dissolved in THF (6ml) in a dry Hypervial. After the reaction mixture was stirred for 90 min. at ambient temperature, collidine (0.9ml) was introduced into the solution, followed by the addition of aqueous iodine solution (0.1M in THF/H_2O, 50/50). The addition of iodine solution stopped when a dark brown color persisted. The solution was stirred for another 10 min. and then worked up as for **14a**. The crude mixture was purified on a flush silica gel column eluted with 2%, 3%, and 4% methanol in CH_2Cl_2. Pure **14d** was obtained

as a white solid in 66% yield (0.78%). m.p.: 103-106°C; U.V. (95% EtOH, max., nm): 208, 264; Rf: 0.37 in solvent A.

h) **15d**: Same as for **14d**. Scale: **13d**, 0.51g; 2',3'-O-bis-(*t*-butyldimethylsilyl)uridine, 0.24g; tetrazole, 0.25g; THF, 2ml. The reaction was carried out in the same manner as **14d**. Chromatographic solvent: 3% methanol in CH_2Cl_2. Pure **15d** was obtained in 76% yield (450mg). m.p.: 120-123°C; U.V. (95% EtOH, max., nm): 208, 263; Rf: 0.27 in solvent A.

6.5.6. Preparation of detritylated dinucleotides (16a-d, 17a-d).

a). **16a**. To a round bottom flask containing **14a** (300mg) was added a solution of 5% trichloroacetic acid in CH_2Cl_2 (25ml). The orange solution was swirled a few times. After the solution was left standing at room temperature for 20 min, it was poured into CH_2Cl_2 (25ml). The organic solution was extracted with 5% aqueous sodium bicarbonate solution (2x50ml), dried over Na_2SO_4, and then concentrated. The residue was dissolved in minimum amount of CH_2Cl_2 and precipitated in Et_2O (50ml) to give pure **16a** as white powders in 61% yield (140mg). m.p.: 130-133°C; U.V. (95% EtOH, max., nm): 210, 278; Rf: 0.65 in solvent B.

b). **17a**. Same as **16a**. Scale: **15a**, 300mg; 5% TCA, 25ml. The reaction mixture was worked up and purified as **16a**. **17a** was obtained in 52% yield (120mg). m.p.: 126-129°C; U.V. (95% EtOH, max., nm): 210, 278; Rf: 0.65 in solvent B.

c). **16b**. Same as **16a**. Scale: **14b**, 120mg; 5% TCA, 25ml. The reaction mixture was worked up as **14a**. The residue was precipitated in hexane/Et_2O (2/1, 30ml) to afford pure **16b** as white powder in 55% yield (50mg). m.p.: 130-133°C; U.V. (95% EtOH, max., nm): 208, 263, 306; Rf: 0.59 in solvent B

d). **17b**. Same as **16a**. Scale: **15b**, 110mg; 5% TCA in CH_2Cl_2, 25ml. The crude reaction mixture was precipitated in 50% ethyl ether in hexane, affording **17b** as white powder in 60% yield (50mg). m.p.: 132-135°C; U.V. (95% EtOH, max., nm): 206, 263, 306; Rf: 0.65 in solvent B.

e). **16c**. Same procedure as that for **16a** was followed. Scale: **14c**, 150mg; 5% TCA in CH_2Cl_2, 25ml. After same work up as for **16a**, the crude product was purified on silica gel column using 5% methanol in CH_2Cl_2 as eluent. The fractions containing the product was concentrated under reduced pressure and precipitated in hexane (50ml) to afford **16c** as white powder in 65% yield (80mg). m.p.: 119-121°C; U.V. (95% EtOH, max., nm): 210, 263; Rf: 0.59 in solvent B.

f). **17c**. Scale: **15c**, 100mg; 5% TCA in CH_2Cl_2, 25ml. The reaction was carried out and worked up as for **16a**. The crude product was precipitated in Et_2O (50ml), giving 40mg of **17c** as white powder (51%). m.p.: 117--120°C; U.V. (95% EtOH, max., nm): 210, 278; Rf: 0.55 in solvent B.

