SYNTHESIS OF SHORT, SELF-COMPLEMENTARY DNA OLIGOMERS

AND

SOLUTION STUDIES OF THEIR DUPLEX STRUCTURE AND FORMATION

SYNTHESIS OF SHORT, SELF-COMPLEMENTARY DNA OLIGOMERS AND SOLUTION STUDIES OF THEIR DUPLEX STRUCTURE AND FORMATION

by

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ABSTRACT

A phosphotriester synthesis for short deoxy oligomers was developed based on the RNA synthesis developed in Neilson's laboratory. This synthesis parallels that of Catlin and Cramer (1973) but differs significantly in a number of procedures and reagents used and represents an overall improvement in every aspect of the previous method. This synthetic procedure allows the synthesis of sufficient quantities of DNA for ^{1}H-NMR analysis. The following sequences were successfully synthesized using this procedure:

d(GCA)
d(AGCT)
d(ACGT)
d(ACGTp)
d(ACGTACGTp).

It was found by variable temperature ^{1}H-NMR that the trimer and the tetramer duplexes had a very low Tm (0-10 $^{\circ}$C) as compared to their RNA counter-parts which had Tms between 29-34 $^{\circ}$C. This demonstrates quantitatively that the short DNA duplex is significantly less stable than the short RNA duplex. Consequently, sequences of at least five or six bases in length will be required for model studies of DNA duplex stabilities using variable temperature NMR methods.

A CD study of d(ACGTACGTp) in conditions of low salt (1M NaCl) and high salt (5M NaCl) demonstrated that a high salt B to Z-helix transition did not occur. Instead, the duplex remained in the right handed B form in both low and high salt.

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ABBREVIATIONS

Å	angstrom (10^{-8} cm)
Ac	acetyl
BBB	2,2,2-trichloroethyl phosphate
Bz	benzoyl
CD	circular dichroism
CE	cyanoethyl
DNA	deoxyribonucleic acid
dA	2'-deoxy adenosine
dC	2'-deoxy cytidine
DCC	N,N'-dicyclohexylcarbodiimide
dG	2'-deoxy guanosine
DMF	N,N-dimethylformamide
D_2O	deuterium oxide
DSS	2,2-dimethyl-2-silapentane-5-sulfonate
dT	2'-deoxy thymidine
EtOH	ethanol
HPLC	high performance liquid chromatography
Hz	hertz (cycles/second)
Ib	isobutyryl
IR	infra-red
J	coupling constant
NMR	nuclear magnetic resonance
MeOH	methanol
MMTr	p-monomethoxytrityl
MS	mesitylenesulfonyl chloride
MST	mesitylenesulfonyl 1,2,4-triazole

MSTet	mesitylenesulfonyl tetrazole
NOE	nuclear overhauser effect
ORD	optical rotatory dispersion
ppm	parts per million
PTSA	p-toluenesulfonic acid
R_f	ratio of distance travelled by solute to that of solvent
RNA	ribonucleic acid
TEA	triethylamine
THF	tetrahydrofuran
Tm	melting temperature of a duplex at which 50% of the strands exist in duplex form.
TPS	2,4,6-triisopropylbenzenesulfonyl chloride
TPSNI	2,4,6-triisopropylbenzenesulfonyl nitroimidazole
TPSTet	2,4,6-triisopropylbenzenesulfonyl tetrazole
tRNA	transfer RNA
UV	ultraviolet
VT-NMR	variable temperature NMR
Zn/Cu	zinc-copper couple

Deoxy mono and oligonucleotides are abbreviated in the standard format (IUPAC-IUB Commission on Biochemical Nomenclature, 1970). d(pA) represents 5' phosphorylated 2'deoxyadenosine, d(Cp) represents 3' phosphorylated 2'-deoxycytidine and d(CpA) represents 2'-deoxycytidylyl (3'-5') 2'-deoxyadenosine. In this text, "d" is replaced with "r" when representing RNA sequences. Abbreviations for synthetic intermediates are described in the text.

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1 INTRODUCTION

The abiltity to synthesize oligonucleotides of defined sequence has formed the basis of many important biological discoveries over the past two decades. The elucidation of the genetic code (Niremberg and Leder, 1964; Khorana et al, 1967) and the characterization of a left handed DNA helix (Pohl and Jovin, 1972; Wang et al, 1979) are just two of many endeavours made possible through the chemical synthesis of RNA and DNA oligomers. Currently the synthesis of oligonucleotides is used to make probes, primers, genes (Khorana et al, 1976; Edge et al, 1981) and provides the means for site directed mutagenesis (Gillam et al, 1975; Smith, 1982). Synthetic DNA and RNA continues to be of great value in improving our understanding of the stucture/function role of many biological molecules and systems.

1.1 Chemical Synthesis of Oligodeoxynucleotides

The challenge of synthesizing DNA can be appreciated by studying the chemical structure of d(AGCT) shown in figure 1.1. The specific 3' to 5' phoshphodiester linkages and the presence of a large number of functional groups demonstrate the need for highly specialized chemical methods. For this reason, the synthesis of DNA oligomers proceeds by means of protecting group chemistry. Certain functional groups are

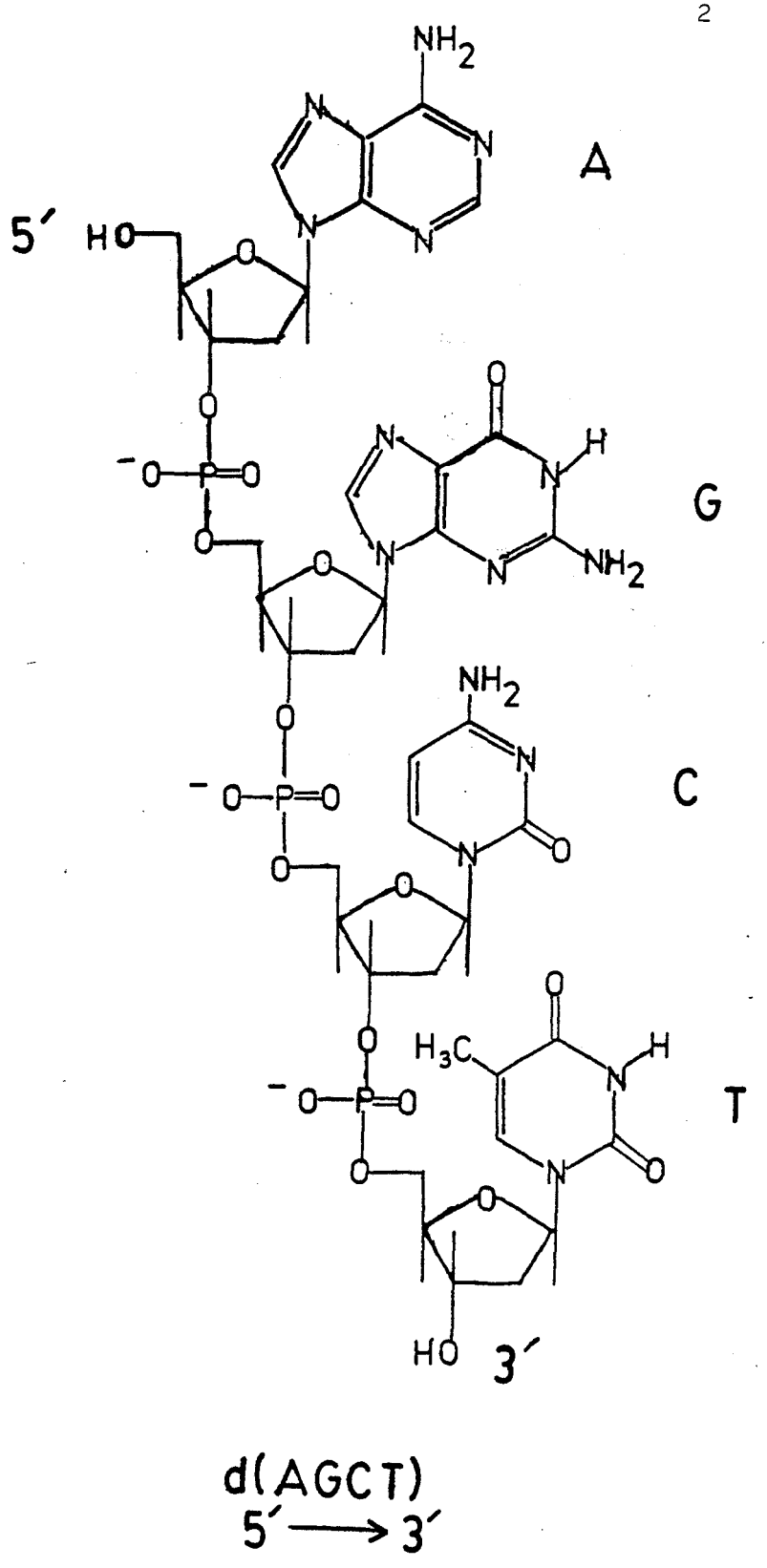


Figure 1.1 - Chemical structure of d(AGCT).

transiently protected during the course of the synthesis so that specific chemical bonds are formed and side reactions and products are minimized. Specifically, it is necessary to deprotect the 5'.hydroxyl and the 3' hydroxyl selectively without affecting the other masking groups. Few protecting groups can meet these criteria.

Two classical methods have been developed. The first, pioneered by Khorana and co-workers in the sixties is the phosphodiester method. This synthesis involves the formation of phosphodiester linkages with an unprotected phosphate. Synthesis of this nature presents many problems. Side products result from branching during synthesis. The increasing polarity of the compound during elongation creates purification problems. The phosphodiester linkages are also rather sensitive to acid and base resulting in strand cleavage. These problems naturally led to the evolution of the phosphotriester method where each internucleotidic phosphodiester function is masked by a protecting group (Neilson, 1969; Eckstein and Rizk, 1969; Letsinger and Ogilvie, 1969; Arentzen and Reese, 1977). The resulting compounds were non-polar, which afforded good solubility in organic solvents. They could therefore be purified by conventional silica gel chromatography and synthesized in relatively large quantities, up to 20g in some cases. The possible formation of pyrophosphates is eliminated. The general scheme for this synthesis involves the

phosphorylation of the 3' hydroxyl of suitably protected nucleosides (or oligonucleotides) and condensation with a 5' hydroxyl of another suitably protected nucleoside (or oligonucleotide).

The strategy was further refined in the "modified" triester approach which included the introduction of another phosphate protecting group (2-cyanoethyl) giving a fully protected phosphate which can be selectively removed (Itakura et al, 1973; Catlin and Cramer, 1973). This modification allowed the synthesis to proceed in either the 3' to 5' or 5' to 3' direction.

Although many variations exist in DNA synthesis, the phosphotriester approach has become the method of choice for the synthesis of sufficient quantities of DNA for physical studies (such as crystallography or NMR).

1.1.1 Protection of the Primary Amino Groups on the Heterobases

The primary amino groups, N^6-A, N^2-G and N^4-G are protected to prevent the formation of phosphoramidate products during the course of the synthesis (dT does not have an amino function and therefore does not require heterobase blocking). Protecting the amino group also reduces the polarity of these compounds. The most commonly used protection is an acyl group forming an amide bond (Khorana et al, 1961). Originally, three different groups were employed: benzoyl for dA, isobutyryl for dG and anisoyl for dC. Recent

approaches have replaced the anisoyl with benzoyl for dC. The fact that the amide bond is more stable to cleavage than the ester bond allows the specific functionalization of the amino group over the 5' and 3' hydroxyls. These protecting groups are base labile and are usually removed by methanolic ammonia.

The acylation of the amino function creates a problem in that the N-glycosidic linkage becomes sensitive to hydrolytic cleavage under acidic conditions. The purines tend to be at greater risk with dA being the most sensitive to acid.

Reactivity at the O6 of dG has been responsible for consistently lower yields for compounds containing dG (Jones et al, 1981). Recent attempts at protecting the O6 of dG with a benzyl (Watkins et al,1982) or 2-phenylthioethyl group (Kuzmich et al., 1982) have been incorporated in the general synthesis of DNA oligomers with some success, however there is some question as to whether the incorporation of these groups and the extra steps involved have resulted in an overall increase in yield.

1.1.2 Blocking of the 5' Hydroxyl Group

The 5' hydroxyl is a primary alcohol whereas the 3' hydroxyl is a secondary alcohol and thus more hindered. This forms the basis of 5' hydroxyl protection in that very large bulky groups will have greater access to the 5' hydroxyl over

the 3' hydroxyl.

The most commonly used protecting group for the 5' hydroxyl is the trityl group or its p-methoxy derivatives (Smith et al, 1962). This protecting group is introduced by means of the alkyl halide to form an ether linkage. This group is acid labile, with cleavage occurring by means of the S_N1 mechanism (see figure 1.2). Careful consideration must be taken since N-protected purines are sensitive to acid as stated earlier.

The addition of one or more methoxy groups on the para positions of the benzene rings reduces the selectivity of the group and increases the acid lability by approximately ten fold for each additional methoxy group. Smith et al have determined that the monomethoxytrityl group provided the proper balance of selectivity and acid lability. The dimethoxytrityl group has since tended to gain in popularity (Narang et al, 1980).

The trityl group also presents an added advantage in that it is distinguished by a yellow-orange color after treatment with acidic ceric sulfate and heated to 150-200°C. This can be utilized for detecting compound containing this group during tlc. One can easily distinguish between compounds containing the trityl group from compounds lacking this group (which usually appear brown to black after similar treatment) even when both components are in the same reaction mixture.

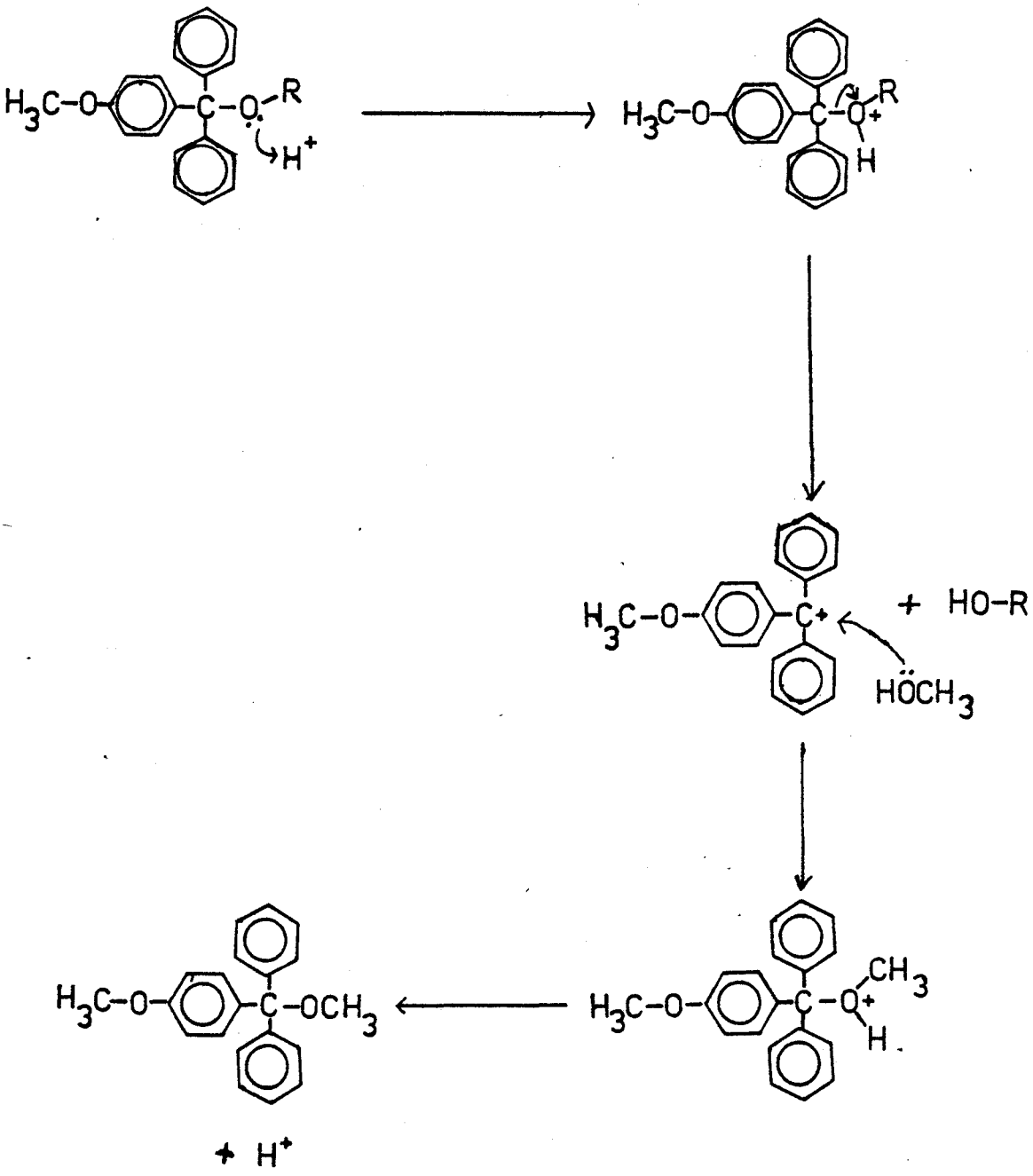


Figure 1.2 - Mechanism of acid catalysed de-tritylation by means of p-toluene sulfonic acid in CH_2Cl_2/CH_3OH (2:1, v/v).

methylene chloride (Koster and Sinha, 1982) and zinc bromide in nitromethane (Matteucci and Caruthers, 1980) have been successfully implemented in selectively removing the trityl group from tritylated compounds. Since these reactions are carried out under neutral conditions, the possibility of glycosidic bond cleavage is minimized.

Other 5' hydroxyl protecting groups have been developed. The levulinyl group (Hassner et al, 1975) has been used successfully and is cleaved by sodium borohydride or hydrazine. Silyl derivatives (Ogilvie et al., 1974) which can be selectively cleaved by tetrabutylammonium fluoride in THF have also been used. However, the reagent also tends to cleave the phosphotriester protecting group.

1.1.3 Blocking of the 3' Hydroxyl

The 3' hydroxyl of non-phosphorylated nucleosides is protected by acylation forming a base labile ester. The most commonly used protecting groups are the benzoyl or acetyl groups. The reagent used is often an acid anhydride. The 3' hydroxyl is usually blocked after the amino group on the heterobase and the 5' hydroxyl group have been protected, giving a fully protected non-polar molecule (see figure 1.3). The 3' acyl blocked hydroxyl cannot be removed selectively without considerable effect on the amide bond on the protected heterobases.

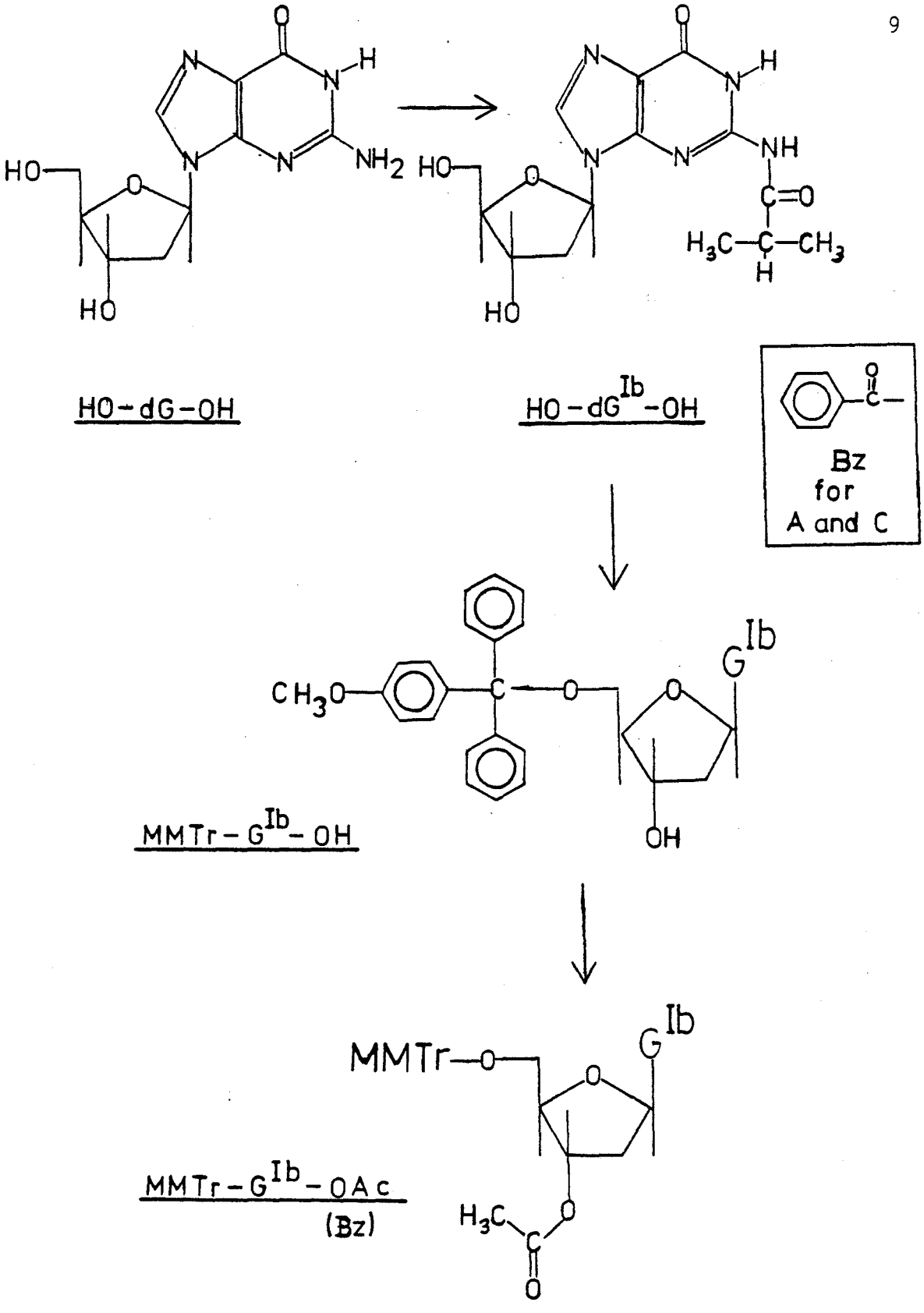


Figure 1.3 - synthesis of fully protected terminal residues. Note that benzoyl (Bz) is used to N-protect dA and dC.

A number of protecting groups have been investigated to serve this function including 2-cyanoethyl (Tener, 1961), 2,2,2-trichloroethyl (Woodward et al, 1966; Eckstein, 1966) and a variety of aryl and alkyl groups. Currently, the most commonly used group is the o- or p-chlorophenyl (Narang et al, 1970; van Boom et al, 1971) for DNA and the 2,2,2-trichloroethyl group for RNA (Neilson, 1969).

The o- and p-chlorophenyl group is often removed by base hydrolysis. A disadvantage resulting from this procedure is the formation of side products and/or strand cleavage. Cleavage occurs by an S_N2 mechanism with the nucleophile attacking the phosphorous giving rise to a trigonal bipyramid intermediate followed by the breaking of a phosphorous oxygen bond at the apical positions. Side products occur depending upon the direction of nucleophilic attack as well as an intramolecular process termed "pseudo-rotation" (Haake and Wertheimer, 1961; Kaiser et al, 1963). These processes allow a variety of trigonal bipyramid intermediates giving rise to different substituents obtaining an apical position.

On the other hand, the 2,2,2-trichloroethyl group is removed by reductive cleavage by zinc/copper couple resulting in a β-elimination reaction as illustrated in figure 1.5a. This cleavage involves a specific carbon-oxygen bond rather than a phosphorous oxygen bond eliminating the possibility of side products.

1.1.5 Phosphorylation of the 3' Hydroxyl

The 3' hydroxyl is phosphorylated by reacting with a protected phosphate in the presence of a condensing agent. The condensing agent is a critical component which determines the efficiency and integrity of the phosphorylation and coupling procedure. A variety of condensing reagents have been developed since the original use of DCC (Gilham and Khorana, 1958).

The reaction proceeds by "activating" the phosphate with a good leaving group followed by the substitution of the leaving group with incoming free 5' hydroxyl (see figure 1.4). DCC was replaced by MS (Jacob and Khorana, 1964) and TPS (Lohrman and Khorana, 1966). The generation of chloride ions resulting from the use of these agents together with their high reactivities resulted in the formation of side products and lower yields. Substantial improvement was made possible by replacing the chloride with imidazolides, triazolides and tetrazolides represented by MST (Narang et al, 1974), MSTet and TPSTet (Stawinski et al, 1977) and TPSNI (van Boom et al, 1977). Although these agents are less reactive than the chloride agents, the yields were much improved. The tetrazolides and their derivatives have greater reactivity than the triazolides. They are also more sensitive to hydrolysis by atmospheric moisture and must be stored at very cold temperatures or made immediately prior to use.

