O^6-ALKYL GUANOSINE RESIDUES IN RNA DUPLEXES

EFFECTS OF O^6-ALKYL GUANOSINE RESIDUES ON RNA DUPLEX STABILITY

by

PATRICIA LOUISE D'ANDREA, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

May, 1984

MASTER OF SCIENCE (1984) McMASTER UNIVERSITY
(Biochemistry) Hamilton, Ontario

TITLE: Effects of O^6-Alkyl Guanosine Residues on RNA Duplex Stability

AUTHOR: Patricia Louise D'Andrea, B.Sc. (McMaster University)

SUPERVISOR: Dr. Thomas Neilson

NUMBER OF PAGES: xx, 178

ABSTRACT

Several short oligoribonucleotide sequences containing modified purine residues of biological significance were synthesized using the phosphotriester method developed in Neilson's laboratory. Variable temperature proton nuclear magnetic resonance (NMR) spectroscopy was used to examine the solution conformations of these oligomers. Studies of the effect of position, type and extent of alkyl modifications on helix structure and stability were undertaken.

The triribonucleotide GpCpA was the first trimer shown to form a stable RNA duplex (Tm 33°C) (Alkema, et al, 1981(a)). This duplex contained two G:C base pairs and two 3'-dangling adenosine residues, and had a stability equal to that of the tetramer duplex UpGpCpA, having four Watson Crick base pairs.

A series of GpCpN trimers was prepared (N= m^6A, $m^6{}_2A$, m^1G, m^6G, e^6G, m^2m^6G), using GpCpA as a reference, to determine how N- or O-alkylation of the dangling residue affected duplex stability. Both the studies of the N-alkylated (N=m^6A, $m^6{}_2A$, m^1G) (D'Andrea, et al, 1983) and O-alkylated (N=m^6G, e^6G, m^2m^6G) sequences (present work) led to the conclusion

that site and degree of modification were important factors for stability. Comparison of N- versus O-alkylated sequences revealed how the hydrophobic regions surrounding the alkylated nitrogen or alkylated oxygen atoms, and the spatial location of these regions, contributed to duplex stability.

Examination of the effect of modified guanosine residues within a short squence, was performed through studies on ApGpNpCpU pentamers (N=m^6G, e^6G, m^2m^6G), having N in internal non-base-pairing and in internal base-pairing positions. No duplex formation was seen, in contrast to studies involving reference compounds : ApGpGpCpU (a qualitative reference), ApGpGpCpU : ApGpUpCpU (Tm 31.4 C), and ApGpGpCpU : ApGpCpCpU (Tm 47.0 C). As no melting temperatures could be calculated for the modified strands, and because their NMR analyses were so similar, no comparisions regarding degree of destabilization, could be made amongst the various modified residues. Never the less, it is clear that O-alkylation of the central G residue significantly disrupts duplex formation, through generation of a centre of great instability. This result sharply contrasts that when the same modified residues are located in terminal, non-bonding positions.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. Thomas Neilson for his guidance and support, to Drs. Dirk Alkema and Donald Hughes for their valuable discussions and technical assistance, and to the members of my supervisory committee, Dr. R.A. Bell, Dr. G.E. Gerber, and Dr. A.B. Futcher, for their suggestions.

I wish to thank Mrs. Dale Tomlinson for help in typing many of the tables.

Special thanks to my future husband , Peter Birkwood, for his love and encouragement.

TABLE OF CONTENTS

Abbreviations

A^a	adenosine
Ac	acetyl
Bz	benzoyl
BzCl	benzoyl chloride
C^a	cytidine
ca	circa
CD	circular dichroism
Cu/Zn	copper-zinc couple
dG^a	2'-deoxyguanosine
D_2O	deuterium oxide
DBU	1,8-diazabicyclo (5.4.0)undec-7-ene
DHP	dihydropyran
DMAP	4,4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DSS	4,4-dimethyl-4-silapentane-1-sulfonate
E	ethyl
e^6G	O^6-ethylguanosine
G^a	guanosine
Hz	hertz (cycles/second)
IR	infra-red
J	coupling constant
Me	methyl
m^1G	N^1-methylguanosine

m^6A	N^6-methyladenosine
m_2^6A	N^6,N^6-dimethylguanosine
m^6G	O^6-methylguanosine
$m^2\,m^6G$	N^2,O^6-dimethylguanosine
mRNA	messenger RNA
MST	mesitylenesulfonyl 1,2,4-triazole
NMR	nuclear magnetic resonance
OD	A_{260}, absorbance units at 260 nm
p	phosphate
p	2,2,2-trichloroethylphosphate
ppm	parts per million
pTSOH	paratoluene sulfonic acid
R_f	ratio of the distance travelled by solute to that of solvent
RNA	ribonucleic acid
T^a	thymidine
TBAF	tetrabutylammonium fluoride
thp or t	tetrahydropyranyl
TIPS	1,1,3,3-tetraisopropyldisiloxane
TIPS-Cl	1,3-dichloro-1,1,3,3-tetrapropyldisiloxane
tlc	thin layer chromatography
Tm	melting temperature of a duplex, at which 50% of the strands exist in duplex form
TMS	tetramethylsilane
TPS-Cl	2,4,6-triisopropylbenzenesulfonyl chloride
TPS-NI	2,4,6-triisopropylbenzene sulfonic acid nitroimidazole

Trac	trityloxacetyl
tRNA	transfer RNA
U[a]	uridine
UV	ultraviolet

[a] Mononucleosides and oligoribonucleotides are abbreviated in the standard format (IUPAC-IUB Commision on Biochemical Nomenclature, 1970). pA represents 5'-adenylic acid, Gp represents 3'-guanylic acid, and ApG represents adenylic (3'-5')guanosine. Oligoribonucleotides are numbered sequentially from the 5' terminus.

LIST OF FIGURES

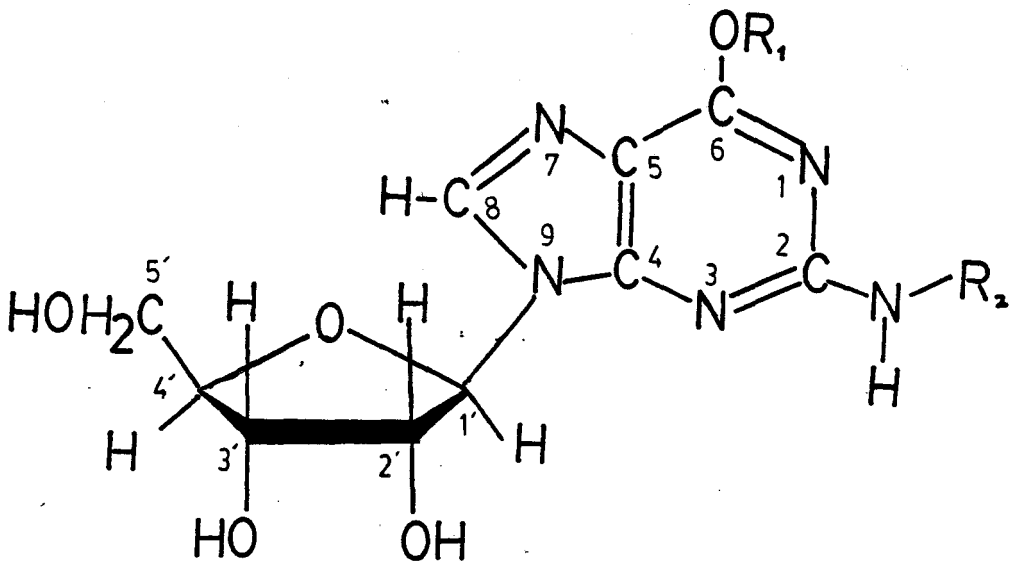
LIST OF TABLES

INTRODUCTION

The work described within this thesis, encompasses variable temperature proton NMR (^{1}H-NMR) studies of the effect of certain purine ribonucleotide heterobase modifications on base stacking and duplex stability of oligomers. Specific areas of interest deal with the insertion of various alkyl groups in the O^6 - position of Guanosine; examination of the stabilizing potential of N - and O- alkylated purines as 3'-danglers (ie. terminal, non-base-paired residues); and investigation of the net result of two opposing effects, in particular, diminished hydrogen bonding capacity versus enhanced hydrophobicity, and of conformational alterations in the modified base residues, when present in base-pairing situations within pentaribonucleotide dimers. Figure 1 illustrates the modified nucleosides studied in this work.

1.1 The Choice of O -Alkylated Guanosine Compounds for Study

The interest in synthesizing O^6-alkylated



O^6-METHYLGUANOSINE (m^6G) $R_1 = CH_3$, $R_2 = H$

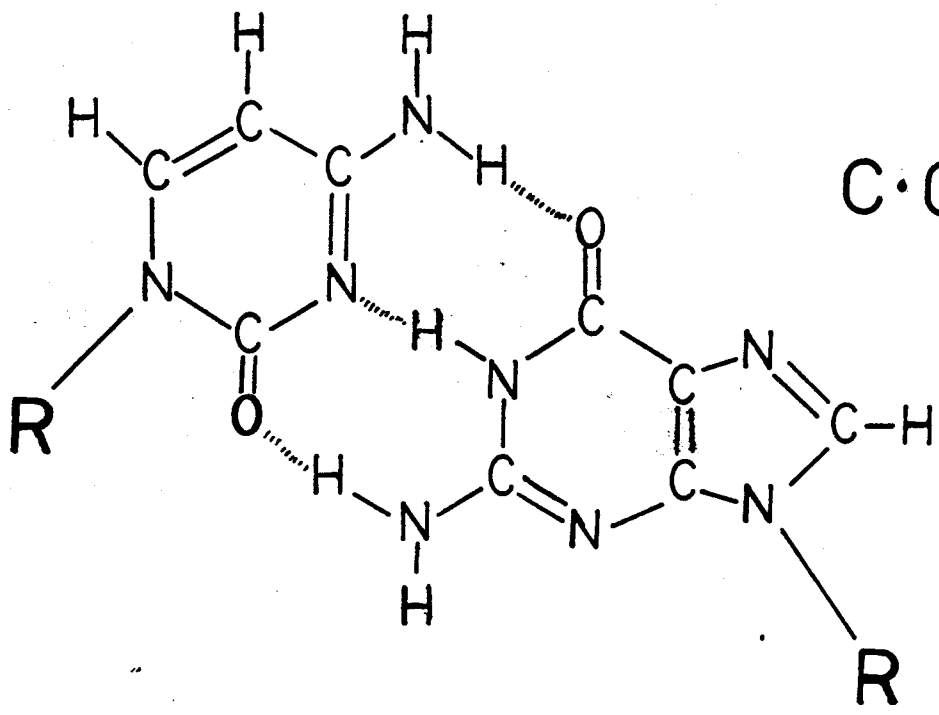
O^6-ETHYLGUANOSINE (e^6G) $R_1 = CH_2CH_3$, $R_2 = H$

N^2, O^6-DIMETHYLGUANOSINE (m^2m^6G) $R_1 = R_2 = CH_3$

Figure 1. O^6-Alkylated Guanosine Nucleosides of Interest

guanosine (G) nucleosides stems from the apparent effect of such residues in nature. The occurence of simple O^6-alkylated guanosine residues in target tissues treated with chemical carcinogens has been correlated with high incidences of tumor formation (Fowler, et al.,1982; Lutz,1982; Hora, et al., 1983; Singer,1979). The actions of simple alkylating agents are thought to be mediated by conversion within the cell to highly reactive alkyl cations which can attack nucleophilic sites in cellular macromolecules, especially DNA (Hora et al., 1983). Alkylation of the dG O^6-oxygen in DNA has been shown to result in both mutagenic and carcinogenic lesions (Medcalf & Lawley, 1981; Pegg,1982; Pettit,1980; Singer,1979; Kuzmich et al., 1983).

The molecular basis for the effect of the alkylation may reside in the ambiguous coding properties of O^6-alkyl dG (Fowler, et al., 1982; Karran & Marinus, 1982; Loveless, 1969; Gerchman & Ludlum, 1973). This modified nucleoside can base pair with thymidine (T) or cytidine (C) in a non-Watson-Crick fashion (see Figures 2 & 4); this may lead to miscoding during replication and ultimately to neoplastic transformation (Loveless,1969; Medcalf & Lawley,1981; Fowler et al., 1982; Bridges & Lehmann,1982). The inability of the E.coli mismatch correction system to alter the rate of



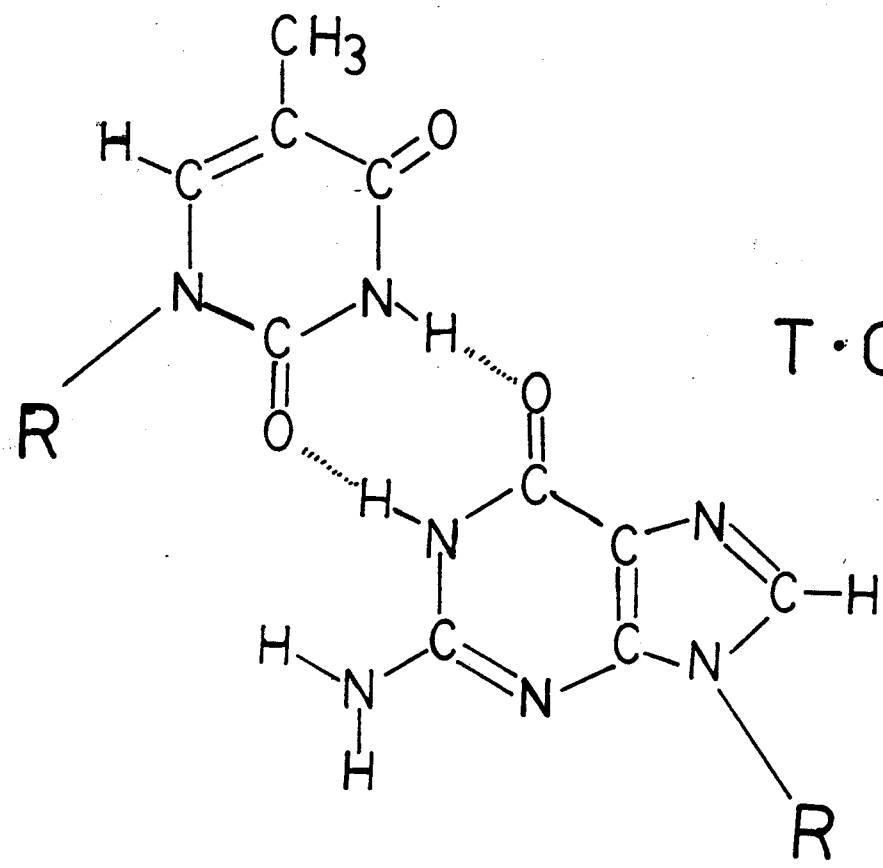
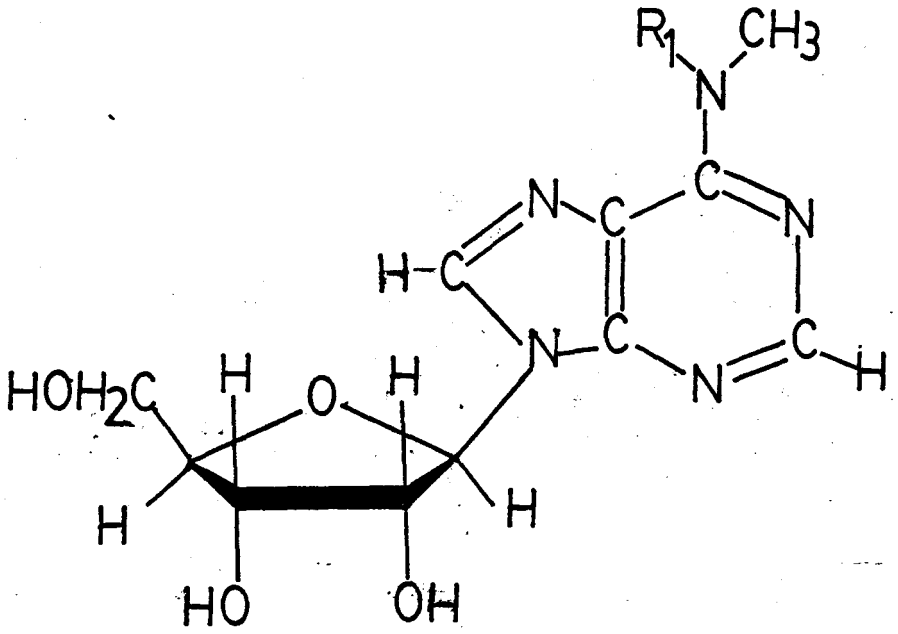
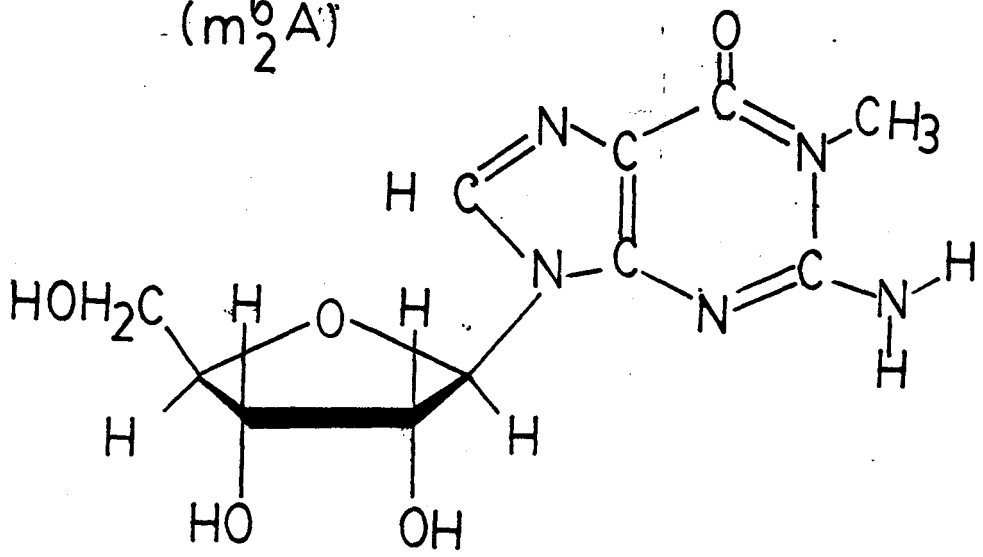


Figure 2. Representation of the C·G and T·G Base Pairs



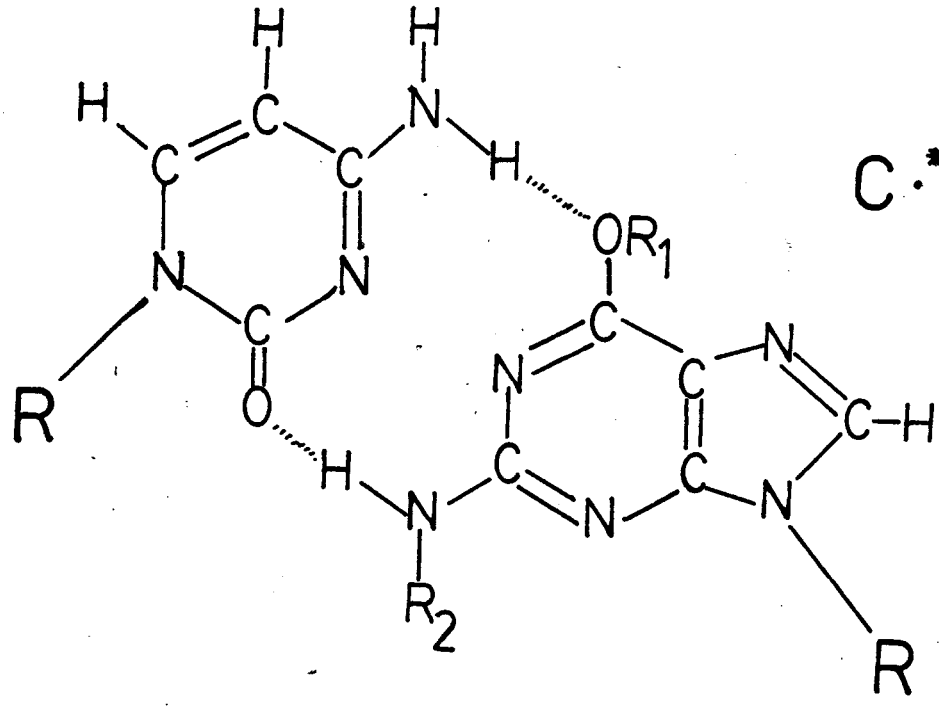
N^6-METHYLADENOSINE (m^6A) $R_1 = H$

N^6, N^6-DIMETHYLADENOSINE (m_2^6A) $R_1 = CH_3$



N^1-METHYLGUANOSINE (m^1G)

Figure 3. N-Alkylated Guanosine Nucleosides of Interest



m^6G $R_1=CH_3$, $R_2=H$

e^6G $R_1=CH_2CH_3$, $R_2=H$

m^2m^6G $R_1=R_2=CH_3$

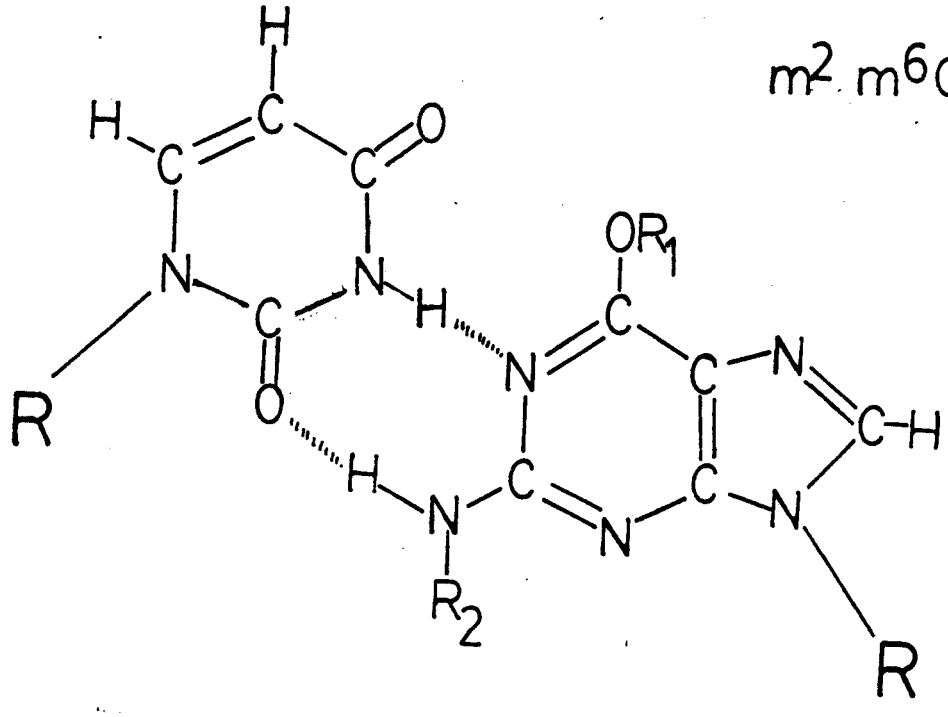


Figure 4. Representation of C·*G and U·*G Base Pairs

O^6-methyl dG -induced mutagenesis, suggest that no satisfactory base pair may exist for this modified residue (Karran & Marinus, 1982). Polymers containing O^6-alkyl G in both ribo- and deoxyribonucleotides have been shown to direct RNA Polymerase incorporation of U (or T) : A, a G → A transition (Kuzmich, et al.,1983). Both O^6-methyl G:C and O^6-methyl G:T base pairs have been shown to be less stable (by UV and CD studies) than their corresponding non-methylated base pairs (Kuzmich et al., 1983). In one instance, a low level of A incorporation, corresponding to a G → T transversion has been reported (Gerchman & Ludlum,1973). This type of mutation has recently been shown to be involved in the activation of a human bladder cancer oncogene (Tabin, et al., 1982; Reddy,et al., 1982).

Besides miscoding, the presence of a modified nucleoside will likely cause some perturbation in the local DNA helix. Other workers have in fact found that O^6-methyl dG decreases the thermal stability of the B DNA helix (Kuzmich, et el., 1983). Factors such as the extent of base stacking, tilt, twist, and sugar conformation may have implications for normal physiological processes such as enzyme recognition, and strand melting for DNA replication (Ehrlich & Wang, 1981; Bridges & Lehmann, 1982).

Certain species are capable of an adaptive

response upon challenge with simple alkylating agents, which results in de-O-alkylation of affected dG residues . The best characterized system is that of rat liver. A protein acting as a methyl-transferase, transfers the O^6-methyl or O^6-ethyl group to a protein-associated cysteine residue (Renard & Verly, 1980; Mehta, et al., 1981; Pegg & Perry, 1981; Hora, et al., 1983). The activity does not require any cofactors or divalent cations; the protein shows preference for double stranded DNA in the kinetics and efficiency of repair, and is inactivated after a single reaction.