g). **16d**. Scale: **14d**, 150mg; 5% TCA in CH_2Cl_2, 25ml. The reaction was performed and worked up as **14a**. The crude mixture from workup was loaded on silica gel column eluted with EtOAc/Et_2O/CH_2Cl_2 (4/1/2) and then 5% methanol in CH_2Cl_2. **16d** was obtained in 82% (95%). m.p.: 122-125°C; U.V. (95% EtOH, max., nm): 208, 264; Rf: 0.59 in solvent B.

h). **17d**. Scale: **15d**, 150mg; 5% TCA in CH_2Cl_2, 15ml. Same procedure as that used for **16a** was followed. The crude product from work up was purified on silica gel column eluted with 4% methanol in CH_2Cl_2. **17d** was obtained in 87% yield (100mg). m.p.: 108-110°C; U.V. (95% EtOH, max., nm): 208, 264; Rf: 0.59 in solvent B.

6.5.7. HPLC analysis of intermediates during the deprotection. The general procedure is illustrated by the deprotection of 16a to give 22a

a). **Removal of methyl phosphate protection (18a-d, 19a-d). 16a** (45mg) was transferred to an Eppendorf tube to which a solution of thiophenoxide (NEt_3/dioxane/thiophenol, 2/2/1, 1.2ml) was added. The solution was left standing for 1 h at room temperature. TLC indicated a very polar product formed (Rf=0.11 in 20% methanol in CH_2Cl_2). The reaction was quenched by the addition of 95% ethanol (1.0ml). The solution was concentrated under reduced pressure. The residue was dissolved in minimum amount of CH_2Cl_2 and precipitated in hexane (50ml). The precipitates were filtered to give white powder as product **18a** (35mg). This was used for ^{31}P measurement, HPLC analysis, and the next deprotection step without further purification. The sample (0.2mg) was dissolved in a solution of CH_3CN and TEAA solution (1/1, 100ul) for HPLC analysis. 10ul was used for each analysis. HPLC conditions: column, Whatman C8 Partisil 5 (4.6x250mm); Solvent, isocratic or gradient CH_3CN in TEAA (0.1M, pH7), flow = 1 ml/min. (Table 8). For ^{31}P NMR the sample (c.a. 15mg) was dissolved in $CDCl_3$ (see Table 8).

b). **Removal of N-acyl protecting group (20a-c, 21a-c). 18a** (ca. 0.5mg) was weighed into a 5ml plastic tube. Methanolic ammonia (4ml) was introduced to the tube. The tube was then capped with rubber septum (Aldrich) and sealed tightly with tape. After the solution was left standing at room temperature for 12h, a needle was inserted to release the pressure

inside the tube before it was exposed to air. The solvent was evaporated by blowing argon over it and then lyophilized on Speed-Vac. The residue was then dissolved in water (1ml) and divided into two Eppendorf tubes (0.5ml each). Part of these was used for the next step. Another part was further diluted with water to 1ml to give a solution of **20a** for HPLC analysis. HPLC conditions: column, Whatman C8 Partisil 5 (4.6x250mm), solvent, isocratic CH_3CN in TEAA (0.1M, pH7), flow = 1ml/min. (see Table 9).

c). **Removal of 2'-silyl protecting group (22a-d, 23a-d).** **20a** obtained above was lyophilized on Speed-Vac. A THF solution of TBAF (100ul) was then added to the sample. After standing for 12h at room temperature, sterile water (0.5ml) was added. The solution was lyophilized and then redissolved in water (1ml) to give the solution for HPLC analysis. 10ul was used for each injection. HPLC conditions: column, Whatman C8 Partisil 5 (4.6x250mm); solvent, isocratic CH_3CN in TEAA (0.1M, pH7), flow = 1ml/min. (see Table 10).

CONTRIBUTIONS TO KNOWLEDGE

The base-labile phenoxyacetyl group has been investigated for the protection of the amino group in oligoribonucleotide synthesis. N-Phenoxyacetyl protected cytidine nucleoside methylphosphoramidite was found not suitable for the present standard RNA synthesis because of its poor solubility in acetonitrile. The use of N-phenoxyacetyl protected adenosine and guanosine methylphosphoramidites in combination with uridine and benzoylated cytidine methylphosphoramidites allows for mild deacylation conditions. These amidites have been successfully used in the synthesis of several oligoribonucleotides. The mild deacylation conditions will probably be useful for the synthesis of oligoribonucleotides containing base labile nucleotides. A 76-unit oligoriboncleotide corresponding to yeast initiator formylmethionine tRNA was also synthesized. The synthetic molecule was found to have 35% of the activity of the natural one.