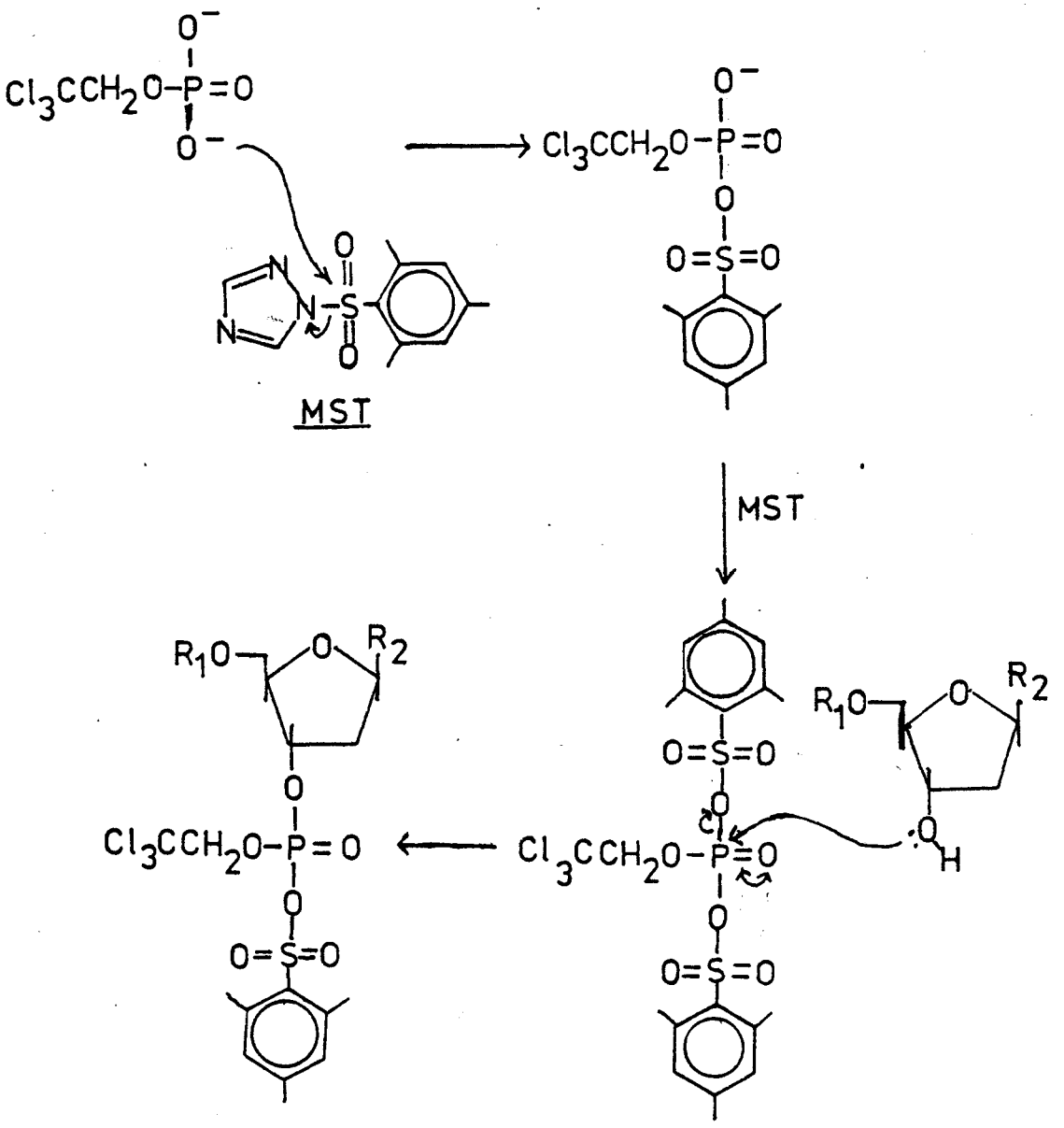


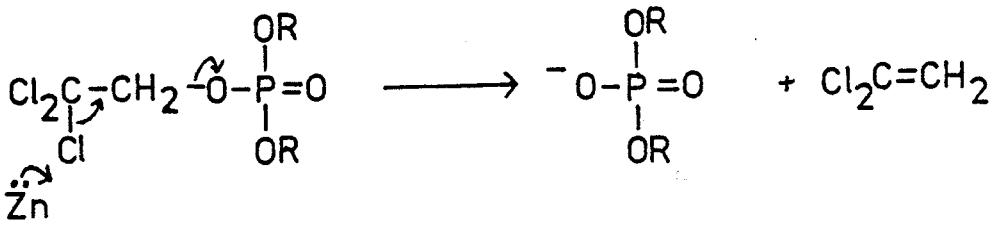
Figure 1.4 - Activation by MST and subsequent condensation of 2,2,2-trichloroethyl-phosphate with a 3'-OH of a suitably protected deoxymononucleoside.

The 3' phosphorylated mononucleotide is transiently protected with the 2-cyanoethyl group (Tener, 1961; Catlin and Cramer, 1973) to yield a completely protected non-polar molecule. This group is removed selectively under mildly basic conditions without affecting the amide bond on the protected heterobase. This group is removed by β-elimination (see figure 1.5b) with the cleavage of a specific carbon-oxygen bond. The reagent often used is anhydrous triethylamine and more recently, t-butyl amine (Hsiung, 1982). The synthesis of fully blocked mononucleotides is illustrated in figure 1.6.

1.1.7 General Strategy for the Synthesis of Oligodeoxynucleotides

The protecting groups used for the synthesis of DNA oligomers are such that the 5' hydroxyl can be removed selectively under anhydrous acidic conditions. Conversely, the cyanoethyl group on the 3' phosphate can be removed selectively under mild basic conditions. This selective deprotection can be carried out without affecting the other protecting groups to any great extent. As a result, one can form specific 3'-5' linkages and construct specifically desired sequences.

a)



b)

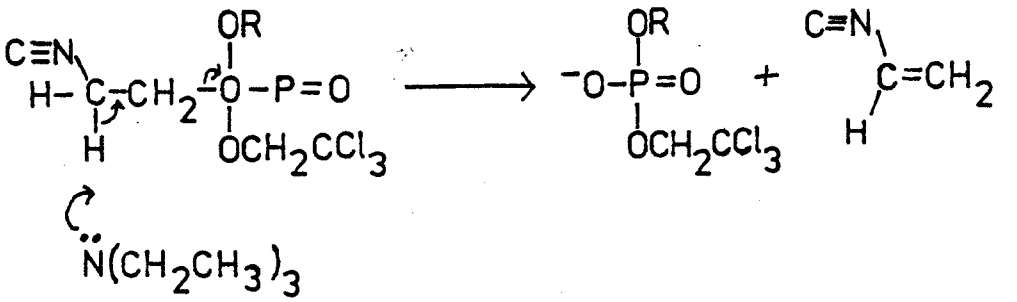


Figure 1.5 - β-elimination reactions by
a) reductive cleavage of the 2,2,2-trichloroethyl group and
b) proton abstraction by mild base on the 2-cyanoethyl group
as a means of selectively deprotecting the phosphotriester protecting groups.

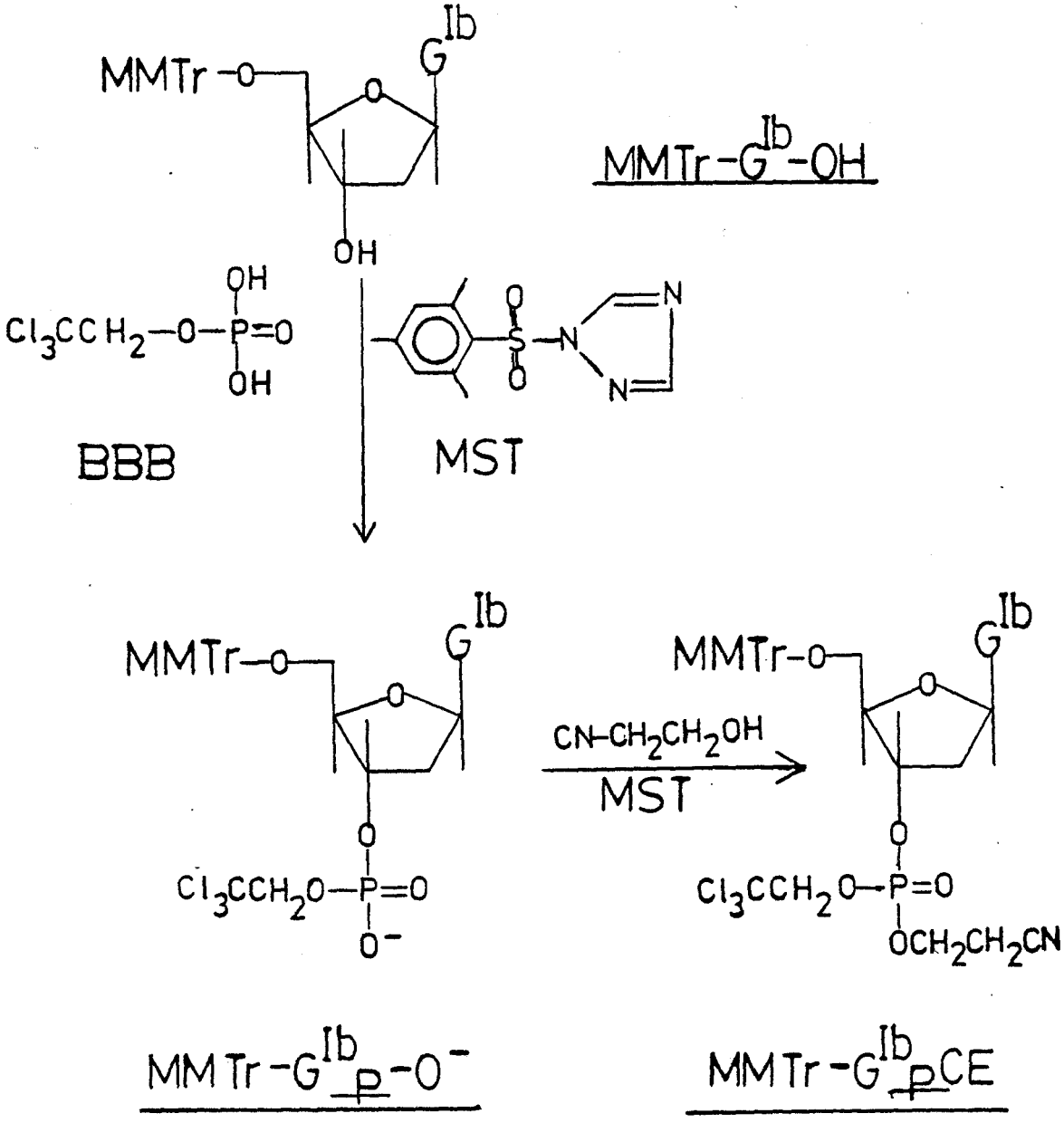


Figure 1.6 - Synthesis of fully protected phosphorylated nucleotides.

the monomethoxytrityl (MMTr) group to protect th 5' hydroxyl and the 2,2,2-trichloroethyl group as the protecting function on the phosphate. Benzoyl groups were used to protect the primary amino groups on dA and dC, isobutyryl for dG.

The general strategy for the synthesis of fully protected DNA oligomers is illustrated graphically in figure 1.7. There are essentially three stages shown in the diagram:

A - the synthesis of fully protected mononucleotides
B - the selective deblocking and coupling of nucleotides and
C - the coupling of terminal non-phosphorylated bases

Synthesis may proceed in either the 5' to 3' direction or 3' to 5' direction and since intermediate nucleotides have a masked phosphate, phosphorylation at each condensation stage is not necessary. The oligomer can be constructed in a stepwise fashion, one base at a time, or by fragment couplings of oligodeoxynucleotides. This is termed "block synthesis".

1.1.8 Other Methods

Other chemical approaches have been devised for the rapid synthesis of small oligonucleotides for use as probes, primers etc. These methods include both liquid and solid phase as well as enzymatic synthesis.

The phosphite triester method (Letsinger and Lunsford, 1976) involves the coupling of nucleosides with a

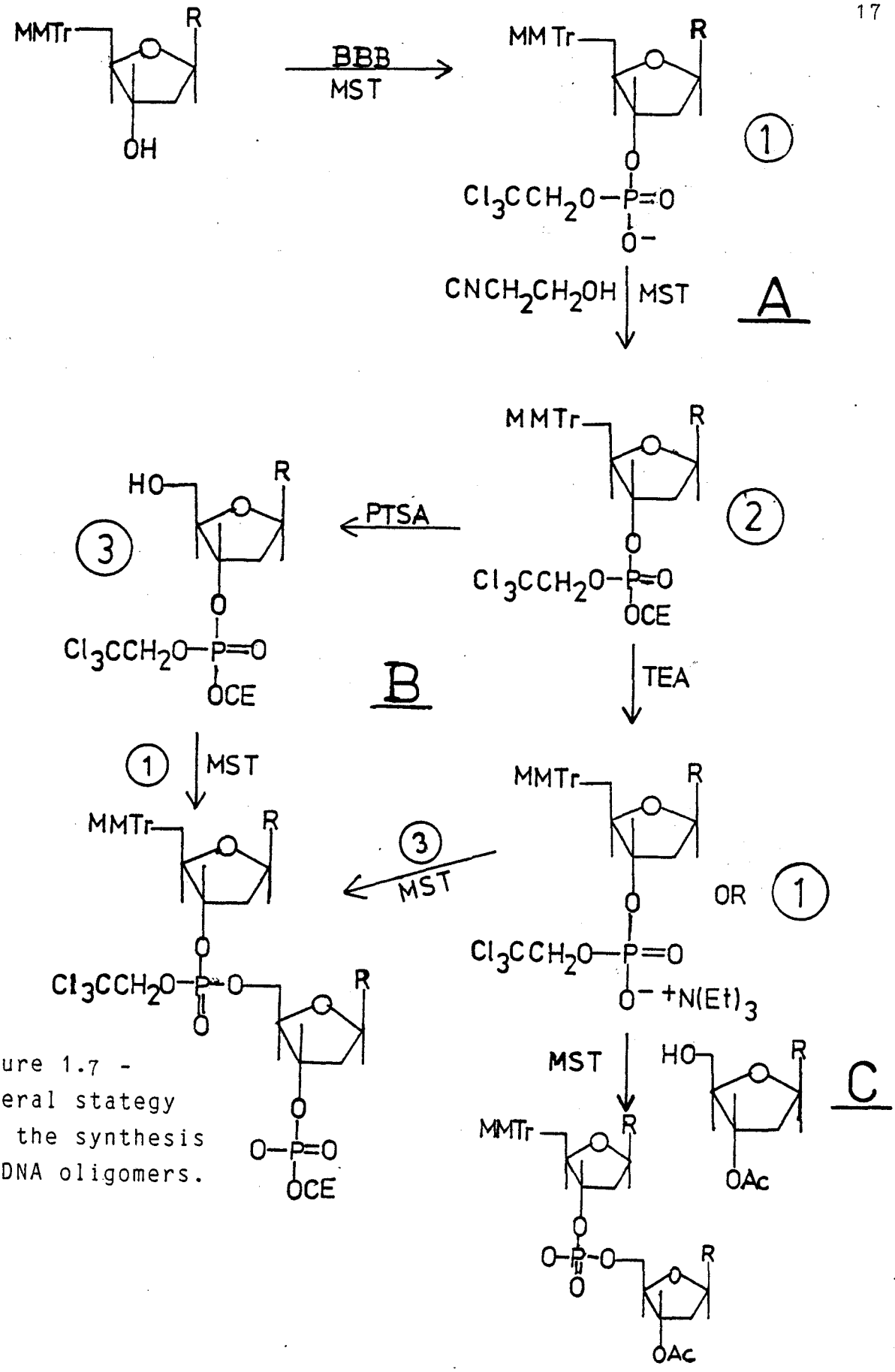


Figure 1.7 - General stategy for the synthesis of DNA oligomers.

desired sequence, the phosphite intermediates are oxidized to phosphotriesters. Since the phosphorus is in a trivalent state it is extremely reactive and thus the coupling time is rather short with relatively high selectivity and good yields. One drawback however, is that these highly reactive species are extremely sensitive to moisture and air oxidation. As a result, it is difficult to store these materials for an extended period of time.

A variation of this synthesis involves phosphoramidites as the key intermediates during the synthesis (Beaucage and Caruthers, 1981). These nucleoside phosphites are relatively stable in normal laboratory conditions and are activated by mild acid treatment. Once again, the advantage of a highly reactive phosphite intermediate is utilized. This particular type of synthesis lends itself very well to solid-phase synthetic methods (Chou et al, 1983).

The chemistry of solid phase synthesis is similar to that of liquid phase synthesis except that one base is bound to a solid support and the sequence is built upon it in a stepwise fashion, frequently one base at a time. Both phosphotriester and phosphite triester approaches have been exploited using a variety of polymer supports including polystyrene (Letsinger and Lunsford, 1976), polyamide (Gait, 1980), enzacryl gel K-2 (Demlek et al, 1981) and silica gel (Chow et al, 1981). Solid phase synthesis allows the rapid

synthesis of large oligomers (up to 20-30 bases) and lends itself to automation however only very small amounts of DNA (50-500ug) can be made at a rather high cost. Such small amounts are useful for microbiological techniques but provides insufficient amounts for physical studies such as NMR. Since the product is not purified after each subsequent reaction the presence of many side products (resulting from less than 100% yield) often creates difficulties in final purification. Also, solid phase synthesis does not lend itself to block synthesis, hence larger oligonucleotides cannot normally be synthesized from previously synthesized smaller oligonucleotides. This is especially true when employing automated systems.

1.2 DNA and RNA Structrure

The information contained within cellular DNA can be thought of as two dimensional: the base sequences contain the coded information from which all cellular polypeptides are derived and the structure itself contains the conformational information by which gene expression is controlled. This conformational information is involved in DNA/protein, DNA/RNA and DNA/DNA interactions. In order to understand the biological activities of DNA at the molecular level, it is necessary to understand the physical structure of DNA and how this structure is sequence dependent.

The present, most important investigative tool used to elucidate nucleic acid structure is X-ray crystallography. It was from the X-ray-diffraction photographs of DNA fibres taken by Rosalind Franklin and Maurice Wilkins that Watson and Crick (1953) based their right-handed double-helix structure. Again, a quarter century later, crystallography provided the means of discovering the left-handed Z-DNA helix (Wang *et al*, 1979).

Data obtained from DNA fibres are limited to values based on the overall structure such as the average number of residues per turn and the average pitch of the helix etc. Improvements in the ability to synthesize specific DNA sequences in sufficient amounts for single crystal studies made it possible to determine finer details such as propeller twist, base roll, sugar conformation etc. for each individual

base pair, advancing our knowledge of DNA structure tremendously.

The chromophoric properties of purine and pyrimidine bases allow the use of many solution state optical techniques such as CD, ORD, UV hypochromicity and temperature jump. Perhaps the most powerful of these techniques is CD from which one can obtain both conformational informaton such as the B to Z transition and thermodynamic information such as duplex stability. Although these studies provide valuable information, the information consists of overall gross parameters regarding the helix in question without providing intimate, in-depth detail.

1.2.1 Nomenclature of Nucleic Acid Conformation

DNA (and RNA) are very flexible molecules and a convention is required to describe precisely the relative positions the atoms adopt in space. A standard IUPAC-IUB convention now exists (Dixon and Davies, 1980). Due to the nature of the molecule, it was decided that an appropriate method of describing the structure is by reporting the various torsion angles along the bonds from one atom to another along the sugar-phosphate backbone. Figure 1.8 illustrates these torsion angles and the symbols used to define them. The signs of the torsion angles are defined according the Klyne-Prelog rule (Klyne and Prelog, 1960). When the bonds are overlapped, the torsion angle is defined as equal to zero and a positive torsion angle is obtained by

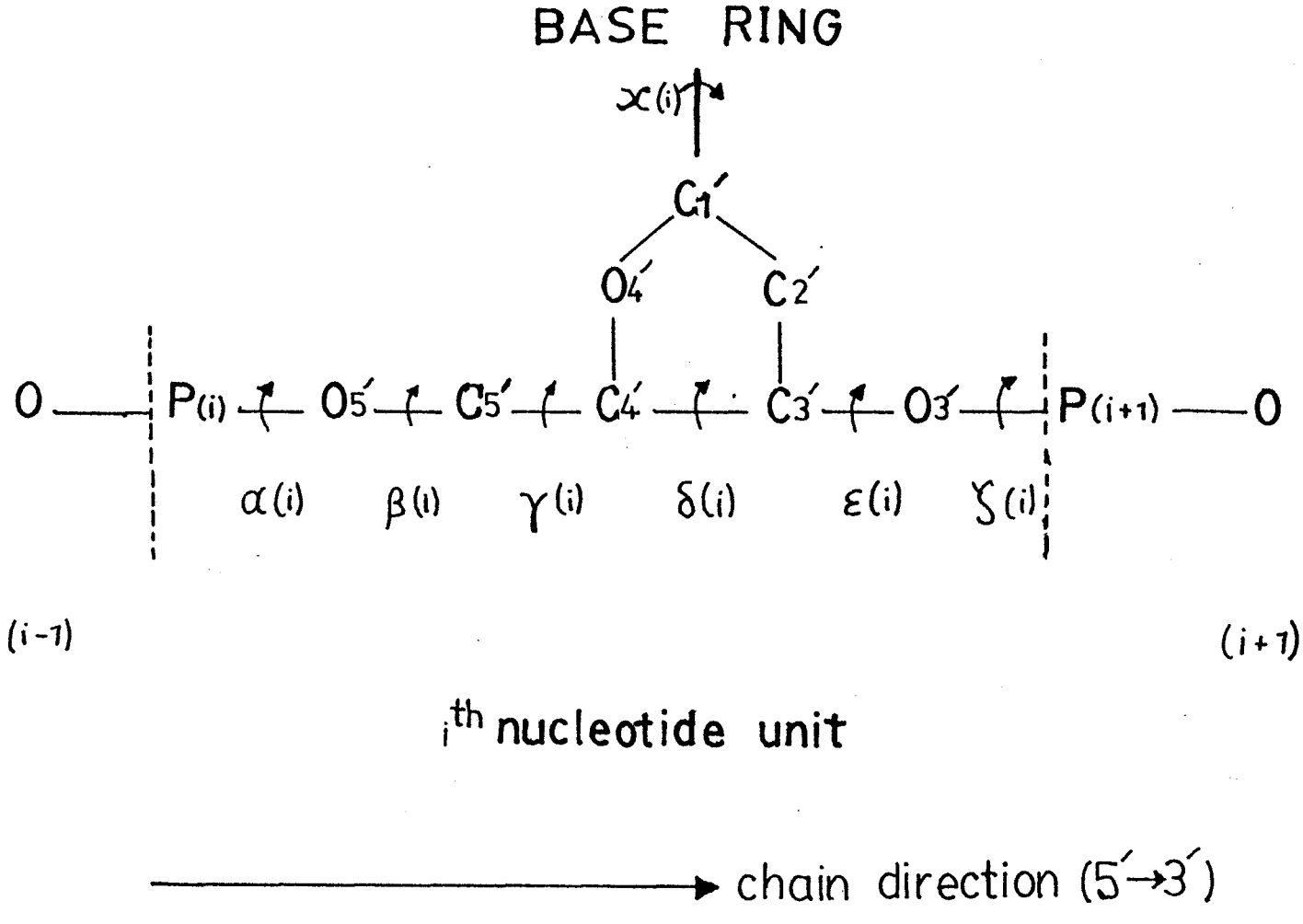


Figure 1.8 - Conformation notation of nucleic acid backbone angles

moving the rear bond clockwise with respect to the front bond when depicted as a standard Newman projection.

The endocyclic torsion angles of the furanose ring are numbered $\tau_0 \ldots \tau_4$ as shown in figure 1.9. A more commonly used decription of the furanose ring in the current literature is the C3'-endo (or N conformation) and the C2'-endo (or S conformation) sugar pucker as shown in figure 1.10. The term "endo" is used to denote that the C3' or the C2' carbons are on the same side of the sugar ring as the C5' carbon. Note that these are idealized and often sugar conformation is stated in terms of the percentage in the N or S conformation (ie. figure 1.9 depicts the furanoside rings in the 100% N and 100% S conformations).

A generalization exists for describing the conformation of the base ring relative to its furanoside ring. When the base ring lies directly above the sugar ring ($\chi = 0^\circ$) the base is said to be in the "syn" conformation. When the base ring is pointed away from the sugar ring ($\chi = 180^\circ$), the base is said to be in the "anti" conformation. These conformations are shown in figure 1.11.

1.2.2 X-Ray Crystallography

Fibre data have shown that DNA assumes a number of conformations depending upon the conditions and solvents present. These right-handed double helices were classified into two major categories, A and B DNA. Drawing DNA fibres

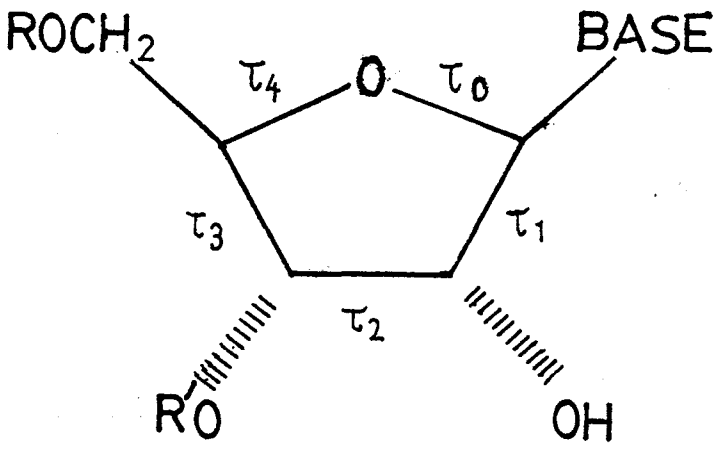
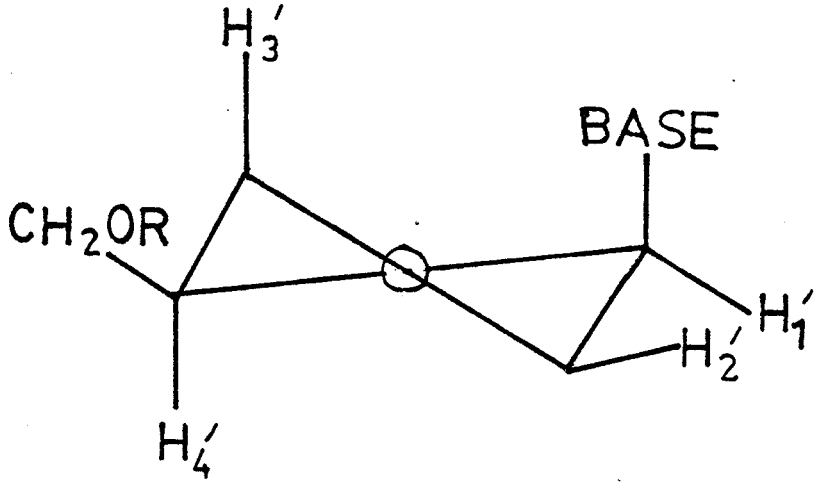


Figure 1.9 - numbering of the endocyclic torsion angles of the furanose ring.

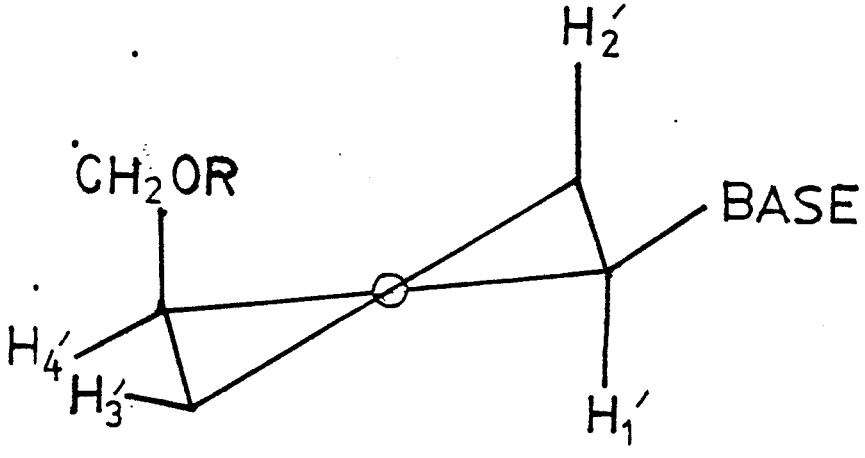


N-conformer

(2′-exo-3′-endo)

C3′-endo

Figure 1.10 - the two major conformations of the furanose ring.