A similar protein has been found in other non-target tissues such as E.coli and human fibroblasts (Olsson & Lindahl, 1980; Medcalf & Lawley, 1981; Bridges & Lehmann, 1982; Karran & Marinus, 1982; Pegg et al., 1982; Sedgewick & Lindahl, 1982; Waldstein, et al., 1982). Repairs are made prior to DNA replication, and thus prior to the production of heritable changes in the cell genome. Tissues such as gerbil, mouse, and hamster liver, which express very low levels of the methyl transferase, are subject to a relatively higher incidence of tumour formation than are the non-target tissues (Bogden, et al., 1981; Bamborschke, et al., 1983). In all cases, O^6-ethyl dG has been found to to be a more mutagenic lesion that O^6-methyl dG, due to the former's comparatively low

capacity to induce protein activity, along with its slow removal by the methyl transferase (Goth & Rajewsky, 1974; Mehta, et al., 1981; Bridges & Lehmann, 1982; Sedgewick & Lindahl, 1982; Heyting, et al., 1983; Muller & Rajewsky, 1983).

The predominance of tumours in particular cell systems reflects a non-uniform transformation risk among potential target cells (a function of their patterns of gene control and expression), at the time of and subsequent to exposure to a particular carcinogen. As well, the relative rates of dealkylation by methyltransferases, DNA replication, mismatch repair, and enzymatic methylation of daughter strands are important (Medcalf & Lawley, 1981; Bridges & Lehmann, 1982; Likhachev, et al., 1983; Muller & Rajewsky, 1983; Pereira, et al., 1983).

Although methylating and ethylating agents yield similar types of base lesions in DNA, it is unclear to what extent the cellular defense mechanisms are able to recognize both methyl and ethyl adducts. This is exemplified in the cases of E.coli, in which O^6-ethyl dG but not O^6-methyl dG can be removed by the uvr^+-dependent excision repair pathway (Sedgewick & Lindahl 1982), and human cells, in which only O^6-ethyl dG may be removed by the same pathway that is utilized in the excision of pyrimidine dimers (Sedgewick & Lindahl, 1982).

While O^6-ethyl dG and O^6-methyl dG are found *in vivo*, the third modified nucleoside of interest, N^2,O^6-dimethyl dG, has been previously isolated only in synthetic polynucleotides *in vitro* (Singer, 1979). Apparently, the exocyclic amino group is reactive only in *in vitro* experiments with simple alkylating agents. Thus, while biological implications may not be directly inferred from studies involving N^2,O^6-dimethyl dG, its biophysical properties are of interest.

The preparation of modified guanosines, rather than 2'-deoxy guanosines, stems from this worker's familiarity with the synthesis (phosphotriester method - England & Neilson, 1976; Werstiuk & Neilson, 1976; Gregoire & Neilson, 1978) and study (by ^{1}H-NMR spectroscopy - Romaniuk, *et al.*, 1978; Neilson, *et al.*, 1980) of oligoribonucleotide sequences, from the greater stability of synthetic RNA duplexes versus DNA duplexes, and from the conformational information readily obtained from the $J_{1',2'}$ coupling constants in the RNA sequences. Results obtained in the well-characterized RNA system should be of a similar nature to those in a DNA system. Thus, study of the relative stability of modified G : N base pairs (where N = U,C) may aid in the understanding of the biophysical effects of alkylation and give additional insights into the mechanisms of action of the

polymerase and mismatch correction systems.

1.2 The GCN System

The self-complementary trimer GCN in earlier studies, provided insight into the function of N-methylated purine nucleotides in native tRNA molecules (D'Andrea, 4th year thesis, 1982; D'Andrea, et al., 1983). Examination of native tRNA sequences, reveals frequent modification of bases immediately adjacent to neck and anticodon regions (McCloskey & Nishimura, 1977; Cedergren et al., 1981; Gauss & Sprinzl, 1981). In the latter, this phenomenon may serve to restrict Watson-Crick base pairing to the anticodon-codon base pairs, thus guarding against potential slippage, and as well, may enhance duplex stability thus making the base pairs involved less susceptible to strand melting. Both of these factors would be important in the prevention of misreading. Similar stabilizing properties would be useful at neck regions, as the loop-helix junction often promotes fraying (Borer, et al., 1975; Patel & Hilbers, 1975; Neilson, et al 1980; D'Andrea, 4th year thesis, 1982; D'Andrea, et al., 1983).

The contribution made by non-base-paired residues to overall duplex stability, has in the past been overlooked by many workers even though these

residues are frequently invariant, stacked, and form single helices which are extensions of one of the duplex strands (Alkema, et al., 1982). In fact, the considerable stabilizing potential of single dangling bases has been demonstrated in several studies (Martin, et al., 1971; Uhlenbeck, et al., 1971; Grosjean, et al., 1976; Neilson, et al., 1980; Alkema, et al., 1981(a), Alkema, et al., 1981(b)).

The ^{1}H-NMR study of GCN, where N = A, revealed through comparison of melting temperatures, that the 3'-dangling residue increases base stacking and duplex stability to the same extent as terminal A : U base pairs (Tm 33°C versus 34°C, respectively), (Alkema, et al., 1981(a); Alkema, et al., 1983). The extent of helical overlap experienced by the 3'-dangling base generates increased aromatic ring-current interaction within a strand, enhancing base stacking, which in turn, strengthens duplex formation. This is an example of a "nearest-neighbor" effect. Other important stabilizing factors may include : Watson-Crick hydrogen bonding, solvation, and entropic effects (Alkema, et al., 1983).

Similar NMR analysis of GCN duplexes in which N = $m^{6}A$, $m^{1}G$, $m^{6}{}_{2}A$ (see Figure 3), revealed that base stacking and duplex stability increase in the order, $A < m^{6}A < m^{1}G < m^{6}{}_{2}A$, thus implying a relation between stabilization, and site and degree of

modification (D'Andrea, 4th year thesis,1982; D'Andrea, et al., 1983). Studies carried out by other workers, of dimers containing A, m^6A, and $m^6{}_2A$, support the following conclusion : introduction of methyl substituents increase dimer melting temperatures, as a result of lowered entropy contributions to the total free energy of the molecule, and as well due to an increased tendency of these hydrophobic residues to promote intramolecular recognition (Olsthoorn, et al., 1980; Tazawa, et al., 1980).

Thus the GCN studies support the postulates that : 1. methylated bases next to duplex regions contribute to overall stability of tRNA molecules, and 2. methylated bases contiguous to the anticodon 3' terminus enhance mRNA - tRNA interactions.

The usefulness of the GCN system to study stacking and stability contributions of 3'-danglers, may be extended to examine O-alkylated purines, specifically, O^6-alkyl G residues. In this manner, it is possible to study how a hydrophobic alkyl group effects two atoms of different electronegativity (oxygen and nitrogen), and to examine the effect of increasing sizes (methyl versus ethyl) and numbers (methyl versus dimethyl) of these groups.

1.3 The AGNCU System

^{1}H-NMR studies of the pentaribonucleotides ApGpNpCpU (N = A, G, C, U) have been carried out by other workers (Alkema, et al., 1982). Of particular interest are the stable duplexes :

as these may serve as parent or reference systems for base stacking and duplex stability studies where N = O^6-methyl G, O^6-ethyl G, and N^2,O^6-dimethyl G. In this way, the effect of G modification on both Watson-Crick (G : C) and Wobble (G : U) base pairing stabilities may be examined.

G : U base pairs were first suggested by Crick (Crick, 1966) to explain the degeneracy of the genetic code. Wobble base pairs were shown to occur in native tRNA's by the discovery of H-bonded imino ring proton resonances and by Nuclear Overhauser Enhancement studies, and are presently an accepted feature of RNA secondary structure and of double helical regions of a number of tRNA's (Robillard, et al., 1976; Johnston & Redfield, 1978,1981; Reid, et al., 1979; Hurd & Reid, 1979: Alkema, et al., 1982).

G : U base pairs may be accomodated internally

within an RNA double helix without causing gross helical distortion, by a little shift the glycosyl torsion angles from those normally associated with Watson-Crick base pairing. The G : U base pair is often a centre of instability within a helix, as a result of this torsion angle shift and its corresponding change in normal duplex stacking interactions, and also because its internal position does not allow it to adopt its preferred conformation (Clarke,1977; Romaniuk, et al., 1979; Alkema, et al., 1982).

In the three alkylated purines examined in this study, the hydrogen bonding potential is diminished (see Figure 4). From the locations of the hydrogen bonds in the modified base pairs, it appears that the Wobble-type pairs (*G : U) may be more stable than the Watson-Crick-type pairs (*G : C). The presence of different sizes and numbers of hydrophobic alkyl pockets may influence the strength of the base pairs formed, and thus affect the overall duplex stability.

EXPERIMENTAL

2.1 Materials and Reagents

All reagents and solvents were commercial reagent grade and used without further purification except for the following : mesitylene sulphonyl chloride (Aldrich) was recrystallized from hot petroleum ether (30°- 60°C); pyridine, dimethyl formamide, and p-dioxane (Baker Analyzed reagents) were dried over Fisher 4Å molecular sieves prior to use.

Silica gel (40-140 mesh, Baker Analyzed) was dried at 80°C over NaOH pellets for one day prior to use in column chromatography. Columns were prepared in CH_2Cl_2 and eluted using a step gradient of CH_3OH in CH_2Cl_2.

Analtech prescored plates were used for tlc and were deveolped in a 5% or 10% CH_3OH/CH_2Cl_2 solution (v/v). The oligoribonucleotides were detected by spraying with 1% $H_4Ce(SO_4)_4$ in 10% sulphuric acid and heated at 300°- 400°C. Compounds generating a trityl cation appeared as yellow spots while others appeared as brown spots.

The deprotected sequences were purified by paper chromatography (descending technique) using Whatman #1 or #40 paper. The solvent system employed was EtOH/NH_4Ac (1 M, pH 7.3), 1:1 (v/v). A short wave UV light was used for detection. In certain cases, oligomers at the fully deprotected stage were purified by HPLC (Waters), using the following conditions :

Pump Controller Conditions

Time (min)	flow (ml min)	%A	%B	Curve
initial	1.00	100	0	*
20.00	1.00	50	50	06
22.00	1.00	30	70	03
27.00	1.00	30	70	01
30.00	1.00	100	0	03
35.00	1.00	100	0	01

solvent A : 0.1 M ammonium acetate buffer, pH = 6.0

solvent B : absolute methanol

Care was taken to ensure that all moisture is excluded from condensation reactions. After evaporation *in vacuo* of the anhydrous pyridine solutions, normal pressure was restored using dry nitrogen. Moisture must also be avoided in the nucleophilic substition reactions used to modify the O^6- position of guanosine residues. All reagent

preparation and reaction steps were performed in a dry nitrogen stream.

2.2 Protected Nucleosides and Coupling Reagents

The unmodified nucleoside derivatives were synthesized and characterized by published procedures : HOUtOH (Griffen, et al., 1968); HOC^{bz}tOH (Neilson & Werstiuk, 1971); TracUtOH (Werstiuk & Neilson,1972); TracA^{bz}tOH and Trac C^{bz}tOH (Werstiuk & Neilson, 1973); HOG^{bz}tOH and HOA^{bz}tOH (Gregoire & Neilson, 1978).

Modification of nucleoside derivatives was achieved via adaptation of published procedures and characterized as above : HOm^{2}m^{6}G^{bz}tOH (Fieser & Fieser,1967; Markiewicz & Wiewiorski, 1978; Ogilvie, et al., 1978; Markiewicz,1979); HOe^{6}G^{bz}tOH and HOm^{6}G^{bz}tOH (Gaffney & Jones, 1982; Markiewicz & Wiewiorowski, 1978; Ogilvie,et al., 1978; Markiewicz,1979).

The coupling reagents were also synthesized and characterized by established procedures : 2,2,2-trichloroethyl phosphate (England & Neilson,1977); MST (Kataguri et al., 1974); TPS-NI (Van Boom, et al., 1977).

2.3 Summary of Preparation and Characterization of Modified Nucleosides

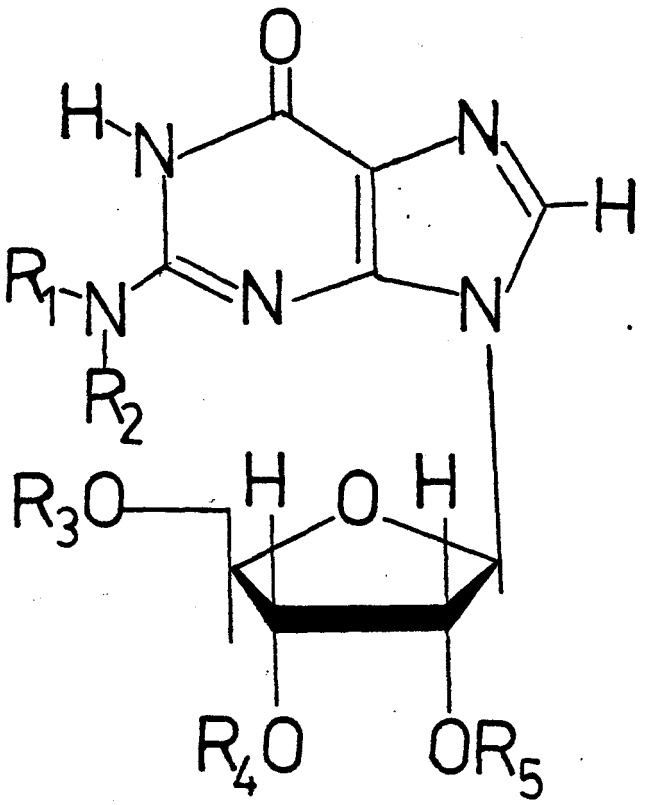
(refer to Figures 5-9, Tables 1a, 1b, 2)

O^6-Alkylated guanosine derivatives (alkyl = methyl 17, ethyl 22) suitable for use in oligoribonucleotide synthesis were prepared using a series of nucleophilic substition reactions (Gaffney & Jones, 1982) at the C^6 position of N^2-Benzoyl-3',5', -O- 1,1,3,3-tetraisopropyl-1,3-disiloxanediyl-2'-O -tetrahydropyranylguanosine 15, using the appropriate alcohol in the final substitution step. Removal of the cyclic disiloxane from the nucleoside was effected by mild treatment with tetrabutylammonium fluoride in tetrahydrofuran (Markiewicz & Wiewiorowski, 1978; Ogilvie, et al., 1978; Markiewicz, 1979) to yield the desired products.

The suitably blocked O^6-methyl G derivative 17 was also prepared from another fully protected nucleoside, N^2-benzoyl-2'-O-tetrahydropyranyl-3',5'-O-diacetylguanosine 19. The same series of nucleophilic substitution reactions followed by base catalyzed de-O-acetylation yielded 17.

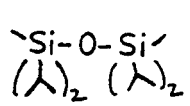
Synthesis of a suitably blocked N^2,O^6-dimethyl G derivative 13 involved diazomethane reaction (CH_2N_2)

Figure 5. Intermediates in the Synthesis of Modified Guanosine Residues



(1) R_1 = -C(=O)-⬡ , R_2 = -H , $R_3 = R_4 = R_5$ = -C(=O)-CH_3

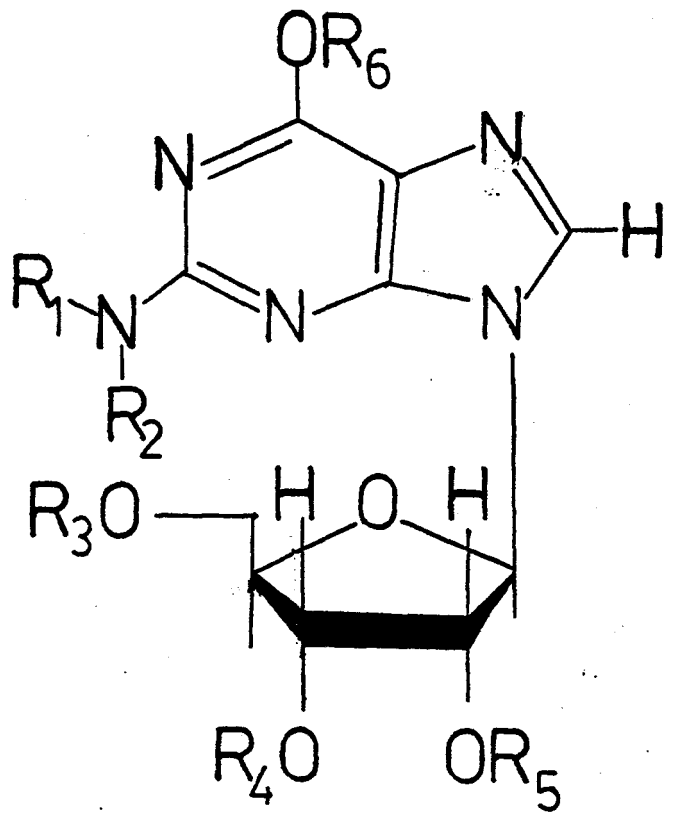
(14) R_1 = -C(=O)-⬡ , $R_2 = R_3 = R_4$ = -H , R_5 = 

(15) R_1 = -C(=O)-⬡ , R_2 = -H , $R_3 + R_4$ =  , R_5 = 

(16) R_1 = -C(=O)-⬡ , $R_2 = R_5$ = -H , $R_3 = R_4$ = -C(=O)-CH_3

(19) R_1 = -C(=O)-⬡ , R_2 = -H , $R_3 = R_4$ = -C(=O)-CH_3 , R_5 = 

Figure 6. Intermediates in the Synthesis of Modified Guanosine Residues



(2) R_1 = -C(=O)-⬡ , R_2 = -H , $R_3 = R_4 = R_5$ = -C(=O)-CH_3 , R_6 = -CH_3

(3) R_1 = -C(=O)-⬡ , $R_2 = R_6$ = -CH_3 , $R_3 = R_4 = R_5$ = -C(=O)-CH_3

(4) R_1 = -C(=O)-⬡ , $R_2 = R_3 = R_4 = R_5$ = -H , R_6 = -CH_3

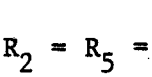
(5) R_1 = -C(=O)-⬡ , $R_2 = R_6$ = -CH_3 , $R_3 = R_4 = R_5$ = -H

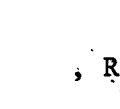
(6) $R_1 = R_2 = R_3 = R_4 = R_5$ = -H , R_6 = -CH_3

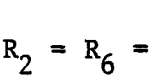
(7) $R_1 = R_3 = R_4 = R_5$ = -H , $R_2 = R_6$ = -CH_3

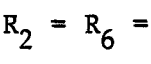
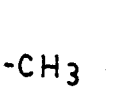
... continued

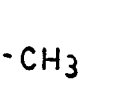
... Figure 6 continued

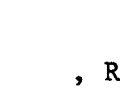
(8) R_1 =  , $R_2 = R_5 = -H$, $R_3 + R_4$ =  ; $R_6 = -CH_3$

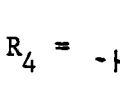
(9) R_1 =  , $R_2 = -H$, $R_3 + R_4$ =  , R_5 =  , $R_6 = -CH_3$

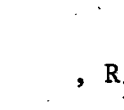
(11) R_1 =  , $R_2 = R_6 = -CH_3$, $R_3 + R_4$ =  , $R_5 = -H$

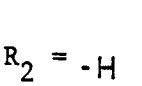
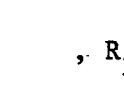
(12) R_1 =  , $R_2 = R_6 = -CH_3$, $R_3 + R_4$ =  , R_5 = 

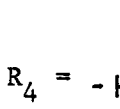
(13) R_1 =  , $R_2 = R_6 = -CH_3$, $R_3 = R_4 = -H$, R_5 = 

(16) R_1 =  , $R_2 = -H$, $R_3 + R_4$ = , R_5 =  , $R_6 = -CH_3$

(17) R_1 =  , $R_2 = R_3 = R_4 = -H$, R_5 =  , $R_6 = -CH_3$

(20) R_1 =  , $R_2 = -H$, $R_3 = R_4$ = , R_5 =  , $R_6 = -CH_3$

(21) R_1 =  , $R_2 = -H$, $R_3 + R_4$ =  , R_5 =  , $R_6 = -CH_2CH_3$

(22) R_1 =  , $R_2 = R_3 = R_4 = -H$, R_5 =  , $R_6 = -CH_2CH_3$

(23) $R_1 = R_2 = R_3 = R_4 = R_5 = -H$, $R_6 = -CH_2CH_3$

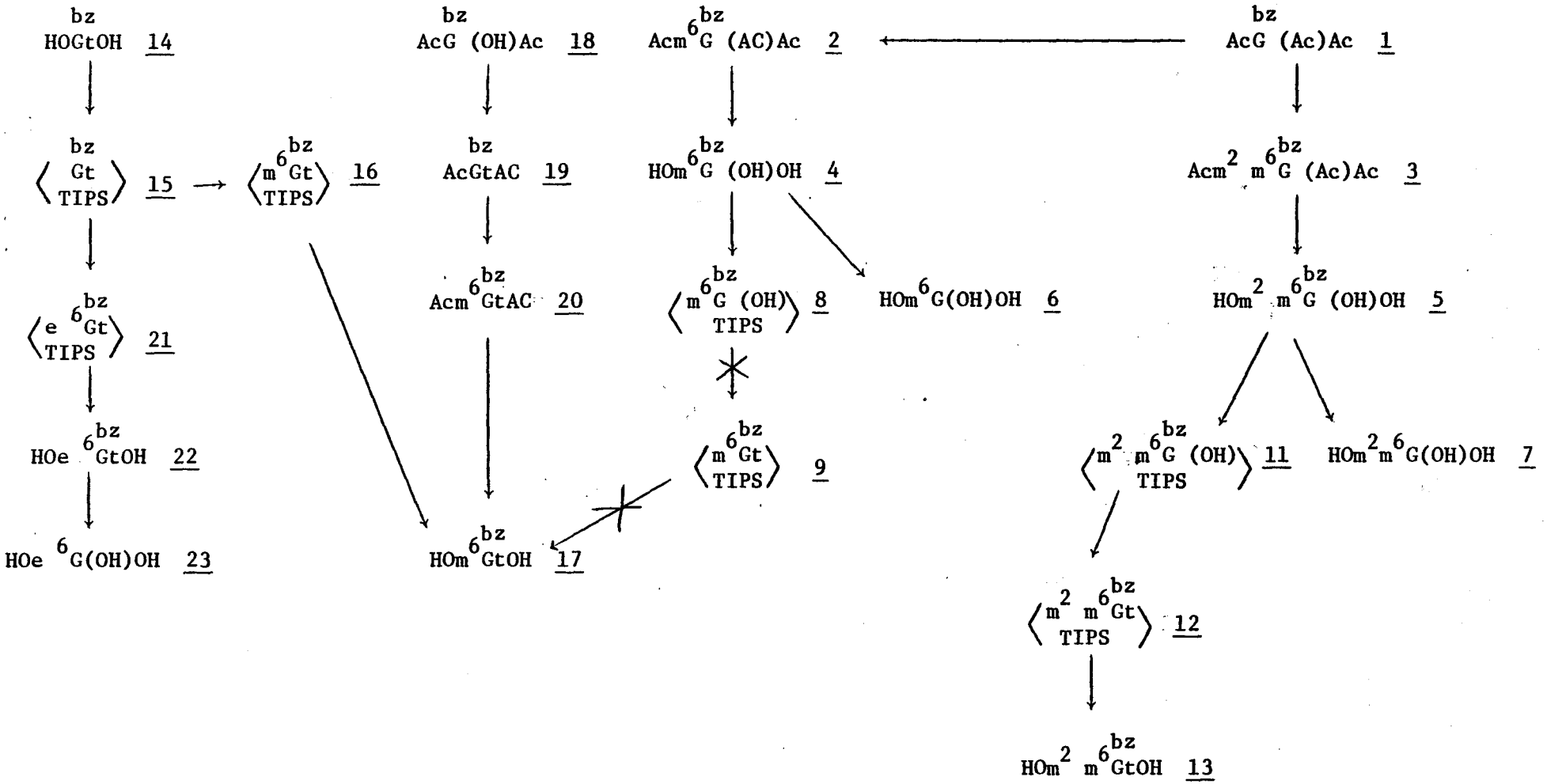


Figure 7. Schematic diagram for the modification of guanosine residues and the corresponding synthesis of suitably protected ribonucleosides, ready for insertion into sequences. In addition to the abbreviations recommended by IUPAC-IUB (1970) the following are used: t, tetrahydropyranyl; Ac, acetyl; Bz, benzoyl; TIPS, 3',5'-O-tetraisopropyldisiloxyl ether; m^6G, O^6-methyl guanosine; e ^6G, O^6-ethyl guanosine; m^2 m^6G, N^2,O^6-dimethyl guanosine.