The standard debenzoylation conditions using aqueous ammonium hydroxide (55°C, 18 h) were found to lead to some cleavage of assembled nucleotide chains in oligoribonucleotide synthesis. The cleavage was brought about by the hydrolysis of the 2'-silyl protecting groups in the ammonium hydroxide solution. The hydrolysis reaction was found to depend on the solvent. It is most severe in aqueous ammonium hydroxide and drastically reduced but still detectable in ammonium hydroxide and ethanol mixture (3/1). The 2'-silyl group is stable in anhydrous methanolic ammonia.

The deacylation conditions that eliminate such side reactions have been developed. This includes the use of methanolic ammonia as the deacylation reagent and the use of the following amino protecting groups: the benzoyl group for cytidine, either the benzoyl or phenoxyacetyl group for adenosine, and the phenoxyacetyl group for guanosine. Benzoyl group remains adequate for the amino protection for adenosine and cytidine. Oligoribonucleotides can be synthesized in higher yields with the new base protection and deprotection procedure since less short default sequences are formed.

A rigorous proof of the fidelity of the alkysilyl groups as 2'-protection in the phosphoramidite coupling procedure in the oligoribonucleotide synthesis has been provided. First of all, it was shown by ^{1}H and ^{31}P NMR that the 2'-silylated nucleoside 3'-methylphosphoramidite, the key intermediate for the synthesis, was prepared free of any contamination with its isomer, the 3'-silylated nucleoside 2'-methylphosphoramidite. This, in turn, confirms that the 2'-silyl groups are stable under the conditions of phosphorylation.

It was then demonstrated that the protected oligoribonucleotides assembled using the 2'-silylated nucleoside 3'-phosphoramidites have the correct 3'-5' phosphate linkages. It has been further elaborated that the 3'-5' phosphate linkages remain intact in the deprotection of the 5'-trityl groups, the methyl phosphate protecting groups, and the N-protecting groups. Finally it has also been shown that the removal of the 2'-silyl protecting group does not cause any cleavage or isomerization of the assembled nucleotide. The synthetic oligoribonucleotides were proven not to have any contamination with the unnatural 2'-5' phosphate linkage. The synthetic oligoribonucleotide products prepared on a solid support using the 2'-silylated nucleoside 3'-phosphoramidites were also confirmed to have the correct phosphate linkages.

Several ribozymes have been chemically synthesized. At this time of the work, this was the first catalytic RNA that was made from a nonbiochemical source. The fact that these synthetic molecules have a biological activity comparable to the biochemical sample has further demonstrated the practicality of the synthetic method.

A general procedure to synthesize mixed DNA-RNA polymers has been developed. The synthesis demonstrated that chemical synthesis not only allows the classic sequence-specific modification of RNA but also makes it possible to carry out the site-specific mutation of RNA, which the *in vitro* biochemical method can not accomplish. Thus we may investigate the specific roles of some functional groups of an RNA.

The use of mixed DNA-RNA polymers has been demonstrated in the study of ribozyme catalysis. Using this technique, we have been able to show, for the first time, the essential role of the 2'-hydroxyl group at the cleavage site for the "hammer-head" ribozyme. The result supports the proposed 2'-nucleophilic attack mechanism of this ribozyme reaction.

The mixed deoxyribo-ribo nucleotide polymers were found to have catalytic activity. This demonstrated, for the first time, that an analogue of a natural ribozyme can also have the catalytic activity. Comparison of the several rationally modified ribozymes with the natural ribozyme suggests that some hydroxyls in the ribozyme are also important for the ribozyme activity. Future work will be directed to defining the specific hydroxyl groups that are related to the ribozyme catalytic activity. The specific chemical modification can probably be extended to the other function groups in a ribozyme. By comparing the modified one with the natural ribozyme, it possible to determine the active sites of a ribozyme. The chemical synthesis of oligoribonucleotides and their analogues will be a

unique approach in the understanding of the fundamental biological processes involving RNA.