S-conformer

(2′-endo-3′-exo)

C2′-endo

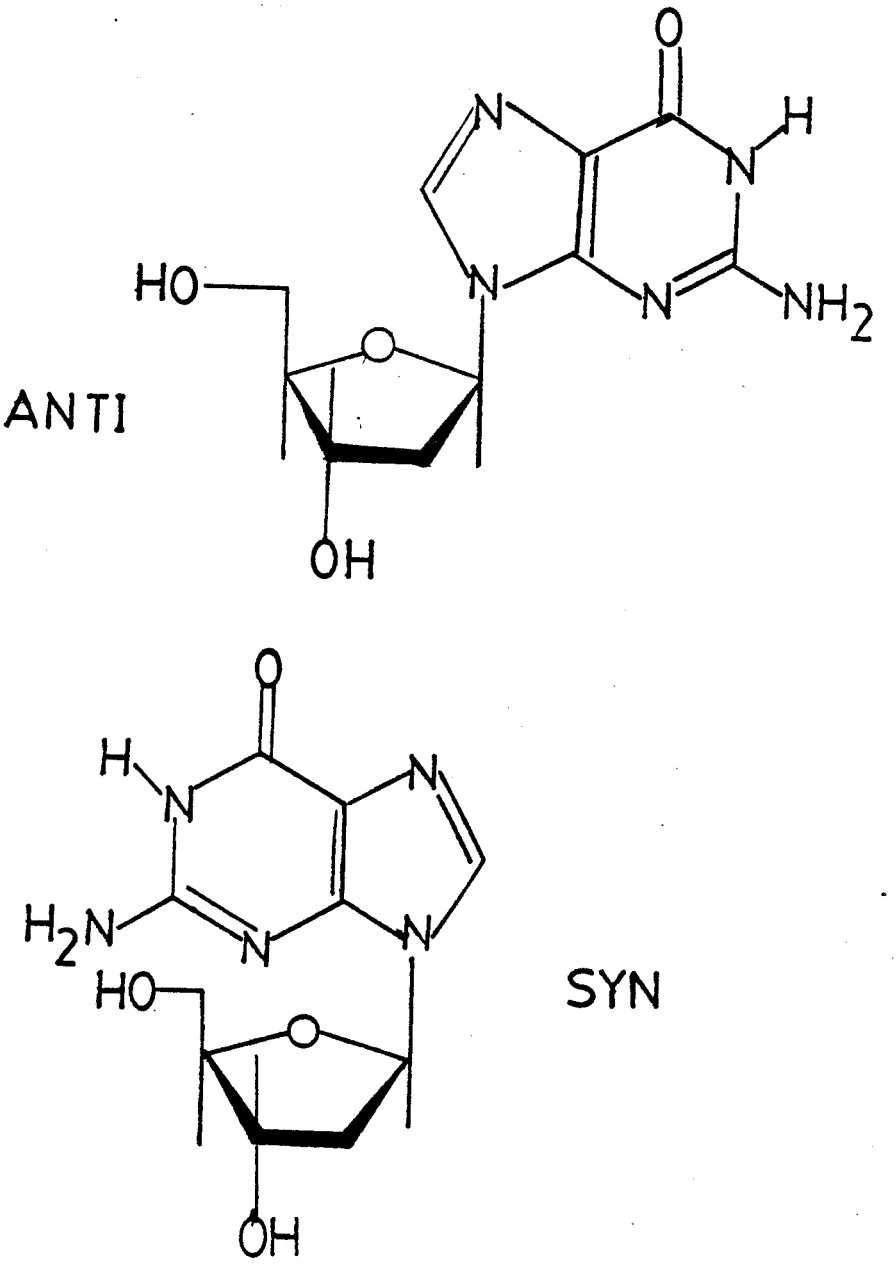


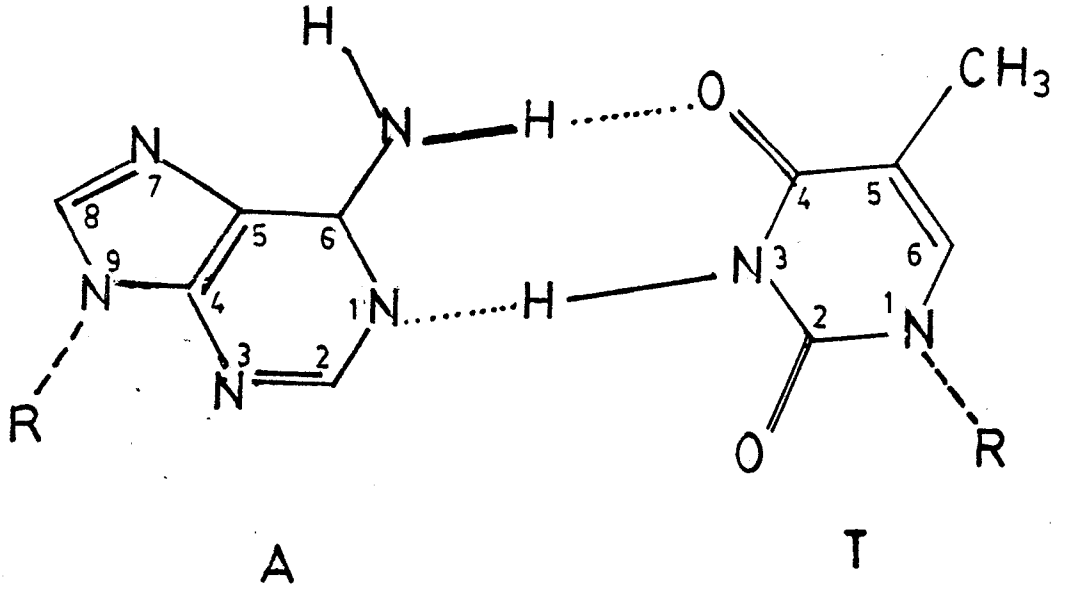
Figure 1.11 - the "anti" and "syn" conformation of dG.

under varying salt, alcohol and humidity conditions, gave rise to the classification of a variety of types of DNA (ie A, B, C, D and E) which differed in certain parameters such as number of base pairs per turn, rotation per base pair and vertical rise per base pair (Watson and Crick, 1953; Langridge et al., 1960; Arnott, 1976;Leslie et al., 1980).

These helices have many common features including standard Watson/Crick hydrogen bonding, the presence of a major and minor groove and all right-handed helices. The major and minor grooves arise due to the fact that the point of attachment of the bases in a base pair do not lie exactly opposite each other as would be necessary to form a perfect helix (see figure 1.12).

Single crystal analysis of specific sequences gave detailed information with regards to A and B DNA. They also led to the discovery of the left-handed "Z-DNA" helix characterized by Wang et al. (1979).

The sequences d(CCGG), d(GGCCGGCC) (Wang et al., 1982a), d(GGTATACC) (Shakked et al., 1983) and r(GCG)d(TATACGC) (Wang et al, 1982b) have been shown to adopt the A conformation. The A-helix is right handed and can be induced by conditions of low humidity (<75%). This helix averages 11 base pairs per helical turn with a 2.9 Å rise between base pairs. A-DNA is characterized by large propeller twists of 15° (see figure 1.13 for a description of propeller twist) and a large base tilt of 13 °.



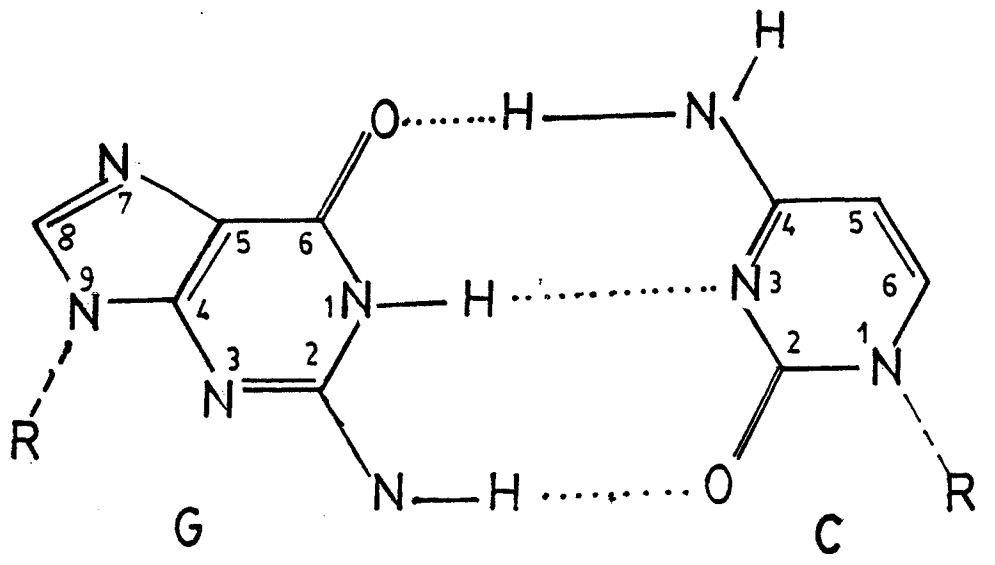


Figure 1.12 - Hydrogen bonded Watson/Crick base pairing.

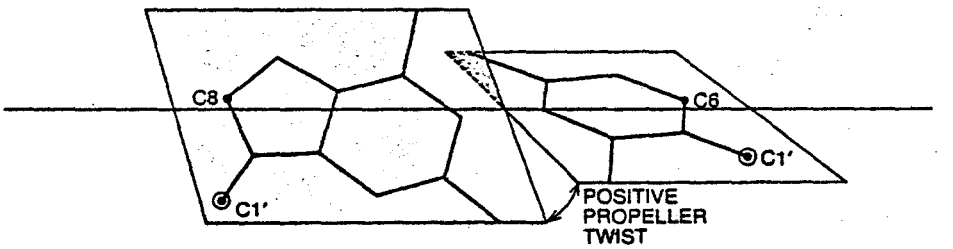


Figure 1.13 - Propeller Twist (Dickerson, 1983)

A positive propeller twist is defined as a clockwise rotation of the nearer base as one looks down the long axis of the base pair in either direction.

Generally, the sugar conformation for A-DNA is C3'-endo, however the central four base pair sugar puckers of d(GGCCGGCC) have an alternating S-type and N-type sugar conformation and termed an "alternating" A-DNA conformation. All of the bases are in the "anti" conformation. A-DNA is more "compact" than B-DNA and has a cavernous major groove with little to no minor groove.

B-DNA is represented by the sequence d(CGCGAATTCGCG) (Dickerson et al., 1981). This conformation has been shown to be the predominant form under physiological conditions (Wang, 1979). The right-handed B-DNA helix averages 10 base pairs per turn with a helical rise of 3.4 Å between base pairs. B-DNA is characterized by a small base tilt of only 2^{o} and an average but highly variable propeller twist of around 12^{o}.

The B helix sugar conformation is C2'-endo or in the N-conformation. There is a distinct major and minor groove with the bases in the "anti" conformation.

Z-DNA is represented by the sequences d(CGCGCG) (Wang et al., 1979) and d(CGCG) (Dickerson, 1981). Z-DNA can best be described as anti A-DNA. This left-handed helix has the purines (G residues) in the syn conformation with a C3'-endo sugar pucker. On the other hand, the pyrimidines (C residues) are in the anti conformation with the furanose in the C2'-endo conformation. This gives rise to a dinucleotide as the assymetric repeating unit in the helix. The Z

conformation is favored by sequences composed largely of alternating purine/pyrimidine residues. Z-DNA appears elongated with 12 base pairs (6 dinucleotide units) per turn. There is a minimal propeller twist of approximately 4.4° and a large major groove with no minor groove.

Fibre studies (Arnott et al, 1969) combined with the analysis of the stacked mini helices r(ApU) (Rosenberg et al., 1973) and r(GpC) (Arnott et al., 1973) gave rise to a number of important parameters concerning RNA helices. RNA exists in what is termed the A helix conformation.

A-RNA is right handed and the general parameters concerning the RNA helix are remarkably similar to that of A-DNA. There are 11 base pairs per helical turn with a rise per residue of 2.8 Å. The bases are in the anti conformation with C3'-endo sugar conformation.

B-RNA has yet to be observed and is unlikely to be found. It has been suggested that this may be due to the 2' hydroxyl group (Kallenbach and Berman, 1977). In the C2'-endo conformation, the 2' hydroxyl cannot be accommodated by the 3' phosphate or the base of the next residue. Instead, the 2' hydroxyl projects out from the helix surface, away from any nearby atoms.

1.2.3 Optical Studies

Although X-Ray structures provide a great deal of information regarding DNA and RNA structure, they do not necessarily represent the actual conformation adopted in solution (Seeman, 1980). Therefore, solution studies are necessary to verify and/or complement the crystal studies.

A number of studies using UV and CD techniques demonstrated that the stability of duplexes containing G.C base pairs was greater than duplexes containing A.U base pairs (Porscke et al., 1973). This is thought to be due to G.C having 3 hydrogen bonds while A.U base pairs have 2 in the duplex state. These studies also demonstrated that sequences containing the same base ratio and length but different base sequences have different stabilities. This demonstrates the sequence dependency of duplex stability.

This sequence dependency is further demonstrated by UV and CD studies showing that two adjacent G.C base pairs are considerably more stable than two G.C base pairs separated by an A.U base pair in a sequence of the same length (Tinoco et al., 1973).

Temperature jump experiments demonstrated duplex formation to be second order with the rate constants dependent on chain length and nucleotide sequence (Porschke et al., 1973).

The investigation of a large number of duplexes (6 to 14 base pairs) using UV and CD methods yielded theoretical

relationships for predicting the Tm of a given sequence (Borer et al., 1973, 1974; Gray et al., 1981). Gray et al summarized these relationships with the following equations:

$$Tm\ (DNA) = 44.0 + 36.1H_G + 9.2H_G^2$$

$$Tm\ (RNA) = 42.8 + 58.4H_G + 11.8H_G^2$$

where H_G is the fraction of G.C base pairs in the sequence. These equations have limited application for short sequences as demonstrated by AGGCU:AGCCU which has a Tm of 54°C (Alkema et al., 1981) rather than the predicted Tm of 82°C. These relationships do not take into account the sequence dependency of duplex stability and is only a gross estimate based on the number of G.C base pairs present in the sequence. It also does no take into account the phenomena resulting from "end effects" such as "fraying".

By using CD, Pohl and Jovin (1972) demonstated that poly d(GC) undergoes a significant conformation change when placed in different salt concentrations. This conformation change is illustrated in figure 1.14 for $d(CG)_3$. Because of this observation, Rich and Dickerson set out to solve the crystal structure of d(CGCGCG) and d(CGCG) in high salt conditions and discovered Z-DNA.

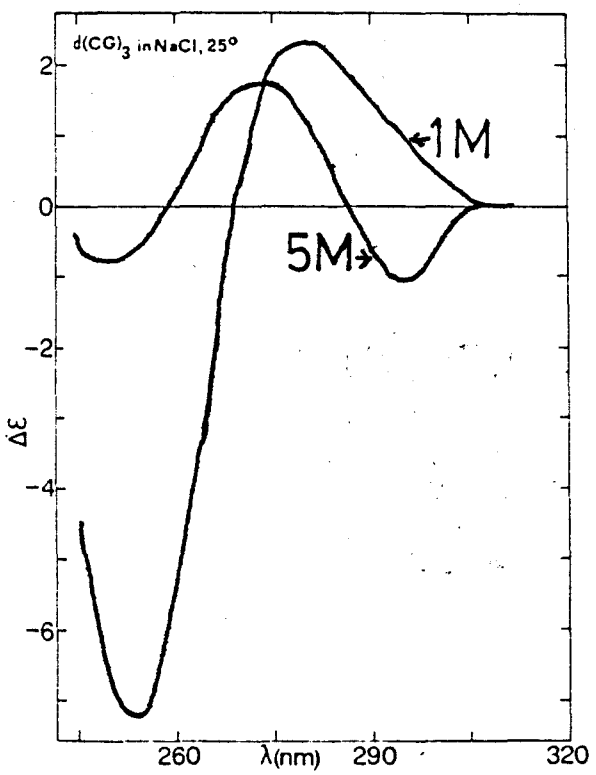


Figure 1.14 - Circular Dichroism spectra of $d(CG)_3$ in 1M NaCl (B-helix) and 5M NaCl (Z-helix). (Quadrigfoglio et al., 1981)

1.2.4 Nuclear Magnetic Resonance Studies

NMR has gained increasing use in this area since the development of effective syntheses of DNA and RNA oligomers in sufficient quantities required for this type of study. By the use of NMR spectroscopy, one can observe changes in local micro-envirnonments of nucleic acid molecules by following the anisotropic shifts of specific resonances. These observations make it possible to observe local modifications which alter stabilities as well as conformational changes.

^{1}H-NMR is particularly useful in determining relative thermodynamic duplex stabilities in solution (Arter et al., 1974; Borer et al., 1974; Krugh et al., 1976 and Romaniuk et al., 1979). By observing the chemical shift of the non-exchangeable protons as a function of temperature, one observes melting curves characteristic for nucleic acid duplexes. These protons include the AH-8, AH-2, GH-8, CH-5, CH-6, T-Me, TH-6, UH-6, UH-5 and the H-1' protons. The resulting melting curves allow one to determine the average Tm of the sequence under investigation. This average Tm is a reflection of the duplex stability. By studying these curves for a variety of sequences, one is able to determine the sequence dependent factors involved in stabilizing the helix.

Conformational information with regard to nucleic acid structure can be obtained from spin-spin coupling constants (Altona, 1982), relaxation data (T_1) (Ts'o *et al*, 1973) and NOE experiments (Kan *et al*, 1982).

Sugar pucker information can be obtained using "generalized Karplus equations" (Altona, 1982). The ribose ring conformation can be deduced from the H1' doublet splitting, where $J_{1'2'}(N) \doteq 1.0$ Hz and $J_{1'2'}(S) \doteq 7.9$ Hz. In the case of the deoxyribose ring, the H1' signal is a triplet from which the sum $J_{1'2'}+J_{1'2''}$ provides conformational information. It is predicted that the value of this summation is in the neighborhood of 9.5-10.5 Hz for the pure N-type conformation and between 15.3-15.8 Hz for the pure S-type (de Leeuw and Altona, 1982).

The exchangeable ring N-H protons involved in the hydrogen bonding of base pair formation can also be observed. These exchangeable protons are normally found in the very low field region (11-15 ppm) of the spectrum (Kearns, 1971, 1976; Hurd and Reid, 1979) as a result of the ring N-H resonances being highly deshielded in the process of hydrogen bonding (Katz and Penman, 1966). An added advantage is provided by the fact that each base pair contains only one ring N-H hydrogen bond and thus one can quantitate base pair formation.

Recently, the observation of these N-H signals was used to demonstrate that strand melting does not occur during

the B to Z transition (Sarma, et al,1983). It was shown that the GN-H1 proton peak in poly d(GC).poly d(GC) did not change in chemical shift or area during the B to Z transition.

Information on the backbone conformation of nucleic acids can also be obtained by observing other nuclei such as ^{13}C and ^{31}P (Patel and Canuel, 1976; Patel, 1980; Learner and Kearns, 1981).

The two distinctly different environments resulting from the dinucleotide unit of Z-DNA creates two distinct ^{31}P resonances which were used to demonstrate the presence of Z-DNA in a plasmid after the insertion of a long poly d(GC) stretch (Singleton, 1982).

1.2.5 Factors Affecting Duplex Stability

The main factors contributing to duplex stability are hydrogen bonding, vertical base stacking interactions and electrostatic charge-charge repulsion between the phosphate groups (Altona, 1982). Conditions or situations which affect these factors will have an effect on the overall duplex stability and conformation.

The effect of hydrogen bonding is apparent from the observations that sequences containing G.C base pairs are more stable than those containing A.T base pairs.

There is often the impression that hydrogen bonding is the most important factor in duplex stability. However, base stacking is more important (Altona, 1982; Dickerson, 1983). The driving force for stacking may lie in the

dipole/induced-dipole interactions between the polar groups and the π-electron clouds of the bases. Base stacking is dependent on the interplanar distances between bases. However, the best geometrical overlap does not necessarily provide the most favorable stacking interaction (Gupta and Sasisekharan, 1978). Thus, factors affecting the ability of the bases to stack will either increase or decrease the duplex stability. An example of this is the propeller twists observed in DNA crystal structures which are thought to enhance stacking interactions (Levitt, 1978; Hogan et al., 1978).

The sequence dependence of duplex stability is probably related to stacking pattern interactions. It is interesting to note that dangling bases increase stability substantially. This is illustrated by r(GCA) which forms a very stable duplex with a Tm of 33^{o}C (Alkema et al., 1981). This duplex consisted of two G.C base pairs and two 3'-dangling adenosine residues. The stability of this trimer is equal to that of the tetramer duplex r(UGCA) which consists of four base pairs. Thus it seems that a 3' dangling adenosine provides the stability of an A.U base pair and that the stability arises from the dangling residues stacking into the duplex.

Hydration also has a significant effect on duplex stability and conformation. A detailed study of hydration in the crystal structure of d(CGCGAATTCGCG) (Kopka, 1983)

demonstrated extensive hydration along the backbone averaging three water molecules per phosphate. There is also a "zig-zag" spine of hydration in the minor groove which is thought to be responsible for stabilizing the sequence into a B helix (see figure 1.15). The spine of hydration is well developed in regions of A.T base pairs but is broken up in the G.C regions. This may be due to the intrusion of the NH_2 on the guanosine residues into the minor groove. This premise is supported by the fact that poly d-(IC) assumes a B-helix conformation (I=inosine which is identical to dG except that it lacks the NH_2 group) (Drew and Dickerson, 1981).

Although a great deal is known about the structure of DNA and RNA, it is not known exactly how these structures are involved in genetic control. Because the B to Z transition is a reversible structural form of DNA, it has been proposed as an excellent candidate for having a regulatory role in genetic activity (Nordheim et al, 1981). The sequence $d(m^5CG)_n$ (a potentially stable Z conformer) occurs frequently in eukaryotic DNA and its presence in a gene has been correlated with the transcriptional inactivity of this gene (Razin and Riggs, 1980). On a contradictory note, it has been proposed that Z-DNA may be involved in transcription enhancement, as demonstrated by the presence of Z-DNA in the transcriptional enhancer region of SV40 (Rich et al, 1983). The actual role of Z-DNA remains obscure.

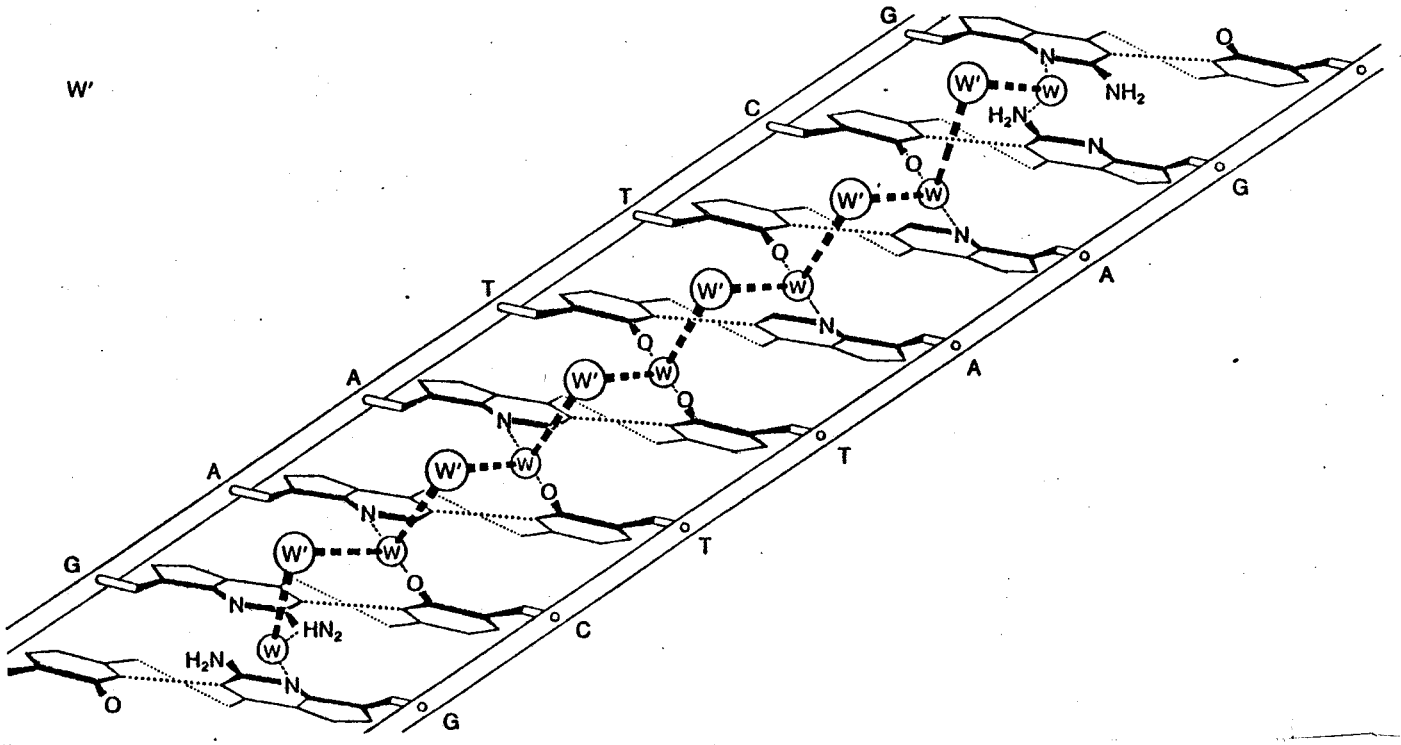


Figure 1.15 - Spine of hydration along minor groove of d(CGCGAATTCGCG). (Dickerson, 1983)

1.3 Objects of Present Study

The purpose of this work was to:

1) develop a practical synthesis of short DNA oligomers (up to 8 bases), in quantities sufficient for ^{1}H-NMR studies.

2) obtain thermodynamic infomation with regards to the ability of these compounds to form duplexes and

3) determine possible secondary structure information from these short oligomers.