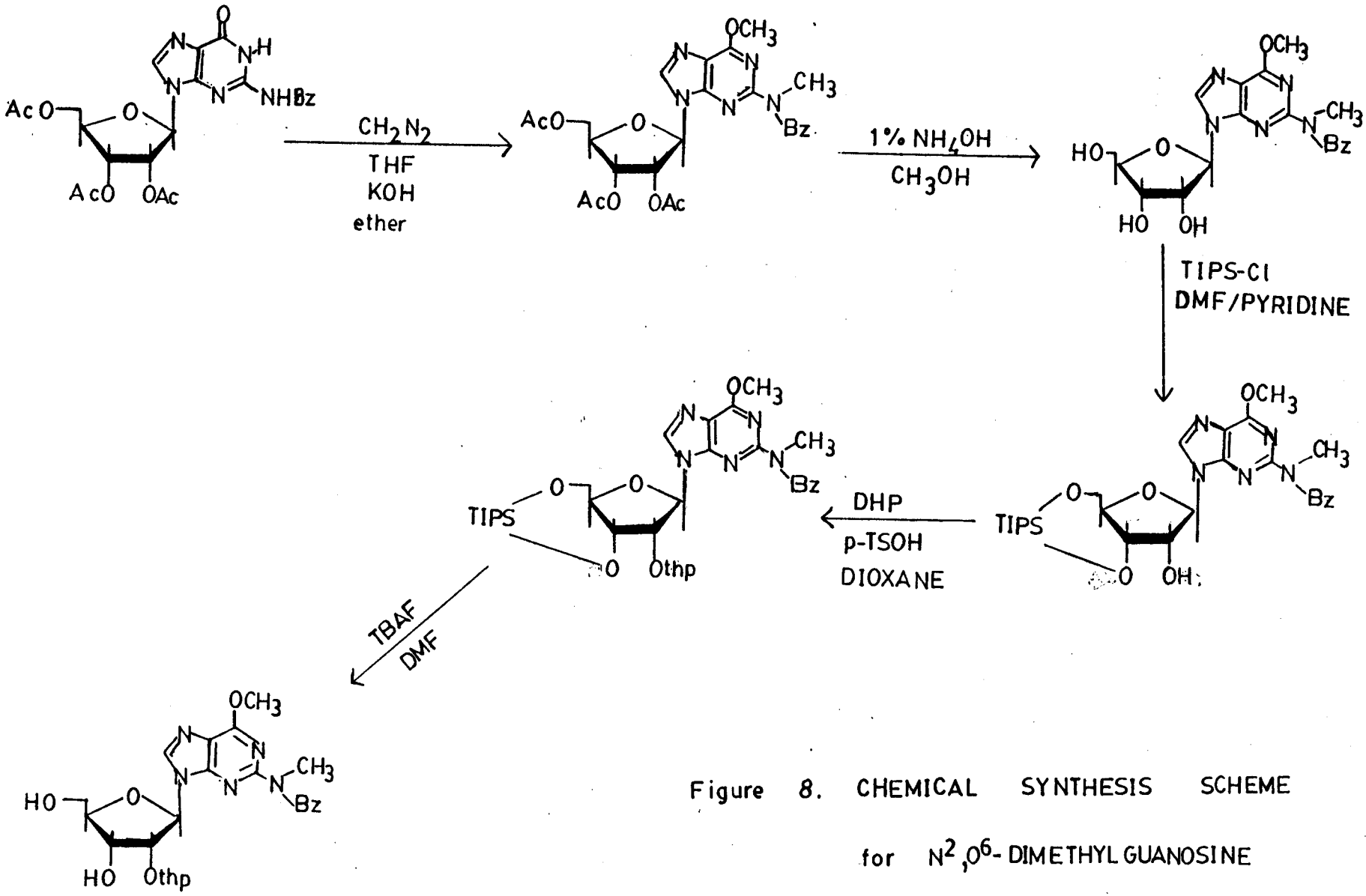


Figure 8. CHEMICAL SYNTHESIS SCHEME

for N^2,O^6- DIMETHYL GUANOSINE

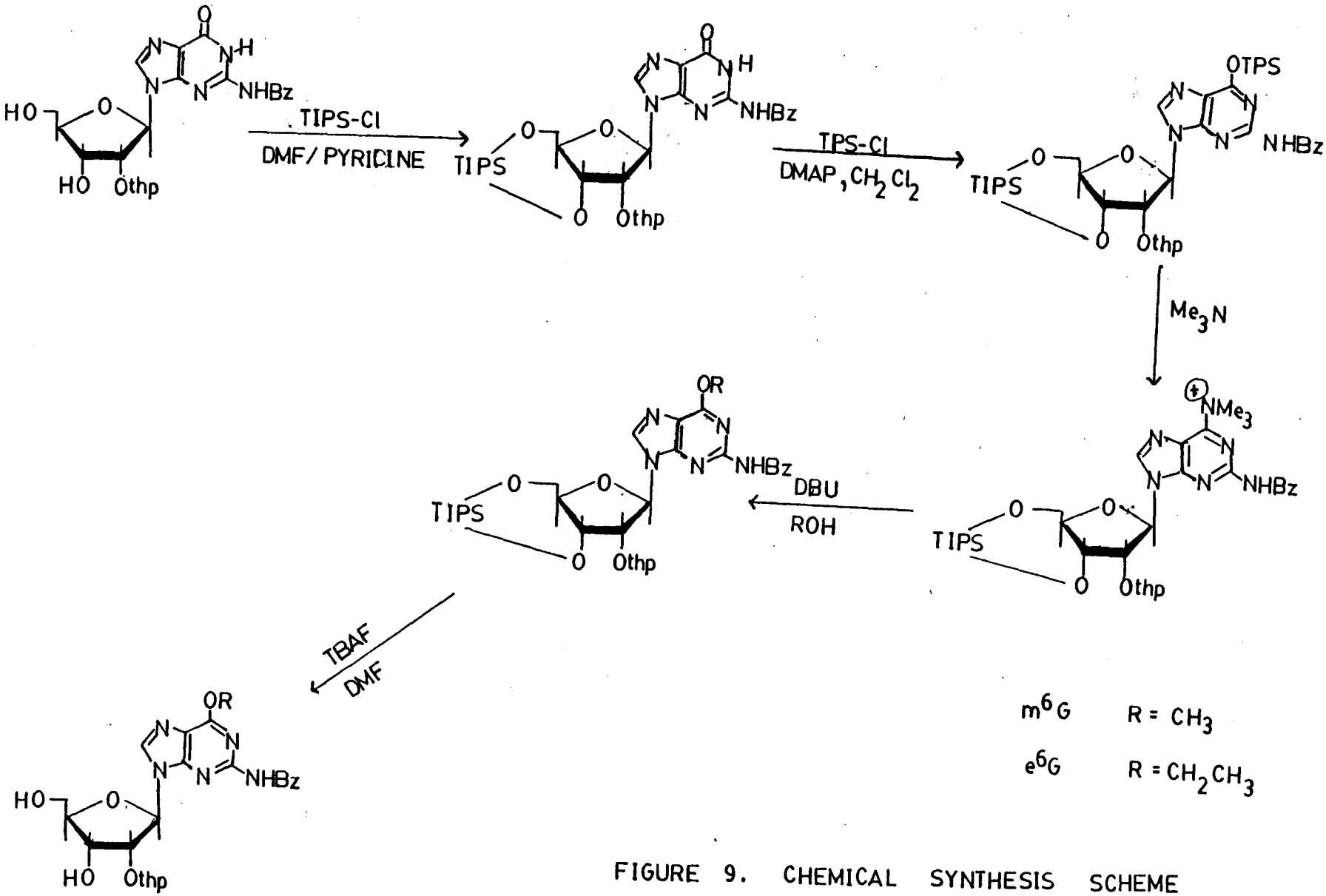
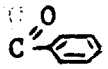
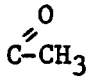


FIGURE 9. CHEMICAL SYNTHESIS SCHEME

for O^6-ALKYLATED GUANOSINE DERIVATIVES

Table 1a. ^{1}H Chemical Shifts and Coupling Constants for Guanosine Derivatives[a]

	Compound						
Proton	(1)	(2)	(3)	(4)	(5)	(6)	(7)
H-8	7.652	7.853	7.939	8.489	8.476	8.087	8.019
NH	9.324	8.593				6.442	6.902
	8.13-8.01 7.60-7.38	7.94-7.90 7.55-7.40	7.43-7.39 7.28-7.20	7.96-7.91 7.62-7.46	7.283 7.265		
H-1'	5.877	6.057	6.003	5.931	5.834	5.770	5.766
H-2'	5.993	5.971	5.910	4.599	4.420	4.450	4.567
H-3'	5.952	6.004	5.654	4.168	4.109	4.079	4.123
H-4'	(4.407)	(4.453)	4.436	3.931	3.912	3.876	3.888
H-5'	4.496	4.504	4.450	3.639	3.619	3.601	3.610
H-5"	4.363	4.446	4.332	3.539	3.520	3.511	3.513
2'-OH				5.515	5.407	5.395	5.386
3'-OH				5.210	5.229	5.126	5.156
5'-OH				5.012	4.979	5.084	
	2.083 2.035 1.952	2.095 2.045 1.999	2.163 2.128 2.109				
6 OCH_3		4.134	3.716	4.045	3.575	3.949	3.942
NCH_3			3.404		3.366		2.791
$^3J_{1'2'}$	4.1	4.1	4.3	6.0	5.6	6.1	6.1
$^3J_{2'3'}$	4.9	5.5	5.5	5.0	5.0	5.0	5.0
$^3J_{3'4'}$	4.8	4.4	5.5	3.3	3.7	3.3	3.3
$^3J_{4'5'}$	2.9	5.5	3.3	4.2	4.4	3.8	4.2
$^3J_{4'5"}$	5.4	4.9	5.7	4.4	4.6	3.9	4.2
$^2J_{5'5'}$	-10.2	-13.4	-13.1	-12.0	-12.0	-12.0	-11.9
$^3J_{OH2'}$				6.0	6.1	6.1	6.1
$^3J_{OH3'}$				4.7	5.1	4.7	4.8
$^3J_{OH5'}$				5.6	5.6	5.6	
$^3J_{NHCH_3}$							4.7

[a] ^{1}H NMR chemical shifts were recorded on a Bruker WM-250 spectrometer at 250.13 MHz (ambient temperature). The following deuterated solvents were used: $CDCl_3$ ((3), 470 µl); $CDCl_3$ + benzene-d^6 ((1), 400 µl + 80 µl; (2), 400 µl + 60 µl); DMSO-d^6 ((4) - (7), 470 µl). Chemical shifts are in ppm relative to TMS, using the residual proton signal $CDCl_3$ (at 7.24 ppm) or DMSO-d^6 (at 2.49 ppm) as internal reference.

Table 1b. ^{13}C Chemical Shifts of Guanosine Derivative[c]

Carbon	1	2	3
1'	87.17	87.18	86.93
2'	73.02	73.47	72.99
3'	70.72	70.88	70.26
4'	79.61	80.26	79.81
5'	62.90	63.38	62.90
2	149.14[b]	152.42[b]	152.07
4	147.97[b]	152.23[b]	156.77
5	122.40	119.18	118.72
6	155.40	161.57	160.14
8	138.41	140.30	140.28
Bz			
C=0	168.02	164.71	172.65
1	136.48	134.70	138.10
2,6	127.97	127.56	127.77
3,5	128.94	128.69	127.95
4	133.67	132.13	129.94
Ac[a]			
C=0			
	170.81	170.38	170.18
	169.88	169.52	169.40
	169.45	169.39	169.14
CH_3			
	20.67	20.66	20.66
	20.55	20.52	20.47
	20.39	20.40	20.39
NCH_3			34.83
OCH_3		54.57	54.02

[a]Cannot be unambigously assigned to a specific 2', 3' or 5' position.

[b]Assignment within a column may be reversed.

[c]62.90 MHz $CDCl_3$

Table 2. ^{1}H Chemical Shifts and Coupling Constants of the Alkylated Guanosine Mononucleosides[a]

Proton	$G^{O^6}CH_3$	$G^{O^6}CH_3,N^2CH_3$	$G^{OCH_2CH_3}$[b]
H-8	8.051	8.019	8.09
H-1'	5.748	5.766	5.94
H-2'	4.450	4.567	4.80
H-3'	4.079	4.123	4.51
H-4'	3.876	3.888	4.20
H-5'	3.601	3.610	3.91
H-5"	3.511	3.510	3.84
OCH_3	3.936	3.942	
NCH_3		2.791	
OCH_2CH_3			4.62
OCH_2CH_3			1.37
$^3J_{1'2'}$	6.2	6.1	
$^3J_{2'3'}$	5.0	5.0	
$^3J_{3'4'}$	3.3	3.3	
$^3J_{4'5'}$	3.8	4.2	
$^3J_{4'5"}$	3.9	4.2	
$^2J_{5'5"}$	-12.1	-11.9	

[a]Spectra recorded on a Bruker WM-250 at 250.13 MHz in DMSO-d6 and 1 drop of D_2O. Chemical shifts are in ppm relative to TMS using the residual proton signal of DMSO-d6 at 2.49 ppm as internal reference.

[b]Solvent: D_2O + x drops of DMSO-d6.

(Fieser & Fieser) on N^2-benzoyl-2',3',5'-O-triacetylguanosine 1, followed by base catalyzed de-O-acetylation, protection of the 3' and 5' hydroxyls via the silyl ether mentioned above, protection of the 2' hydroxyl by a tetrahydropyranyl group, and then removal of the cyclic disiloxane as described above. A similar route was attempted to make the O^6-methyl G derivative but without success. It is believed that the acidic conditions used to facilitate the tetrahydrofuran reaction cleaved the silyl ether bonds to create di- and/or tri-tetrahydropyran -substituted molecules.

Many of the intermediate products were characterized by some or all of the following physical techniques : ^{1}H NMR, UV, IR, and Mass spectroscopy, and combustion analysis. Use of ^{1}H-NMR for chemical shift determination allowed for verification of the integrity of the compounds, and for anticipation of the contribution of the alkylated purines to the overall oligomer spectra.

The ^{1}H NMR spectra were recorded as described previously. UV spectra were obtained over a range of 320 - 220 nm on a Cary 118 spectrometer. Mass spectra were recorded on a V.G. Micromass Ltd. 7070F spectrometer at an ionization energy of 70 ev. A Perkin Elmer 283 spectrometer was used to obtain the IR spectra. Combustion analyses were performed by

Galbraith Laboratory, Knoxville, Tennessee; samples were dried under vacuum for 16 hours at 40°C in a drying pistol containing phosphorous pentoxide.

2.4 N^2-Benzoyl-N^2,O^6-dimethyl-2'-O-tetrahydropyranyl-guanosine (Figures 5-8)

Preparation of Diazomethane (Fieser & Fieser, 1967) :

A 40% aqueous potassium hydroxide solution (30 ml) was added to anhydrous ether (100 ml) and the stirred mixture was cooled to 0°C. N-Methyl-N'-nitro-N-nitrosoguanidine (10 g,0.07 mol) was added in portions. After 15 minutes, the yellow ether layer (75 ml) was decanted and stored over KOH. Solution (0.05 M) should contain 2.1 g of diazomethane.

2.4.1.0 N^2-Benzoyl-N^2,O^6-dimethyl-2',3',5'-O-triacetylguanosine (3)

N^2-Benzoyl-2',3',5'-O-triacetylguanosine (1) (Gregoire & Neilson,1978) (5.0 g,9.7 mmol) was dissolved in tetrahydrofuran (100 ml) and the stirred solution was cooled to 5°C. The ethereal solution of diazomethane (29.1 ml,19.4 mmol) was added slowly, and the reaction stirred at 5°C for two days. An additional 29.1 ml of the diazomethane solution was

added and the reaction was stirred under the same conditions for another two days. Reaction progress was monitored daily by tlc. Following a four day reaction period, the solution was allowed to stand open to the atmosphere at room temperature for 18 hours, to permit evaporation of the ether and any unreacted diazomethane. Residual solvents were evaporated under reduced pressure to produce an orange-yellow oil, which was submitted to column chromatography. The product was eluted with 0.5 - 1.5% CH_3OH/CH_2Cl_2 as a light yellow foam (2.34 g, 4.30 mmol, 44.6%, R_f 0.66 in 5% CH_3OH/CH_2Cl_2).

^1H and ^{13}C chemical shifts - Tables 1a and 1b

IR ($CHCl_3$) V_{max} : 1755, 1660, 1605, 1595, 1470, 1450, 1380, 1240, 1040 cm^{-1}

Mass Spectrum : m/e (relative intensity) : 541 (m^+,7), 254 (12), 139 (26), 105 (100), 77 (68), 51 (28)

Anal. Calculated for $C_{25}H_{27}N_5O_9$: C 55.45 ,H 5.04, N 12.94

Found : C 55.22, H 5.12, N 12.73

2.4.1.1 <u>N^2-Benzoyl-N^2,O^6-dimethylguanosine (5)</u>

N^2-Benzoyl-N^2,O^6-dimethyl-2',3',5'-O-triacetylguanosine <u>(3)</u> (2.0 g, 3.68 mmol) was

dissolved in 48 ml of a 1% NH_4OH/CH_3OH (v/v) solution (Werstiuk & Neilson,1976). Reaction was allowed to stand at room temperature for 1 day whereupon the solution was evaporated to dryness under reduced pressure. This was followed by azeotroping with two 10 ml portions of CH_2Cl_2. Product was purified by column chromatography (eluted with 7-10% CH_3OH/CH_2Cl_2) and was isolated as a solid white foam (1.38 g, 2.55 mmol, 90%, R_f 0.30 in 5% CH_3OH/CH_2Cl_2).

^{1}H-NMR chemical shifts - Table 1a

IR (KBr) V_{max} : 3380, 2930, 1656, 1600, 1515, 1470, 1440, 1390, 1255, 1040, 790, 720, 690 cm^{-1}

Mass Spectrum : m/e (relative intensity) : 415 (m^+,4), 283 (14), 282 (10), 254 (35), 179 (27), 165 (21), 105 (100), 77 (66)

Anal.calculated for $C_{19}H_{21}N_5O_6 \cdot H_2O$: C 52.65, H 5.35, N 16.16

Found : C 52.94, H 5.41, N 16.14

2.4.1.2 <u>N^2,O^6-Dimethylguanosine (7)</u>

N^2-Benzoyl-N^2,O^6-dimethyl-2',3',5'-O-triacetylguanosine (<u>3</u>) (0.20 g, 0.37 mmol) was dissolved in 6 ml of a 50% NH_4OH/CH_3OH solution (v/v) (Werstiuk & Neilson,1976). The solution was allowed to stand at room temperature for two days, during

which time a crystalline product formed. Product was collected and recrystallized from 50% NH_4OH /CH_3OH (v/v) as white needles (0.085 g, 0.03 mmol, 75%, R_f 0.25 in 5% CH_3OH/CH_2Cl_2).

^{1}H-NMR - Table 2

Melting point : 125-127°C (decomposition)

IR (KBr) V_{max} : 3510, 3370, 2960, 1620, 1600, 1565, 1490, 1385, 1255, 1080, 1065, 900, 785 cm^{-1}

Mass Spectrum m/e (relative intensity) : 311 (m^+, 10), 288 (10), 180 (21), 179 (100), 178 (16), 151 (18), 151 (28), 135 (15), 105 (14),57 (16)

Anal. calculated for $C_{12}H_{17}N_5O_6 \cdot 1.5\ H_2O$: C 42.60, H 5.96, N 20.70

Found : C 42.71, H5.73, N 20.72

2.4.2 <u>N^2-Benzoyl-N^2,O^6-dimethyl-3',5'-O-tetraisopropyldisiloxylguanosine (11)</u>

To a stirred solution of N^2-benzoyl-N^2, O^6-dimethylguanosine (5) (1 g, 2.41 mmol) in 20 ml dry pyridine was added 1,3-dichloro-1,1,3,3,-tetra-isopropyldisiloxane (1 ml, 3.17mmol) (Markiewicz & Wiewiorowski, 1978; Markiewicz,1979). The reaction went to completion in 1.5 hr (R_f 0.42 → 0.78 in 5% CH_3OH/CH_2Cl_2), was quenched with ice and extracted into CH_2Cl_2 (3 x 20 ml). Following extraction, the

product was washed with water (20 ml), solvents evaporated *in vacuo* to produce a yellow oil and azeotroped *in vacuo* with toluene (3 x 10 ml). Column chromatography on silica gel was performed. The product (11) eluted with 1.5 - 2% CH_3OH/CH_2Cl_2 and was reduced *in vacuo* to a light yellow foam (1.45 g, 2.21 mmol, 91%).

2.4.3 N^2Benzoyl-N^2,O^6-dimethyl-2'-O-tetrahydropyranyl-3',5'-O-tetraisopropyldisiloxylguanosine (12)

(Gregoire & Neilson, 1978)

A stirred solution of p-toluene sulfonic acid monohydrate (302.4 mg, 1.6 mmol) in dry p-dioxane (14.98 ml) containing molecular sieves was cooled to solidification. 2,3-Dihydropyran (3.54 ml, 42.5 mmol) was added portionwise with stirring. N^2-Benzoyl-N^2,O^6-dimethyl-3',5'-O -tetraisopropyl-disiloxylguanosine (11) (1.4 g, 2.13 mmol) was dissolved in p-dioxane (9.94 ml) cooled on ice, and added portionwise with stirring to the reaction mixture. After standing at 0°C for one hour, the mixture was allowed to warm to room temperature and stirred overnight. After one day the reaction was complete (R_f 0.58 → 0.96, in 2% CH_3OH/CH_2Cl_2). The mixture was neutralized with 7 N NH_4OH. filtered, evaporated *in vacuo* and azeotroped with CH_2Cl_2 (3 x 25

ml) to a yellow oil. The product (12) eluted with 1 - 2% CH_3OH/CH_2Cl_2, and was evaporated *in vacuo* to a solid yellow foam (1.36 g, 1.83 mmol, 1.83 mmol, 86%).

2.4.4 N^2-Benzoyl-N^2,O^6-dimethyl-2'-O-tetrahydropyranyl guanosine (13)

N^2-Benzoyl-N^2,O^6-dimethyl-2'-O-tetrahydropyranyl -3',5'-O-tetraisopropyldisiloxylguanosine (12) (1.36 g, 1.83 mmol) was dissolved in tetrahydrofuran (20 ml) with stirring. Tetrabutylammonium fluoride (3.66 ml, 3.66 mmol) was added portionwise (Markiewicz & Wiewiorowski, 1978; Ogilvie, *et al.*, 1978; Markiewicz, 1979). The reaction went to completion (R_f 0.96 → 0.38 and 0.27 in 5% CH_3OH/CH_2Cl_2) in 0.5 hr, was quenched with excess water, extracted into CH_2Cl_2 (3 x 20 ml) and evaporated *in vacuo* to a yellow oil. Column chromatography on silica gel yielded two products (high and low isomers of (13)), which eluted with 2 - 3% and 3 - 5% CH_3OH/CH_2Cl_2, respectively. Both products were evaporated *in vacuo* to solid white foams (200 mg, 0.40 mmol, and 352 mg, 0.70 mmol, total 62%) respectively.

2.5 N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl-guanosine (17) (Figures 5-7,9)

2.5.1.1 N^2-Benzoyl-O^6-methyl-2',3',5'-O-triacetylguanosine (2)

(A). N^2-Benzoyl-O^6-methyl-2',3',5'-O-triacetylguanosine (2) was isolated by column chromatography (eluted with 2 - 3% CH_3OH/CH_2Cl_2) as a minor product from the diazomethane reaction which produced (3). The product (2) was obtained as a pale yellow foam (0.31 g, 0.58 mmol, 6.08%, R_f 0.98 in 5% CH_3OH/CH_2Cl_2).

(B). (adapted from Gaffney & Jones, 1982)

Triethylamine (5.58 ml, 40.0 mmol) and 4,4-dimethyl aminopyridine (0.061g, 0.50 mmol) were dissolved in stirring CH_2Cl_2 (25 ml) containing molecular sieves.
N^2-Benzoyl-2',3',5'-O-triacetylguanosine (5.13 g, 10.0 mmol) and then 2,4,6-triisopropylbenzenesulfonyl chloride (6.06 g, 20.0 mmol) were added in portions and the total reaction volume increased to 60 ml by the addition of CH_2Cl_2. Reaction was stirred at room temperature for 2.5 hr, during which time reaction progress was monitored by tlc (R_f 0.24 → solvent front in 5% CH_3OH/CH_2Cl_2).