The following sequences were made using the strategy outlined in section 1.1.7:

1. d(GCA) (56)

2. d(AGCT) (52)

3. d(ACGT) (55)

4. d(ACGTACGTp) (59)

The sequence d(GCA) was synthesized in order to compare it to r(GCA) which is known to form a very stable duplex (Alkema *et al*, 1981). Sequences d(AGCT) and d(ACGT) were synthesized to demonstrate the integrity of the synthesis as these sequences incorporated all four bases. The sequence d(ACGTACGTp) demonstrated the ability to synthesize octamers. Sequences 3 and 4 were also interesting in that they may be useful in studying the B to Z transition. This is due to the fact that they consist of alternating purine/pyrimidine residues an important structural property concerning the B to Z transition.

2 EXPERIMENTAL AND RESULTS

This section will outline in detail the synthetic protocols and physical procedures used to synthesize and characterize the following deoxy oligomers:

d(GCA) (56)
d(AGCT) (52)
d(ACGT) (55)
d(ACGTp) (60)
d(ACGTACGTp) (59)

Due to the complexity of the synthetic intermediates, abbreviated representations are used to illustrate them. These abbreviations are shown in figures 1.3 and 1.6. To facilitate the visualization of the chemical procedures outlined, the reactions are illustrated by equation form followed by the protocol itself.

To avoid redundancy, procedures which are used repetitively are presented in detail once, together with an appropriate example.

Procedures used for obtaining variable temperature proton NMR and circular dichroism spectra will be outlined in detail as well as the resulting data.

2.1 General Procedures and Reagents

All reagents and solvents were commercial reagent grade and used without further purification with the following exceptions: mesitylenesulphonyl chloride (Aldrich) was recrystallized from hot petroleum ether (30-60°C);

pyridine and N,N-dimethylformamide were stored over Fischer 4Å molecular sieves prior to use. Triethylamine was distilled and stored over sieves. Nucleosides were obtained from Vega Chemicals.

The compound 2,2,2-trichloroethylphosphate was synthesized and characterized by an established procedure (England & Neilson, 1977) as was MST (Katagiri et al., 1974).

Emulsions frequently obtained during methylene chloride extractions of aqueous pyridine solutions were broken by the addition of saturated sodium chloride solution (2-3 ml).

Phosphorylations and couplings required anhydrous conditions. This was achieved by evaporation in vacuo of reactants from anhydrous pyridine several times to give a minimal volume. The final volume was also kept to a minimum. In all cases, pressure was restored to the system using dry nitrogen.

Selective protection and deprotection of nucleosides and coupling reaction mixtures were monitored by thin layer chromatography using pre-scored Silica Gel G plates (Analtech) resolved in 5% or 10% methanol in methylene chloride. Detection was accomplished by spraying with 1% ceric sulphate in 10% sulphuric acid followed by heating to 150-200°C. Compounds containing p-monomethoxytrityl groups appeared yellow-orange, dimethoxytrityl groups appeared red, and those without trityl groupings appeared brown. Polar

phosphorylated compounds appeared as a "cusp" extending from the origin.

Silica gel (40-140 mesh, Baker Analysed) used in column chromatography was dried at 80°C over solid NaOH for one day prior to use. Columns were prepared in dichloromethane and eluted using a step gradient of methanol (1 to 10%) in dichloromethane.

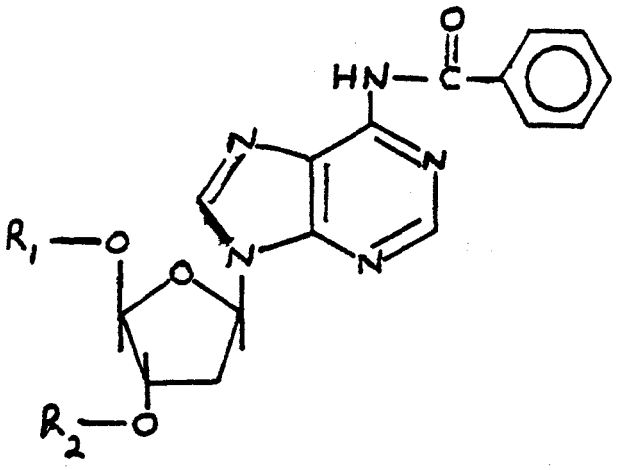
Elemental analyses were performed by Galbraith Laboratories, Knoxville Tennessee.

The proton NMR spectra of nucleoside derivatives were obtained on a Varian EM390 at room temperature and the signals reported in ppm relative to tetramethylsilane (TMS). The spectra were run in deuterated dimethylsulfoxide (DMSO-d_6).

The fully deprotected deoxy sequences were purified by descending paper chromatography on Whatman #1 and #40 papers. The solvent system employed consisted of ethanol/NH_4OAc (1M, pH 7.3), 1:1 (v/v). A short wave UV lamp was used to detect deprotected deoxy oligomers.

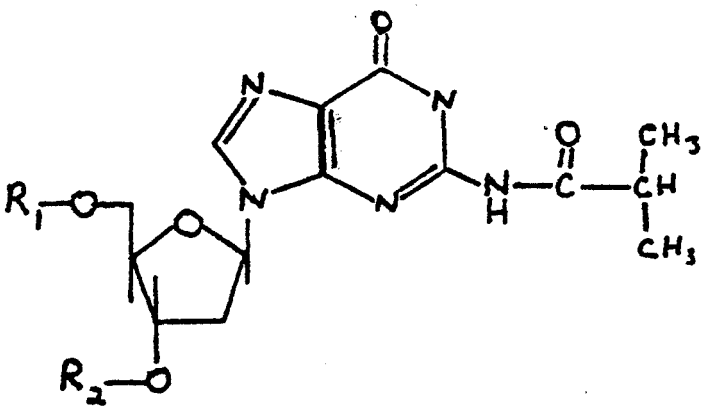
2.2 Procedures for the Synthesis of Deoxymononucleoside Derivatives

The synthesized deoxymononucleoside derivatives are presented in figures 2.1-2.4. Several of these procedures are specific for a particular nucleoside while others were



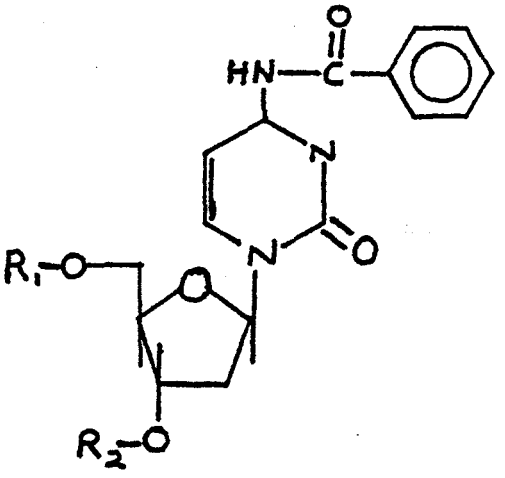
No.	R_1	R_2
1	H	H
2	MMTr	H
3	MMTr	Bz
4	MMTr	$-P(=O)(O^-)-OCH_2CCl_3$
5	MMTr	$-P(OCH_2CH_2CN)-OCH_2CCl_3$
6	DMTr	H
7	DMTr	$-P(=O)(O^-)-OCH_2CCl_3$

Figure 2.1 - Deoxyadenosine derivatives sythesized.



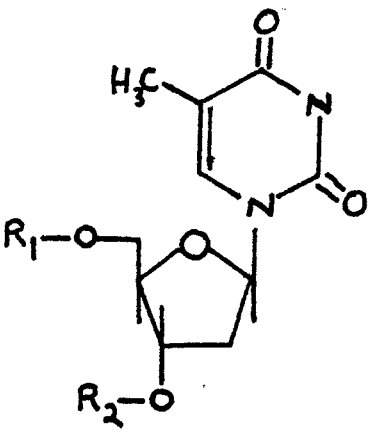
No.	R_1	R_2
8	H	H
9	MMTr	H
10	MMTr	$-P(=O)(O^-)-OCH_2CCl_3$
11	MMTr	$-P(OCH_2CH_2CN)-OCH_2CCl_3$
12	H	$-P(OCH_2CH_2CN)-OCH_2CCl_3$

Figure 2.2 - Deoxyguanosine derivatives sythesized.



No.	R_1	R_2
13	H	H
14	MMTr	H
15	MMTr	$-P(=O)(O^-)-OCH_2CCl_3$
16	MMTr	$-P(-OCH_2CH_2CN)(=O)-OCH_2CCl_3$
17	H	$-P(-OCH_2CH_2CN)(=O)-OCH_2CCl_3$

Figure 2.3 - Deoxycytidine derivatives sythesized.



No.	R_1	R_2
18	H	H
19	MMTr	H
20	MMTr	Ac
21	H	Ac
22	MMTr	$-P(=O)(O^-)-OCH_2CCl_3$
23	MMTr	$-P(OCH_2CH_2CN)-OCH_2CCl_3$
35	H	$-P(OCH_2CH_2CN)-OCH_2CCl_3$

Figure 2.4 - Deoxythymidine derivatives synthesized.

used for all four nucleosides. To avoid repetition, procedures common to all four nucleosides (sections 2.2.2 - 2.2.5) are illustrated by a general equation and described by the synthesis of a particular compound as an example. Tables are presented within some of these sections summarizing the synthetic reactions carried out and the yields obtained.

2.2.1 Synthesis of N-protected Deoxymononucleosides

2.2.1.1 N^6-Benzoyldeoxyadenosine (1) (Schaller, H. et al., 1963)

HO-A-OH --> BzO-A^{Bz}-OBz --> HO-A^{Bz}-OH (1)

2'-Deoxyadenosine (1.07 g, 4.25 mmole) was dried by evaporation in vacuo from anhydrous pyridine (3x20 ml) and reduced to a final volume ca. 10 ml. The suspension was cooled to 0°C in an ice bath with continuous stirring. Benzoyl chloride (4.4 ml, 37.8 mmole) was added dropwise to the solution over a 30 min. period. The reaction was then brought to room temperature. The course of the reaction was followed by tlc on SiO_2 in $MeOH/CH_2Cl_2$ (1:10) for the formation of the product (R_f: 0.0 --> 0.9). The reaction was complete after 1 hour and stopped by the addition of ice (ca. 2 g). The mixture was partitioned with dichloromethane (3x25 ml) and the combined organic layers were washed with water (30 ml), a saturated aqueous solution of sodium bicarbonate (30 ml) and water (30 ml). Dichloromethane was evaporated in vacuo and residual pyridine was then removed by azeotroping

in vacuo with toluene (3x15 ml) to yield $\underline{N}^6$, $\underline{O}^{3'}$, $\underline{O}^{5'}$-tribenzoyl-2'-deoxyadenosine as a yellow oil.

The yellow oil was dissolved in EtOH:pyridine (2:1 v/v, 10 ml) and cooled to 0°C in an ice bath and a mixture of EtOH:2M NaOH (1:1 v/v, 10 ml) was then added with continuous stirring. The reaction was monitored by tlc on SiO_2 in MeOH/CH_2Cl_2 (1:10) for the formation of the product (R_f: 0.9 --> 0.4). After 7 min., the reaction was neutralized by the addition of Dowex 50W-X8 (pyridinium form, ion exchange resin, *ca.* 2 g).

The dowex was removed by filtration and washed with ethanol (3x10 ml). The combined filtrate and washings were evaporated *in vacuo* to a yellow oil. The oil was dissolved in dichloromethane (*ca.* 20 ml) and applied to a silica column (*ca.* 10 g) and the product eluted with 5% MeOH in CH_2Cl_2. The eluant was evaporated *in vacuo* to yield $\underline{N}^6$-benzoyl-2'-deoxyadenosine (1) (586 mg, 39% yield) as a white powder.

Proton NMR spectra in DMSO-d_6 included the following signals (δ(TMS), ppm.): 8.96 (1H, singlet, H(8)); 8.89 (1H, singlet, H(2)); 8.3-7.6 (5H, multiplet, $\underline{N}^6$-benzoyl protons); 6.68 (1H, triplet, H(1')) in addition to the normal nucleoside signals.

2.2.1.2 N^2-Isobutyryl-2'-deoxyguanosine (8)

(Stawinski, J. et al., 1977)

HO-G-OH --> IbO-G^{Ib}-OIb --> HO-G^{Ib}-OH (8)

2'-Deoxyguanosine (1 g, 3.75 mmole) was dried by evaporation in vacuo from anhydrous pyridine (3x20 ml) and then reduced to a final volume ca. 10 ml. The suspension was cooled to 0°C in an ice bath with continuous stirring. Isobutyryl chloride (3.7 ml, 35.3 mmole) was added dropwise over a 30 min. period. The reaction mixture was brought to room temperature and the course of the reaction followed by tlc on SiO_2 in 10% MeOH-CH_2Cl_2 for the formation of the product (R_f: 0.0 --> 0.8). The reaction was complete after 2 hours and stopped by the addition of ice (ca. 2 g). The mixture was partitioned with dichloromethane (3x25 ml) and the combined organic layers were washed with water (30 ml), a saturated aqueous solution of sodium bicarbonate (30 ml) and water (30 ml). Dichloromethane was evaporated in vacuo and residual pyridine was then removed by azeotroping with toluene (3x15 ml) in vacuo to yield N^2, $O^{3'}$, $O^{5'}$-triisobutyryl-2'-deoxyguanosine as a brown oil.

The brown oil was dissolved in EtOH:pyridine (2:1 v/v, 40 ml) and cooled to 0°C on an ice bath with continuous stirring. EtOH:2M NaOH (1:1 v/v, 8 ml) was added slowly over a period of 1 hour. The reaction was monitored by tlc on SiO_2 in MeOH/CH_2Cl_2 (1:10) for the formation of the product (R_f: 0.8 --> 0.1). The reaction was neutralized by the

addition of Dowex 50W-X8 (pyridinium form, ion exchange resin, ca. 2 g).

The dowex was removed by filtration and washed with ethanol (3x10 ml). The combined filtrate and washings were evaporated in vacuo to a brown oil. The oil was dissolved in hot ethanol (20 ml) and allowed to crystallize for 16 hours at 4°C. The resulting product was filtered and washed with petroleum ether (5x20 ml) to yield N^2-isobutyryl-2'-deoxyguanosine (8) as white crystals (1.01 g, 80% yield).

Proton NMR spectra in DMSO-d_6 included the following signals (δ(TMS), ppm.): 8.2 (1H, singlet, H(8)); 6.18 (1H, triplet, H(1')); 1.1 (6H, doublet, methyl protons on the isobutyrl group) in addition to the other normal nucleoside signals.

2.2.1.3 N^4-Benzoyl-2'-deoxycytidine (18)

(Otter and Fox, 1973)

HO-C-OH --> HO-C^{Bz}-OH (18)

2'-Deoxycytidine (1 g, 4.41 mmole) was suspended in absolute ethanol (100 ml), and warmed to the point of refluxing with continuous stirring. To the refluxing mixture was added benzoic anhydride (1 g). After one hour, additional benzoic anhydride (1 g) was added to the mixture and again after two hours. The reaction was allowed to proceed for another hour until complete, and was monitored by tlc on SiO_2 in $MeOH/CH_2Cl_2$ (1:10) for the formation of the product (R_f: 0.0-->0.4). The solution was allowed to cool

slowly to room temperature. The ethanol was removed by evaporation *in vacuo* to yield a white powder. The residue was swirled in hot diethyl ether (3x50 ml) which was subsequently removed by decantation and the residue allowed to dry. The product was recrystallized from water. The "gummy" white crystals were collected and dried to a cake *in vacuo* over solid NaOH at room temperature for 18 hours to yield N^4-benzoyl-2'-deoxycytidine (18) (1.34 g, 92% yield).

Proton NMR spectra in DMSO-d_6 included the following signals (δ (TMS), ppm.): 7.9 (1H, doublet, H(6)); 7.6-7.2 (5H, multiplet, N^4-benzoyl protons); 6.08 (1H, triplet, H(1')); 5.16 (1H, doublet, H(5')) in addition to the normal nucleoside signals.

2.2.2 Selective Tritylation of the 5'-OH Group of N-protected Deoxymononucleosides

(Smith, M. *et al.*, 1963)

General Reaction: HO-X^*-OH --> MMTr-X^*-OH

(X^*=A^{Bz} (2), G^{Ib} (9), C^{Bz} (14) or T (19))

Example: HO-C^{Bz}-OH (13) --> MMTr-C^{Bz}-OH (14)

N^4-Benzoyl-2'-deoxycytidine (13) (1.341 g, 4.05 mmole) was dried by repeated evaporations *in vacuo* from anhydrous pyridine (3x20 ml). Pyridine was added to a final volume of 20 ml under N_2. *p*-Monomethoxytrityl chloride (1.39g, 4.5 mmole) was added to this solution, and placed in the dark for 16 hours. The reaction was monitored by tlc on

SiO_2 in $MeOH/CH_2Cl_2$ (1:10) for the formation of the product (R_f: 0.3-->0.8). Upon completion, the reaction was stopped by the addition of ice (ca. 2 g). The product was partitioned into dichloromethane (3x30 ml) and the combined organic extracts washed with water (30 ml), a saturated aqueous solution of sodium bicarbonate (30 ml) and water (30 ml). Dichloromethane was removed by evaporation in vacuo and residual pyridine was then removed by azeotroping in vacuo with toluene (3x15 ml) to yield a yellow foam. The foam was dissolved in dichloromethane (ca. 30 ml) and applied to a silica gel column. The product was eluted with 3% MeOH in CH_2Cl_2 yielding N^4-benzoyl-$O^{5'}$-p-monomethoxytrityl-2'-deoxycytidine (2.312 g, 95% yield) as a yellow foam.

Proton NMR spectra in DMSO-d_6 included the following signals (δ(TMS), ppm.): 7.9 (1H, doublet, H(6)); 7.6-7.2 (complex multiplet, trityl and benzoyl protons); 6.85 (1H, doublet, H(5)); 6.1 (1H, triplet, H(1')); 3.67 (3H, singlet, p-methoxy protons on the trityl group) in addition to the normal nucleoside signals.

A summary of all the reactions using this tritylation procedure is presented in Table 2.1.

Table 2.1

Calculated yields for the $O^{5'}$ protection reactions carried out as described in section 2.2.2.

Starting Material	Quantity (mg)	(mmole)	Product	Quantity (mg)	Yield (%)
HO-A^{Bz}-OH (1)	586	1.65	MMTr-A^{Bz}-OH (2)	655	64
HO-A^{Bz}-OH (1)	1000	2.82	DMTr-A^{Bz}-OH (6)	938	51
HO-G^{Ib}-OH (8)	1200	3.56	MMTr-G^{Ib}-OH (9)	1460	67
HO-C^{Bz}-OH (13)	1341	4.05	MMTr-C^{Bz}-OH (14)	2312	95
HO-T-OH	1000	4.13	MMTr-T-OH (19)	1858	88

2.2.3 Phosphorylation of the 3'-OH of the $O^{5'}$-tritylated, N-protected mononucleosides with 2,2,2-trichloroethylphosphate.

General Reaction: MMTr-X^{*}-OH --> MMTr-X^{*}p-O^{-}

(X^{*}=A^{Bz} (4), G^{Ib} (10), C^{Bz} (15) or T (22))

Example: MMTr-C^{Bz}-OH (14) --> MMTr-C^{Bz}p-O^{-} (15)

2,2,2-Trichlorethylphosphate (840 mg, 3.7 mmole) was converted to its pyridinium salt by repeated evaporations in vacuo from pyridine (3x20 ml) to a final volume ca. 10 ml. To this suspension was added MST (1.83 g, 7.3 mmole) and the mixture dried by repeated evaporations in vacuo from pyridine (3x20 ml) to a final volume ca. 10 ml and sealed under dry nitrogen. The reaction mixture was then warmed to 40°C until the MST went into solution (ca. 2 hours).

N^{4}-Benzoyl-$O^{5'}$-p-monomethoxytrityl-2'-deoxycytidine (14) (1.108 g, 1.84 mmole) was dried by repeated evaporations in vacuo from anhydrous pyridine (3x20 ml) to a final volume ca. 10 ml. This was added to the MST activated 2,2,2-trichloroethylphosphate, and the reaction mixture was concentrated to half volume (ca. 10 ml), sealed under dry nitrogen and stored in the dark.

The formation of the product was monitored by tlc on SiO_2 in MeOH/CH_2Cl_2 (1:10) (R_f: 0.8 --> "cusp" 0.1-0.4). The reaction was complete after 4 days and stopped by the addition of ice (ca. 2 g). The product was partitioned into dichloromethane (5x30 ml) and the combined organic extracts

were washed with water (2x30 ml). Dichloromethane was removed by evaporation *in vacuo* and the resulting oil was dried by repeated evaporation *in vacuo* with anhydrous pyridine (3x20 ml). The oil, $\underline{N}^4$-benzoyl-$\underline{O}^{5'}$-*p*-monomethoxytrityl-$\underline{O}^{3'}$-(2,2,2-trichloroethylphosphate)-2'-deoxycytidine (15) in a minimal volume of pyridine (*ca.* 3 ml) was used immediately without further purification or characterization.

2.2.4 *Treatment of $\underline{O}^{3'}$-phosphorylated, $\underline{O}^{5'}$-tritylated, N-protected Deoxymononucleosides with 2-Cyanoethanol to yield Fully Protected Deoxymononucleosides.*

(Catlin and Cramer, 1973)

General Reaction: $MMTr\text{-}X^{*}\underline{p}\text{-}O^{-}$ --> $MMTr\text{-}X^{*}\underline{p}\text{-}OCE$

($X^{*}=A^{Bz}$ (5), G^{Ib} (11), C^{Bz} (16) or T (23))

Example: $MMTr\text{-}C^{Bz}\underline{p}\text{-}O^{-}$ (15) --> $MMTr\text{-}C^{Bz}\underline{p}\text{-}OCE$ (16)

$\underline{N}^4$-Benzoyl-$\underline{O}^{5'}$-*p*-monomethoxytrityl-$\underline{O}^{3'}$-(2,2,2-trichloroethylphosphate)-2'-deoxycytidine (15) (1.84 mmole theoretical) was dried by repeated evaporations *in vacuo* from anhydrous pyridine (3x20 ml) and reduced to *ca.* 10 ml. MST (510 mg, 2.03 mmole) was added to this solution and warmed to 40°C for *ca.* 1 hour and 2-cyanoethanol (1.5 ml, 22 mmole, Aldrich gold label) was added to the solution. The reaction mixture was dried by repeated evaporations *in vacuo* from anhydrous pyridine (2x20 ml), concentrated to a minimum volume (*ca.* 8 ml) and sealed under dry nitrogen. The reaction mixture was stored in the dark.

The reaction was monitored by tlc on SiO_2 in $MeOH/CH_2Cl_2$ (1:10) for the product (R_f: 0.1-0.3 "cusp" --> 0.9). The reaction was complete after 8 days and quenched by the addition of ice (ca. 2 g). The product was partitioned into dichloromethane (3x30 ml) and the combined organic extracts washed with water (2x30 ml). Dichloromethane was removed by evaporation in vacuo and the residual pyridine then removed by azeotroping with toluene (3x15 ml) in vacuo to yield a yellow foam. The foam was dissolved in dichloromethane (ca. 30 ml) and applied to a silica gel column. Elution with 4% MeOH in CH_2Cl_2 yielded the product N^4-benzoyl-$O^{5'}$-p-monomethoxytrityl-$O^{3'}$-(2,2,2-trichloroethyl-2-cyanoethylphosphate)-2'-deoxycytidine (16) (950mg, 60% yield).

The yields for the synthesis of the fully protected mononucleosides are presented in Table 2.2.

The fully protected derivatives of dA, dG and dC were analysed by means of elemental analysis. These analyses are presented in Table 2.3.

Table 2.2

Calculated yields for the synthesis of fully protected phosphorylated deoxymononucleosides using the procedures outlined in sections 2.3 and 2.4.

Starting Material	Quantity (mg)	(mmole)	Product	Quantity (mg)	Yield (%)
MMTr-A^{Bz}-OH (2)	200	0.32	MMTr-A^{Bz}pCE (5)	200	72
MMTr-G^{Ib}-OH (9)	500	0.82	MMTr-G^{Ib}pCE (11)	427	60
MMTr-C^{Bz}-OH (14)	1108	1.84	MMTr-C^{Bz}pCE (16)	950	60
MMTr-T-OH (19)	3000	5.48	MMTr-TpCE (23)	3896	86

Table 2.3

Elemental analyses of fully protected dA, dG, and dC.

MMTr-A^{Bz}p-OCE ($C_{42}H_{38}N_6O_8PCl_3$, mol. wt. 892.18)

Calculated:	%C=56.57,	%H=4.30,	%N=9.42,	%P=3.47,	%Cl=11.89
Found:	%C=56.87,	%H=4.52,	%N=8.92,	%P=3.40,	%Cl=11.49

MMTr-G^{Ib}p-OCE ($C_{39}H_{40}N_6O_9PCl_3$, mol. wt. 874.17)

Calculated:	%C=53.58,	%H=4.62,	%N=9.62,	%P=3.54,	%Cl=12.17
Found:	%C=54.64,	%H=5.26,	%N=9.39,	%P=3.38,	%Cl=11.59

MMTr-C^{Bz}p-OCE ($C_{41}H_{38}N_4O_9PCl_3$, mol. wt. 868.15)

Calculated:	%C=59.48,	%H=4.91,	%N=7.18,	%P=2.98,	%Cl=10.17
Found:	%C=56.72,	%H=4.42,	%N=6.46,	%P=3.57,	%Cl=12.25

2.2.5 Acylation of the 3'-OH of $O^{5'}$-tritylated, N-protected Mononucleosides to yield Fully Protected Terminal Mononucleoside Derivatives.

General Reaction: MMTr-X^{*}-OH --> MMTr-X^{*}-OAc (or MMTr-X^{*}-OBz)

(X^{*}=A^{Bz} (3), G^{Ib}, C^{Bz} or T (20))

Example: MMTr-T-OH (19) --> MMTr-T-OAc (20)

$O^{5'}$-p-Monomethoxytrityl-2'-deoxythymidine (19) (1.858 g, 3.62 mmole) was dried by repeated evaporations in vacuo from pyridine (3x30 ml) and reduced to a final volume ca. 20 ml. Acetic anhydride (0.5 ml, 5.14 mmole) was added to the solution with continuous stirring. The reaction mixture was heated to 60°C and formation of the product was monitored by tlc on SiO_2 in MeOH/CH_2Cl_2 (1:20) (R_f: 0.2 --> 0.75). The reaction was complete after 1.5 hours and stopped by the addition of ice (ca. 3 g). The product was partitioned into dichloromethane (3x30 ml) and the combined organic extracts washed with water (30 ml), a saturated aqueous solution of sodium bicarbonate (30 ml) and water (30 ml). Dichloromethane was removed in vacuo with residual pyridine then removed by azeotroping in vacuo with toluene (3x20 ml) to a white foam. The white foam was dissolved in dichloromethane (ca. 35 ml) and applied to a silica gel column. The product was eluted with 2% MeOH in CH_2Cl_2 to yield $O^{5'}$-p-monomethoxytrityl-$O^{3'}$-acetyl-2'-deoxythymidine (20) (1.768 g, 88% yield) as a white foam.

Proton NMR spectra in DMSO-d_6 included the following signals ((TMS), ppm.): 6.9 (1H, doublet, H(6)); 7.6-7.3 (complex multiplet, trityl protons); 6.5 (1H, triplet, H(1')); 3.85 (3H, singlet, p-methoxy protons); 2.15 (3H, singlet, acetate protons); 1.88 (3H, singlet, C5-methyl protons) in addition to the normal nucleoside signals.

The derivatives synthesized by this procedure are summarized in Table 2.4.

2.3 Selective Removal of Protecting Groups

Synthesis of deoxy oligomers requires selective deprotection of the 5' hydroxyl and the 3' phosphates on different residues to achieve a specific 3' to 5' linkage. The protecting groups employed obey this requirement since the 5' MMTr protecting group can be removed using anhydrous acidic conditions and the 3' 2-cyanoethyl group on the phosphate can be removed using mild basic conditions.

Once again, these procedures are repetitively used in the synthesis of deoxyoligomers and therefore are presented as a general reaction and described by a particular example.

Table 2.4

Calculated yields for the synthesis of fully protected terminal deoxymononucleosides using the acylation procedure outlined in section 2.2.5

Starting Material	Quantity (mg)(mmole)	Product	Quantity (mg)	Yield (%)
MMTr-T-OH (19)	1858 3.62	MMTr-T-OAc (20)	1768	88
MMTr-A^{Bz}-OH (2)	1100 1.75	MMTr-A^{Bz}-OBz (3)*	936	73

* In this case, a 3-fold molar equivalent of benzoic anhydride was substituted for acetic anhydride.

2.3.1 Selective Removal of the $O^{5'}$ p-Monomethoxytrityl Group

(Stawinski, T. et al., 1977)

General Reaction: MMTr-X^*-OR --> HO-X^*-OR

(X^*=A^{Bz}, G^{Ib}, C^{Bz} or T)

Example: MMTr-C^{Bz}p-OCE (16) --> HO-C^{Bz}p-OCE (34)

N^4-Benzoyl-$O^{5'}$-p-monomethoxytrityl-$O^{3'}$-(2,2,2-trichloroethyl-2-cyanoethylphosphate)-2'-deoxycytidine (16) (1.6 g, 1.85 mmole) was dissolved in CH_2Cl_2/MeOH (2:1 v/v, 30 ml). A solution of p-toluenesulfonic acid monohydrate (PTSA) (320 mg) in methanol (1 ml) was added with continuous stirring at room temperature. The formation of the product was monitored by tlc on SiO_2 in MeOH/CH_2Cl_2 (1:10) (R_f:0.9 yellow-orange --> 0.75 black).

The reaction was complete after 45 minutes. The excess acid was neutralized by the addition of a saturated solution of sodium bicarbonate (30 ml). The product was partitioned into dichloromethane (5x30 ml) and the combined organic layers washed once with water (30 ml). The organic phase was evaporated *in vacuo* to yield a foam. The foam was dissolved in dichloromethane (*ca.* 30 ml) and applied to a silica gel column. Elution with 5% MeOH in CH_2Cl_2 yielded N^4-benzoyl, $O^{3'}$-(2,2,2-trichloroethyl-2-cyanoethylphosphate)-2'-deoxycytidine (943 mg, 86% yield) as a white foam. The material was used without further characterization.

A summary of all the reactions for which this procedure was used is presented in Table 2.5.

Table 2.5

Calculated yields for the detritylation of the $O^{5'}$ MMTr groups on fully protected oligodeoxynucleotides by the procedure outlined in section 2.3.1.

Starting Material*	Quantity (mg)	(mmole)	Product	Quantity (mg)	Yield (%)
MMTr-GpCE (11)	427	0.49	HO-GpCE (28)	284	88
MMTr-CpCE (16)	1600	1.85	HO-CpCE (34)	943	86
MMTr-T-OAc (20)	1000	1.80	HO-T-OAc (21)	363	71
MMTr-TpCE (23)	2000	2.57	HO-TpCE (35)	1176	90
MMTr-CT-OAc (30)	230	0.21	HO-CT-OAc (32)	136	81
MMTr-GT-OAc (44)	370	0.34	HO-GT-OAc (45)	190	68
MMTr-A-OBz (3)	936	1.28	HO-A-OBz (24)	378	64
MMTr-CA-OBz (25)	628	0.50	HO-CA-OBz (26)	343	70
MMTr-GTpCE (37)	2547	1.95	HO-GTpCE (39)	1382	68
MMTr-ACGTpCE(40)	700	0.29	HO-ACGTpCE (42)	412	69

* All bases are N-protected: Ib for G, Bz for A and C.

2.3.2 Selective Removal of the Cyanoethyl group from Fully Protected Nucleotides

(Adamiak, R. W. et al., 1976, Sood & Narang, 1977)

General Reaction: $RO\text{-}X^{*}\underline{p}CE \dashrightarrow RO\text{-}X^{*}\underline{p}\text{-}O^{-+}N(Et)_3$

($X^{*}=A^{Bz}$ (4), G^{Ib} (10), C^{Bz} (15) or T)

Example: $MMTr\text{-}A^{Bz}\underline{p}CE$ (5) $\dashrightarrow$ $MMTr\text{-}A^{Bz}\underline{p}\text{-}O^{-+}N(Et)_3$ (4)

$\underline{N}^{6}$-Benzoyl-$\underline{O}^{5'}$-$\underline{p}$-monomethoxytrityl-$\underline{O}^{3'}$-(2,2,2-trichloroethyl-2-cyanoethylphosphate)-2'-deoxyadenosine (5) (1.358 g, 1.52 mmole) was dried by repeated evaporations in vacuo from pyridine (3x20 ml) and concentrated to a final volume of ca. 10 ml. Anhydrous triethylamine (3 ml, 21.6 mmole) was added with continuous stirring at room temperature. The reaction was monitored by tlc on SiO_2 in $MeOH/CH_2Cl_2$ (1:10) for the formation of the product (R_f: 0.8 $\dashrightarrow$ 0.3 "cusp") and judged to be complete after 3 hours. The reaction mixture was azeotroped in vacuo with toluene (3x15 ml) to yield $\underline{N}^{6}$-benzoyl-$\underline{O}^{5'}$-$\underline{p}$-monomethoxytrityl-$\underline{O}^{3'}$-(2,2,2-trichloroethylphosphate)-2'-deoxyadenosine (4) as a brown oil. The product was used immediately without further characterization or purification.

2.4 Synthesis of Fully Protected Oligonucleotides

Once selective deprotection of the 5' hydroxyl of one compound and the phosphate of another has been achieved, the compounds can be "coupled" in the presence of MST to yield a 3'-5' phosphodiester linkage. This new compound can be deprotected by the procedures outlined in section 2.3

allowing it to be further coupled with additional nucleosides to yield a growing oligonucleotide sequence. The procedures for the selective deprotection of fully protected deoxy oligomers are identical to the selective deprotection of the mononucleotides.

2.4.1 Coupling of Selectively Deprotected Residues

General Reaction: $R_1\underline{p}\text{-}O^- + HO\text{-}R_2 \rightarrow R_1\underline{p}R_2$

R_1 and R_2 are summarized in Table 2.6.

Example: MMTr-$A^{Bz}\underline{p}$-O^-(4) + HO-$C^{Bz}\underline{p}$CE(34)-->MMTr-$A^{Bz}\underline{p}C^{Bz}\underline{p}$CE(36)

$\underline{N}^6$-Benzoyl-$\underline{O}^{5'}$-$\underline{p}$-monomethoxytrityl-$\underline{O}^{3'}$-(2,2,2-trichloroethylphosphate)-2'-deoxyadenosine (2.22 mmole theoretical) was dried by repeated evaporations *in vacuo* from anhydrous pyridine (3x20 ml) and concentrated to a final volume *ca.* 10 ml. To this solution was added MST (557 mg, 2.22 mmole) and the mixture was warmed to 40 °C for 1 hour.

$\underline{O}^{3'}$-(2,2,2-trichloroethyl-2-cyanoethylphosphate)-2'-deoxycytidine (34) (943 mg, 1.58 mmole) was dried by repeated evaporations *in vacuo* from anhydrous pyridine (3x20 ml) and concentrated to a volume of *ca.* 10 ml. This solution was added to the MST activated compound (4) solution. The reaction mixture was further evaporated *in vacuo* from anhydrous pyridine (3x20 ml) and concentrated to a minimum volume (*ca.* 8 ml).

The reaction was monitored by tlc on SiO_2 in MeOH/CH_2Cl_2 (1:10) for the formation of the dinucleotide

product (R_f: incoming $O^{3'}$-(2,2,2-trichloroethyl-2-cyanoethyl-phosphate)-2'-deoxycytidine (34) black 0.75 --> orange 0.8 (36)). Upon completion (9 days), the reaction was stopped with ice (ca. 1 g) and the product partitioned into dichloromethane (3x30 ml). The combined organic extracts were washed with water (2x30 ml). The dichloromethane was removed by evaporation in vacuo with the residual pyridine then removed by azeotroping in vacuo with toluene (3x15 ml) to yield a reddish-brown oil. The oil was dissolved in dichloromethane (ca. 20 ml) and applied to a silica gel column. Elution with 4% MeOH in CH_2Cl_2 yielded the product (36) (1.392 g, 74% yield) as a brown foam. The product was not further characterized at this point. Instead, 11 mg was deprotected by the procedure described in detail in section 2.5.

The integrity of this compound and the other protected deoxyoligomers was verified by the H^1-NMR of the deprotected material and is described in detail in section 2.7. Protected oligomers were not directly characterized.

2.4.2 Synthetic Strategies for the Preparation of Fully Protected Oligonucleotides

The strategies employed for the synthesis of fully protected d(GCA), d(AGCT) and d(ACGTACGTp) are illustrated in figures 2.5-2.7. d(GCA) was synthesized stepwise 3' to 5' while d(AGCT) and d(ACGTACGTp) were prepared using a "block" strategy. Table 2.6 summarizes the coupling reactions and yields obtained for the synthesis of all the deoxyoligomers.

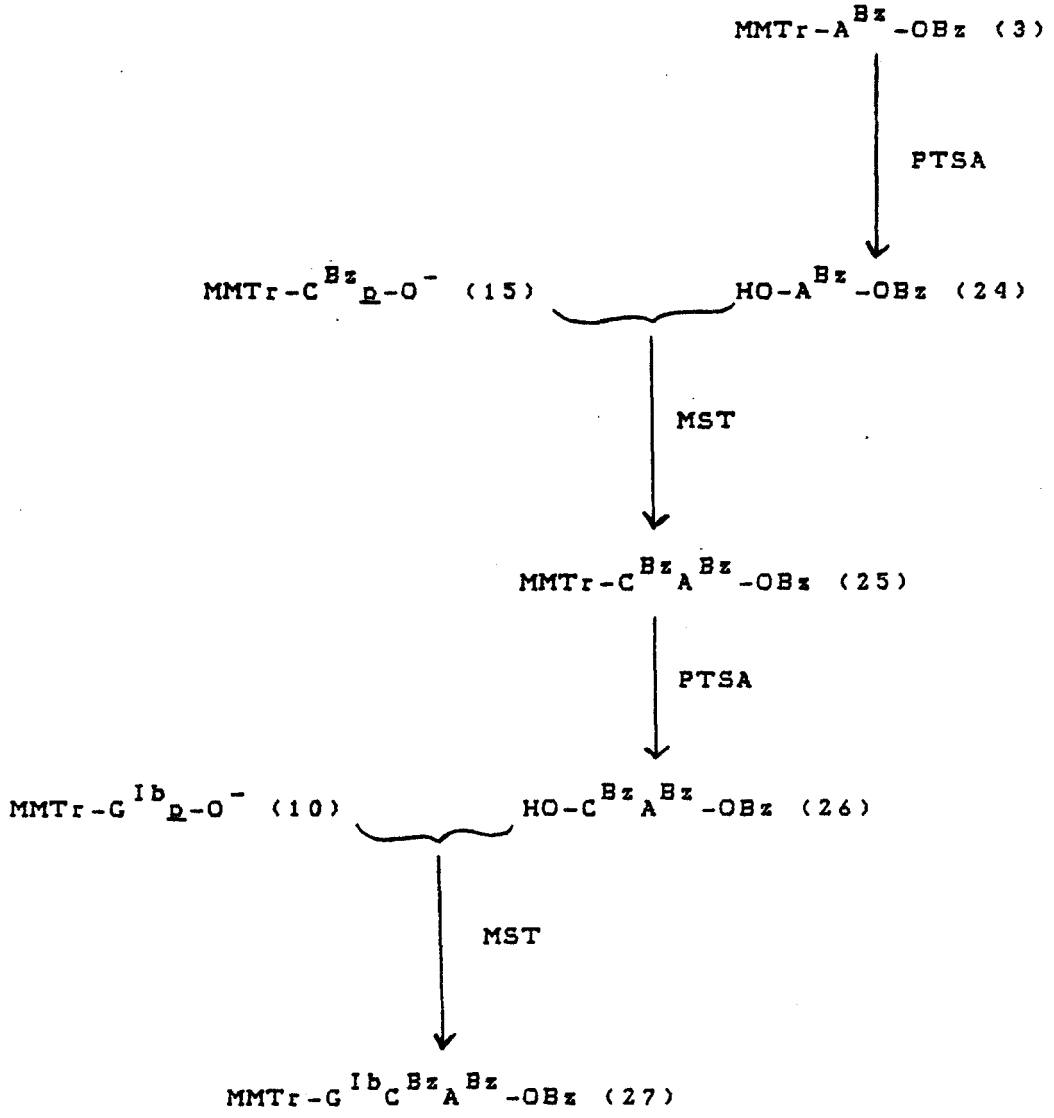


Figure 2.5- Stepwise synthesis of fully protected d(GCA)(27).

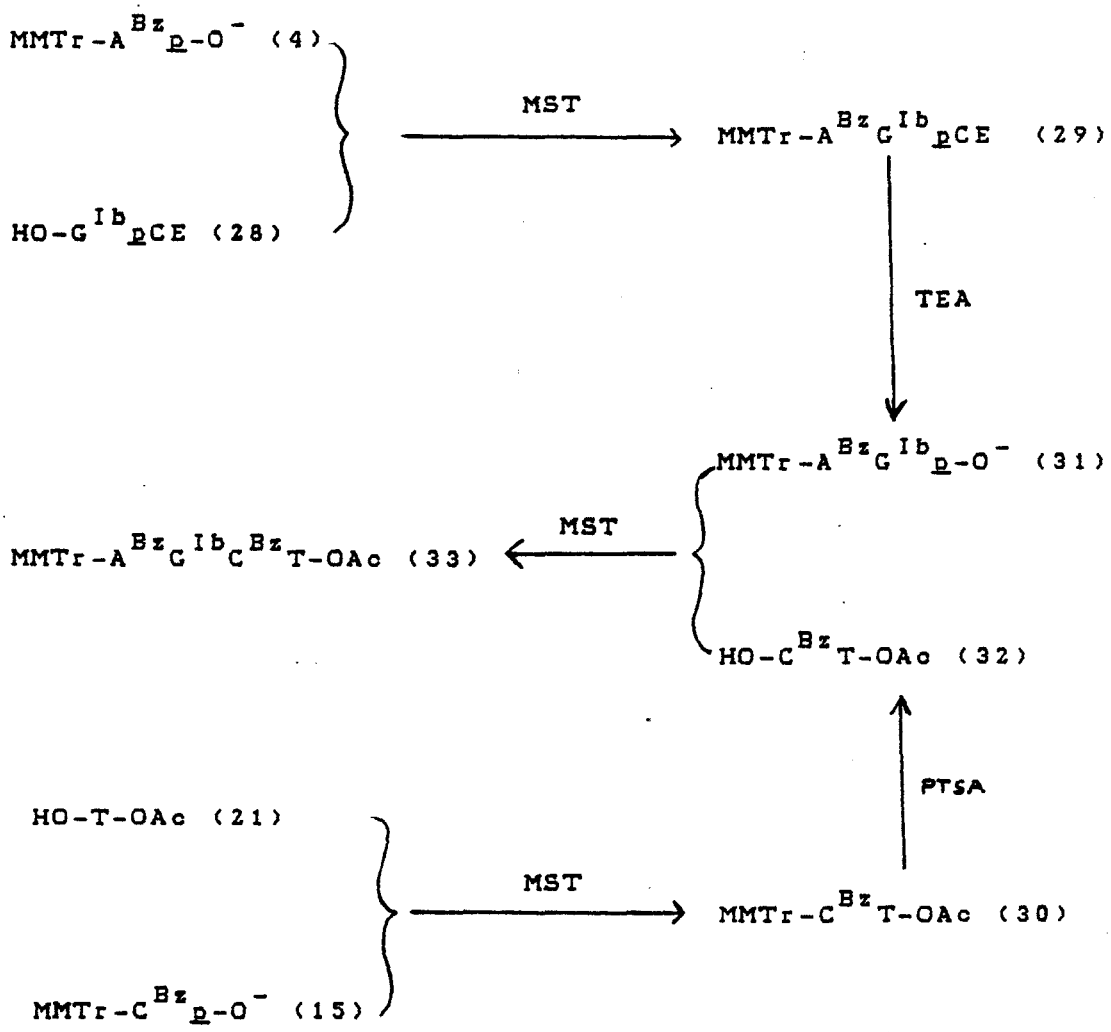


Figure 2.6 - Synthesis of fully protected d(AGCT) (33) using a "block" synthetic strategy.

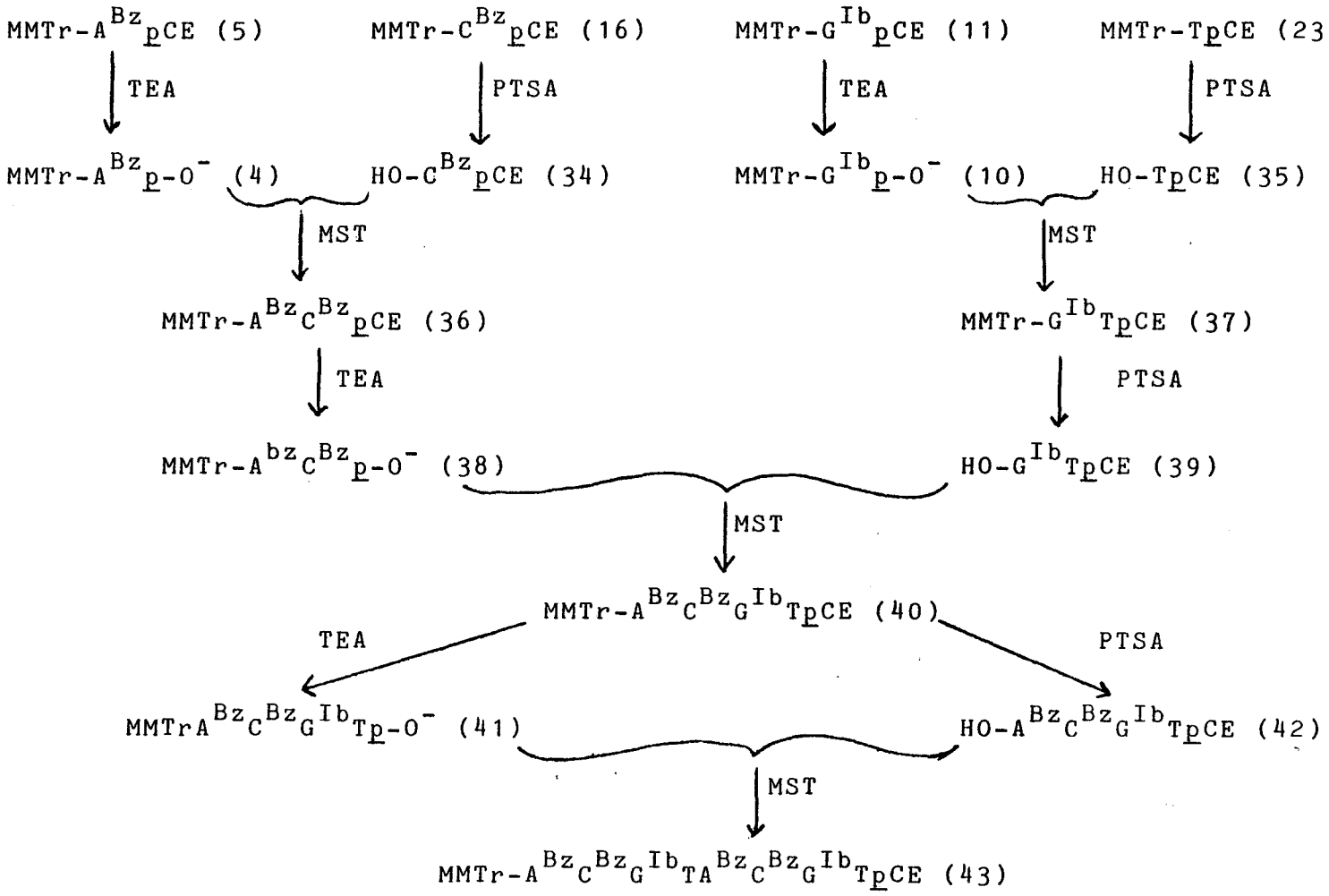


Figure 2.7 - Synthesis of fully protected d(ACGTACGTp) using a "block" synthetic strategy.

Table 2.6

Calculated yields for coupling reactions carried out in the synthesis of deoxyoligomers.

Starting Materials*	Quantity (mg)	Quantity (mmole)	Product	Quantity (mg)	Yield (%)
DMTr-A-OH (6) HO-G<u>p</u>CE (28)	94	0.16	DMTr-AG<u>p</u>CE (46)	90	38
MMTr-C-OH (14) HO-T-OAc (21)	185	0.65	MMTr-CT-OAc (30)	485	69
MMTr-A-OH (2) HO-G<u>p</u>CE (28)	284	0.47	MMTr-AG<u>p</u>CE (29)	482	72
MMTr-AG<u>p</u>CE (29) HO-CT-OAc (32)	114	0.14	MMTr-AGCT-OAc (33)	100	36
MMTr-G-OH (9) HO-T-OAc (21)	122	0.43	MMTr-GT-OAc (47)	382	81
MMTr-A-OH (2) MMTr-C<u>p</u>CE (34)	133	0.22	MMTr-AC<u>p</u>CE (36)	265	86
MMTr-AC<u>p</u>CE (36) HO-GT-OAc (45)	190	0.23	MMTr-ACGT-OAc (48)	240	48
MMTr-C-OH (14) HO-A-OBz (24)	378	0.82	MMTr-CA-OBz (25)	628	61
MMTr-G-OH (9) HO-CA-OBz (26)	343	0.35	MMTr-GCA-OBz (27)	262	43
MMTr-A<u>p</u>CE (5) HO-C<u>p</u>CE (34)	698	1.17	MMTr-AC<u>p</u>CE (36)	1392	84
MMTr-G<u>p</u>CE (11) HO-T<u>p</u>CE (25)	1176	2.32	MMTr-GT<u>p</u>CE (37)	2547	84
MMTr-AC<u>p</u>CE (36) HO-GT<u>p</u>CE (39)	732	0.71	MMTr-ACGT<u>p</u>CE (40)	1292	76
MMTr-ACGT<u>p</u>CE (40) HO-ACGT<u>p</u>CE (42)	412	0.20	MMTr-ACGTACGT<u>p</u>CE(43)	458	55

* All bases are N-protected: Ib for G, Bz for A and C.
** Based on the amount of 5' deprotected incoming nucleoside.

2.5 Complete Deprotection of the Fully Protected Oligonucleotides to yield Target Oligonucleotides.

All glassware used in the following procedures was acid washed. The 2,2,2-trichloroethyl blocking group was removed by reductive cleavage with Zn/Cu couple which was prepared from a previously established procedure (Le Goff, E., 1964).

Deblocking fully protected deoxy oligomers involves three distinct stages:

1. removal of the 2,2,2-trichloroethyl blocking group,
2. removal of the base labile groups (Bz, Ib, CE and Ac) and
3. removal of the acid labile MMTr group.

The two strategies employed for deprotecting fully protected deoxy oligomers are illustrated in figure 2.8. The difference involves the removal of the acid labile MMTr group last in aqueous acidic media (Strategy 1) or cleaving it first in an anhydrous acidic media (Strategy 2).

Deprotected oligodeoxynucleotides were characterized by ^{1}H-NMR. This will be described in detail in section 2.7.