To the stirred nucleoside solution were added two equal volumes (50 ml) of a solution containing trimethylamine* (2.88 g, 15.0 mmol) and CH_3OH (9.38 ml, 0.94 mmol) at one hour intervals. Reaction was completed in two hours. Reaction progress, as monitored by tlc showed that the spot migrated from the solvent front to the origin in 5% CH_3OH/CH_2Cl_2. The mixture was then cooled to 5°C and two equal amounts of 1,8-diazabicyclo (5.4.0) undec-7-ene (2.24 ml, 15.0 mmol) were added dropwise at 0.5 hr intervals. The reaction was completed in overnight indicated by tlc (R_f origin → 0.58 in 5% CH_3OH/CH_2Cl_2). Solid NaCl generated by the reaction between the trimethylamine hydrochloride and sodium methoxide, was removed by vacuum filtration. The solvents were evaporated under reduced pressure to yield a thick red oil which azeotroped in vacuo from CH_2Cl_2 (2 x 20 ml) and then toluene (20 ml). Column chromatography initially yielded one mixed fraction. A second column provided a pure sample of the product which eluted with 2 - 3% CH_3OH/CH_2Cl_2. The product was obtained as a yellow foam, following evaporation in vacuo (2.3 g, 4.29 mmol, 44.7%, R_f 0.75 in 5% CH_3OH/CH_2Cl_2).

^1H and ^{13}C chemical shifts - Tables 1a and 1b

IR ($CHCl_3$) ν_{max} : 3450, 3000, 2955, 1755, 1705, 1610,

1515, 1460, 1395, 1360, 1230, 1070 cm^{-1}

Mass Spectrum m/e (relative intensity) : 527 (m^+, 5), 259 (16), 139 (49), 105 (100), 77 (63), 51 (15)

Anal. Calculated for $C_{24}H_{25}N_5O_9$: C 54.65, H 4.78, N 13.28

Found : C 54.47, H 4.82, N 13.06

*The trimethylamine solution was prepared by adding CH_3OH (30 ml, 0.74 mol) to a mixture of trimethylamine hydrochloride (15.01 g, 0.16 mol) and sodium methoxide (8.48 g, 0.16 mol). Upon reaction completion, the volume was increased to 160 ml by the addition of CH_2Cl_2.

2.5.1.2 N^2-Benzoyl-O^6-methylguanosine (4)

N^2-Benzoyl-O^6-methyl-2',3',5'-O-triacetylguanosine (0.15 g, 0.28 mmol) was dissolved in 6 ml of a 1% NH_4OH/CH_3OH (v/v) solution (Werstiuk & Neilson, 1976). The reaction was allowed to stand at room temperature for two days, during which time the product crystallized out of solution. The product was isolated as white needles (0.087 g, 0.22 mmol, 77%, R_f 0.26 in %5 CH_3OH/CH_2Cl_2).

^{1}H-NMR - Table 1a

Melting point : 280 -210°C (decomposition)

IR (KBr) V_{max} : 3380, 2940, 1695, 1610, 1460, 1395,

1240, 1045, 790, 705 cm^{-1}

Mass Spectrum m/e (relative intensity) : 401 (m^+,1), 298 (11), 269 (9), 254 (16), 240 (21), 165 (17), 105 (100), 77 (62), 51 (14)

Anal. calculated for $C_{18}H_{19}N_5O_6 \cdot H_2O$: C 53.86, H 4.77, N 17.45

Found : C 53.66, H 4.95, N 17.18

2.5.1.2.1 O^6-Methylguanosine (6)

N^2-Benzoyl-O^6-methyl-2',3',5'-O-triacetylguanosine (2) (0.40 g, 0.76 mmol) was dissolved in 6 ml of a 50% NH_4OH/CH_3OH (v/v) solution (Werstiuk & Neilson, 1976). Reaction was allowed to stand at room temperature for two days. The solution was evaporated under vacuum to a colourless gum which was partially soluble in CH_2Cl_2. Methanol was added to this gum to effect solution at which time silica gel was added to the solution. The solvents were evaporated to dryness under reduced pressure in order to adhere the product to the silica gel. This material was subjected to three evaporations from 30 ml portions of CH_2Cl_2 and then was placed onto a silica column for chromatography. The product, which eluted with 6 - 8% CH_3OH/CH_2Cl_2, was isolated as a pale yellow foam (0.18 g, 0.61 mmol, 80.5%, R_f 0.20 in 5% CH_3OH/CH_2Cl_2).

^{1}H-NMR - Table 2

IR (KBr) V_{max} : 3370, 2930, 1615, 1595, 1480, 1395, 1255, 1070, 785 cm^{-1}

Mass Spectrum m/e (relative intensity) : 297 (m^+,11), 208 (17), 194 (16), 166 (28), 165 (100), 164 (18), 136 (17), 135 (19), 108 (12), 105 (9), 57 (13)

Anal. calculated for $C_{11}H_{15}N_5O_5$: C 44.44, H 5.09, N 23.56

Found : C 44.72, H 5.51, N 23.35

2.5.1.3 N^2-Benzoyl-O^6-methyl-3',5'-O-tetraisopropyl-disiloxylguanosine (8)

Reaction procedure was similar to that described for the synthesis of N^2-Benzoyl-N^2,O^6-dimethyl-3',5'-O-tetra-isopropyldisiloxyguanosine (11).

Reagent	Amount	mmol
$m^6G^{bz}OH$	1.0g	2.49
dry pyridine	20 ml	
TIPS	1.0 ml	3.17

The reaction was complete in 1.5 hr (R_f 0.38 → 0.60 in 5% CH_3OH/CH_2Cl_2), and was worked up as previously described. A methanol rinse was required

to transfer the product residue from the reaction flask to the separatory funnel. The product, isolated by column chromatography, eluted with 2 - 2.5% CH_3OH/CH_2Cl_2 and was reduced *in vacuo* to a sticky yellow foam (1.30 g, 2.02 mmol, 81.0%).

2.5.1.4 N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl -3',5'-O-tetraisopropyldisiloxylguanosine (9)

The reaction was performed in a similar manner to that used for the synthesis of N^2-benzoyl-N^2,O^6-dimethyl-2'-O-tetrahydropyranyl- 3',5'-O-tetraisopropyldisiloxylguanosine (12).

Reagent	Amount	mmol
p-TsOH	265 mg	1.4
dry p-dioxane	12.84 ml	
2,3-dihydropyran	3.1 ml	37.2
m^6G^{bz}TIPS (8)	1.2 g	1.86
dry p-dioxane	8.52 ml	

The reaction appeared sluggish (ie. proceeded only *ca* 10%, R_f 0.38 → 0.92 in 2% CH_3OH/CH_2Cl_2) and was allowed to stir for a total of four days. As no further reaction was detected at this time, an excess of 2,3-dihydropyran (4.65 ml, 55.8 mmol) was added. The reaction was stirred for four more days, at which time it "appeared" 80% complete (by tlc), and was

worked up as discussed for compound (12). Column chromatography on silica gel yielded the crude product (9) which eluted with 0.5 - 1.5% CH_3OH/CH_2Cl_2 and was evaporated *in vacuo* to a thick yellow oil (2.25 g) having a dihydropyran-like odor.

2.5.1.5 N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl-guanosine (17)

The reaction was performed similarly to that described for the synthesis of N^2-benzoyl-N^2,O^6-dimethyl-2'-O-tetra hydropyranylguanosine (13).

Reagents	Amount	mmol
crude m^6G^{bz} + TIPS (9)	2.0 g	2.7
THF	20 ml	
TBAF	2 x 5.4 ml	2 x 5.4

No reaction was evident after four hours. The reaction mixture was worked up and quenched as previously described. The reaction was repeated using forcing conditions (ie. tetrabutylammonium fluoride 2 x (16.2 ml, 16.2 mmol)) at one hour intervals, without success. Since better methods to synthesize the desired product (17) were later devised, this method was abandoned.

2.5.2.1 N^2-Benzoyl-2'-O-tetrahydropyranyl-

3',5'-O-diacetyl guanosine (19)

A solution of p-toluene sulphonic acid monohydrate (101.6 mg, 0.54 mmol) in dry p-dioxane (10.7 ml) containing molecular sieves, was cooled on ice to solidification. 2,3-Dihydro-4H-pyran (3.6 ml, 42.85 mmol) was added dropwise with stirring. N^2-Benzoyl-3',5'-O-diacetylguanosine (18) (1 g, 2.14 mmol) suspended in dry p-dioxane (7.1 ml) was added portionwise with stirring. The mixture was chilled for one hour and then warmed to room temperature and stirred overnight. Tlc analysis indicated reaction completion (R_f 0.40 → 0.80 in 10% CH_3OH/CH_2Cl_2). After brief chilling the mixture was neutralized with 7N NH_4OH, filtered, and azeotroped in vacuo from CH_2Cl_2 (3 x 20 ml) to a yellow oil. Column chromatography was performed; the product eluted with 3 - 4% CH_3OH/CH_2Cl_2 and was reduced in vacuo to a solid yellow foam (1.08 g, 1.96 mmol, 91%).

2.5.2.2 N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl -3',5'-O-diacetylguanosine (20)

The reaction was done similarly to that for the synthesis of compound (2).

Reagents	Amount	mmol
TEA	0.60 ml	7.26

4,4-DMAP	11 mg	0.09
$AcG^{bz}tAc$ (19)	1.0 g	1.81
TPS-Cl	1.1 g	3.63

The reaction mixture was stirred for 3 hr, at which time the reaction appeared to have proceeded 85 - 90% (R_f 0.50 → solvent front in 5% CH_3OH/CH_2Cl_2).

Reagents	Amount	mmol
TMA*	2 x 0.59 g	2 x 10
CH_3OH	2 x 3.84 ml	2 x 386

The reaction went to completion in two hours (R_f solvent front → origin in 5% CH_3OH/CH_2Cl_2).

Reagent	Amount	mmol
DBU	1.2 ml	7.24

The reaction was chilled for 0.5 hr, then allowed to warm room temperature. The reaction went to completion overnight (R_f origin → 0.66 in 5% CH_3OH/CH_2Cl_2). Following workup (see section 2.5.1.1), the product eluted with 3 - 4% CH_3OH/CH_2Cl_2 during column chromatography, and was isolated *in vacuo* as a solid yellow foam (460 mg, 0.77 mmol,45%).

*The trimethylamine solution was prepared by adding CH_3OH (20 ml,2.01 mmol) to a mixture of trimethylamine hydrochloride (5 g, 52.1 mmol) and

sodium methoxide (2.82 g, 52.1 mmol) under a stream of dry nitrogen. The total volume was made up to 52.1 ml by the addition of CH_2Cl_2.

2.5.2.3 N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl-guanosine (17)

Two pilot reactions were performed to determine the better method for de-O-acetylation of compound (20).

1. N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl-3',5'-O-diacetylguanosine (20) (30 mg, 0.05 mmol) was dissolved in a mixture of absolute ethanol (0.36 ml) and dry pyridine (0.30 ml) with stirring. To this solution was added a solution of absolute ethanol (0.18 ml) and 2 N NaOH (0.18 ml). The reaction went to completion (R_f 0.96 → 0.68 (high isomer) and 0.62 (low isomer) in 10% CH_3OH/CH_2Cl_2) in 0.25 hr. Workup of the reaction involved neutralization with Dowex (NH_4^+ form), filtration, and azeotroping from CH_2Cl_2 (2 x 3 ml) in vacuo.

2. Compound (20) (30 mg, 0.05 mmol) was dissolved in 4 ml of a 10% NH_4OH/CH_3OH solution (1 ml/9 ml). The reaction went to completion in 1.0 hr as indicated by

tlc (see 1.). Workup consisted of evaporation of the solvents followed by azeotroping with CH_2Cl_2 (2 x 3ml) *in vacuo*.

Method 2 was chosen to de-O-acetylate the remainder of compound (20), because of its milder conditions and its more convenient work up conditions.

N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl -3',5'-O-diacetylguanosine (20) (400 mg, 0.67 mmol) was dissolved in 40 ml of a 10% NH_4OH/CH_3OH solution with stirring. After 1 hr, the reaction was worked up and column chromatography was done. The high R_f isomer eluted with 2 - 2.5% CH_3OH/CH_2Cl_2 while the low R_f isomer did so with 3 - 4% CH_3OH/CH_2Cl_2. The products were isolated as solid white foams, following evaporation *in vacuo* (high isomer - 134 mg, 0.28 mmol, low isomer - 80 mg, 0.17 mmol, total yield 66%).

2.5.3.1 N^2-Benzoyl-2'-O-tetrahydropyranyl-3',5-O-tetraisopropyldisiloxylguanosine (15)

N^2-Benzoyl-2'-O-tetrahydropyranylguanosine (14) (5 g, 10.5 mmol) was dissolved in 70 ml of dry pyridine with stirring at room temperature. 1,3-Dichloro-1,1,3,3 -tetraisopropyldisiloxane (5 ml, 15.9 mmol) was added portionwise with stirring (Markiewicz & Wiewiorowski, 1978; Ogilvie, *et al*, 1978; Markiewicz, 1979). The reaction went to

completion in 2.5 hr (R_f 0.63 → 0.88 in 10% CH_3OH/CH_2Cl_2). It was quenched with excess ice, extracted from CH_2Cl_2 (3 x 40 ml), washed with water (20 ml) and evaporated *in vacuo* to a yellow oil. Following column chromatography, the product, which eluted with 2 - 4% CH_3OH/CH_2Cl_2, was isolated as a light yellow foam, *in vacuo* (6.60 g, 9.24 mmol, 88%).

2.5.3.2 N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl-3',5'-O-tetraisopropyldisiloxylguanosine (16)

The procedure was similar to that followed for the synthesis of compound (2) (method B). Performing the reaction steps in a dry nitrogen stream along with use of one equivalent of 4,4-dimethylaminopyridine yielded purer, quicker reactions, having higher overall yields.

Reagent	Amount	mmol
TEA	1.4 ml	16.8
4,4-DMAP	256.2	2.1
G^{bz} + TIPS (15)	1.5 g	2.1
TPS-Cl	1.3 g	4.2

The reaction was stirred for 0.75 hr and reaction progress was monitored by tlc (R_f 0.30 → 0.95 in 2.5% CH_3OH/CH_2Cl_2).

Reagent	Amount	mmol

TMA*	2 x 0.88 g	2 x 15
CH_3OH	2 x 5.76 ml	2 x 579

The reaction went to completion in 3.5 hrs (R_f 0.95 → origin in 2.5% CH_3OH/CH_2Cl_2).

Reagent	Amount	mmol
DBU	1.96 ml	12.6

The reaction was chilled for 0.5 hr, warmed to room temperature and stirred for 3 hr at which time it had gone to completion (R_f origin → 0.35 in 2.5% CH_3OH/CH_2Cl_2). Following workup (see section 2.5.1.1), the product (16) was subjected to column chromatography (eluted with 2 - 3% CH_3OH/CH_2Cl_2) was reduced *in vacuo* to a solid yellow foam (1.38 g, 1.9 mmol, 90%).

* The trimethylamine solution was prepared by adding CH_3OH (20 ml, 2.01 mmol) to a mixture of trimethylamine hydrochloride (5 g, 52.1 mmol) and sodium methoxide (2.82 g, 52.1 mmol) under a stream of dry nitrogen. The total volume was made up to 52.1 ml by the addition of CH_2Cl_2.

2.5.3.3 N^2-Benzoyl-O^6-methyl-2'-O-tetrahydropyranyl-guanosine (17)

Compound (16) (1.3 g, 1.79 mmol) was dissolved in tetrahydrofuran (15 ml) at room temperature. Tetrabutylammonium fluoride (3.58 ml,3.58 mmol) was added portionwise with stirring. The reaction went to completion in 0.5 hr (R_f solvent front → 0.46 in 10% CH_3OH/CH_2Cl_2) was quenched with excess ice, extracted into CH_2Cl_2 (3 x 20 ml), washed with water (15 ml), and reduced to a yellow oil in vacuo. The product was subjected to column chromatography (eluted with 3 - 4% CH_3OH/CH_2Cl_2) and was evaporated in vacuo to a solid white foam (765 mg, 1.58 mmol, 88%).

2.6 N^2-Benzoyl-O^6-ethyl-2'-O-tetrahydropyranyl-guanosine (22) (Figures 5-7, 9)

2.6.1 N^2-Benzoyl-O^6-ethyl-2'-O-tetrahydropyranyl-3',5'-O-tetraisopropyldisiloxylguanosine (21)

The reaction was performed similarly to that for the synthesis of compound (16).

Reagent	Amount	mmol
TEA	1.4 ml	16.8
4,4-DMAP	256 mg	2.1
$G^{bz}t$ TIPS (15)	1.5 g	2.1
TPS-Cl	1.3 g	4.2

The reaction was stirred for 0.75 hr, and was monitored by tlc (R_f 0.30 → 0.97 in 2.5% CH_3OH/CH_2Cl_2).

Reagent	Amount	mmol
TMA*	2 x 0.88 g	2 x 15
CH_3CH_2OH	2 x 5.76 ml	2 x 838

The reaction went to completion in 2.5 hr (R_f 0.97 → origin in 2.5% CH_3OH/CH_2Cl_2).

Reagent	Amount	mmol
DBU	1.96 ml	12.6

The reaction mixture was cooled for 0.5 hr, then allowed to warm to room temperature and stirred for 3 hr to completion (R_f origin → 0.80 in 2.5% CH_3OH/CH_2Cl_2). Following workup (see section 2.5.1.1), column chromatography was performed to isolate (21), which eluted with 1 - 1.5% CH_3OH/CH_2Cl_2 and was reduced *in vacuo* to a thick yellow oil (1.36 g, 1.83 mmol, 87%).

*Sodium Hydride (NaH) (2 g - 60% dispersion in mineral oil, 83.3 mmol, or effectively, 50 mmol) was washed with petroleum ether (4 x 25 ml) and maintained

under a stream of dry nitrogen. Absolute ethanol (40 ml, 686 mmol) was added portionwise with stirring. The yellow suspension was stirred under a dry nitrogen stream for 0.5 hr. Trimethylamine hydrochloride (5 g, 52.1 mmol) was added to the suspension, stirred for 0.5 hr and than made up to a final volume of 52.1 ml by the addition of CH_2Cl_2.

2.6.2 N^2-Benzoyl-O^6-ethyl-2'-O-tetrahydropyranyl-guanosine (22)

The reaction was performed similarly to that for the synthesis of compound (17) using Method B.

Reagent	Amount	mmol
e^6G^{bz}tTIPS (21)	1.3g	1.75
THF	20 ml	
TBAF	4.38 ml	4.38

The reaction went to completion in 0.5 hr (R_f solvent front → 0.56 in 10% CH_3OH/CH_2Cl_2). Following workup (see section 2.5.1.1), the product was subjected to column chromatography and eluted with a trace of by-product (R_f 0.53). The pure product was obtained after a second column (eluted with 3 - 4% CH_3OH/CH_2Cl_2) and was isolated as a yellow-white foam *in vacuo* (742 mg, 1.48 mmol, 86%).

2.6.2.1 O^6-ethylguanosine (23)

Deprotection of N^2-benzoyl-O^6-ethyl-2'-O-tetrahydropyranylguanosine (22) was effected by modification of the published procedure (England & Neilson, 1976). Methanolic ammonia ($CH_3OH/H_2O/NH_4OH$, 1/1/2 (v/v/v)) was added, the reaction vessel sealed tightly, and left to stir for one day at room temperature.

The partially deblocked nucleoside was purified by paper chromatography on Whatman #1 paper (descending technique) in CH_3CH_2OH /50 % NH_4OH (1/1, v/v). The appropriate black band (R_f 0.90) was cut out, and elution with glass distilled water (descending technique) followed.

The solution was acidified to pH = 2.0 with 2 N HCl and stood for two days at room temperature. Neutralization with 1N NH_4OH followed, and the solution was evaporated to dryness in vacuo. Purification was achieved through paper chromatography using Whatman #1 paper, in the same manner as before. The desired black-blue band (R_f 0.30) was desalted, dried and eluted as before. The yield was calculated from the UV absorbance maximum (277.4 nm) to be 78%.
^{1}H-NMR - Table 2

2.7 Preparation of Oligoribonucleotides

(Tables 3a - 10, Figures 10 - 11)

Table 3a and 3b provides the complete details for the synthesis of the sequences used in this study. All sequences were made using published techniques (England & Neilson, 1976; Westiuk & Neilson, 1976). The stepwise synthesis of $ApGpm^2m^6G$ is detailed below to illustrate the general procedure. A summary of the stepwise preparation of compounds may be found in Tables 3a and 3b. Characterizations of these compounds are located in Tables 4 - 7.

2.7.1 Synthesis of $TracA^{bz}tpG^{bz}tpm^2\ m^6G^{bz}tOH$ (Figure 10)

2.7.1.1 N^6-Benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyl-adenlyl (3'(2,2,2-trichloroethyl) -5') -N^2-benzoyl-2'-O-tetrahydropyranylguanosine ($TracA^{bz}tpG^{bz}tOH$)

The free acid of 2,2,2-trichloroethylphosphate (England & Neilson, 1976) (2.64 g, 11.93 mmol, 3 equiv.) was evaporated *in vacuo* from anhydrous pyridine (3 x 20 ml) to convert it to the pyridinium salt. MST (6 g, 23.88 mmol, 6 equiv.) was added to the pyridinium solution (*ca* 15 ml), and the phosphate mixture was heated (40°C) for two hours to achieve

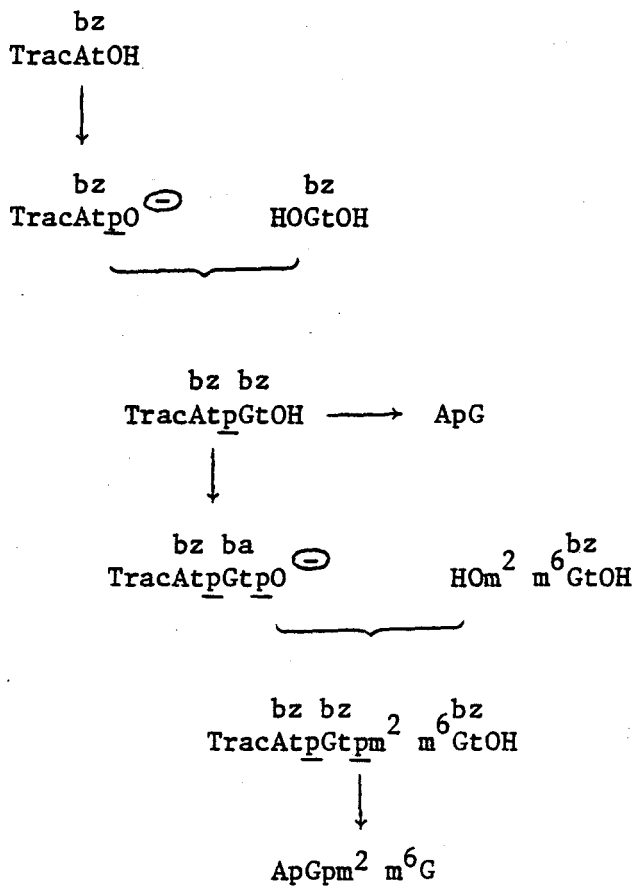


Figure 10. Schematic diagram for the stepwise chemical synthesis of the triribonucleotide ApGpm^2,m^{6}G by the phosphotriester method. In addition to the abbreviations recommended by IUPAC-IUB (1970) the following are also used: Trac, triphenylmethoxyacetyl; t, tetrahydropyranyl; pO$^-$, 3'-O-(2,2,2-trichloroethyl) phosphate; p between two characters, 3',5'-(2,2,2-trichloroethyl) phosphate; m^2 m^{6}G, N^2,O^6-dimethylguanosine.

Table 3a. Summary of the Stepwise Preparation of Protected Oligoribonucleotides[a]

Reactants						Products		
	Quantity			Quantity			Quantity	
Compound	mg	mmol	Compound	mg	mmol	Compound	mg	% Yield
A	3000	3.58	G	1880	3.98	AG	4260	74
AG	500	0.34	C	148	0.34	AGC	600	83
AG	500	0.34	m^6G	167	0.34	AGm^6G	498	67
AG	500	0.34	e ^6G	172	0.34	AGe ^6G	620	83
AGe ^6G	440	0.20	C	87	0.20	AGe ^6GC	320	60
AGe ^6GC	300	0.11	U	35	0.11	AGe ^6GCU	258	72
AG	500	0.34	m^2 m^6G	172	0.34	AGm^2 m^6G	581	78
AGm^2 m^6G	440	0.20	C	87	0.20	AGm^2 m^6GC	360	63
AGm^2 m^6GC	300	0.11	U	35	0.11	AGm^2 m^6GCU	320	8.9
AGGC[b]	110	0.04	U	16	0.05	AGGCU[b]	61	46
AGUC[b]	213	0.08	U	33	0.10	AGUCU[b]	127	50

[a]Column 1 contains the 5'-trityloxyacetyl reactants and A is $TracA^{bz}tOH$. Column 4 contains the incoming nucleoside, all of which are protected at the 2'-OH group with a tetrahydropyranyl residue and all except U are protected at their NH_2 groups with a benzoyl residue. Column 7 contains the trityloxyacetyl products and for example, AG stands for $TracA^{bz}tpG^{bz}tOH$. Three equivalents of pyridinium mono 2,2,2-trichloroethyl phosphate activated by six equivalents of mesitylene sulphonyl 1,2,4-triazolide (MST) in anhydrous pyridine are used in each phosphorylation step. The coupling step to the incoming nucleoside derivative is driven by 1.3 equivalents of MST.