2.5.1 Deprotection of Fully Protected Oligodeoxynucleotides using Strategy 1

Fully protected oligodeoxynucleotide (10 to 20 mg.) was dissolved in 0.5 to 1 ml of DMF. Zn/Cu couple (ca. 20 mg.) was added with continuous stirring. The suspension was heated to ca. 55-60°C for 2 to 10 hrs. The reaction was

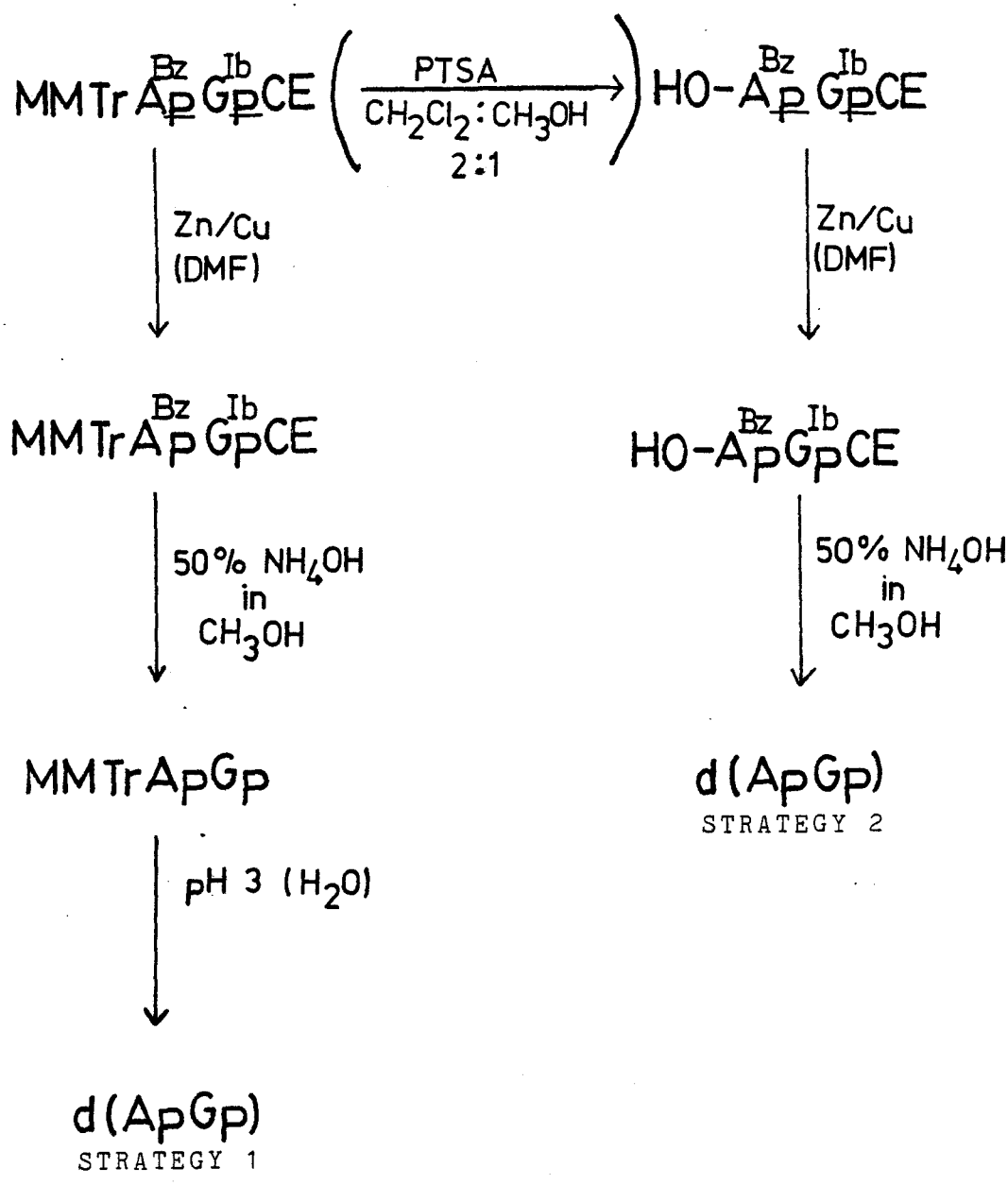


Figure 2.8 - Two strategies for deblocking fully protected deoxy oligomers.

monitored by tlc on SiO_2 in $MeOH/CH_2Cl_2$ (1:10) (R_f: 0.3-0.8 --> 0.0). Methanol (2 ml) and concentrated ammonia (2 ml) were added to the reaction vial which was then sealed with parafilm and a screw cap. The mixture was allowed to stir at room temperature for two days. The Zn-Cu couple was removed by filtration and washed with 1M ammonia (3x5 ml). The combined filtrate and washings were stirred with chelex-100 (ammonium form, ca. 1 g) for 1 hour. The chelex was removed by filtration and washed with 1M ammonia (3x5 ml). The combined filtrate and washings were evaporated in vacuo to dryness. The resulting product 5'-MMTr-oligodeoxynucleotide was dissolved in deionized water (ca. 0.5 ml) and isolated by descending chromatography on Whatman #1 filter paper as outlined in section 2.1. Detection was facilitated by short wave UV and the resulting band cut out and immersed in absolute ethanol (ca. 30 ml) for 1 hour followed by immersion in diethylether (ca. 30 ml) for 20 min. The paper was dried and the product eluted in a minimum volume of 10 ml with deionized water by descending chromatography for 16 hours. The solution was brought to pH 3 with 2M HCl and left for 2 days, neutralized with 1M NH_4OH and evaporated in vacuo to dryness. The product, completely deprotected oligodeoxynucleotide was purified by descending chromatography on Whatman #1 or #40 paper as outlined in section 2.1. The product was detected by short wave UV, the band cut out and immersed in absolute ethanol for 1 hour

(2x*ca.* 30 ml) followed by diethylether (*ca.* 30 ml) for 20 minutes. The paper was allowed to dry and the oligodeoxynucleotide was eluted in a minimum volume of 10 ml with deionized water by descending chromatography for 16 hours. The UV absorbance of an appropriate dilution of the eluant was determined at 260 nm. From this measurement, and the known dilution, the total optical density (OD_{260}) of free oligonucleotide was determined. The water was removed by lyophilization to yield a white residue.

2.5.2 Deprotection of Detritylated Protected Oligodeoxynucleotides using Strategy 2

During the synthesis of deoxyoligomers, compounds were selectively de-tritylated as outlined in section 2.3.1 so that they could be coupled to another compound. These compounds were deprotected after they were de-tritylated since this allowed the elimination of the aqueous acid treatment stage outlined in section 2.5.1.

5'-Deprotected oligonucleotide (10 to 20 mg.) was dissolved in 0.5 to 1 ml of DMF. Zn/Cu couple (*ca.* 20 mg.) was added with continuous stirring. The suspension was heated in a water bath to *ca.* 55-60°C for 2 to 10 hrs. The reaction was monitored by tlc on SiO_2 in $MeOH/CH_2Cl_2$ (1:10) (R_f: 0.2-0.5 --> 0.0). Methanol (2 ml) and concentrated ammonia (2 ml) were added to the reaction vial which was then sealed with parafilm and a screw cap. The mixture was allowed to stir at room temperature for two days. The Zn-Cu

couple was removed by filtration and washed with 1M ammonia (3x5 ml). The combined filtrate and washings were stirred with chelex-100 (ammonium form, ca. 1 g) for 1 hour. The chelex was removed by filtration and washed with 1M ammonia (3x5 ml). The combined filtrate and washings were evaporated in vacuo to dryness. The product, fully deprotected oligodeoxynucleotide was purified by descending chromatography on Whatman #1 or #40 paper as outlined in section 2.1. The product was detected by short wave UV, the band cut out and immersed in absolute ethanol for 1 hour (2x ca. 30 ml) followed by diethylether (ca. 30 ml) for 20 minutes. The paper was allowed to dry and the completely deprotected oligomer was eluted in a minimum volume of 10 ml with deionized water by descending chromatography for 16 hours. The UV absorbance of an appropriate dilution of the eluant was determined at 260 nm. From this measurement, and the known dilution, the total optical density (OD_{260}) of free deoxy oligomer was determined. The water was removed by lyophilization to yield a white residue.

2.5.3 Deprotection of Fully Protected d(ACGTACGTp) using a Procedure That is Similar to Strategy 2

Fully protected d(ACGTACGTp) (30 mg) was dissolved in 1 ml of 2% p-toluenesulfonic acid in methanol. The reaction was monitored by tlc on SiO_2 in $MeOH/CH_2Cl_2$ (1:10) for the formation of the product, $O^{5'}$ detritylated protected

d(ACGTACGTp) (R_f: 0.4 (orange) --> 0.3 (black)). Upon completion (30 min.), the excess acid was neutralized with saturated sodium bicarbonate (3 ml). The product was partitioned into dichloromethane (3x2 ml) and the combined organic extracts washed once with water (2 ml). The extraction and washing procedure was carried out in a 15x100mm centrifuge tube with the resulting emulsion broken by centrifugation. The organic extract was evaporated *in vacuo* to dryness. Without further purification, the resulting residue, 5'-detritylated d(ACGTACGTp), was then completely deprotected using strategy 2 outlined in section 2.5.2.

The ^{1}H-NMR study of deprotected d(ACGTACGTp) demonstrated the presence of two distinct species of molecules after the chromatographic purification. This will be discussed in detail in the Discussion, section 3.1.2. The octamer was further purified by HPLC on a Waters C-18 reverse-phase column. Initial conditions were 85% CH_3OH (HPLC grade) and 15% NH_4OAc (0.1M, pH 6.0). Final conditions were 50% CH_3OH and 50% NH_4OAc attained after 20 minutes using an automated parabolic gradient (Waters Associates gradient curve 9 as described in "Model 720 System Controller - Preliminary Instruction Manual", Manual No. 82477, March 1980, pages 3-28). Technical data, in the form of a "WISP" report concerning the HPLC conditions and resulting analysis are presented in Appendix A.

The HPLC trace is presented in figure 2.9 with the largest peak having a retention time of 19.6 and assumed to represent deprotected d(ACGTACGTp). Characterization of the purified compound by ^{1}H-NMR demonstrated that the major peak was purified d(ACGTACGTp). The characterization of this octamer by means of ^{1}H-NMR is discussed in detail in section 2.7.

2.6 Determination of Deprotection Yields Based on the Total Optical Density of Free Oligodeoxynucleotide.

The molar extinction coefficient at 260 nm of the oligodeoxynucleotide was estimated from nearest neighbor interactions as outlined by Cantor *et al*, 1970. The yield of deprotected oligonucleotide was calculated using the equation:

$$\% \text{ YIELD} = OD_{260}/E_{260}\text{max}/PO/MWPO*100$$

where PO is the amount of protected oligomer starting material and MWPO is the molecular weight of the protected oligomer. Table 2.7 summarizes the deprotecting yields for a number of blocked deoxy oligomers including the deprotecting strategy used.

2.7 NMR Studies

The various ^{1}H-NMR spectra of deprotected deoxy oligomers were generated on Bruker WM-250 and WH-400 MHz spectrometers operating in Fourier Transform mode and equipped with quadrature detection. The field/frequency lock

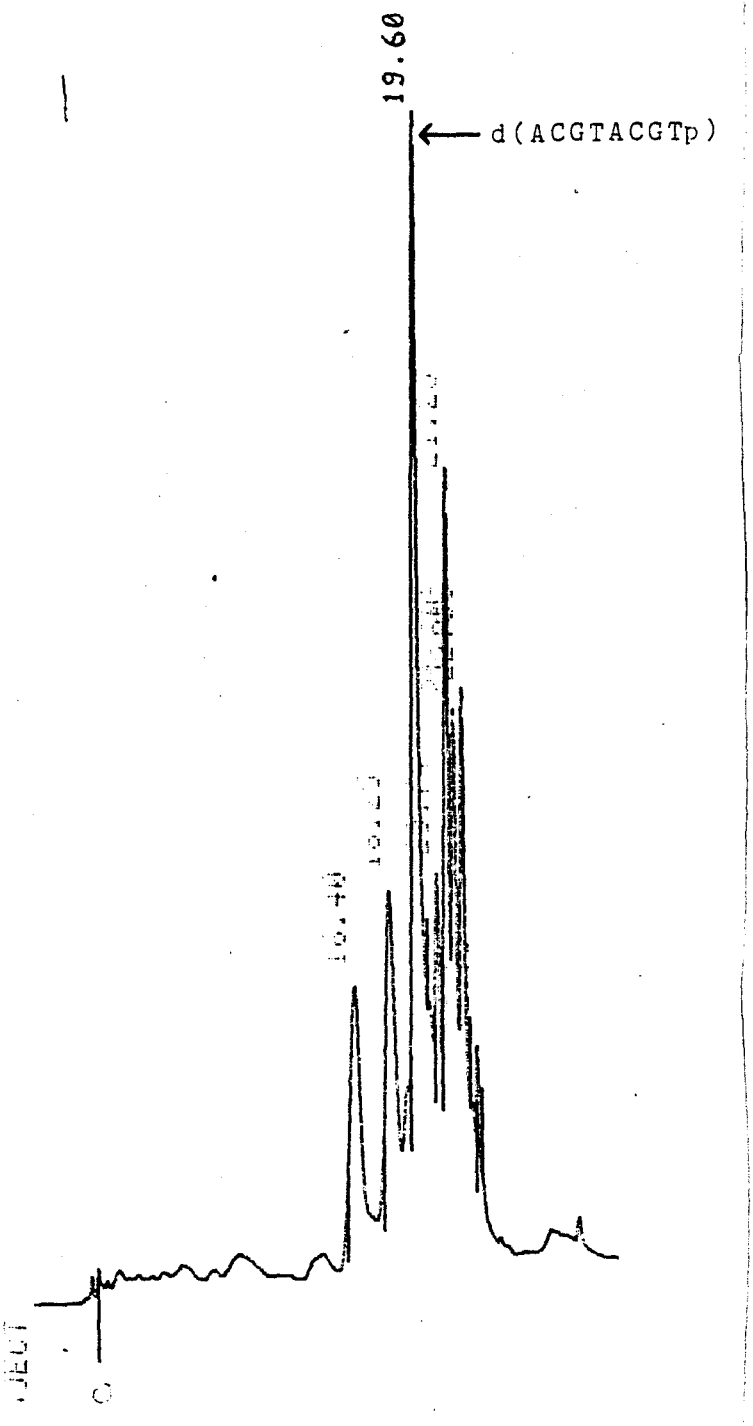


Figure 2.9 - HPLC trace of d(ACGTACGTp) after deprotection procedure outlined in section 2.5.3.

Technical detail is presented in section 2.5.3 and Appendix A.

Protected[a] Compound	Amount (mg)	(umole)	Strategy[b]	R_f[c]	OD_{260}	$E_{260}max$[d] ($X10^4$)	Yield (%)	Product
HO-CT-OAc (30)	20	24.7	2	0.56	344	1.62	86	(49)
DMTr-AGpCE (46)	15	10.3	1	0.36	50	2.78	17	(50)
MMTr-AGpCE (29)	15	10.6	1	0.36	100	2.78	34	(50)
MMTr-AGCT-OAc (33)	21	9.7	1	0.38	79	4.09	20	(52)
MMTr-ACpCE (36)	11	7.8	1	0.38	47	2.28	27	(53)
MMTr-GT-OAc (47)	10	9.2	1	0.40	52	2.04	28	(54)
MMTr-ACGT-OAc (48)	20	9.3	1	0.38	45	4.01	12	(55)
MMTr-GCA-OBz (27)	20	11.2	1	0.43	46	3.25	13	(56)
HO-GTpCE (39)	12	9.2	2	0.44	140	2.04	75	(57)
MMTr-ACpCE (23)	23	16.3	1	0.39	28	2.28	8	(53)
MMTr-ACGTpCE (49)	32	13.4	2	0.23	34	4.01	17	(58)
MMTr-ACGTACGTpCE (43)	30	6.8	2	0.14	34	7.94	5	(59)

a All bases N-protected (except T residues), all phosphates fully protected.

b Deprotection strategies are outlined in section 2.5.

c Chromatography carried out on Whatman #1 or #40 as described in section 2.1.

d Calculated on basis of nearest neighbor interactions (section 2.6).

Table 2.7 - Calculated yields for the deprotection reactions carried out as described in section 2.5.

was provided by the deuterium signal of D_2O. The 250 MHz spectra were obtained using a ^1H probe and 400 MHz spectra were obtained using a ^{13}C probe tuned for protons. Probe temperatures were maintained by variable temperature units to within 1^oC and verified by thermocouple measurements. The internal reference was ^{t}-butanol-OD at 1.231 ppm relative to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS). All chemical shifts are reported relative to DSS.

2.7.1 Sample Preparation for NMR Studies

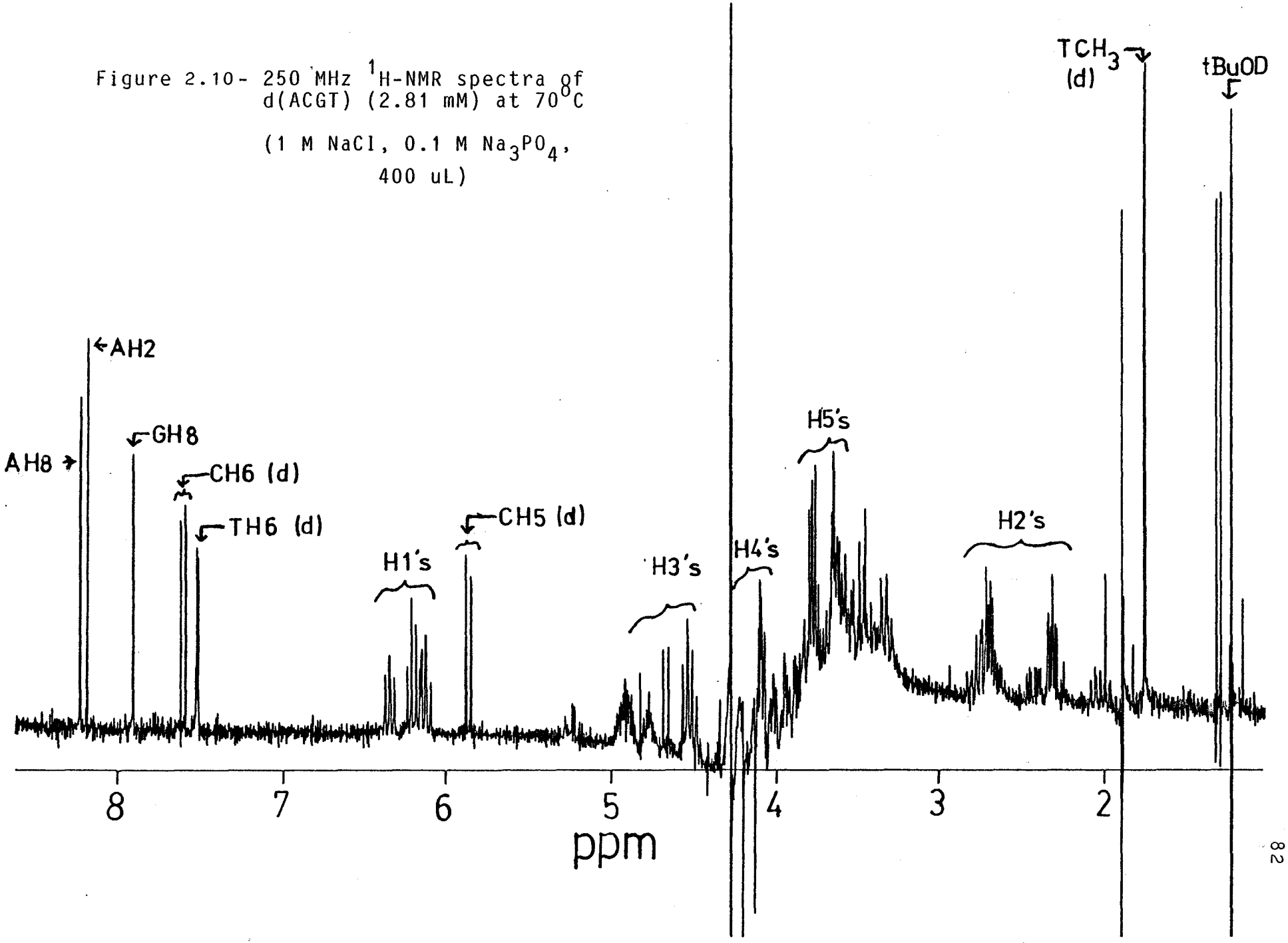
Completely deprotected oligodeoxynucleotides were lyophilized twice from redistilled D_2O (Aldrich), dissolved in NMR buffer (400 ul, 1M NaCl, 0.01M Na_3PO_4 and t-butanol-OD (5 ul) in D_2O, pD ca. 7.2) and transferred into an NMR sample tube (Wilmad, 5mm).

2.7.2 Characterization of Deoxy Oligomers

Sequence integrity was determined by ^1H-NMR at 70^oC (90^oC in the case of d(ACGTACGTp)) to ensure minimal secondary structure interactions. Base ratio information was provided by the characteristic non-exchangeable proton peaks AH-8, AH-2, GH-8, CH-5, CH-6, TH-6, and T-CH_3. Correct base ratios for the oligomers and the intermediate oligomers (ie. tetramers and dimers) from which they were derived, coupled with the knowledge of the synthetic stategy employed, formed the basis for verifying the integrity of synthesized sequences. Fig. 2.10 represents a typical spectrum and

Figure 2.10- 250 MHz ^{1}H-NMR spectra of d(ACGT) (2.81 mM) at 70^{0}C

(1 M NaCl, 0.1 M Na_3PO_4, 400 uL)



illustrates the regions containing all the non-exchangeable protons of interest.

The proton peaks used to characterize the deoxyoligomers are listed in the 70° or 90°C columns in Tables 2.8-2.11. These signals were all found to represent the correct base ratios for all of the deprotected oligomers.

2.7.3 Variable Temperature-PMR Studies

Tables 2.8 to 2.11 list the chemical shift of the various non-exchangeable protons for d(GCA), d(ACGT), d(AGCT) and d(ACGTACGTp) over a range of temperatures. Duplex formation was studied by plotting the resultant chemical shifts as a function of temperature for particular resonances. Figs 2.11 to 2.16 represent the melting profiles of the above sequences.

The melting curves were computer fitted to a fifth order differential equation from which the first derivative minimum was obtained. For a sigmoidal relationship, this minimum is the point of inflection in the curve, which represents the Tm of the system, where one half of the molecules exist in the duplex state. The Tms calculated for the various curves were averaged to give an average Tm for that particular sequence.

TEMP (^{o}C)

	70.3	60.8	50.2	40.3	35.4	30.3
AH-8	8.317	8.319	8.318	8.317	8.315	8.312
AH-2	8.166	8.154	8.137	8.118	8.106	8.901
GH-8	7.878	7.878	7.879	7.88	7.88	7.884
CH-6	7.605	7.592	7.571	7.553	7.543	7.533
CH-5	5.886	5.872	5.851	5.83	5.816	5.799
AH-1'	6.399	6.394	6.386	6.378	6.373	6.368
CH-1'	6.133	6.125	6.113	6.103	6.097	6.089
GH-1'	6.133	6.119	6.108	6.096	6.088	6.083

TEMP (^{o}C)

	25.3	20.5	15.6	10.7	1.0
AH-8	8.309	8.303	8.295	8.285	8.259
AH-2	8.073	8.048	8.018	7.979	7.892
GH-8	7.887	7.892	7.901	7.914	7.949
CH-6	7.532	7.511	7.499	7.489	7.474
CH-5	5.781	5.757	5.728	5.691	5.613
AH-1'	6.363	6.355	6.347	6.336	6.309
CH-1'	6.083	6.076	6.07	6.063	6.05
GH-1'	6.076	6.071	6.063	6.035	6.025

Table 2.8: Chemical Shift (ppm) of non-exchangeable protons for d(GCA) (3.5 mM, 1M NaCl) at various temperatures.

TEMP (°C)

	70.8	61.2	51.5	46.4	40.5	35.6
AH-2	8.162	8.149	8.132	8.122	8.106	8.09
AH-8	8.113	8.102	8.09	8.084	8.075	8.067
GH-8	7.914	7.914	7.915	7.916	7.917	7.918
CH-6	7.721	7.708	7.691	7.68	7.664	7.647
CH-5	5.9	5.88	5.852	5.833	5.804	5.774
TH-6	7.57	7.573	7.575	7.577	7.578	7.581
TM-5	1.832	1.826	1.822	1.817	1.811	1.805
AH-1'	6.226	6.203	6.188	6.2	6.189	6.188
TH-1'	6.209	6.206	6.19	6.178	6.159	6.143
GH-1'	6.197	6.198	6.2	6.203	6.209	6.215
CH-1'	6.076	6.052	6.024	6.024	5.985	5.964

TEMP (°C)

	30.5	25.7	20.7	14.7	9.8	0.3
AH-2	8.069	8.046	8.017	7.972	7.931	7.834
AH-8	8.06	8.052	8.044	8.032	8.024	8.01
GH-8	7.919	7.918	7.915	7.903	7.891	7.853
CH-6	7.628	7.608	7.587	7.557	7.535	7.511
CH-5	5.737	5.696	5.65	5.867	5.534	5.426
TH-6	7.581	7.576	7.572	7.554	7.53	7.461
TM-5	1.797	1.738	1.764	1.728	1.686	1.571
AH-1'	6.183	6.171	6.161	6.14	6.118	6.063
TH-1'	6.122	6.081	6.077	6.046	6.024	5.993
GH-1'	6.215	6.222	6.229	6.233	6.233	6.215
CH-1'	5.942	5.919	5.894	5.868	5.809	5.796

Table 2.9: Chemical Shift (ppm) of non-exchangeable protons for d(AGCT) (4.8 mM, 1N NaCl) at various temperatures.

TEMP (°C)

	70.3	60.8	50.2	40.3	35.4	30.3
AH-8	8.224	8.22	8.214	8.206	8.202	8.197
AH-2	8.181	8.169	8.152	8.13	8.115	8.096
GH-8	7.903	7.901	7.902	7.905	7.909	7.916
CH-6	7.6	7.582	7.557	7.592	7.512	7.493
CH-5	5.851	5.831	5.803	5.769	5.747	5.719
TH-6	7.514	7.508	7.5	7.491	7.485	7.478
TM-5	1.751	1.739	1.723	1.708	1.7	1.689
AH-1'	6.342	6.333	6.321	6.305	6.297	6.283
GH-1'	6.207	6.207	6.206	6.206	6.207	6.209
CH-1'	6.118	6.104	6.088	6.074	6.067	6.061
TH-1'	6.149	6.14	6.119	6.08	6.056	5.997

TEMP (°C)

	25.3	20.5	15.6	10.7	1.0
AH-8	8.195	8.193	8.195	8.198	8.216
AH-2	8.078	8.052	8.016	7.979	7.914
GH-8	7.923	7.932	7.939	7.941	7.914
CH-6	7.483	7.456	7.431	7.408	7.364
CH-5	5.689	5.647	5.594	5.535	5.486
TH-6	7.467	7.448	7.415	7.368	7.249
TM-5	1.677	1.655	1.609	1.548	1.387
AH-1'	6.27	6.252	6.233	6.207	6.178
GH-1'	6.211	6.213	6.21	6.207	6.178
CH-1'	6.058	6.054	6.048	6.041	6.028
TH-1'	5.943	5.861	5.766	5.686	5.546

Table 2.10: Chemical Shift (ppm) of non-exchangeable protons for d(ACGT) (2.8 mM, 1N NaCl) at various temperatures.

TEMP (°C)

	90.6	85.6	80.4	74.5	70.4	65.0
A(5)H-8	8.253	8.25	8.248	8.242	8.238	8.233
A(1)H-8	8.217	8.216	8.214	8.213	8.21	8.206
A(1)H-2	8.819	8.183	8.178	8.168	8.158	8.14
A(5)H-2	8.123	8.114	8.106	8.092	8.072	8.027
G(7)H-8	7.898	7.901	7.898	7.9	7.9	7.896
G(3)H-8	7.874	7.876	7.876	7.877	7.877	7.882
C(6)H-6	7.613	7.601	7.589	7.57	7.552	7.523
C(2)H-6	7.601	7.592	7.584	7.57	7.552	7.523
T(8)H-6	7.539	7.536	7.533	7.529	7.526	7.509
T(4)H-6	7.307	7.303	7.296	7.284	7.275	7.559
C(5)H-6	5.871	5.863	5.852	---	---	---
T(8)CH3	1.744	1.738	1.729	1.715	1.703	1.685
T(4)CH3	1.721	1.713	1.706	1.693	1.679	1.654

TEMP (°C)

	60.3	55.3	50.6	45.6
A(5)H-8	8.223	8.228	8.235	8.242
A(1)H-8	8.151	8.144	---	---
A(1)H-2	8.113	8.082	8.053	8.033
A(5)H-2	7.949	7.844	7.75	---
G(7)H-8	7.887	7.886	7.877	7.874
G(3)H-8	7.887	7.886	7.888	7.885
C(6)H-6	7.493	7.465	7.446	7.436
C(2)H-6	7.493	7.465	7.446	7.436
T(8)H-6	7.496	7.479	7.46	7.446
T(4)H-6	7.24	7.224	7.213	7.211
C(5)H-6	5.715	5.661	5.609	5.573
T(8)CH3	1.665	1.644	1.627	1.615
T(4)CH3	1.619	1.578	1.544	1.521

Table 2.11: Chemical Shift (ppm) of non-exchangeable protons for d(ACGTACGTp) (1.1 mM, 1N NaCl) at various temperatures.

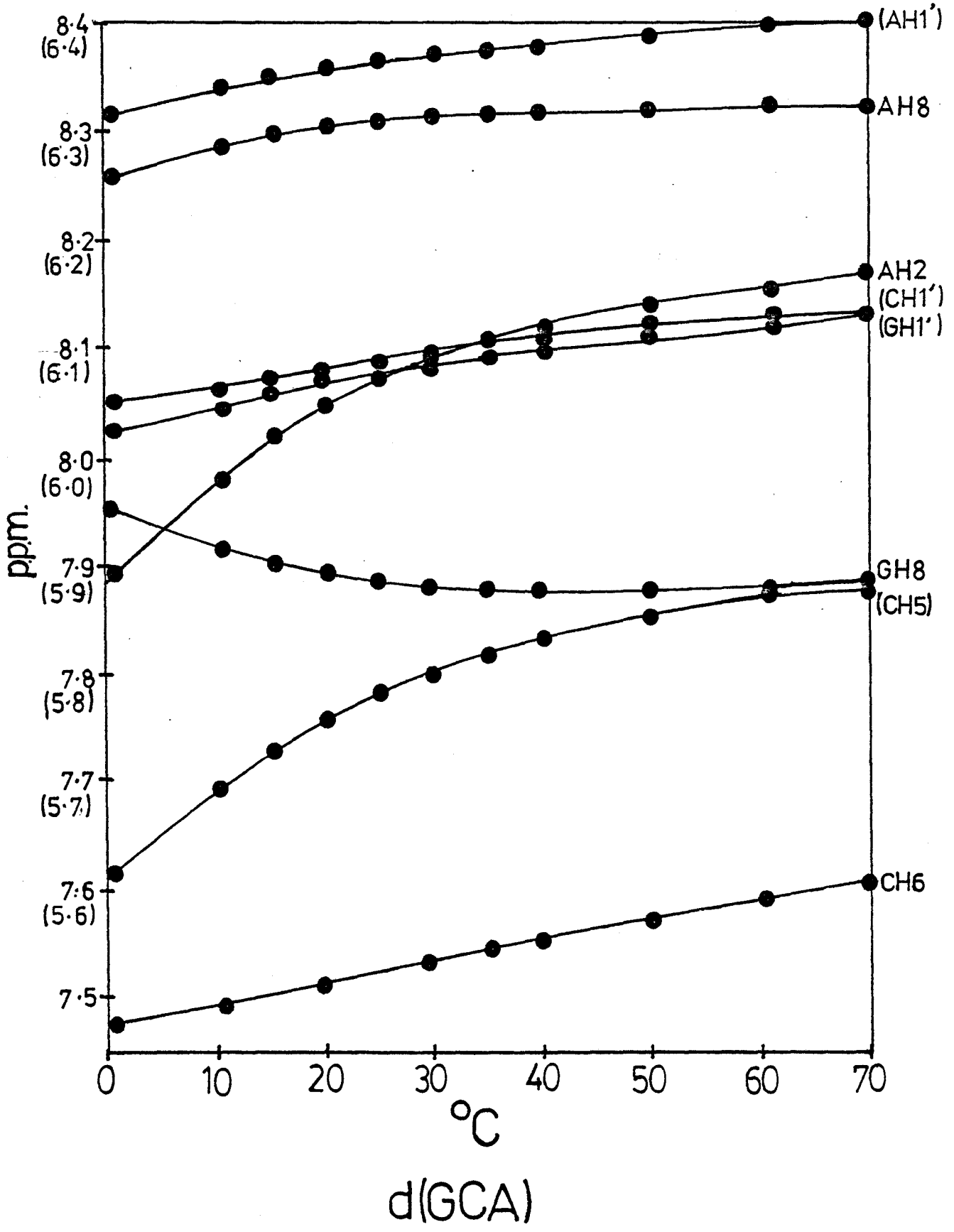


Figure 2.11- Melting curves for d(GCA) (3.5 mM)

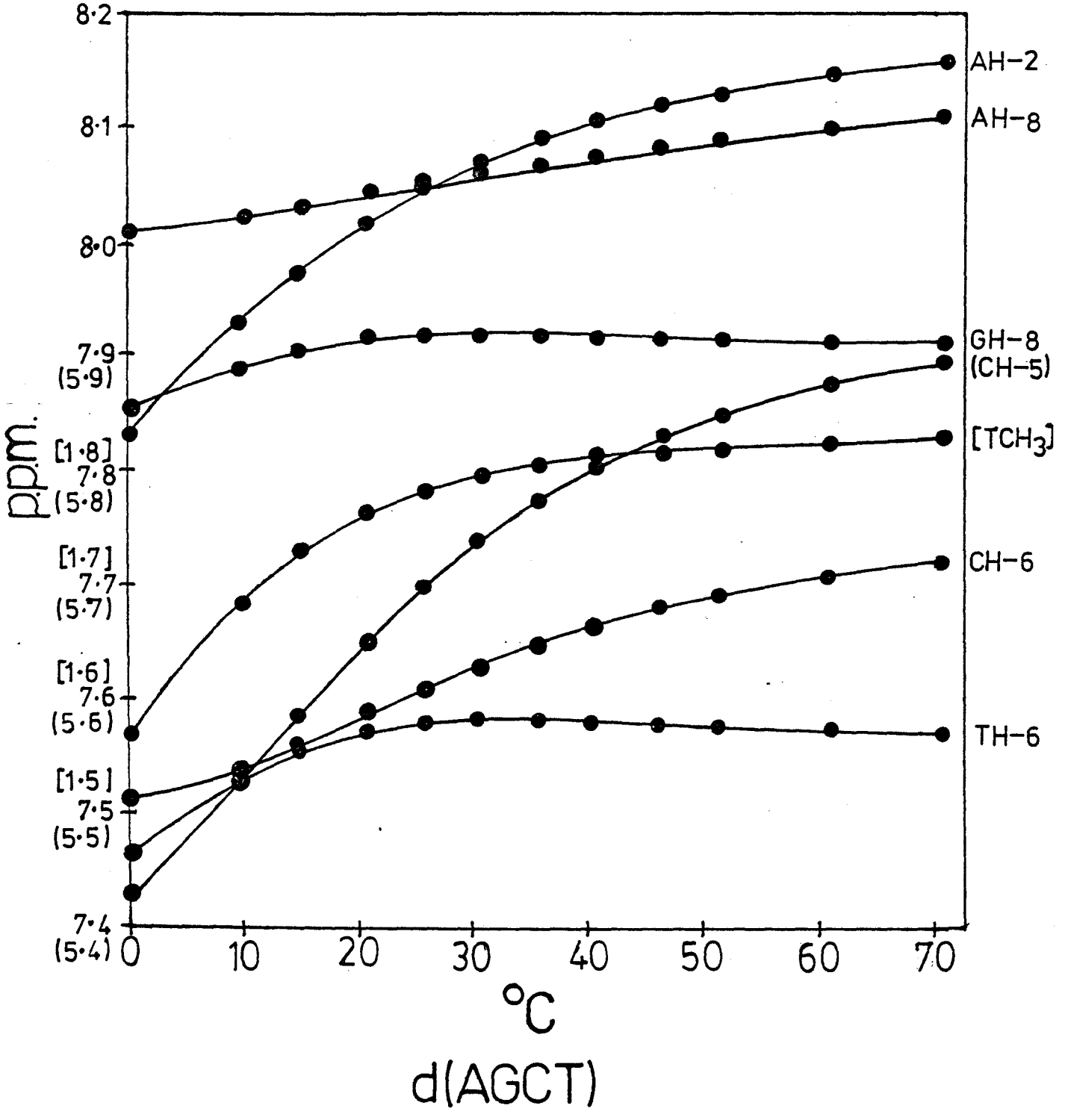


Figure 2.12 - Melting curves from the aromatic and methyl protons of d(AGCT) (4.8 mM).

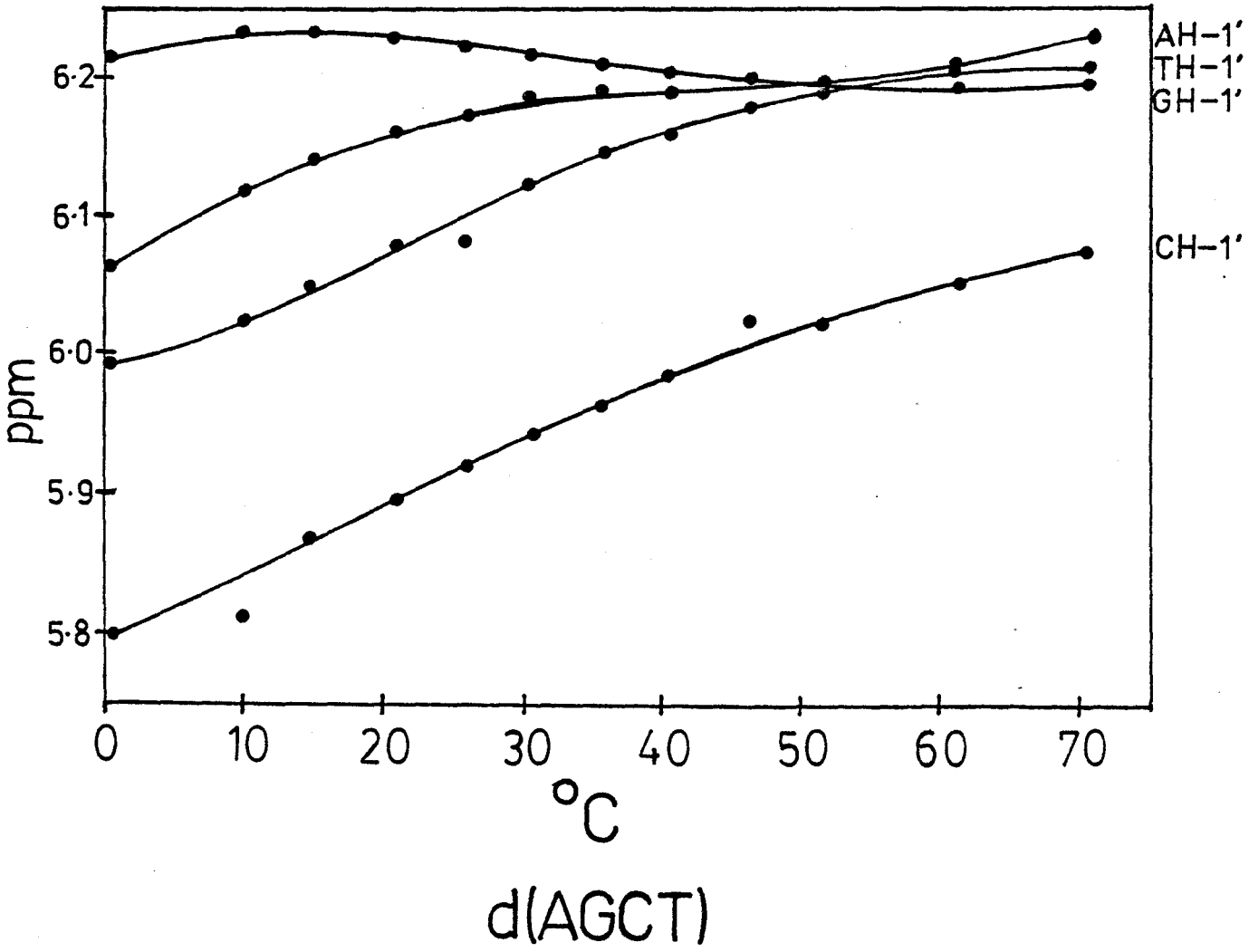


Figure 2.13 - Melting curves from the H1's of d(AGCT)

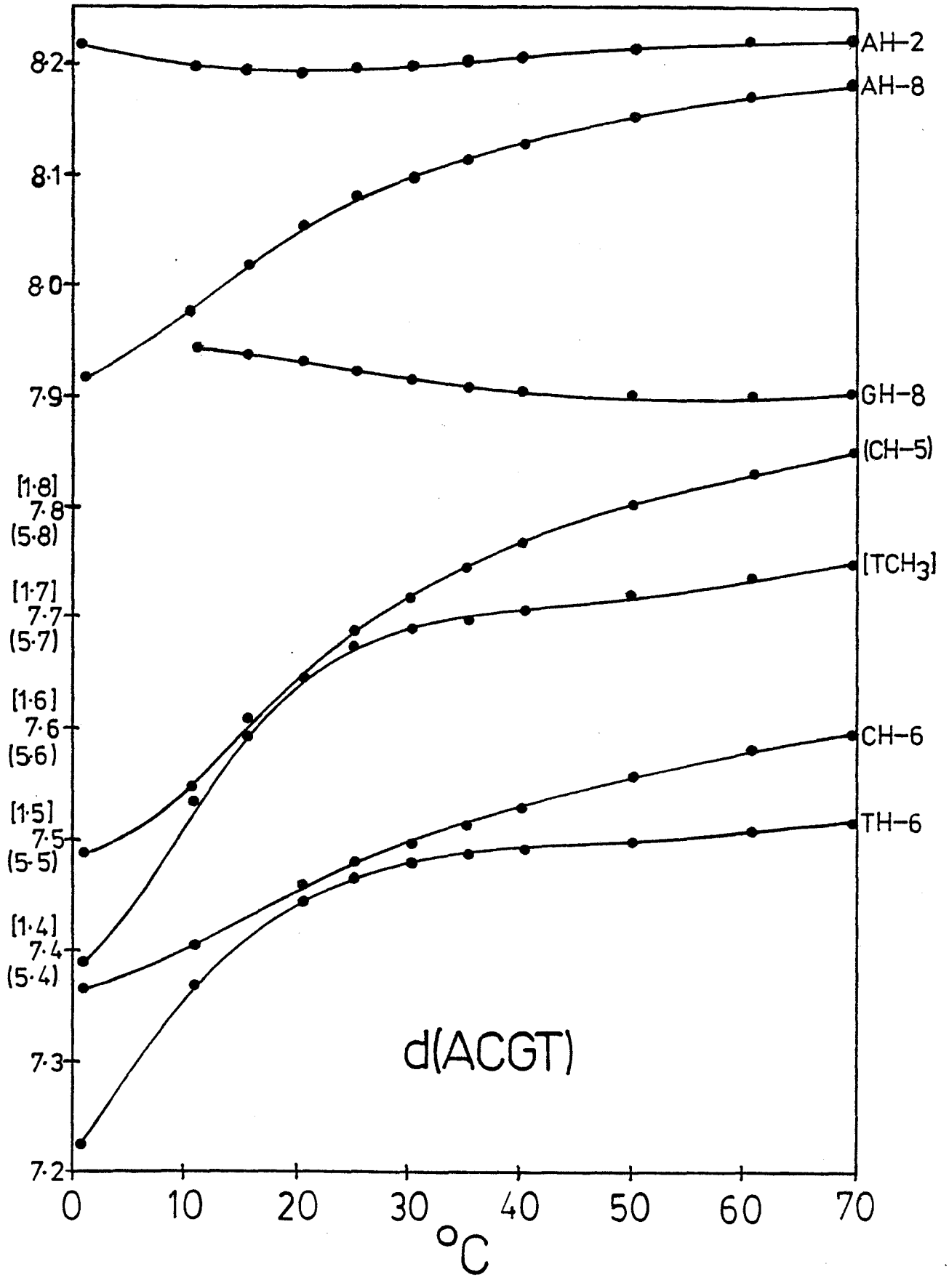


Figure 2.14 - Melting curves from the aromatic and methyl protons of d(ACGT) (2.8 mM).

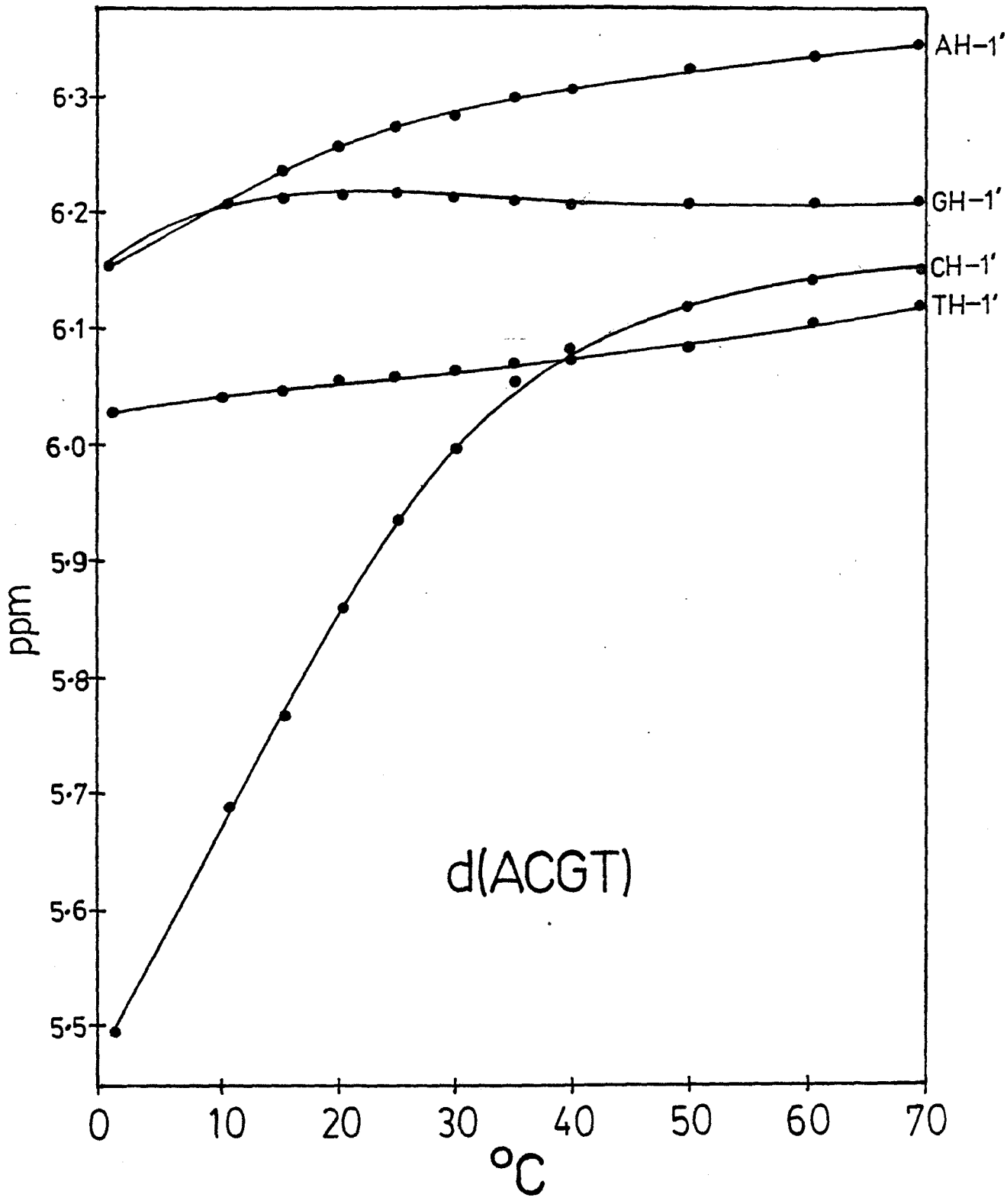


Figure 2.15 - Melting curves from the H1' protons of d(ACGT) (2.8 mM).

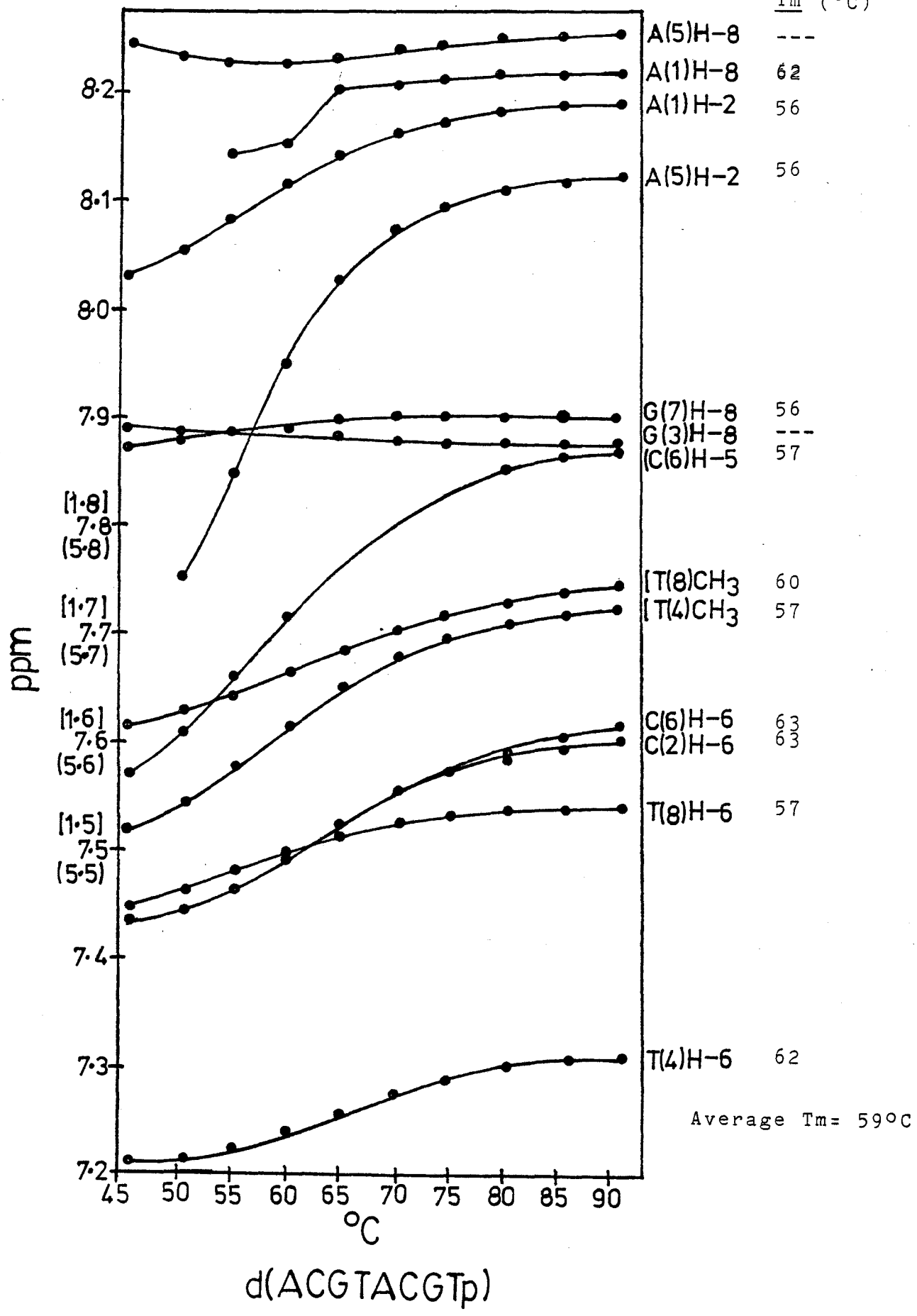


Figure 2.16 - Melting curves for d(ACGTACGTp) (1.1 mM),

The following table summarizes the average Tms for thes sequences:

d(GCA) (56)	3.5 mM	0 °C
d(AGCT) (52)	4.8 mM	0-10 °C
d(ACGT) (55)	2.8 mM	0-10 °C
d(ACGTACGTp) (59)	1.1 mM	59 °C

2.8.0 Optical Studies

Circular dichroism studies were carried out on a Cary 61 spectrometer. The CD spectra for d(ACGTACGTp) (86 uM) are presented in figure 2.17. The spectra were virtually identical in both 1M and 5M NaCl demonstrating that a B to Z transition does not occur at 5M salt. This spectrum is that of a typical B helix (Quadrigfoglio et al, 1981). The decreased response at 40 °C relative to 0 °C indicates that the deoxy oligomer is duplexed at 0 °C. An illustration of the type of response expected for a B to Z transition was given previously in figure 1.16.