[b]Compounds prepared by Dr. Dirk Alkema.

Table 3b. Summary of the Stepwise Preparation of Protected Oligoribonucleotides[a]

Reactants						Products		
	Quantity			Quantity			Quantity	
Compound	mg	mmol	Compound	mg	mmol	Compound	mg	% Yield
C	3000	4.10	U	1340	4.10	CU	4150	79
G	3000	3.89	C	1680	3.89	GC	3620	65
GC	250	0.18	m^6A	106	0.18	GCm^6A	140	36
GC	250	0.18	m_2^6A	68	0.18	GCm_2^6A	128	36
GC	250	0.18	m^1G	106	0.18	GCm^1G	134	34
GC	400	0.28	m^6G	136	0.28	GCm^6G	311	52
GC	400	0.28	e^6G	140	0.28	GCe^6G	303	50
GC	400	0.28	$m^2 m^6G$	140	0.28	$GCm^2 m^6G$	320	53

[a]For synthetic details, see footnote to Table 3a.

[b]Synthesized during the course of 4th year thesis work, 1981-1982.

Table 4. ^{1}H Chemical Shifts and Coupling Constants for CpU at a Temperature of 71.2°C ± 1.0°C[a]

Resonance	CpU
C(1)H-6	7.794
U(2)H-6	7.819
C(1)H-5	6.036
U(2)H-5	5.870
C(1)H-1'	5.862
U(2)H-1'	5.896
Coupling Constants	
$^{3}J_{1'2'}$	
C(1)H-1'	4.3
U(2)H-1'	4.8

[a] Spectra recorded on a Bruker WM-250 at 250.13 MHz in D_2O containing 0.01 M sodium phosphate buffer and 1.0 M NaCl at pH = 7.0. Chemical shifts are in ppm relative to DSS using t-butanol-OD as an internal reference.

Table 5a. ^{1}H Chemical Shifts of the Alkylated Guanosine Triribonucleotides of GpCpN Series at 70.5° ± 1.0°C[a]

Resonance	GpC	GpCpG[b]	GpCpm^{6}G	GpCpe^{6}G	GpCpm^{2} m^{6}G
G(3)H-8		7.965	8.069	8.063	7.992
G(1)H-8	7.966	7.935	7.919	7.922	7.920
C(2)H-6	7.780	7.712	7.693	7.685	7.651
G(3)H-1'		5.855	5.929	5.930	5.954
C(2)H-5	5.910	5.862	5.874	5.892	5.908
C(2)H-1'	5.913	5.895	5.867	5.866	5.834
G(1)H-1'	5.874	5.832	5.808	5.809	5.798
O^{6}CH$_3$			4.005		3.969
O^{6}CH$_2$CH$_3$				4.425	
O^{6}CH$_2$CH$_3$				1.348	
N^{2}CH$_3$					2.894
$^{3}J_{1',2'}$ Coupling Constants					
G(1)H-1'		4.768	4.998	4.833	4.680
C(2)H-1'		4.549	4.339	5.278	5.275
G(3)H-1'		4.826	4.862	4.998	4.913
C(2)H-6			7.567	7.532	7.573
C(2)H-5			7.772	7.681	7.519

[a]Spectra were recorded on a Bruker WM-250 spectrometer at 250.13 MHz in D_2O containing 0.01 M sodium phosphate buffer and 1.0 M NaCl at pD = 7.0. Chemical shifts are in ppm relative to DSS using t-butanol-OD as an internal reference.

[b]Prepared by Dr. Dirk Alkema.

Table 5b. ^{1}H Chemical Shifts of the Alkylated Guanosine Triribonucleotides of GpCpN Series at 70.5° ± 1.0°C[a]

Resonance	GpC	GpCpA[b]	GpCpm^{6}A[c]	GpCpm^{1}G[c]	GpCpm$_2^6$A[c]
A(3)H-8		8.344	8.273		8.232
A(3)H-2		8.185	8.178		8.095
G(3)H-8				7.946	
G(1)H-8	7.966	7.918	7.905	7.932	7.892
C(2)H-6	7.780	7.701	7.686	7.704	7.683
C(2)H-5	5.910	5.827	5.841	5.844	5.852
A(3)H-1'		6.059	6.051		6.053
G(3)H-1'				5.848	
C(2)H-1'	5.913	5.872	5.855	5.880	5.844
G(1)H-1'	5.874	5.803	5.785	5.824	5.761
m^{6}CH$_3$			3.026		
m^6(CH$_3$)$_2$					3.303
m^{1}CH$_3$				3.380	
$^3J_{1',2'}$ Coupling Constants					
G(1)H-1'					
C(2)H-1'					
G(3)H-1'					

[a]See footnote at the bottom of Table 5a.

[b]Synthesized by Dr. Dirk Alkema.

[c]Synthesized during 4th year thesis work, 1981-1982.

Table 6. ^{1}H Chemical Shifts of the Alkylated Guanosine Triribonucleotides of the AGN Series at 70.5° ± 1.0°C[a]

Resonance	ApG	ApGpG	ApGpGO^6CH_3	ApGpGN^2CH_3 O^6CH_3	ApGpGO^6E
A(1)H-8	8.238	8.212	8.195	8.191	8.196
A(1)H-2	8.186	8.167	8.141	8.144	8.146
G(2)H-8	7.942	7.935	7.871	7.851	7.882
G(3)H-8		7.906	8.032	7.965	8.040
A(1)H-1'	5.967	5.946	5.923	5.929	5.923
G(2)H-1'	5.848	5.837	5.707	5.665	5.711
G(3)H-1'		5.785	5.904	5.910	5.907
O^6CH_3			3.952	3.919	
N^2CH_3				2.881	
OCH_2CH_3					1.308
$^3J_{1',2'}$ Coupling Constants					
A(1)H-1'	5.0	5.3	4.8	4.5	5.4
G(2)H-1'	4.7	5.0	4.7	5.2	4.9
G(3)H-1'		5.0	5.1	5.3	5.4

[a]Spectra recorded on a Bruker WM-250 at 250.13 MHz in D_2O containing 0.01 M sodium phosphate buffer and 1.0 M NaCl at pH = 7.0. Chemical shifts are in ppm relative to DSS using t-butanol-OD as an internal reference.

Table 7. ^{1}H Chemical Shifts of the Alkylated Guanosine Tetramers of the Series ApGpNpC at 70.5° ± 1.0°C[a]

Resonance	ApGpGpC[b]	ApGpe^{6}GpC	ApGpm^{2}m^{6}GpC
A(1)H-8	8.206	8.1963	8.187
A(1)H-2	8.150	8.1512	8.140
G(2)H-8	7.916	7.8917	7.875
G(3)H-8	7.929	8.0327	7.966
C(4)H-6	7.750	7.7021	7.685
C(4)H-5	5.871	5.8386	5.796
A(1)H-1'	5.939	5.9305	5.914
G(2)H-1'	5.770	5.7156	5.686
G(3)H-1'	5.816	5.8815	5.911
C(4)H-1'	5.888	5.8606	5.819
O^6CH_3			3.931
N^2CH_3			2.871
$O\underline{CH_2}CH_3$		*	
$OCH_2\underline{CH_3}$		1.324	
$J_{1',2'}$ Coupling Constants			
A(1)H-1'	5.403	5.507	3.988
G(2)H-1'	5.186	5.686	5.377
G(3)H-1'	4.549	4.192	5.290
C(4)H-1'	4.349	3.397	3.805
C(4)H-6		7.428	7.492
C(4)H-5		7.594	7.793

[a]Spectra recorded on a Bruker WM-250 at 250.13 MHz in D_2O containing 0.01 M sodium phosphate buffer and 1.0 M NaCl at pH = 7.0. Chemical shifts are in ppm relative to DSS using t-butanol-OD as an internal reference.

[b]Synthesized by Dr. Dirk Alkema.

activation.

The activated phosphate solution was added to a pyridinium solution (ca 20 ml) containing TracA^{bz}tOH (3.0 g, 3.98 mmol, 1 equiv.), and the total volume reduced in vacuo to ca 15 ml, sealed under nitrogen and let stand at room temperature in the dark. The phosphorylation reaction was monitored by tlc and had proceeded ca 70% after 1 day (R_f 0.80 → 0.30). Additional MST (100 mg, 0.40 mmol) was added daily until the reaction went to completion in three days. The reaction was quenched with ice (ca 10 g) and was allowed to sit at room temperature for 30 min. The reaction mixture was poured into icewater (150 ml) and extracted into CH_2Cl_2 (4 x 60 ml). The combined organic extracts were washed with water (50 ml) and evaporated in vacuo to a yellow oil.

TracA^{bz}tpO$^-$ was evaporated from anhydrous pyridine (3 x 40 ml) to a final volume of ca 30 ml, and MST (1.25 g, 5.17 mmol, 1.3 equiv.) was added. The mixture was heated gently (30°C) for 0.5 hr. A pyridine solution (ca 15 ml) of the high R_f isomer of HOG^{bz}tOH (1.88 g, 3.98 mmol, 1 equiv.) was added to the activated complex, the final volume reduced in vacuo to ca 25 ml, the reaction mixture sealed under dry nitrogen, and allowed to stand at room temperature in the dark.

Daily monitoring by tlc followed the coupling

reaction (R_f 0.30 → 0.70). MST (50 mg, 0.20 mmol) was added daily to push the reaction to completion. The coupling appeared to be complete after 7 days, and was quenched and worked up as in the previous step. The combined organic extracts underwent co-distillation with toluene (3 x 20 ml) *in vacuo* to remove any remaining pyridine, to produce a sticky yellow foam.

Silica gel chromatography (50 g) was used to purify the foam, with pure $TracA^{bz}tpG^{bz}tOH$ eluting with 4 - 6% CH_3OH/CH_2Cl_2. The product was obtained as a sticky orange foam following evaporation *in vacuo* (4.26 g, 2.93 mmol, 74%).

2.7.1.2 N^6-Benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyl-adenyl (3'(2,2,2-trichloroethyl)-5') N^2-benzoyl-2'-O-tetrahydropyranylguanlyl (3'(2,2,2-trichloroethyl)-5') N^2-benzoyl-N^2,O^6-dimethyl-2'-O-tetrahydropyranylguanosine ($TracA^{bz}tpG^{bz}tpm^2\ m^6G^{bz}\ tOH$)

2,2,2-trichloroethylphosphate (238 mg, 1.03 mmol, 3 equiv.) was converted to its pyridinium salt through evaporation *in vacuo* from anhydrous pyridine (3 x 15 ml). MST (519 mg, 2.06 mmol, 6 equiv.) was added to the pyridinium solution (*ca* 10 ml) and the solution heated (40°C) to activate the phosphate.

After 2 hr the activated solution was added to a pyridinium solution (ca 15 ml) of TracA^{bz}t$\underline{p}G^{bz}$tOH (500 mg, 0.344 mmol, 1 equiv). The total volume was reduced to ca 15 ml, sealed under nitrogen in vacuo and let stand in the dark at room temperature. After two days, and daily additions of MST (25 mg, 0.10 mmol) the phosphorylation reaction was complete (R_f 0.70 → 0.35). The reaction was quenched with ice (ca 5g), stood at room temperature for 0.5 hr,and then poured into ice water (30 ml) and extracted into CH_2Cl_2 (4 x 25 ml). The combined organic extracts were washed with water (25 ml) and reduced to a yellow oil in vacuo.

TracA^{bz}t$\underline{p}G^{bz}$t$\underline{p}O^-$ was evaporated from anhydrous pyridine (3 x 20 ml), final volume ca 15 ml. MST (104 mg, 0.413 mmol, 1.3 equiv.) was added, and the mixture heated (30°C) for 0.5 hr. A pyridine solution (ca 10 ml) containing the low R_f isomer of HO$m^2m^6G^{bz}$tOH (172 mg, 0.344 mmol, 1 equiv.) was added, the final volume reduced to ca 15 ml in vacuo, the reaction sealed under nitrogen and let stand in the dark at room temperature.

The coupling was monitored daily by tlc; MST (20 mg, 0.1 mmol) was added daily to drive the reaction to completion. After 14 days, the reaction appeared ca 85% complete (R_f 0.35 → 0.62) and was quenched and worked up as before. Evaporation from

toluene (3 x 10 ml) removed any traces of pyridine, to yield a yellow oil.

Silica gel column chromatography (15 g) was used to purify the product. Pure TracA^{bz}tpG^{bz}tpm^{2},m^{6}G^{bz}tOH eluted with 3 - 6% CH_3OH/CH_2Cl_2, and was obtained as a sticky yellow foam after evaporation in vacuo (581 mg, 0.27mmol, 78%).

2.7.2 Synthesis of TracA^{bz}tpG^{bz}tpm^{6}G ^{bz}tpC^{bz}tpUtOH

Block Synthesis (see Figure 11, Tables 8 - 10)

Stepwise synthesis is very useful in the production of short nucleotide sequences; however, as sequence length increases, final yields of the desired oligomer decrease, partially due to incomplete coupling and /or pure product isolation after each coupling step, and partially due to the slowing reaction rates as molecular size of the reactants increase. Thus in the preparation of longer sequences (5 or more nucleotides) it is often useful to use the block synthesis technique (Werstiuk & Neilson, 1972) (see Figure 11, Table 8).

To synthesize AGm^{6}GCU in this manner, it is first necessary to synthesize the building blocks, TracA^{bz}tpG^{bz}tpm^{6}G^{bz}tOH and TracC^{bz}tpUtOH by the stepwise method.

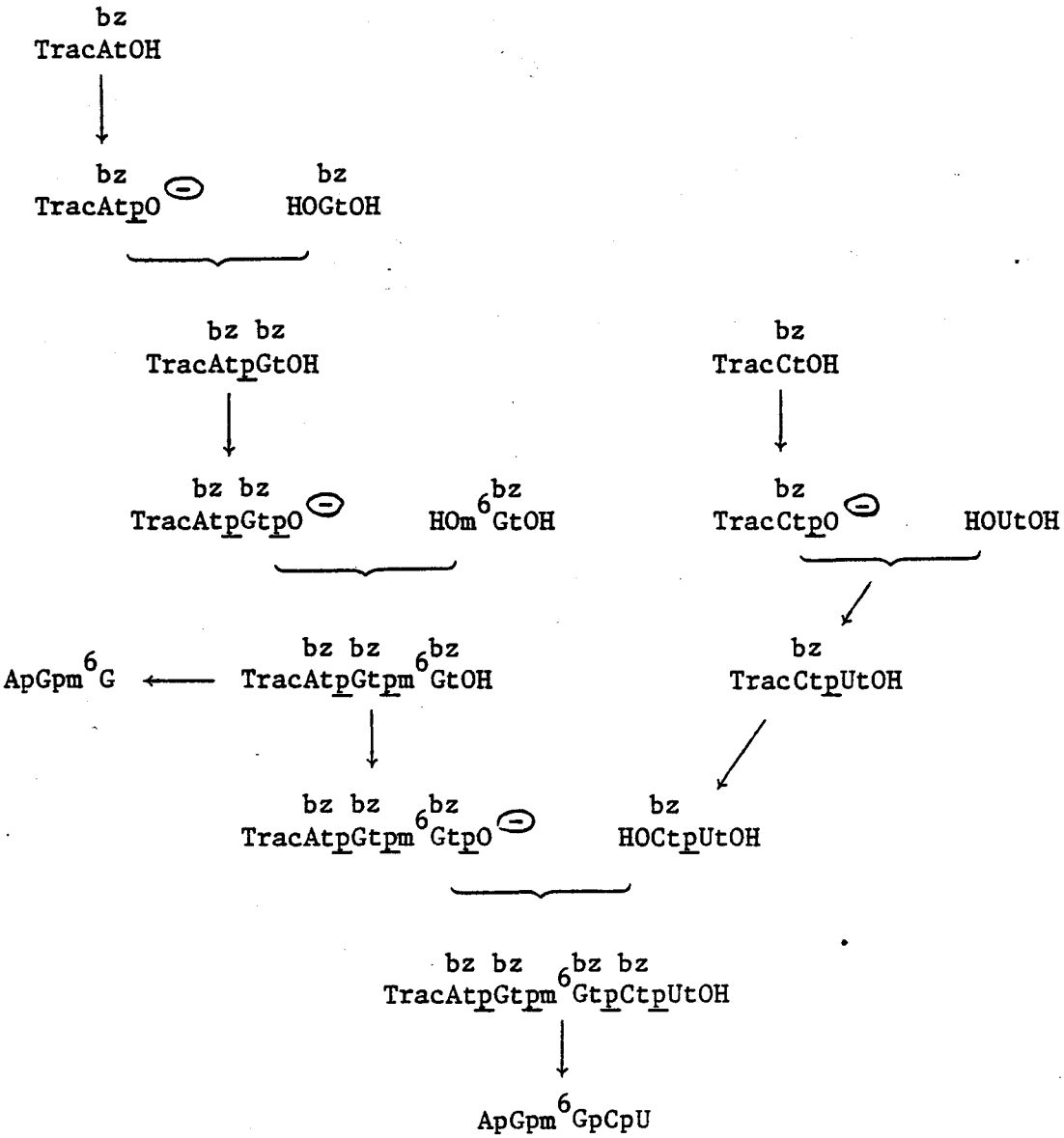


Figure 11. Schematic representation for the chemical block synthesis of the pentaribonucleotide ApGpm^{6}GpCpU. Abbreviations as in Figure 10 and in addition m^{6}G, O^6-methylguanosine.

Table 8. Summary of the Block Synthesis of Protected Oligoribonucleotides[a]

Reactants						Products		
	Quantity			Quantity			Quantity	
Compound	mg	mmol	Compound	mg	mmol	Compound	mg	% Yield
AGC	600	0.28	CU	280	0.28	AGCCU	594	54
AGm^6G	300	0.14	CU	136	0.14	AGm^6GCU	155	33
$AGe\ ^6G$	300	0.14	CU	136	0.14	$AGe\ ^6GCU$	160	34
$AGm^2\ m^6G$	300	0.14	CU	136	0.14	$AGm^2\ m^6GCU$	210	45

[a]Column 1 contains the 5'-trityloxyacetyl reactants and A is $TracA^{bz}tOH$. Column 4 contains the incoming nucleoside, all of which are protected at the 2'-OH group with a tetrahydropyranyl residue and all except U are protected at their NH_2 groups with a benzoyl residue. Column 7 contains the trityloxyacetyl products and for example, AG stands for $TracA^{bz}tpG^{bz}tOH$. Three equivalents of pyridinium mono 2,2,2-trichloroethyl phosphate activated by six equivalents of mesitylene sulphonyl 1,2,4-triazolide (MST) in anhydrous pyridine are used in each phosphorylation step. The coupling step to the incoming nucleoside derivative is driven by 1.3 equivalents of MST.

[b]Compounds prepared by Dr. Dirk Alkema.

Table 9. ^{1}H Chemical Shifts of the Alkylated Guanosine Pentamers of the Series ApGpNpCpU at 70.5° ± 1.0°C[a]

Resonance	ApGpGpCpU	ApGpm^{6}GpCpU	ApGpe ^{6}GpCpU	ApGpm^2 m^{6}GpCpU
A(1)H-8	8.210	8.199	8.199	8.189
A(1)H-2	8.164	8.154	8.152	8.156
G(2)H-8	7.914	7.887	7.896	7.877
G(3)H-8	7.928	8.017	8.029	7.960
C(4)H-6	7.752	7.687	7.700	7.670
U(5)H-6	7.773	7.749	7.751	7.740
C(4)H-5	5.853	5.829	5.822	5.759
U(5)H-5	5.823	5.797	5.802	5.791
A(1)H-1'	5.942	5.927	5.929	5.909
G(2)H-1'	5.780	5.710	5.710	5.689
G(3)H-1'	5.839	5.868	5.880	5.891
C(4)H-1'	5.910	5.853	5.856	5.816
U(5)H-1'	5.892	5.868	5.871	5.840
O^{6}CH$_3$	-	3.959	-	3.933
O^{6}CH$_2$CH$_3$	-	-	*	
O^{6}CH$_2$CH$_3$	-	-	1.322	
N^{2}CH$_3$	-	-	-	2.869
$J_{1',2'}$ Coupling Constants				
A(1)H-1'		5.415	5.544	5.035
G(2)H-1'		5.357	5.674	5.501
G(3)H-1'		3.252	4.184	3.852
C(4)H-1'		4.142	3.434	4.438
U(5)H-1'		3.252	4.096	3.420
C(4)H-5		7.775	7.640	7.429
C(4)H-6		7.499	7.631	7.592
U(5)H-5		8.291	8.145	8.135
U(5)H-6		8.161	8.093	8.062

[a]Spectra recorded on a Bruker WM-250 at 250.13 MHz in D_2O containing 0.01 M sodium phosphate buffer and 1.0 M NaCl at pH = 7.0. Chemical shifts are in ppm relative to DSS using t-butanol-OD as an internal reference.

*Beneath D_2O peak.

Table 10. ^{1}H Chemical Shifts of ApGpCpCpU in D_2O at 70.5° ± 1.0°C[a]

Resonance	ApGpCpCpU
A(1)H-8	8.245
A(1)H-2	8.170
G(2)H-8	7.926
C(3)H-6	7.713
C(4)H-6	7.809
U(5)H-6	7.796
A(1)H-1'	5.977
G(2)H-1'	5.780
C(3)H-1'	5.844
C(4)H-1'	5.892
U(5)H-1'	5.912
C(3)H-5	5.799
C(4)H-5	5.979
U(5)H-5	5.843

[a]Chemical shifts were recorded on a Bruker WM-250 spectrometer at 250.13 MHz and are reported in ppm relative to DSS using t-butanol-OD as an internal reference.

TracA^{bz}t$\underline{p}$G^{bz}t$\underline{p}$m^{6}G^{bz}tOH was phosphorylated as described above for the stepwise synthesis. In order to couple the required dimer to this trimer however, the 5'-O-triphenylmethoxyacetyl (Trac) blocking group must first be removed selectively from TracC^{bz}t$\underline{p}$UtOH. Dissolving of the sequence which is to be deprotected in a 1% NH_4OH/CH_3OH solution (v/v, 1 ml solution per 10 mg of oligomer) achieves this requirement.

The specific deprotection is followed by tlc, as the yellow spot generated by the Trac group, moves to the solvent front, and a brown spot appears below the yellow spot of the fully protected sequence (R_f 0.72 → 0.46 and 0.42 (isomers)). The reaction is quenched after approximately 1.5 hrs, or at the point at which the N-benzoyl blocking groups begin to be cleaved (R_f 0.34), by evaporation $\underline{in\ vacuo}$. The dimer HOC^{bz}t$\underline{p}$UtOH is purified by silica gel chromatography. The free 5'-hydroxyl moiety of the dimer is now free to couple with the phosphorylated trimer, to yield the desired pentamer TracA^{bz}t$\underline{p}$G^{bz}t$\underline{p}$m^{6}G^{bz}t$\underline{p}$C^{bz}t$\underline{p}$UtOH.

While theoretically, block synthesis should have produced the desired pentamers in higher yields, in practice, it was found that improved yields were produced in instances in which the oligomers were synthesized in a stepwise fashion (see Tables 3 and 8).

2.8 Deprotection (Table 11)

All protected oligoribonucleotides were deprotected in a similar fashion (England & Neilson, 1976). Ten or twenty milligrams of the protected oligomer was dissolved in 1 ml of dry dimethylformamide and Cu/Zn couple (ca 10-20 mg). The reactions were stirred for 2 hr at 50°C and monitored by tlc. The reaction was considered to be complete when the tlc spot did not move off the origin (R_f 0.0). Methanolic ammonia (CH_3OH/concNH_4OH, 1/1, v/v or CH_3OH/concNH_4OH/H_2O, 1/1/1, v/v/v) was added, the reaction vessel sealed tightly, and allowed to stir at room temperature for one day. The choice of solvent depended on the identity of the compound being deblocked. Those compounds containing O^6-ethyl or N^2,O^6-dimethyl residues were deprotected better in a more aqueous system.