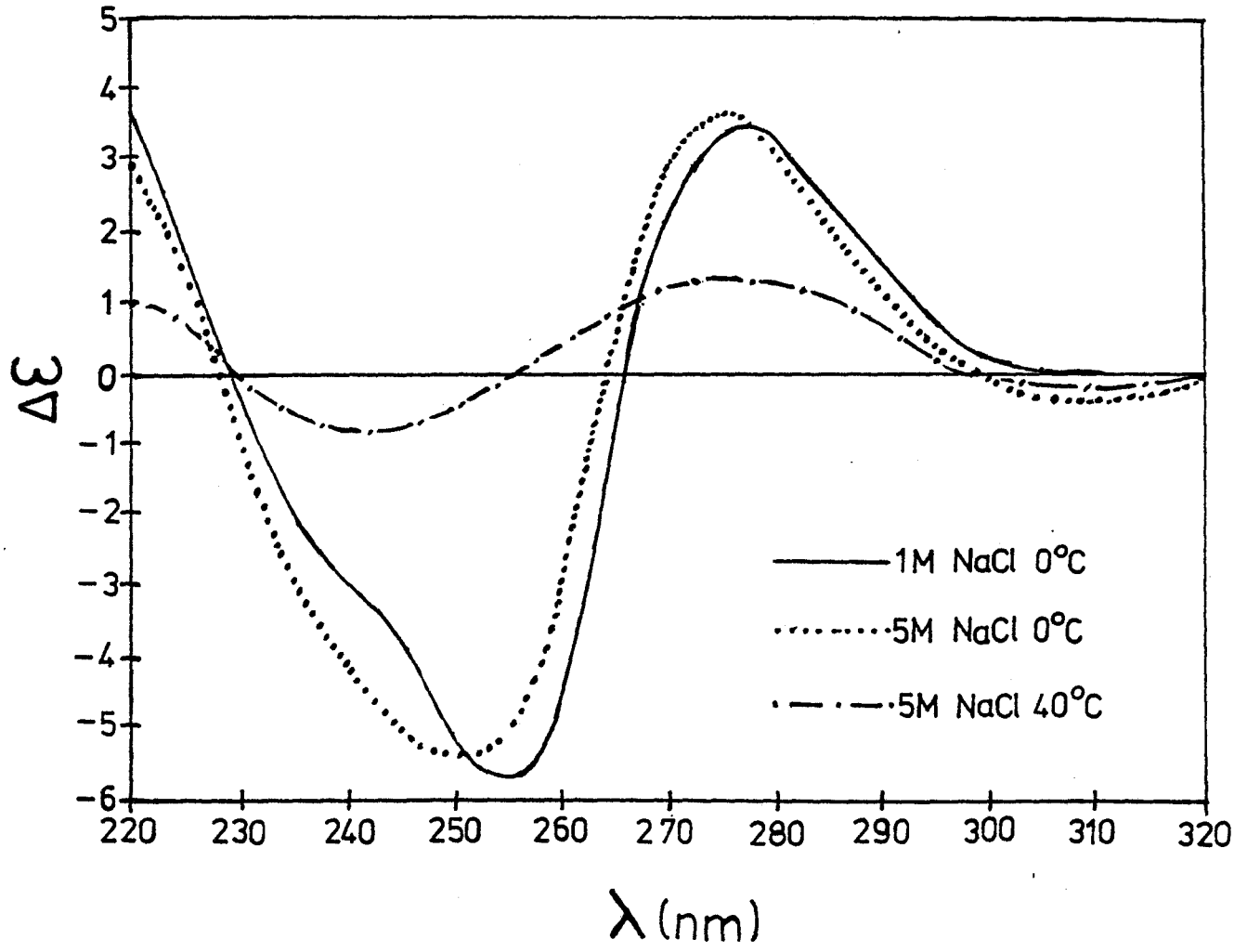


Figure 2.17 - Circular dichroism spectra of d(ACGTACGTp) at 0°C in 1M NaCl; 5M NaCl and at 40°C.

3 Discussion

3.1 Synthesis of DNA oligomers

The synthesis presented in this work was based on the synthesis of RNA oligomers developed in Neilson's laboratory. This synthesis also parallels that of Catlin and Cramer (1973) but differs significantly in a number of points:

1) The isobutyryl blocking group and benzoyl group were used for the N-protection of dG and dC respectively, not acetyl and anisoyl.

2) MST was employed as the condensing agent, not TPS.

3) The cyanoethyl group used to protect the phosphate group, was removed with freshly distilled TEA, not 0.1M NaOH in aqueous pyridine.

4) PTSA in CH_3OH/CH_2Cl_2 (1:2) was used to remove the MMTr group used to protect the 5'-OH, not 1% trifluoroacetic acid in CH_2Cl_2.

5) The three stage deblocking procedure used involved removing the trichloroethyl group first, followed by the base labile groups (Bz, Ib, and CE) and finally, the acid labile group (MMTr) last in aqueous acidic conditions. It was hoped that this strategy would minimize depurination which can occur during acid treatment when the purines are N-protected. Catlin's deblocking procedure involved 4 stages. The acid labile MMTr group was removed first, followed by the cyanoethyl group, then the trichloroethyl group and finally

the base labile groups.

These improvements resulted in a superior procedure for the synthesis of deoxy oligomers. This was verified by direct comparison of a number of obtained yields for the synthesis of a particular compound.

The use of MST rather than TPS resulted in considerable improvement in coupling yields especially in cases where dG is present. This is due to elimination of the side reactions such as the acylation of the O^6 of dG which results from the high reactivity of TPS. On comparison of the yields for a number of deoxy dimers, it is observed that Catlin and Cramer (1973) achieved an average yield of 41% with a yield of 7% for fully protected d(GGp) and 40% for fully protected d(GTp). The average yield presented in this work is 71% with a yield of 84% for fully protected d(GTp). Catlin synthesized two tetramers, neither of which contained a G residue. On the other hand the procedure used in this work was successful in synthesizing an octamer (fully protected d(ACGTACGTp)) which contained two G residues and still resulted in a yield of 55%. This demonstrates considerable improvement in the synthesis of fully protected DNA oligomers.

During the selective deprotection of the 5' hydroxyl under acidic conditions, depurination may occur as a side reaction. The yields obtained by Catlin and Cramer (1973), from the selective 5' deprotection of deoxy dimers using

triflouroacetic acid demonstrates this problem. Catlin achieved an average yield of 58% for the de-tritylation of 11 deoxy dimers. Depurination is apparent from yields of 13% and 32% for blocked d(ACp) and d(GTp), respectively. The present work suggests that the use of PTSA in organic solvents constitutes a significant improvement in the detritylation procedure. Using this approach, average yields of 76% was achieved for 9 dimers (see Table 2.5) with the lowest yield being 68% for MMTr-$G^{Ib}T\underline{p}CE$. A yield of 69% was achieved for the tetramer MMTr-$A^{Bz}C^{Bz}G^{Ib}T\underline{p}CE$ which contains two purines, one a 5'-adenosine.

Significant improvement was also achieved in deblocking the fully protected oligomers. Catlin obtained an average overall yield of 10%. An overall average yield of 22% was obtained in this work using the strategy of removing the acid labile MMTr group last by means of aqueous acidic conditions described in section 2.5.1. Although these yields were low, they were sufficient for the purposes of NMR investigation.

Two deblockings were carried out after first removing the MMTr group with PTSA in anhydrous conditions, followed by the trichloroethyl group and finally the base labile acyl groups (see section 2.5.2). This resulted in an overall yield of 70% for d(CT) and 51% for d(GTp) which is considerably better. The deblocking procedure will be discussed in more detail later in this section.

The comparisons between the phosphotriester synthesis described by Catlin and Cramer (1973) and the synthesis described in this thesis demonstrates that the modifications incorporated resulted in an overall improvement in most aspects of the phosphotriester synthesis.

3.1.1 Blocking of the amino function on heterobases

Originally, attempts were made using the acetyl and benzoyl blocking groups for the protection of the amino function on dG. The acetyl group proved too unstable and the N-acetyl product was never isolated. The benzoyl derivative also proved unsuccessful, yielding undesireable di-benzoyl products. Subsequent attempts using the isobutyrl blocking group were successful with good yield (80%). This group offered good stability and the final product could be easily monitored by tlc (see section 2.2.1.2).

To date, the most commonly employed method for synthesizing N^4-benzoyl dC, involves two steps: reaction with benzoyl chloride and subsequent removal of the benzoyl function from the 5' and 3' hydroxyls by base hydrolysis (Narang et. al., 1980). The synthesis described in this thesis employed the method of Otter and Fox (1973). This one step procedure involves refluxing dC in the presence of benzoic anhydride in ethanol. This procedure was highly successful with excellent yield (92%) (see section 2.2.1.3).

3.1.2 Synthesis of Blocked Nucleotides

Tables 2.1-2.6 lists the yields for the reactions carried out in the synthesis of d(GCA), d(AGCT), d(ACGT) and d(ACGTACGTp). In general, the yields are very good. The most popular 5' protecting group in use today is the di-p-methoxytrityl (DMTr) group which is less stable than the MMTr group to acidic cleavage (see section 1.1.2). The synthesis of fully protected d(AGp) was carried out using the DMTr group in the hope that milder acid conditions could be employed for its removal during the course of the synthesis and deblocking procedure. Problems arose, however, in that the group was so acid sensitive that it was cleaved during purification on the mildly acidic silica gel column. This lowered the yield and introduced impurities that were difficult to remove. Also, there was no improvement in the deblocking yields for compounds with 5' DMTr protection (see Table 2.7). The MMTr group proved more successful and afforded very good yields.

During the course of tetramer synthesis, yields were lower for block couplings using dimers with the cyanoethyl group selectively removed. This may have been due to impurities and/or moisture in the triethylamine used to remove this group. This problem was overcome by incorporating two changes to the procedure. Freshly distilled triethylamine was used rather than reagent grade. Pyridine was distilled *in vacuo* to remove any particulate

impurites, immediately prior to use. Subsequent improvement in yield was demonstrated by the synthesis of the tetramer d(ACGT) from the two corresponding dimers, with a 48% yield compared to that of 76% for d(ACGTp) (see figures 2.6 and 2.7 and Table 2.6). Improvement was also noted in the coupling efficiency. The coupling was essentially complete after three days, as compared to the four to ten days previously required for coupling reactions.

As stated earlier, the deblocking yields were quite low except for the dimers which were de-tritylated prior to deblocking. Deblocking the octamer proved to be difficult. The original strategy of removing the acid labile group last, (strategy 1 in section 2.5.1), failed to isolate any free octamer. An attempt was made to detritylate the octamer first, followed by the next two deblocking stages without prior purification of the detritylated intermediate (see section 2.5.3). The resulting product was purified by paper chromatography (see section 2.1); however, NMR analysis indicated the presence of two distinct octamers. Subsequent HPLC analysis confirmed the presence of a mixture of products (see figure 2.9). Fortunately, sufficient completely deblocked octamer was obtained through further purification by HPLC for the purpose of variable temperature ^{1}H-NMR and CD analysis.

In order to resolve this deblocking problem in the hope of improving the yields, 10 mg of fully protected

octamer was deprotected as described in section 2.5.1, up to the point of the final aqueous acid treatment and the product d(MMTr-ACGTACGTp) analyzed by HPLC. All HPLC analyses were carried out using gradient 9 and the same conditions described in section 2.5.3 and Appendix A. Analysis confirmed the presence of a multitude of products. It was concluded that the products consisted of octamers with one or more trichloroethyl groups still present. The procedure was repeated with another 10 mg of fully protected octamer. The compound was treated with Zn/Cu couple for 8 hours rather than 2 to 4 hrs. HPLC analysis showed one major product which was thought to represent 5'-MMTr octamer. The material was purified by HPLC and placed in water, pH 3 for the final removal of the 5' MMTr group. After 5 days, there was no reaction and the pH was lowered to 2.5. After 2 days HPLC anaylsis indicated that the material was breaking down without formation of any free octamer (expected retention time of 19.6 minutes). The sensitivity of these compounds to aqueous acid may account for the low deblocking yields obtained for the shorter oligomers. This possiblity is further substantiated by the good yield obtained from dimers where the 5' MMTr group was removed under anhydrous conditions prior to deblocking with Zn/Cu and methanolic ammonia. This may represent the best deblocking approach.

Despite some problems with the deblocking procedure, DNA oligomers (including the octamer) were successfully

synthesized using the procedures outlined in this thesis, and in quantities suitable for NMR analysis. Since the sequences in this study incorporated all four bases associated with DNA, this synthetic method can be used generally to build any desired sequence of eight bases or less in sufficient quantities for physical studies.

3.2 ^{1}H-NMR Analysis

3.2.1 Peak Assignments

Peak assignments were determined by method of incremental analysis (Borer et al., 1975). This method is particularly helpful for longer sequences where a nucleotide resonance is likely to be repeated. The chemical shifts of the proton signals in the oligonucleotides at high temperature closely resemble those of the mononucleotides.

Peak assignments for the dimers, trimer and tetramers were straightforward with the exception of d(AGCT). One normally observes the AH-8 resonance at a lower field than AH-2. The T_1 relaxation time of AH-2 is longer than that of AH-8 (Ts'o et al., 1973). This is due to the interaction of A-H8 with the deoxy ribose ring nuclei. Examination of the relaxation times of the two low field peaks demonstrated that the lowest field peak was actually the AH-2 peak with the AH-8 peak slightly upfield.

The NMR analysis of d(pAGCT) by Young and Kallenback (1980) reported the reverse, ie. the AH-8 proton is the

lowest field proton with the AH-2 proton slightly upfield. This demonstrates the paramagnetic shielding effect of the 5' phosphate on the AH-8 proton when the nucleoside is in the anti conformation. This phosphate has no effect on the AH-2 since the chemical shift for this proton is practically equivalent for d(pAGCT) and d(AGCT) at 60°C. This demonstrates the shielding effect of the 3' G residue flanking the A residue in d(AGCT) and the deshielding effect obtained from the 5' phosphates.

The H1's of the tetramers were assigned using parameters derived from 500 MHz ^{1}H-NMR studies of similar sequences (Tran-Dinh et al., 1983).

The shielding effects on a particular proton are dependent on the nearest neighbor and next to nearest neighbor residues. The greatest shielding is observed for adjacent purine residues (A)G). Less shielding is observed from adjacent pyrimidines (C)T) (Geissner-Prettre et al, 1976; Lee et al, 1976 and Ezra et al., 1977). These principles were applied in assigning the non-exchangeable proton peaks in d(ACGTACGT).

By studying this sequence, it can be seen that the first two residues (A1 and C2) and the last two residues (G7 and T8) would have resonances very similar to the tetramer d(ACGT) and are assigned accordingly. The internal four bases would experience internal neighbor and nearest neighbor effects which result in the upfield shifting of their proton

resonances, thus the higher field resonances were assigned to them. Note however that the internal A5 residue has a 5' phosphate that deshields the H-8 proton and is therefore assigned as the lowest field proton.

Due to considerable peak overlap in the H1' region, the H1' and C(2)H-5 peaks could not be assigned.

3.2.2 Variable Temperature NMR Analysis

The melting curves for d(GCA), d(AGCT), d(ACGT) and d(ACGTACGTp) are presented in figures 2.6 to 2.9. Analysis of these curves demonstrates that only the octamer has a definitive average Tm (59°C). Based on the trends observed from the curves for the two tetramers, it appears that their Tms lie between 0-10°C. The trimer d(GCA) may have a Tm around 0°C. Young and Kallenbach (1980) do not state a Tm for d(pAGCT) but do state that "most protons underwent a transition in the range of 10-30°C." On studying the melting profile provided, it appears that d(pAGCT) may have a Tm of about 18°C (9 mM, no buffer). Future model studies on DNA duplex stability by means of VT-NMR will require sequences of at least 5 to 6 bases in length in order to achieve Tms in the temperature range of 30-50°C.

It is interesting to compare these Tms with those of their RNA counterparts:

r(AGCU) 34° (Neilson et al., 1980)

r(ACGU) 29° (Neilson et al., 1980)

r(GCA) 34° (Alkema et al., 1981)

It is quite evident from these data that the RNA duplex has greater stability than the DNA duplex of the same sequence.

This trend in stability was predicted by Gray's equations (1981, see section 1.2.3). Using these relationships, $r(GC)_{10}$ would have a predicted Tm of 112° and $d(GC)_{10}$ a Tm of 89°, a difference of 20% in stability.

The data just presented may be a reflection of the relative stabilities of the A and B helix. All of the DNA duplexes would have adopted the B-helical form under the conditions of the experiments (Wang, 1979; Dickerson et al., 1982, 1983). The RNA duplexes would have adopted the A-helical form.

It has been shown that propeller twist enhances stacking in a duplex (Levitt, 1978; Dickerson et al., 1982; 1983) and that stacking may be the most important factor in stabilizing nucleic acid duplexes (Altona, 1982). It may be more accurate to state that propeller twisting results from the duplex obtaining a maximum stacking conformation. Calladine (1982) has shown that propeller twisting in B-DNA gives rise to cross-chain purine repulsions. These clashes, resulting from the steric hindrance of cross chain purine

residues, decrease the amount of propeller twist possible in B-DNA. On the other hand, these cross-chain repulsions do not occur in A-DNA (Calladine, 1982). Although a number of possible mechanisms have been proposed to limit these cross-chain clashes (Calladine, 1982), recent modelling studies suggest that these clashes can be minimized by increasing the base role angle (Dickerson, 1983). This is illustrated in figure 3.1.

Putting this all in perspective, hydration of the minor groove of DNA promotes the stabilization of the B-helix. However, the resulting cross-chain purine interactions generate clashes; and in the process of minimizing these clashes, the ability of the bases to achieve maximum base stacking is reduced. This does not occur in A-DNA and as a result the A helix may represent a helix which is maximally stacked and duplex stabilized. This is corroborated by the fact that the residues in an A-helix are packed closer together.

Since the presence of the 2' hydroxyl prevents the formation of a B-helical RNA, the RNA duplex maintains the A form and may explain why the RNA duplex is more stable than that of DNA. The 2' hydroxyl also "sticks out" into the backbone of the helix and as a result, may be involved with hydrogen bonding to water molecules and the phosphate backbone. This increased "hydration" may also contribute to the stabilization of the RNA duplex.

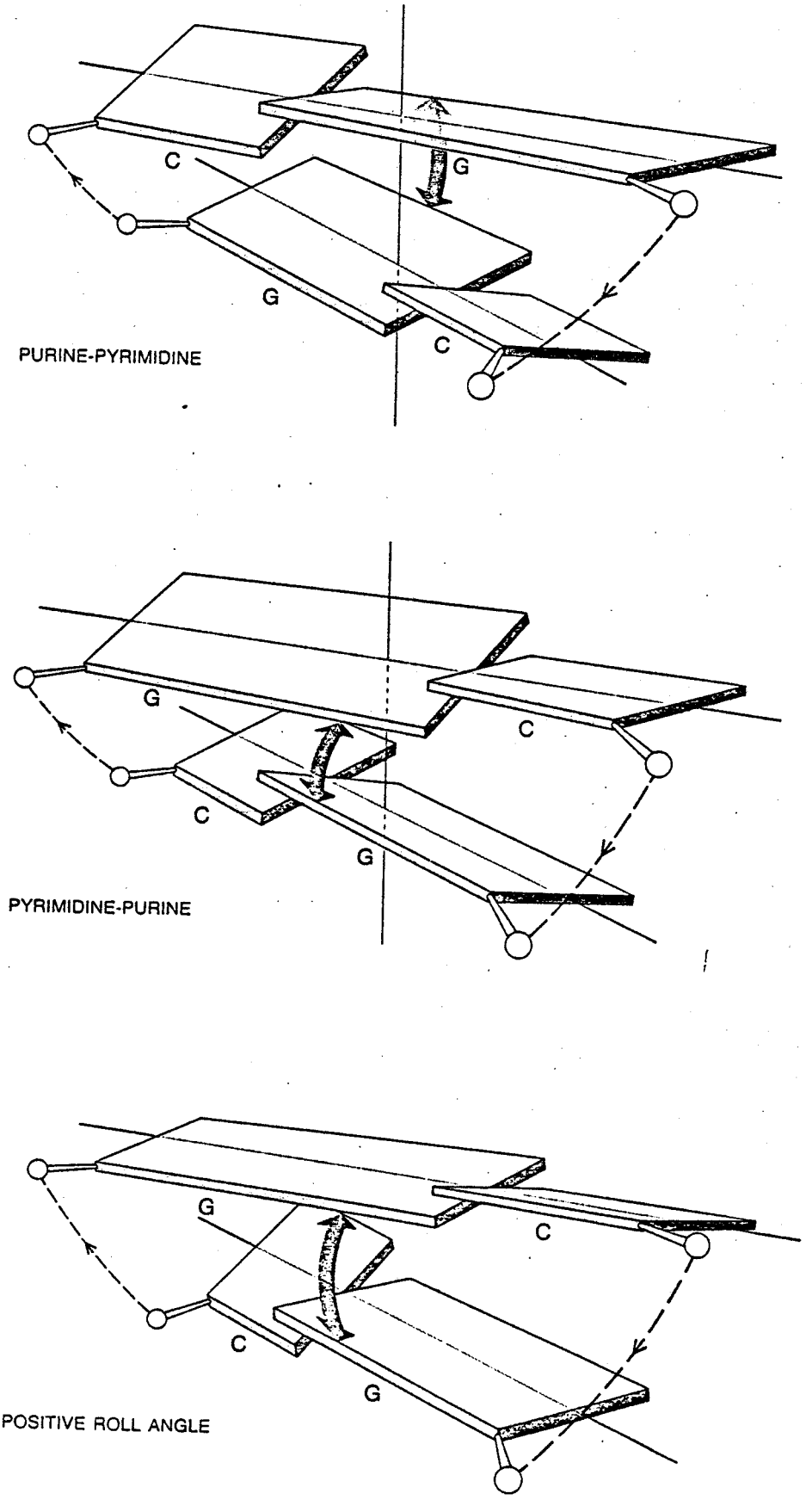


Figure 3.1 - Cross-chain purine clashes resulting from propeller twist and compensation by increasing base roll. (Dickerson, 1983)

3.3 The CD study of d(ACGTACGTp)

Since Z-DNA was discovered by Wang et al. in 1979, considerable work has been carried out to determine the sequence dependency and factors required for the B to Z transition. To date, only the following sequences have been found to adopt the Z-form in solution:

poly d(GC) (Pohl and Jovin, 1972)

$d(CG)_3$ (Quadrigfoglio et al, 1981)

$d[T(2\text{-amino A})]_3$ (Gaffney et al., 1982)

poly d(AC).poly d(GT) (Jovin et al., 1983, only in negatively supercoiled closed circular DNA).

The following sequences do not undergo B to Z transition in solution:

poly d(AT) (Dickerson, 1982; Jovin et al., 1983)

poly d(IC) (Dickerson, 1982)

$d(GC)_3$ (Albergo et al., 1981; Marky et al., 1982)

$d(CG)_2$ (Quadrigfoglio et al., 1981)

$d(AT)_3$ (Quadrigfoglio et al., 1981)

$d(AT)_3d(CG)_3$ (Quadrigfoglio et al., 1981)

d(CGTACG) (Kuzmich, 1982)

It is apparent that alternating purine/pyrimidine residues does not constitute a general requirement for B to Z transition. The above sequences demonstrate that AT residues interfere with the transition process.

There has been some question concerning the length requirement for sequences to undergo the B to Z transition.

Rich (1983) has proposed that eight base pairs should be sufficient. Bearing this in mind, it was interesting to see if d(ACGTACGTp) would undergo transition in high salt conditions. This octamer is simply the above hexamer d(CGTACG) with an extra A.T base pair at each end.

CD has been used to investigate the B to Z transition in all of the above sequences and particularly the low salt (1M NaCl) to high salt (5M NaCl) right handed to left handed transition. The CD analysis is presented in figure 2.10. The results indicate that the sequence remains in the right-handed B-helical form with no transition to the Z-form in high salt.

After consideration of the above sequences which do not undergo B to Z transition, the finding that d(ACGTACGTp) does not "flip" is not surprising. One reason may be that the presence of A.T base pairs maintains the stability of the B form due to hydration of the minor groove.

Calladine (1982) has proposed that cross-chain purine repulsions may be involved in the B to Z process since maximum clash occurs with the sequence $d(CG)_n$. This is further demonstrated by the fact that poly [T(2-amino A)] does undergo transition. The analog 2-amino A mimics G and the amino group is intimately involved in the cross-chain purine clashes. The amino group may also intrude into the minor groove interrupting the spine of hydration responsible for stabilizing the B conformation, as the amino group of dG

does in d(CGCGAATTCGCG) (Dickerson, 1983).

It appears that much more experimentation will be required before the mechanism and requirements of B to Z transition can be fully understood.

4 Conclusions

The phosphotriester synthesis described in this work resulted in the successful synthesis of deoxyoligomers of up to eight bases in length and in sufficient quantities for NMR investigations. This synthesis represents an improvement in many aspects of the phosphotriester synthesis developed by Catlin and Cramer (1973). The synthesis could probably be used to synthesize any desired DNA sequence up to eight bases in length.

Short RNA duplexes are more stable than short DNA duplexes of similar sequence. The difference in stability is quite pronounced over a temperature range of 30°C. DNA duplex sequences of four base pairs or less containing two G.C base pairs do not display a Tm of greater than 10°C, if any at all, in the concentration range of 2 to 5 mM. As a result, future model studies on DNA duplex stability using VT-NMR will require sequences of at least five to six bases in order to observe Tms in the range of 30°-50°C for stability comparisons.

The octamer duplex $d(ACGTACGTp)_2$ does not flip into the Z-conformer in high salt (5 M NaCl) conditions, but instead, the duplex remains in a right handed B-helical form, consistent with other studies of DNA oligomers containing A.T base pairs.

Appendix A - "WISP" Report for figure 2.9.


```
---------- SYSTEM CONTROLLER CONDITIONS ---------
OPERATION NO. 05
METHOD: 11                          OPERATOR:
PUMP SET: 11                        DETECTOR 1:  ST85ATO
COLUMN: GRAD AN                     DETECTOR 2:  50A

SAMPLE NAME: OCTAMER SAMPLE
CONC UNITS: 10 ULS

---------- PUMP CONTROLLER CONDITIONS ---------

   STANDBY CONDITIONS FOR RUN SET  11

   INITIAL CONDITIONS/GRADIENT TABLE
      TIME      FLOW        %A          %B          %C          CURVE
      INITIAL   1.00        85          15          0           *
      20.00     1.00        50          50          0           09
      30.00     1.00        50          50          0           01
      35.00     1.00        30          70          0           06
      40.00     1.00        30          70          0           06
      45.00     1.00        85          15          0           06
      50.00     1.00        85          15          0           06


   EXTERNAL EVENTS
      TIME        NO.       STATUS


SAMPLE POSITION              05      SINGLE MODE        WISP REPORT

INJECTION VOLUME           0010
NUMBER OF INJECTIONS          1      INJECTIONS REMAINING          0
RUN TIME                   00:30     EQUILIBRATION DELAY       00:20
NON-DEFAULT SYS MSG'S: 6500-0030 PSI,8701
WISP CODES GENERATED:

 NOV. 28, 1983   18:31:34     CHART  0.25 CM/MIN
                              RUN #35                      CALC #0
 COLUMN                       SOLVENT                 OPR ID:      S

 EXTERNAL STANDARD QUANTITATION
 SAM AMT       0.10000    IS AMT       0.10000    SF        0.01000


  PEAK#          AMOUNT      RT      EXP RT      AREA              RF
               151.07700     19.60            15107860 L    0.000000E0
                14.51040     20.74             1451050 L    0.000000E0
                67.10120     21.25             6710131 F    0.000000E0
                43.47670     21.60             4347693 F    0.000000E0
                43.76840     22.06             4376868 L    0.000000E0
                 7.67925     22.73              767925 L    0.000000E0
 TOTAL         327.61200
```

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