The mixture was filtered to remove the Cu/Zn couple, and washed with 1 N NH_4OH and water. Chelex-100 (NH_4^+ form) was added to the combined filtrate washes, and stirred for 1 hr. The Chelex was filtered off and washed with 1 N NH_4OH and water. The combined filtrate and washings were evaporated in vacuo to a waxy residue. The partially deprotected oligomer was purified by paper chromatography

Table 11. Chromatographic Data and Deprotection Yields

Compound[a]	R_f[b]	% Yield[c]
ApG	0.40	75.0
CpU	0.67	67.5
GpC	0.50	41.4
ApGpC	0.36	60.0
ApGpm^{6}G	0.27[d]	38.9
ApGpe ^{6}G	0.29[d]	36.7
ApGpm^2 m^{6}G	0.40[d]	42.2
GpCpm^{6}A	0.45	41.1
GpCpm$_2^6$A	0.48	47.0
GpCpm^{1}G	0.19	47.8
GpCpm^{6}G	0.32[d]	37.8
GpCpe ^{6}G	0.43[d]	36.1
GpCpm^2 m^{6}G	0.55[d]	33.3
ApGpCpCpU	0.17	75.0
ApGpm^{6}GpCpU[e]	0.15	33.2
ApGpe ^{6}GpCpU[e] (block)	0.15	11.7
ApGpm^2 m^{6}GpCpU[e] (block)	0.13	23.3
ApGpe ^{6}GpC (stepwise)	0.30	30
ApGpe ^{6}GpCpU[e] (stepwise)	0.15	
ApGpm^2 m^{6}GpC (stepwise)	0.31	65
ApGpm^2 m^{6}GpCpU[e] (stepwise)	0.13	36

[a]Refers to the free, deblocked, oligoribonucleotide.

[b]Chromatography system: 1.0 M ammonium acetate/ethanol (50/50, v/v) on Whatman #1 or #40 paper.

[c]Calculated from UV spectrophotometric data assuming a 90% hypochromicity factor.

[d]Fluorescent blue bands.

[e]Purified by chromatography on Whatman #1 initially, and then subjected to HPLC purification.

(descending technique) on Whatman #1 paper * using CH_3CH_2OH/1 M NH_4Ac (pH 7.3) (1/1,v/v). The appropriate band was desalted for 1 hr in absolute ethanol, immersed in anhydrous ether for 15' for drying, and then eluted with glass distilled water (descending technique) overnight. The optical density of the eluted solutions was checked, and the solutions acidified to pH 2 with 2N HCl and allowed to stand for two days at room temperature.

The solutions were neutralized with 1N NH_4OH, evaporated to dryness in vacuo, and purified on Whatman # 1 or 40 paper**, as described earlier.

The pentaribonucleotides obtained were further purified using a Waters HPLC unit (see page 17 for details).

*In instances in which the partially deprotected material was difficult to elute from the chromatography paper with water, due to solubility problems, this step of the purification was eliminated. Instead, following evaporation to dryness in vacuo the residue was resuspended and acidified as described above.

**The choice of Whatman #1 or #40 paper in the final purification step depended on the time at which a particular deblocking was done. The quality of Whatman #40 paper changed during the second year of thesis work; thereafter only Whatman #1 paper for

purification.

2.9 Nature of the ^{1}H-NMR Studies

The ^{1}H-NMR spectra required for this study were obtained from a Bruker WM-250 spectrometer or a WH-400 spectrometer, operating in the Fourier Transform (FT) mode, and equipped with quadrature detection. Probe temperatures were maintained to within $\pm$ 1 C by Bruker Variable Temperature units and calibrated by thermocouple measurements. The samples were lyophilized twice from D_2O and then dissolved in 100% D_2O (Aldrich) containing 0.01 M sodium phosphate buffer (pD *ca* 7.2) and 1.0 M sodium chloride.

t-Butanol-OD was used as an internal reference (1.231 ppm) and chemical shifts reported relative to sodium 2,2-dimethyl -2-disilapentane-5-sulphonate (DSS). The field/frequency lock was provided by the deuterium signal of D_2O.

The main advantage of ^{1}H-NMR spectroscopy over other solution techniques such as CD or UV spectroscopy, is the ability to monitor changes in local microenvironments by recording molecular events at specific atomic sites within the molecule, rather than by monitoring the molecule as a whole (Martin, *et al.*, 1971; Borer, *et al.*, 1975; Grosjean, *et al.*, 1976; Romaniuk, *et al.*, 1979; Alkema, PhD. thesis,

1982).

The absorbance of electromagnetic energy by protons in a strong magnetic field yields a particular chemical shift, whose value is influenced by the nucleus' enviroment. Fourier Transform (FT), a form of broad band NMR spectroscopy, allows all nuclei to be excited simultaneously and through a mathematical operation converts magnetic free induction decay on a time domain to a frequency domain spectrum, which is plotted to yield chemical shift values for the nuclei studied (Silverstein & Bassler,1967; Abraham & Loftus. 1979). Examination of chemical shifts, coupling constants, line widths, and peak assignments provide conformational information about the changing enviroment of each individual base pair involved in the coil → helix transition (70^{o} - 0^{o}C) (Romaniuk,et al.,1978; Neilson, et al., 1980). Peak assignments of the non-exchangeable proton resonances (GH-8, AH-2,H-8, CH-5,H-6, UH-5,H-6, H-1's of the ribose moieties, and alkyl group protons) are made possible through use of incremental analysis : the comparison of a sequence-related series of oligomers, where each member of the series is incremented one nucleotide unit from the previous one. Changes in spectra from one oligomer to the next larger one, are due to the appearance of the resonances of the additional nucleoside and its shielding effects on the

neighboring protons (Borer, et al., 1975; Alkema, PhD thesis, 1982; Alkema, et al., 1982).

The base ratios of the oligoribonucleotide sequences are available from the distinct aromatic signals at low field. Sequence integrity is checked by incremental analysis (Borer, et al., 1975), which involves checking each intermediate in the stepwise synthesis of a sequence. Since sequences are built in the 5' → 3' direction only, there is no chance of an incorrect sequence being made. NMR analysis also provides a method for checking purity of a compound.

In order to ensure that no secondary structure interactions occured, the samples were checked at 70°C. The spectra were taken at intervals over the temperature range 70° - 0°C in order to study duplex formation. Graphs of "chemical shift" versus "temperature" yield families of melting curves characteristic of the compound being studied. Upfield shifts are usually indicative of general strand stacking, while downfield shifts usually imply looping out.

Duplex formation is indicated by a series of sigmoidal melting curves signifying upfield movement of chemical shifts with decreasing temperature, and by approach of $J_{1',2'}$ coupling constants to zero (Romaniuk, et al., 1978; Neilson, et al., 1980).

3. Results and Discussion

3.1 Preparation of O^6-Alkylated Guanosine Derivitives Ready for Sequence Insertion

(refer to Figures 5 - 9)

N^2-Benzoyl-N^2,O^6-dimethyl-2',3',5'-O-triacetyl guanosine (3) was prepared by the addition of a cold ethereal diazomethane solution to a stirring solution of N^2-benzoyl-2',3',5'-O-triacetylguanosine (1), at 0°C. Characterization of (3) by mass spectroscopy showed the molecular ion at m/e 541; combustion analysis produced the expected atomic ratio : C 55.22, H 5.12, N 12.73.

(3) was subjected to very mild base hydrolysis conditions (1% concNH_4OH/CH_3OH, v/v) for one day to facilitate de-O-acetylation, to give N^2-benzoyl-N^2, O^6-dimethylguanosine (5). The presence of the molecular ion at m/e 415 and the combustion analysis (C 52.94, H 5.41, N 16.14) served to identify the product.

(3) was also subjected to more severe base hydrolysis conditions (50% concNH_4OH/CH_3OH, v/v) for two days to facilitate full deprotection, to produce

N^2,O^6-dimethyl guanosine (7). The molecular ion at m/e 311, along with the combustion analysis (C 42.71,H 5.73, N 20.72) characterized the product, which crystallized with 1.5 moles of water.

The 3',5'-disiloxanedilyl nucleoside (11) was prepared by the addition of 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane to a stirred solution of N^2-benzoyl-N^2,O^6 dimethylguanosine(5) in dry pyridine.

Reaction of (11) with dihydropyran in dioxane containing p-toluene sulfonic acid monohydrate, yielded the corresponding 2'-O-tetrahydropyranyl derivitive (12). Despite the presence of two diastereomers, due to the generation of a chiral center at C-1' only one spot was seen on the tlc plate.

The 3',5'-O-disiloxanedilyl group was removed by reaction with tetrabutylammonium fluoride in tetrahydrofuran, to yield two diastereomers of N^2,O^6-dimethylguanosine (13).

N^2-Benzoyl-O^6-methyl-2',3',5'-O-triacetyl-guanosine (2) was isolated using two different procedures.

A. The first involved diazomethane treatment of N^2-Benzoyl-2',3',5'-O-triacetylguanosine (1), as described for the synthesis of the corresponding N^2,O^6-dimethyl compound.

B. Product (2) was more successfully isolated

through a series of nucleophilic substitutions at the O^6 position of (1). This set of reactions was adapted from Gaffney,B.L. & Jones,R.A., 1982, and modified to improve overall yield (ie. one equivalent of 4,4-dimethylaminopyridine, was used, all reactions were performed under a dry nitrogen stream).

Reaction of (1) with 2,4,6-triisopropylbenzene sulfonyl chloride and 4,4-dimethyl aminopyridine produced the corresponding O^6-2,4,6-triisopropyl-benzene sulfonyl-nucleoside. Addition of a methylene chloride solution containing trimethylamine and absolute methanol facilitated production of the charged C^6-trimethylammonium species. Addition of 1,8-diazabicyclo-(5.4.0)-undec-7-ene facilitated displacement of the trimethylamino group by methanol, to give the desired product (2). The mass spectrum of (2) revealed the expected molecular ion at m/e 527; combustion analysis further characterized the product (C 54.47, H 4.82, N 13.06).

Compound (2) was subjected to mild basic conditions (1% concNH_4OH/CH_3OH, v/v) for two days to allow de-O-acetylation to occur. The product N^2-benzoyl- O^6-methylguanosine (4) was identified by mass spectroscopy (molecular ion at m/e 401); combustion analysis revealed that the compound crystallized with one mole of water (C 53.66, H 4.95, N 17.18).

(2) was also subjected to 50% concNH_4OH/CH_3OH (v/v) for two days to facilitate full deprotection. The product, O^6-methylguanosine (6) was characterized by the presence of its molecular ion at m/e 297, and by combustion analysis (C 44.72, H 5.51, N 23.35).

The 3',5'-disiloxanedilyl derivative of N^2-benzoyl-2' -O-tetrahydropyranylguanosine (15) was prepared by the addition of TIPS-Cl to a stirred solution of (14) in dry pyridine, to yield N^2-benzoyl-2'-O-tetrahydropyranyl-3',5'-O -tetraisopropyldisiloxylguanosine (15).

Three routes were attempted to prepare N^2-benzoyl -O^6-methyl-2'-O-tetrahydropyranylguanosine (17).

A). N^2-Benzoyl-O^6- methylguanosine (4) was blocked at the 3' and 5' hydroxyls in a manner similar to that described for compound (5), to yield N^2-benzoyl-O^6-methyl-3',5'-O -tetraisopropyldisiloxylguanosine (8).

(8) was protected at the 2' hydroxyl by a tetrahydropyranyl blocking group, in conditions similar to those described for compound (11). The reaction was very sluggish however; excess 2,3-dihydropyran was added to force the reaction (R_f 0.38 → 0.92, in 2% CH_3OH/CH_2Cl_2). Attempts to remove the 3',5'-O-disiloxanedilyl group from the crude product were futile, despite the addition of a vast

excess of fluoride reagent (TBAF). It was thought that the acidic conditions used to facilitate thp addition lead to cleavage of one or both silyl ether bonds, followed by nucleophilic attack by the free hydroxyl(s) on 2,3-dihydropyran molecules. As better methods were devised to synthesize the desired compound(17), this route was abandoned.

B). N^2-Benzoyl-2'-O-tetrahydropyranyl-3',5'-O-tetraisopropyl disiloxylguanosine (15) was subjected to a series of nucleophilic substitution displacement reactions at C^6, similar to those described in the synthesis of N^2-benzoyl-O^6-methyl-2',3',5'-O-triacetylguanosine (2) from compound (1), to produce N^2-benzoyl-O^6-methyl-2'-O-tetrahydropyranyl-3',5'-O-tetraisopropyl-disiloxylguanosine (16).

Removal of the cyclic disiloxane from compound (16) was facilitated by addition of tetrabutylammonium fluoride to a stirring nucleoside-THF solution, to obtain product (17).

C). N^2-Benzoyl-3',5'-O-diacetylguanosine (18) was dissolved in a dioxane solution containing p-TSOH and 2,3-DHP to yield a mixture of diastereomers of (19), N^2-benzoyl-2'-O-tetrahydropyranyl-3',5'-O-diacetyl guanosine. The isomers of (19) underwent a series of nucleophilic displacements at C^6, as described for the synthesis of (2), to produce

N^2-benzoyl-O^6-methyl-2'-O-tetrahydropyranyl -3',5'-O-diacetylguanosine (20).

(20) was de-O-acetylated using two different methods (pilot reactions). In the first, a 50/50 (v/v) mixture of absolute ethanol/2 N NaOH was added to a stirring ethanol/pyridine solution containing compound (20), to isolate the two isomers of (17). The second method employed a stirring methanol/ammonia solution (10% concNH_4OH/CH_3OH, v/v) to dissolve and de-O-acetylate the blocked nucleoside (20). As the conditions employed in the second method were milder, the methanolic/ammonia procedure was used to de-O-acetylate the remainder of compound (20), to yield the isomers of (17).

N^2-Benzoyl-2'-O-tetrahydropyranyl-3',5'-O-tetraisopropyldisiloxylguanosine (15) underwent a series of nucleophilic substitution reactions, as in the synthesis of compound (17), to produce the corresponding O^6-ethyl guanosine derivative (21). The choice of alcohol in the final substitution step determined the identity of the product. Removal of the cyclic disiloxane protecting group from (21) yielded N^2-benzoyl-O^6-ethyl-2'-O-tetrahydro-pyranylguanosine (22).

3.2 Methylated Bases Stabilize Short RNA Duplexes

The triribonucleotide GpCpA forms a stable duplex containing two G:C Watson-Crick base pairs and two 3'- dangling adenosine residues (Alkema, et al., 1981(a); Alkema, et al., 1981(b)). Dangling bases have been found to increase base stacking and thus improve overall duplex strength (Alkema, et al., 1981(a); Alkema, et al., 1981(b); Martin, et al., 1971:; Neilson, et al., 1980). In an earlier study, GpCpN sequences (where N = m^6A, $m^6{}_2A$, m^1G) were used as model systems to examine the effect of purine heterobase N-alkylation on duplex stability (D'Andrea, 1982; D'Andrea, et al., 1983). The results indicated that the stability of the RNA duplexes

$$\begin{array}{l} 5'\text{-}\overrightarrow{GpCpN}\text{-}3' \\ \quad\vdots\quad\vdots \\ 3'\text{-}N\underleftarrow{pCpG}\text{-}5' \end{array}$$

increased in the order N = A < m^6A < m^1G < $m^6{}_2A$ (see Tables 12 - 15, Figure 12). These studies implied that methylation of the 3'-dangling residue enhanced base stacking and duplex stability, with the site and degree of modification being significant factors.

The present study extends this model system to investigate the consequences of guanosine O^6-alkylation on duplex stability. Examination of GpCpN sequences in which N = m^6G, e^6G, m^2m^6G, revealed increasing stabilizing potential in the order N = m^6G

Table 12. ^{1}H NMR Chemical Shift Assignments for GCA (7.3 mM) over the Temperature Range 70°-0°C

Resonance	69.7°	59.9°	50.0°	45.0°	40.3°	36.0°	30.1°	19.6°	7.8°	0.0°	T_m, °C
AH-8	8.344	8.338	8.321	8.302	8.282	8.255	8.220	8.158	8.107	8.055	30.0°
AH-2	8.185	8.159	8.108	8.061	8.007	7.944	7.861	7.720	7.621	7.541	32.6°
GH-8	7.918	7.922	7.932	7.942	7.957	7.972	7.994	8.029	8.050	8.078	32.4°
CH-6	7.701	7.696	7.687	7.678	7.671	7.663	7.654	7.640	7.630	7.626	39.3°
AH-1'	6.059	6.054	6.048	6.042	6.039	6.035	6.031	6.024	6.014	6.000	NSB[a]
CH-1'	5.872	5.856	5.826	5.796	5.769	5.734	5.686	5.611	5.553	5.524	32.6°
GH-1'	5.803	5.798	5.796	5.796	5.800	5.805	5.813	5.823	5.824	5.815	31.1°
CH-5	5.827	5.781	5.706	5.645	5.583	5.515	5.431	5.304			37.8°
											av 33.7°

[a]NSB = no sigmoidal behaviour.

Table 13. ^{1}H NMR Chemical Shift Assignments for GCm^{6}A (7.3 mM) over the Temperature Range 70°-0°C

Resonance	69.7°	59.9°	50.0°	45.0°	40.3°	36.0°	30.1°	19.6°	7.8°	0.0°	T_m, °C
m^{6}AH-8	8.273	8.264	8.245	8.223	8.199	8.174	8.132	8.041	8.040	8.016	33.2°
m^{6}AH-2	8.178	8.147	8.092	8.035	7.977	7.915	7.828	7.705	7.629	7.565	34.9°
GH-8	7.905	7.908	7.923	7.938	7.955	7.976	8.002	8.082	8.069	8.079	33.2°
CH-6	7.686	7.681	7.675	7.669	7.662	7.657	7.645	7.639	7.637	7.637	37.3°
m^{6}AH-1'	6.051	6.045	6.041	6.036	6.033	6.032	6.023	6.024	6.022	6.018	NSB
CH-1'	5.855	5.838	5.808	5.778	5.744	5.706	5.645	5.573	5.515	5.515	32.9°
GH-1'	5.785	5.780	5.782	5.786	5.793	5.801	5.811	5.820	5.827	5.822	34.3°
CH-5	5.841	5.796	5.724	5.658	5.590	5.522	5.427	5.313	5.241	5.188	37.6°
CH_3	3.026	3.012	2.993	2.976	2.960	2.944	2.919	2.882	2.850	2.838	33.8°
											av 34.7°

Table 14. ^{1}H NMR Chemical Shift Assignments for GCm_2^6A (7.3 mM) over the Temperature Range 70°-0°C

Resonance	69.7°	59.9°	50.0°	45.0°	40.3°	36.0°	30.1°	19.6°	7.8°	0.0°	T_m, °C
m_2^6AH-8	8.232	8.222	8.197	8.175	8.153	8.135	8.109	8.036			41.9°
m_2^6AH-2	8.095	8.055	7.975	7.909	7.844	7.788	7.713	7.602			41.4°
GH-8	7.892	7.900	7.920	7.940	7.960	7.982	8.006	8.078			41.4°
CH-6	7.683	7.679	7.668	7.658	7.647	7.639	7.626	7.611			39.1°
m_2^6AH-1'	6.053	6.047	6.040	6.035	6.031	6.029	6.025	6.018			56.0[a]
CH-1'	5.844	5.825	5.783	5.745	5.702	5.662	5.609	5.534			38.0°
GH-1'	5.761	5.758	5.762	5.767	5.772	5.778	5.778	5.771			43.8°
CH-5	5.852	5.802	5.703	5.623	5.548	5.477	5.390	5.280			41.4°
$(CH_3)_2$	3.303	3.286	3.257	3.237	3.218	3.201	3.183	3.161	3.323,	2.908	45.3°
											av 41.5°

[a]Not included.

Table 15. ^{1}H NMR Chemical Shift Assignments for GCm^{1}G (7.3 mm) over the Temperature Range 70°-0°C

Resonance	69.7°	59.9°	50.0°	45.0°	40.3°	36.0°	30.1°	19.6°	7.8°	0.0°	T_m, °C
m^{1}GH-8	7.946	7.935	7.898	7.863	7.823	7.782	7.731	7.661	7.613	7.582	36.4°
GH-8	7.932	7.939	7.958	7.976	7.994	8.012	8.037	8.067	8.084	8.090	38.1°
CH-6	7.704	7.697	7.683	7.672	7.661	7.652	7.643	7.634	7.632	7.631	45.7°
CH-1'	5.880	5.861	5.822	5.784	5.748	5.715	5.672	5.622	5.594	5.583	39.9°
m^{1}GH-1'	5.848	5.843	5.835	5.834	5.830	5.834	5.830	5.832	5.835	5.837	NSB
GH-1'	5.824	5.821	5.822	5.825	5.830	5.834	5.840	5.844	5.835	5.837	NSB
CH-5	5.844	5.793	5.690	5.610	5.531	5.458	5.368	5.264		5.170	40.8°
CH_3	3.380	3.364	3.327	3.295	3.261	3.228	3.185	3.129	3.089	3.070	38.1°
											av 39.8°

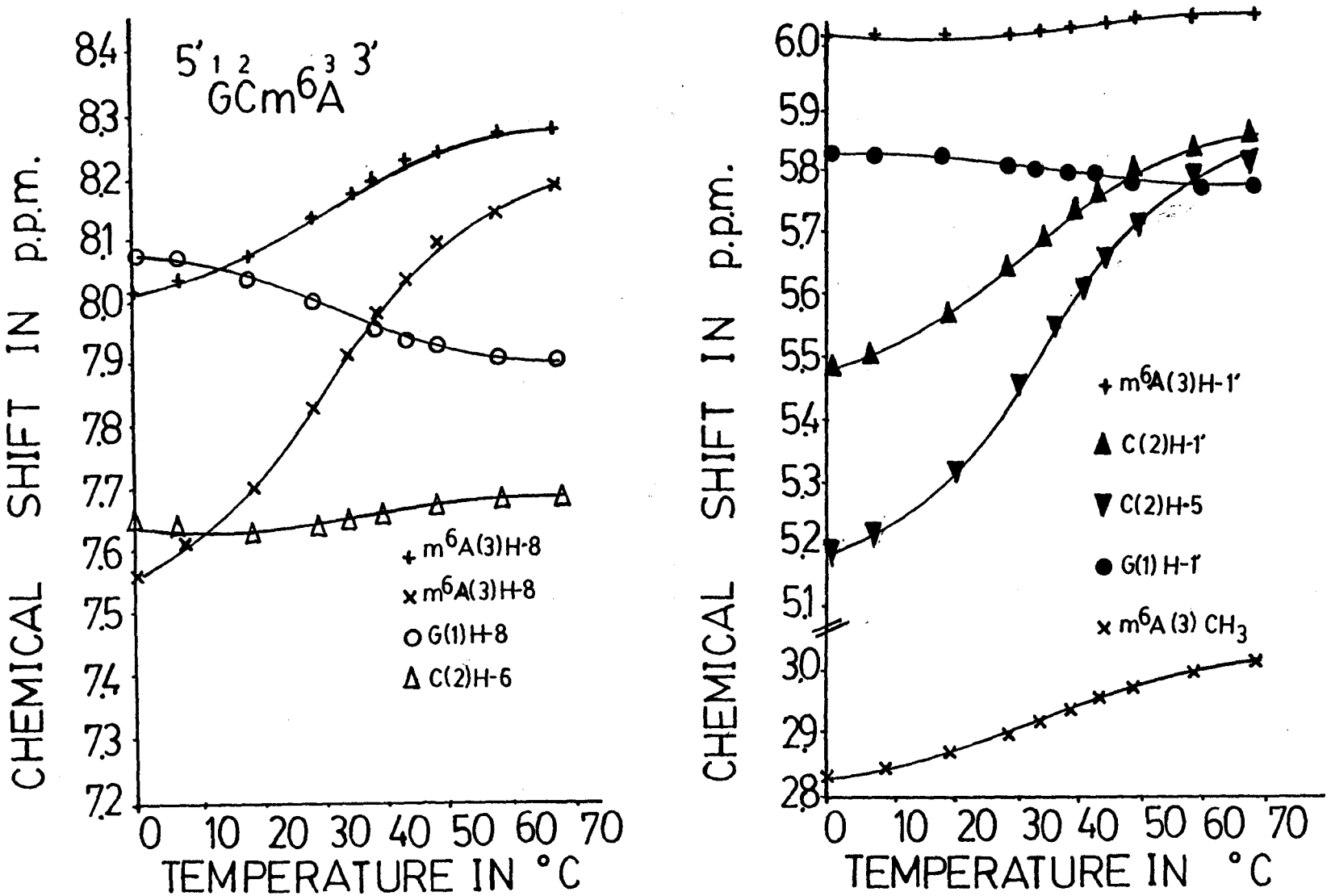


Figure 12. Variable Temperature Study of GCm^6A

< e^6G < m^2m^6G. These experiments support the conclusions of the earlier N-alkylation studies, and in addition, imply that the size of the hydrophobic substituent appears important.

3.2.1 Results

The oligoribonucleotides, GCm^6G, GCe^6G, and GCm^2m^6G were synthesized using the phosphotriester method. Their duplex formation was monitored by variable temperature ^1H NMR spectroscopy (250.13 MHz) and was analyzed by examination of the sigmoidal curves generated by the aromatic, alkyl, and ribose H-1' protons of the nucleoside residues, on "Chemical Shift versus Temperature" plots (see Tables 17 - 19, Figures 13-16). The chemical shift values were assigned at 70°C by comparison to those of GpC and of the appropriate free modified nucleoside. The average melting temperature (Tm) values, obtained at concentrations between 8.0 and 8.8 mM, were as follows : $GpCpm^6G$, 36.2°, $GpCpe^6G$, 37.1°, $GpCpm^2m^6G$, 38.9°C. Melting temperatures were obtained by computer analysis using the first derivative of a fifth-order equation, which is fitted the "chemical shift versus temperature" data points.

Melting temperature (Tm) is the temperature at which the compound under study exists 50% in single

Table 16. ^{1}H NMR Chemical Shift Assignments for GCG over the Temperature Range 70°-0°C

Resonance	70.0°	60.0°	50.8°	45.0°	41.0°	36.0°	31.2°	25.9°	20.0°
G(1)H-8	7.935	7.940	7.949	7.954	7.960	7.967	7.974	7.986	7.995
G(3)H-8	7.965	7.959	7.941	7.928	7.916	7.902	7.886	7.914	7.847
C(2)H-6	7.712	7.705	7.693	7.688	7.680	7.673	7.665	7.660	7.650
C(2)H-1'	5.895	5.877	5.850	5.826	5.812	5.789	5.771	5.748	5.728
G(3)H-1'	5.855	5.848	5.842	5.837	5.834	5.831	5.829	5.827	5.818
G(1)H-1'	5.832	5.827	5.821	5.818	5.815	5.814	5.813	5.820	5.818
C(2)H-5	5.862	5.815	5.741	5.698	5.656	5.611	5.569	5.519	
$J_{1',2'}$ Coupling Constants in Hz									
G(1)H-1'	4.76	5.14	4.95	4.43	4.14	3.84	3.53	3.12	1.50
C(2)H-1'	4.54	5.32	3.77	4.00	2.56	2.95	2.34	1.50	1.50
G(3)H-1'	4.82	4.64	5.39	5.12	5.17	4.38	4.10	3.22	1.50

Table 17. ^{1}H NMR Chemical Shift Assignments for GCm^{6}G (8.0 mM) over the Temperature Range 70°-0°C (105 OD's)

Resonance	70.5°	60.8°	55.0°	49.9°	45.2°	40.0°	35.0°	30.0°	25.1°	20.4°	15.3°	10.2°	0.6°	Tm, °C
G(3)H-8	8.069	8.057	8.044	8.024	7.998	7.964	7.926	7.887	7.854	7.822	7.797	7.777	7.751	34.7°
G(1)H-8	7.918	7.923	7.928	7.934	7.944	7.958	7.974	7.991	8.006	8.019	8.035	8.053	8.065	29.0°
C(2)H-6	7.692	7.686	7.682	7.675	7.668	7.660	7.651	7.644	7.638	7.634	7.630	7.628	7.625	41.5°
G(3)H-1'	5.929	5.923	5.919	5.915	5.910	5.906	5.901	5.897	5.894	5.890	5.887	5.887	5.880	*
CH-5	5.873	5.824	5.782	5.729	5.666	5.589	5.508	5.432	5.370	5.315	5.268	5.244	5.187	38.1°
CH-1'	5.866	5.846	5.827	5.803	5.773	5.738	5.700	5.663	5.633	5.606	5.584	5.568	5.548	37.8°
GH-1'	5.807	5.799	5.794	5.788	5.785	5.782	5.781	5.781	5.784	5.797	5.809	5.818	5.813	*
$O^{6}CH_3$	4.004	3.988	3.976	3.963	3.949	3.933	3.917	3.902	3.890	3.879	3.869	3.860	3.846	43.8°[a]
													avg Tm	36.2°C
$J_{1',2'}$ Coupling Constants in Hz														
G(3)H-1'	4.99	4.91	4.77	4.52	4.27	3.50	3.04	3.18	2.75	2.48	2.11	0	0	
CH-1'	4.33	3.85	4.19	3.52	3.25	2.90	2.31	1.60	0	0	0	0	0	
GH-1'	4.86	4.85	4.66	4.16	3.79	3.52	2.93	1.03	0	0	0	0	0	
CH-6	7.56	7.59	7.56	7.57	7.58	7.57	7.56	7.61	7.54	7.58	7.27	6.87	6.59	
CH-5	7.77	7.38	7.57	7.55	7.58	7.50	7.54	7.50	7.37	7.36	-	-	-	

* non sigmoidal behavior
[a] not used in calculation

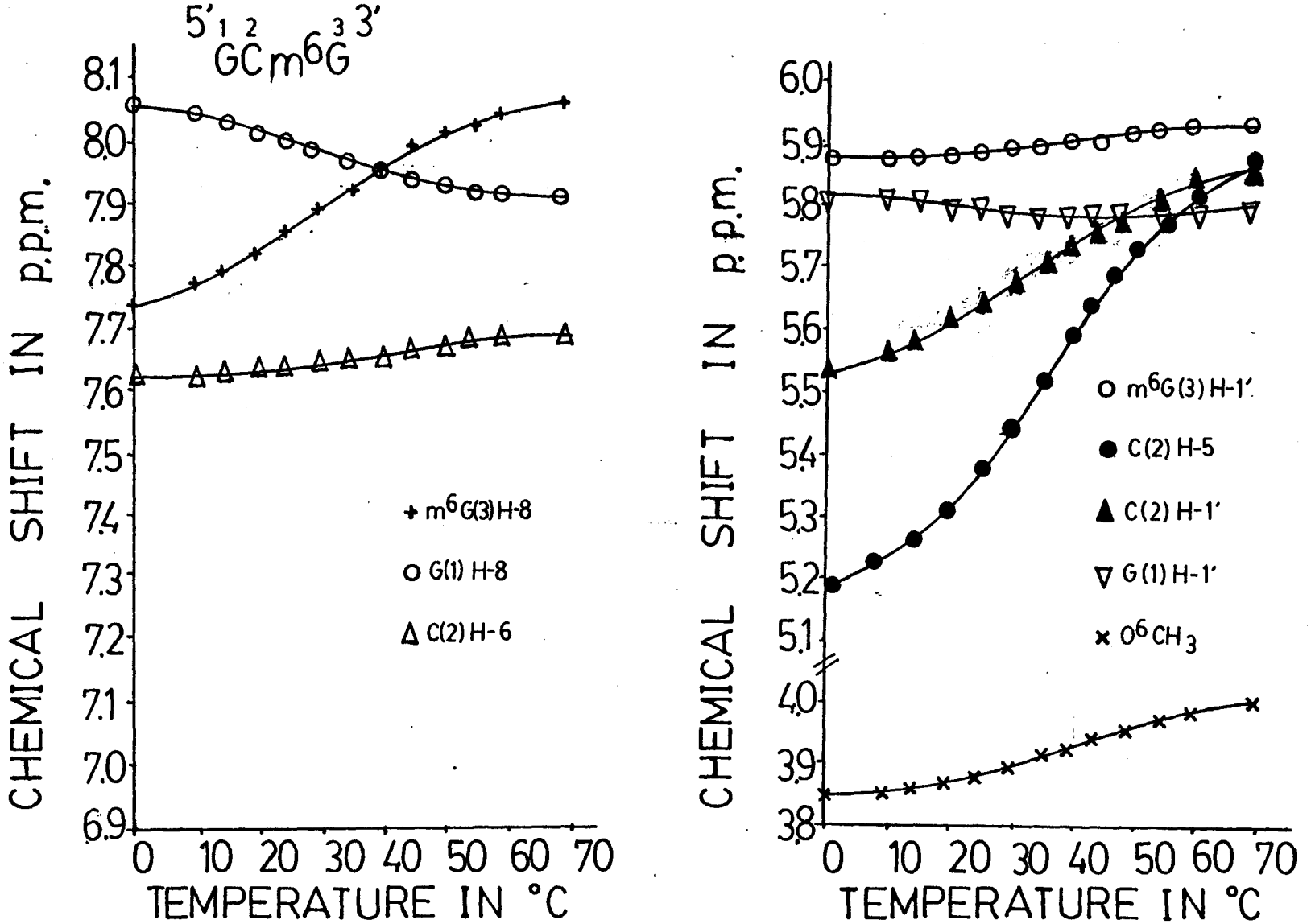


Figure 13. Variable Temperature Study of GCm^{6}G

Table 18. ^{1}H NMR Chemical Shift Assignments for GCe^{6}G over the Temperature Range 70°-0°C

Resonance	70.2°	60.0°	55.3°	50.3°	45.6°	40.7°	35.6°	30.8°	25.8°	21.1°	10.2°	0.8°	Tm, °C
G(1)H-8	7.921	7.924	7.924	7.928	7.932	7.941	7.952	7.964	7.976	7.990	8.036	8.047	*
e G(3)H-8	8.063	8.050	8.036	8.018	7.990	7.956	7.914	7.874	7.835	7.802	7.748	7.725	34.4°
C(2)H-6	7.684	7.676	7.672	7.667	7.659	7.653	7.644	7.637	7.631	7.627	7.624	7.624	40.0°
e G(3)H-1'	5.929	5.923	5.919	5.916	5.910	5.907	5.902	5.898	5.894	5.891	5.886	5.886	*
C(2)H-5	5.892	5.847	5.807	5.759	5.695	5.621	5.535	5.457	5.386	5.326	5.241		38.0°
C(2)H-1'	5.866	5.843	5.830	5.810	5.780	5.748	5.708	5.672	5.639	5.610	5.568	5.554	36.1°
G(1)H-1'	5.808	5.796	5.785	5.775	5.762	5.748	5.735	5.729	5.714	-	-		*
$OCH_2\underline{CH_3}$	1.347	1.340	1.334	1.331	1.325	1.320	1.315	1.312	1.309	1.307		1.305	*
$O\underline{CH_2}CH_3$	4.425	4.399	-	4.366	4.346	4.327	4.307	4.290	5.549	5.326			*
												avg Tm	37.1°C
$J_{1',2'}$ Coupling Constants in Hz													
G(1)H-1'	4.83	4.70	4.28	4.39	4.96	3.38	2.44	1.57	-	-	-		
C(2)H-1'	5.27	5.16	4.36	4.22	4.19	3.38	2.63	1.92	0	0	0		
etG(3)H-1'	4.99	4.82	4.76	4.57	4.29	3.89	2.84	3.00	2.86	2.55	-		
C(2)H-6	7.53	7.62	7.56	7.58	7.64	7.62	7.61	7.60	7.57	7.70	7.01		
C(2)H-5	7.68	7.04	7.03	7.61	7.52	7.59	7.69	7.67	-	-	-		

*non sigmoidal behavior

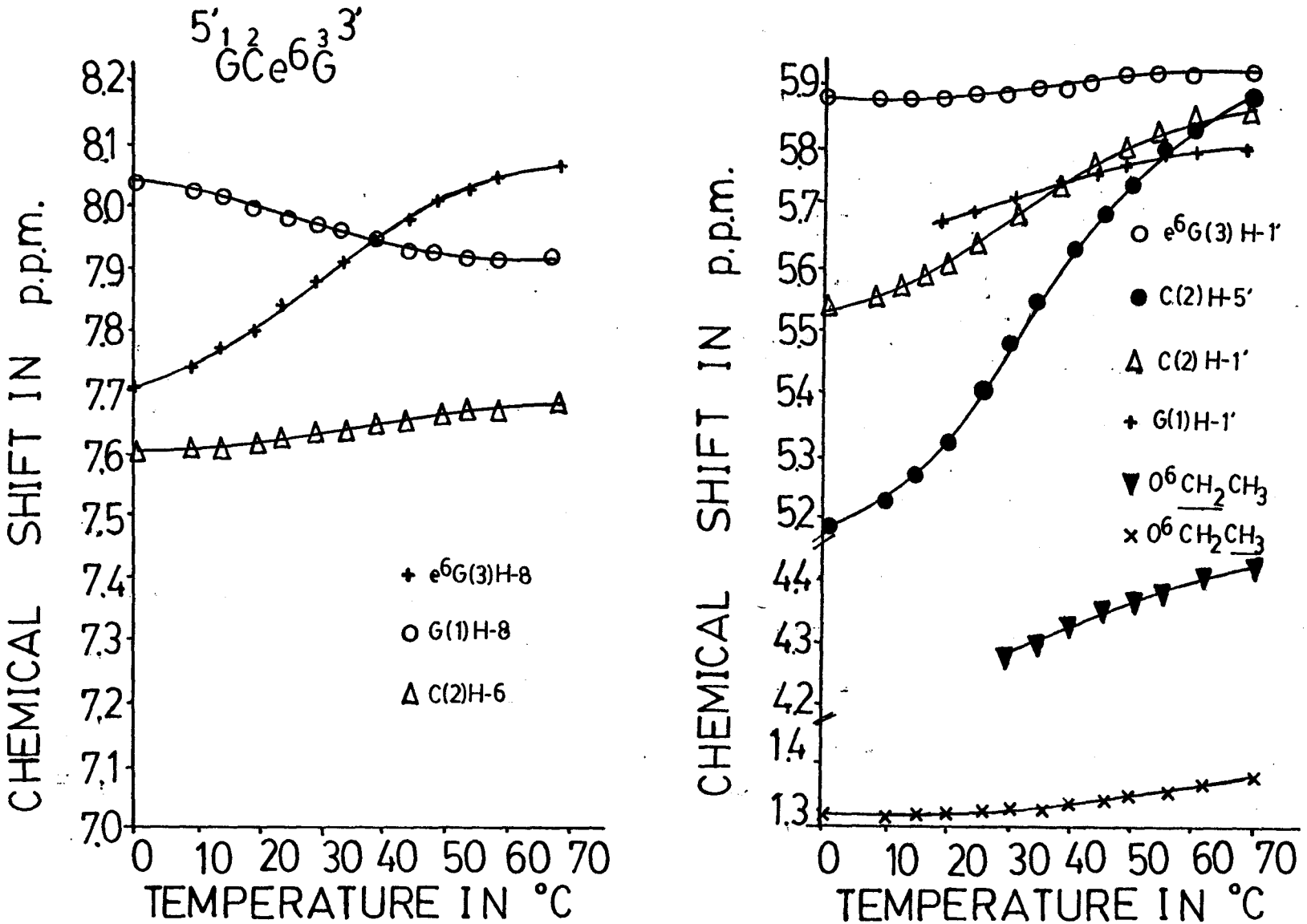


Figure 14. Variable Temperature Study of GCe6G

Table 19. ^{1}H NMR Chemical Shift Assignments for $GCm^2\ m^6G$ over the Temperature Range 70°-0°C (105 OD's)

Resonance	70.5°	60.8°	55.0°	49.9°	45.2°	40.0°	35.0°	30.0°	25.1°	20.4°	15.3°	10.2°	0.6°	Tm, °C
G(3)H-8	7.991	7.978	7.963	7.946	7.916	7.882	7.847	7.812	7.785	7.761	7.738	7.719	7.696	37.3°
G(1)H-8	7.920	7.926	7.935	7.942	7.962	7.982	8.004	8.026	8.044	8.061	8.076	8.086	8.103	35.6°
CH-6	7.650	7.645	7.641	7.636	7.631	7.625	7.619	7.613	7.610	7.607	7.605	7.604	7.602	42.7°
G(3)H-1'	5.953	5.949	5.947	5.945	5.943	5.941	5.939	5.936	5.935	5.933	5.933	5.929	5.923	*
CH-5	5.908	5.860	5.814	5.754	5.683	5.597	5.507	5.426	5.362	5.306	5.252	5.214	5.160	39.8°
CH-1'	5.833	5.813	5.791	5.771	5.741	5.705	5.668	5.633	5.606	5.583	5.563	5.549	5.532	39.0°
GH-1'	5.797	5.793	5.791	5.790	5.792	5.797	5.803	5.810	5.820	5.836	5.835	5.830	5.865	*
O^6CH_3	3.968	3.950	3.940	3.930	3.921	3.913	3.906	3.900	3.896	3.892	3.888	3.883	3.879	59.8°[a]
N^2CH_3	2.894	2.864	2.833	2.790	2.738	2.670	2.600	2.545	2.477	2.427	2.384	2.349	2.292	37.3°[a]
													avg Tm	38.9°C
$J_{1',2'}$ Coupling Constants in Hz														
G(3)H-1'	4.68	4.56	4.38	4.15	3.80	2.89	2.80	2.68	2.29	2.03	0	0	0	
CH-1'	5.27	5.19	4.44	4.70	3.79	3.10	2.39	1.58	0	0	0	0	0	
GH-1'	4.91	5.03	4.44	4.73	4.04	3.52	2.94	1.93	1.12	0	0	0	0	
CH-6	7.57	7.58	7.58	7.57	7.56	7.57	7.58	7.55	7.50	7.58	7.45	7.72	7.33	
CH-5	7.51	7.55	7.17	7.50	7.58	7.43	7.45	7.45	7.20	-	-	-	-	

*non sigmoidal behavior

[a]not used in calculation

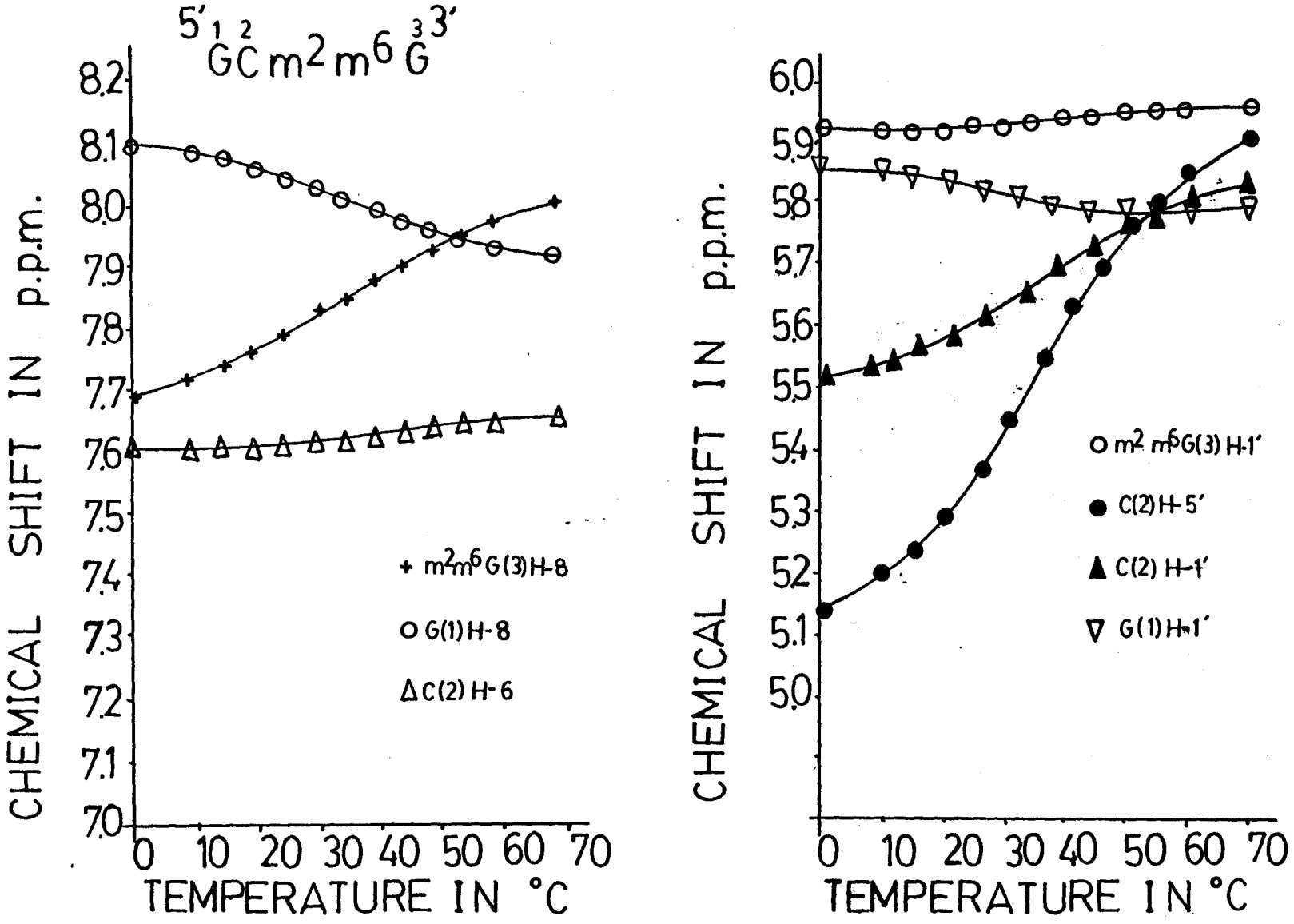


Figure 15a. Variable Temperature Study of GCm^2m^6G

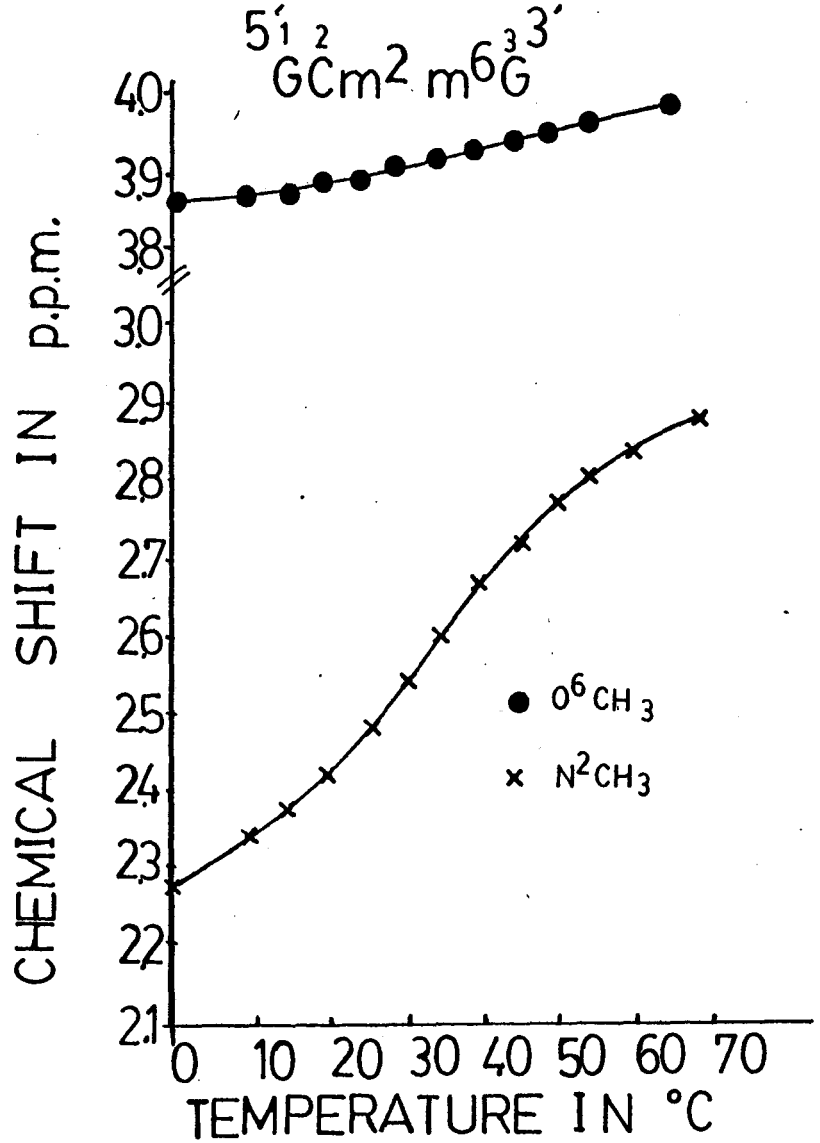


Figure 15 b. Variable Temperature Study of GCm2 m6G

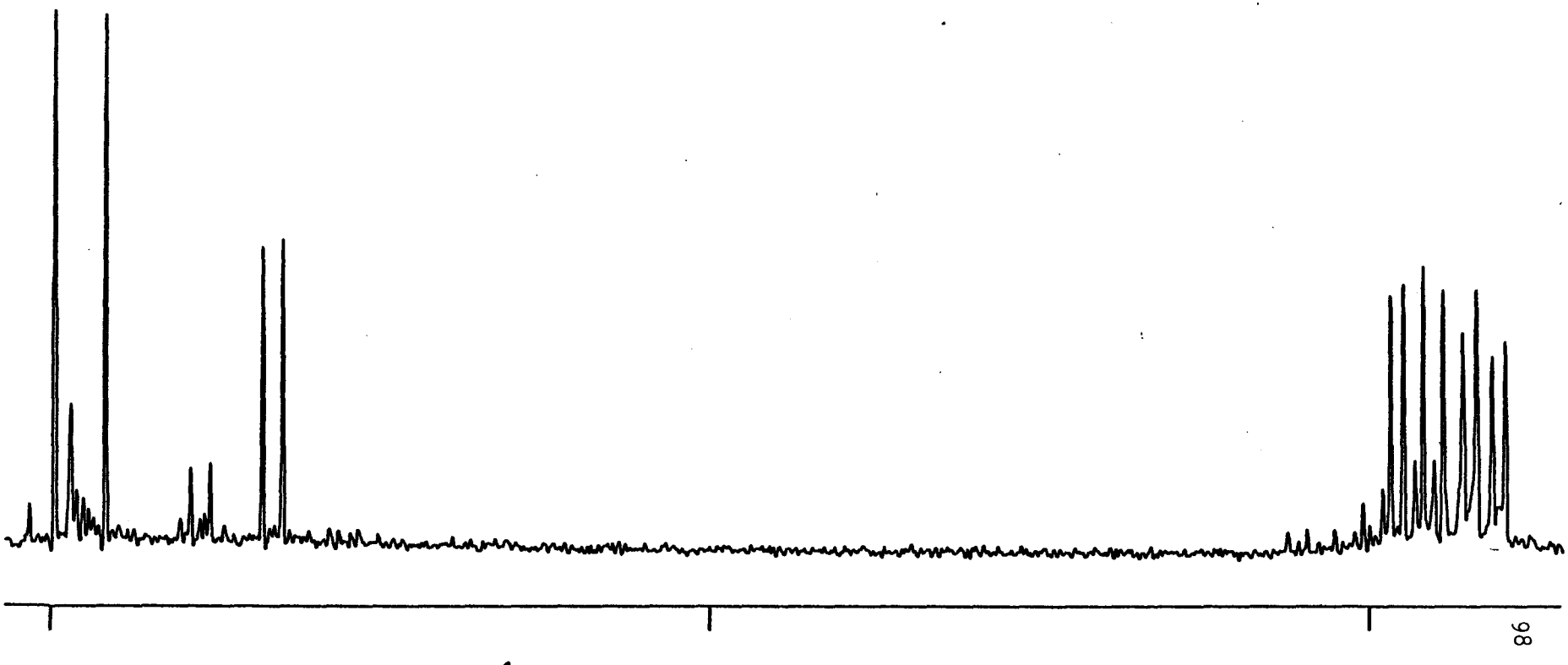


Figure 16a. 70°C ^{1}H-NMR Spectrum of GCm^{2}m^{6}G

stranded form and 50 % in duplex form. In UV experiments Tm represents the melting temperature of the compound as a whole. In NMR experiments however, individual nonexchangeable protons are monitored, and as a result a Tm is obtained for each resonance. Each Tm value reflects the microenvironment surrounding its corresponding proton resonance. The numerical mean of these melting temperatures (average Tm) is equivalent to the Tm value obtained by UV measurements.

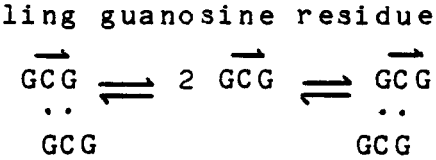
The upfield chemical shift change seen in the C(2)H-5 resonances (70°- 0°C) in all three modified duplexes (0.629, 0.651, 0.694 ppm, respectively) indicate that normal Watson-Crick base pairs were formed (Alkema, et al., 1981(a); Alkema,1982). Upfield chemical shift changes (0.292,0.315, 0.272 ppm, respectively) for all modified G(3)H-8 resonances revealed increased shielding of the dangling base with increased duplex formation. These changes are similar to those seen in the corresponding resonances when N = m^1G (0.333 ppm, Tm = 39.8°C). The chemical shifts of all alkyl groups in the modified duplexes also experienced upfield movement over this temperature range.

The $J_{1',2'}$ coupling constants for the G and C residues in GpCpm^{6}G and GpCpe^{6}G were reduced to < 0.5 Hz near 25°C while those for the dangling residue remained > 1.0 Hz until near 10°C. In the

case of GpCpm^{2}m^{6}G, the corresponding resonances collapsed to < 0.5 Hz at temperatures of 25°C (C), 20° C (G), and 15°C (m^{2}m^{6}G). $J_{1',2'}$ values are indicative of the extent of base stacking (Altona,1975; Ezra, et al., 1977; Romaniuk, et al., 1978). In all instances then, the G and C residues, which can form Watson-Crick base pairs, were stacked at temperatures at which the dangling residue still retained some flexibility. The comparative rates of $J_{1',2'}$ value decline for the various ribose H-1' protons parallel the relative order of duplex stability, indicated by the Tm values.

3.2.2 Discussion

The parent system for the GCN trimers (where N is a modified residue) is GCG, which apparently does not form a unique duplex with decreasing temperature (70°- 0°C) (Alkema and coworkers, unpublished) (Table 16). GCG may form a duplex containing a 5' or a 3' dangling guanosine residue, as shown :



Examination of the melting curves for GCG reveals general upfield movement of the chemical shifts with

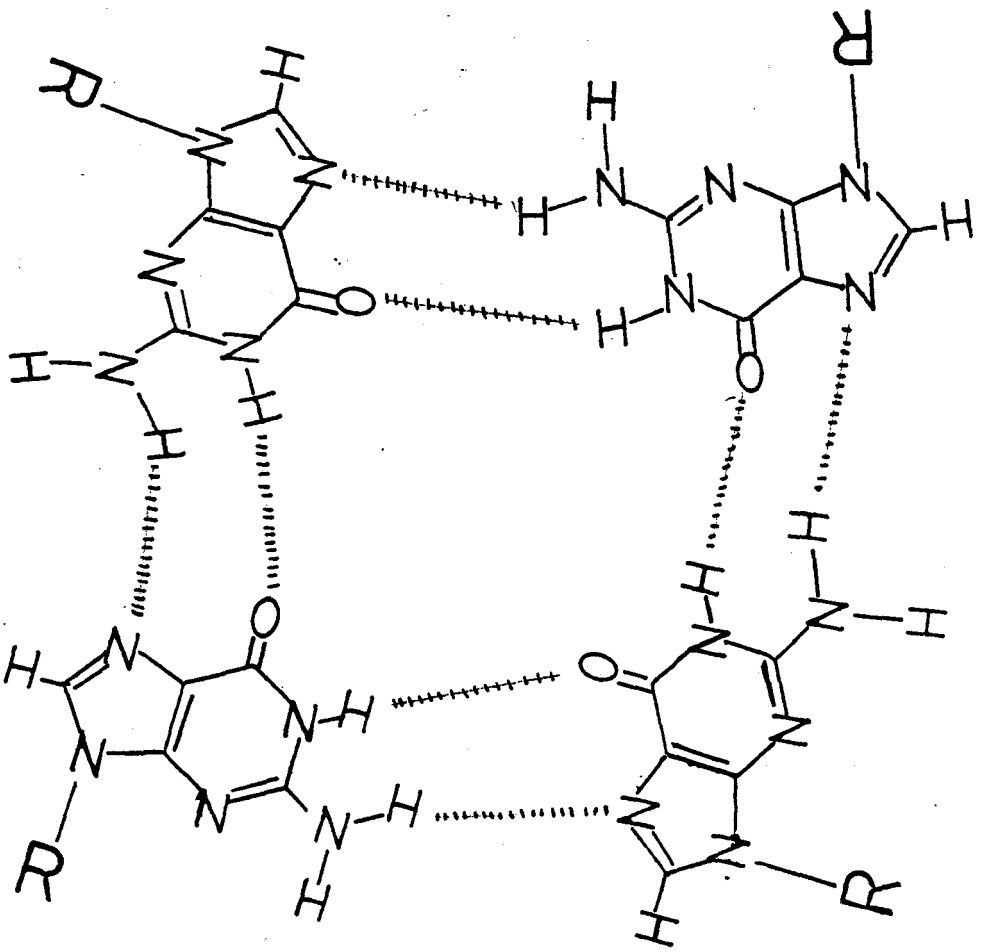


Figure 17 Possible "G" Aggregate Formed with GCG

decreasing temperature (see Table 16); however, below 20°C, the proton resonances become so broad as to be undiscernable. In addition, other peaks of unknown origin appear in the spectrum at 20°C.

As GCG appears to exist in at least a three state equilibrium at low temperatures, no Tm can be calculated for this oligomer. It has been speculated that GCG may be forming some sort of tetramer aggregate, similar to that postulated by other workers for fibers of Gmp (Gellert, et al., 1962) (see Figure 17). To investigate this possibility, further studies must be done, to determine the molecular weight of GCG at low temperatures,and to solve the crystal structure of this compound if this is possible.

Substitution of an alkyl group at the N^1, O^6, or N^2 and O^6 postions of the 3'-terminal guanosine residue (N = m^1G, m^6G, e^6G, m^2m^6G) allows GCN to form a unique duplex, whose Tm can be determined. If GCG does form a hydrogen-bonded, self-aggregate at low temperatures, then modification of potential hydrogen bonding sites (N^1, N^2, O^6, N^7) would prevent formation of such a complex, and allow a simple two state equilibrium to exist. This would explain the sigmoidal melting curves obtained in the present studies.

Given that GCG is an unsatisfactory reference, and that the melting temperature of GCA : GCA may be

determined (Tm = 33.7°C), the latter has been used as the control for the GCN trimers studied. Alkylation at the O^6 position of guanosine (m^6G, e^6G, m^2m^6G) results in complete aromatization of the six membered portion of the guanosine ring, as is the case of the adenosine ring. This phenomenon allows some level of comparison to be drawn between GCA and the GCN duplexes studied in this work.

In earlier studies, methylation of the N^6 position of A (GCm^6A) was found to increase duplex stability over that of GCA (Tm = 34.7°vs 33.7°). Methylation at the O^6 position of guanosine (GCm^6G) had a similar but more intense stabilizing effect (Tm = 36.2°). It might be expected that m^6G and m^6A would have a comparable strengthening effect, since in space, the methyl groups should be in similar positions relative to the central duplex, thus allowing for similar extensions of the hydrophobic core. Although the aromatic G residue is not fully equal in character to the A, the levels of stabilization produced by m^6G and m^6A are similar, especially when compared with those achieved by the other modified purines studied.

Study of the effect of a larger alkyl group at the O^6 position of G (e^6G vs m^6G) reveals a slight increase in duplex melting temperature (37.1°vs 36.2° C, respectively). As Tm values are only accurate to

within ± 1°C, this difference may not be significant. Thus although the expanse of the entire base pair core is increased (ethyl vs methyl) there may be no real resultant effect on duplex stability. Perhaps the size difference of the two alkyl groups is not significant with respect to stabilization, or perhaps the spatial location of the larger alkyl group does not allow for sufficient extension into the hydrophobic core region to give it any enhanced stabilizing potential.

The addition of a second alkyl group to the guanosine residue (m^2m^6G vs m^6G) causes a further increase of nearly 3°C in the melting temperature of the resultant duplex (38.9°vs 36.2°C). These results imply that the increased number and/or the new location of alkyl substituents significantly affects duplex strength, and as well, parallels the situation involving comparison of $GCm^6{}_2A$ and GCm^6A. A study of GCm^2G would determine which of these factors is more significant in generating stabilizing potential.

The similar nature of the Tm's of GCm^2m^6G and GCm^1G (38.9°and 39.8°C, respectively) implies that alkyl substitution in the N^1-N^2 spatial region has a more stabilizing effect than does alkylation in the O^6 region. Perhaps the spatial locations of the N-substituted positions allows for comparatively greater extension of the hydrophobic core as duplex

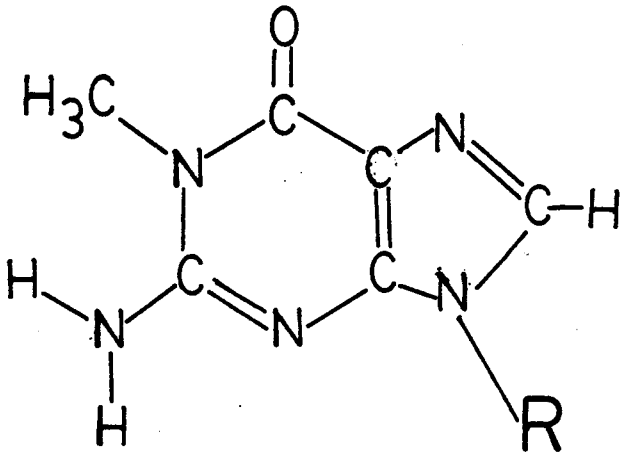
formation occurs. Two factors support this hypothesis : (1). the magnitudes of the upfield chemical shift changes of the alkyl groups with decreasing temperature (70° - 0°C):

compound	alkyl group	Δ ppm
m^1G	N^1-CH_3	0.310
m^6G	O^6-CH_3	0.158
m^2m^6G	O^6-CH_3	0.090
	N^2-CH_3	0.601

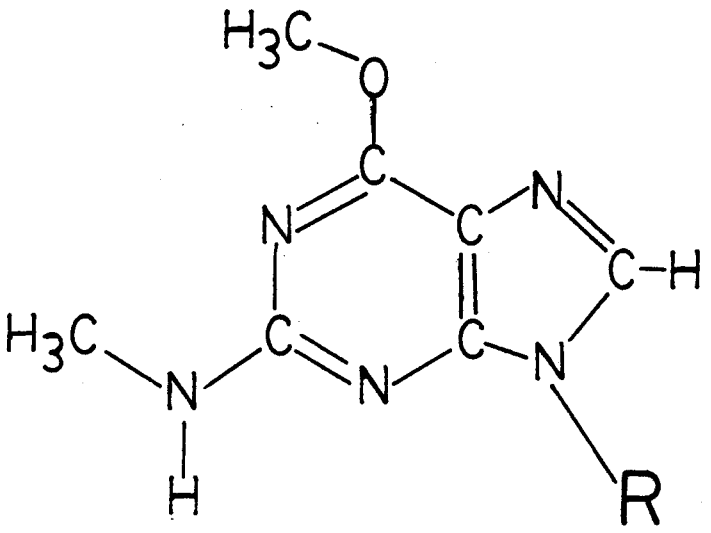
and (2). the crystal structure of m^2m^6G (Allore,B.D., Lock,C.J.L., Hughes,D.W., D'Andrea,P.L., Bell,R.A., and Neilson,T. , submitted), which indicates that the two methyl groups in this compound are in a similar spatial region as the methyl group of m^1G.

3.3 Study of Internal Modified G : U, G : C, and Non-Bonded Base Pairs Within a Short RNA Duplex

Earlier studies (Alkema, et al, 1982) involving a series of pentaribonucleotides AGNCU (where N=A,G,C,U) examined the stability of AGNCU : AGNCU duplexes, containing central, non-base-paired oppositions, and compared the stabilities of AGN_1CU : AGN_2CU (where N_1 : N_2 = A:U, G:U, G:C) with that of the parent AGCU : AGCU (Neilson, et al, 1980), and with each other. These studies served as models for the experiments performed by this worker.



m^1G



m^2m^6G

Figure 16b. Illustration of the Conformation of m^1G and m^2m^6G

Nonbonded base oppositions are a common feature of RNA secondary structure (Lomant & Fresco, 1975; Clarke,1977; Alkema, 1982; Alkema, et al., 1982). As a result, formation of duplexes containing non-bonded base pairs has been considered important by several workers (Lomant & Fresco, 1975; Lee & Tinoco, Jr., 1978; Haasnoot, et al., 1979,1980; Romaniuk, et al., 1979; Alkema, et al., 1982). In this study, the sequence AGGCU (Alkema,et al,1982) has been used as a reference, to look at the effect of G(3) modification (ie. N=m^6G, e^6G, m^2m^6G) on the formation of central, non-bonded bases in a pentamer duplex.

Alkylation of the O^6 oxygen of deoxyguanosine has been shown to result in both mutagenic and carcinogenic lesions (Medcalf & Lawley, 1981; Pegg, 1982; Pettit,1980; Singer, 1979; Kuzmich, et al, 1983). These lesions may, in part, be a consequence of the ambiguous coding nature of O^6-alkyl dG (Fowler, et al, 1982; Karran & Marinus, 1982; Loveless,1969; Gerchman & Ludlum, 1973). In this study, the stabilities of duplexes containing internal modified N : U (N=m^6G,e^6G,m^2m^6G) and modified N : C (N=m^2m^6G) base pair oppositions were compared with those of reference duplexes AGGCU : AGUCU and AGGCU : AGCCU. These experiments sought to provide some insight into similar in vivo situations often found in tumorous target tissues, following treatment with chemical

carcinognens (Fowler *et al*, 1982; Lutz, 1982; Hora, *et al*, 1983; Singer, 1979).

3.3.1 Results

Each of the three pentaribonucleotides was studied by ^{1}H-NMR spectroscopy at concentrations between 1.72 and 1.80 mM. ^{1}H-NMR analysis was used to check sequence integrity and to observe changes in conformation as a function of temperature. Mixing experiments were done at single strand concentrations of 1.72-1.80 mM. As melting temperature is concentration dependent, the duplexes AGGCU : AGUCU, AGGCU : AGCCU, were studied at similar concentrations to be valid reference systems for the modified pentamers.

Prior to assignment of low field nonexchangable proton chemical shifts of the pentamers, it was necessary to assign corresponding values for the alkylated AGN triribonucleotides and AGNC tetranucleotides. These assignments were made via incremental analysis (Borer, *et al*., 1975; Everett, *et al*., 1980) using chemical shift values obtained for the dimer AG, and from the corresponding AGN trimer, respectively, (see Tables 20 - 24, Figures 18 - 23).

The alkylated G(3)H-8 signal appears downfield

Table 20. ^{1}H NMR Chemical Shift Assignments for AGG over the Temperature Range 70°-0°C (27 OD's)

Resonance	71.6°	62.5°	52.0°	41.8°	31.3°	20.0°
AH-8	8.212	8.209	8.206	8.202	8.193	8.183
AH-2	8.167	8.147	8.128	8.102	8.069	8.023
G(3)H-8	7.935	7.926	7.919	7.906	7.883	7.851
G(2)H-8	7.906	7.893	7.880	7.864	7.835	7.795
AH-1'	5.946	5.935	5.926	5.920	5.905	5.887
G(3)H-1'	5.837	5.827	5.817	5.806	5.785	5.764
G(2)H-1'	5.785	5.773	5.754	5.730	5.695	5.653

Table 21. ^{1}H NMR Chemical Shift Assignments for AGm^{6}G over the Temperature Range 70°-0°C (56 OD's)

Resonance	70.8°	60.8°	50.2°	40.3°	30.3°	20.5°	10.7°	1.0°
A(1)H-8	8.195	8.192	8.188	8.182	8.177	8.173	8.164	8.155
A(1)H-2	8.141	8.122	8.097	8.066	8.032	7.995	7.946	7.903
G(2)H-8	7.871	7.862	7.847	7.830	7.809	7.788	7.759	7.731
G(3)H-8	8.032	8.025	8.012	7.996	7.977	7.957	7.929	7.896
A(1)H-1'	5.923	5.915	5.905	5.895	5.884	5.875	5.861	5.846
G(2)H-1'	5.707	5.690	5.668	5.646	5.622	5.598	5.569	5.542
G(3)H-1'	5.904	5.896	5.886	5.876	5.865	5.854	5.837	5.821
$O^{6}CH_3$	3.952	3.941	3.928	3.915	3.904	3.894	3.879	3.863
$J_{1',2'}$ Coupling Constants in Hz								
A(1)H-1'	4.8	4.9	5.0	5.1	5.2	5.0	4.5	4.3
G(2)H-1'	4.7	4.4	4.7	4.3	4.0	3.6	3.0	2.7
G(3)H-1'	5.1	4.8	4.5	4.6	4.8	5.5	4.2	3.0

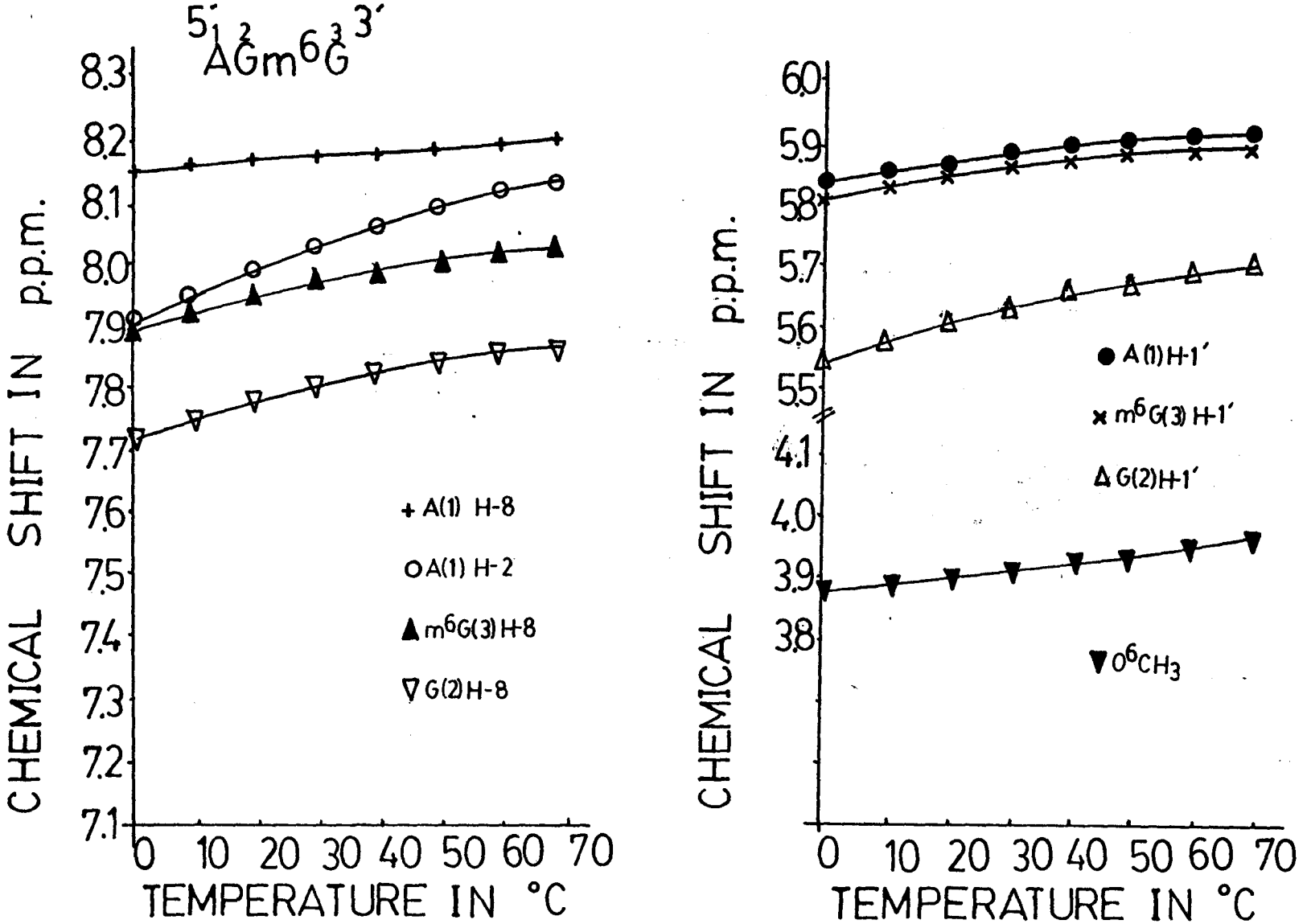


Figure 18. Variable Temperature Study of AGm^{6}G

Table 22. ^{1}H NMR Chemical Shift Assignments for AGe^{6}G over the Temperature Range 70°-0°C (52 OD's)

Resonance	70.6°	60.0°	50.4°	40.3°	30.6°	20.0°	10.2°
A(1)H-8	8.197	8.194	8.190	8.186	8.181	8.175	8.168
A(1)H-2	8.150	8.131	8.111	8.087	8.058	8.020	7.979
G(2)H-8	7.881	7.872	7.861	7.848	7.830	7.807	7.782
G(3)H-8	8.040	8.034	8.026	8.015	8.000	7.980	7.958
A(1)H-1'	5.923	5.915	5.907	5.899	5.891	5.877	5.868
G(3)H-1'	5.912	5.905	5.898	5.890	5.880	5.868	5.855
G(2)H-1'	5.710	5.693	5.677	5.660	5.640	5.617	5.594
$O\underline{CH_2}CH_3$	4.367						
$OCH_2\underline{CH_3}$	1.309	1.306	1.305	1.306	1.308	1.312	1.316
$J_{1',2'}$ Coupling Constants in Hz							
A(1)H-1'	5.33	5.32	5.18	5.29	5.14	5.06	4.64
G(3)H-1'	4.99	4.84	4.77	4.87	4.65	4.53	4.51
G(2)H-1'	5.31	5.19	5.00	4.86	4.57	4.24	3.58

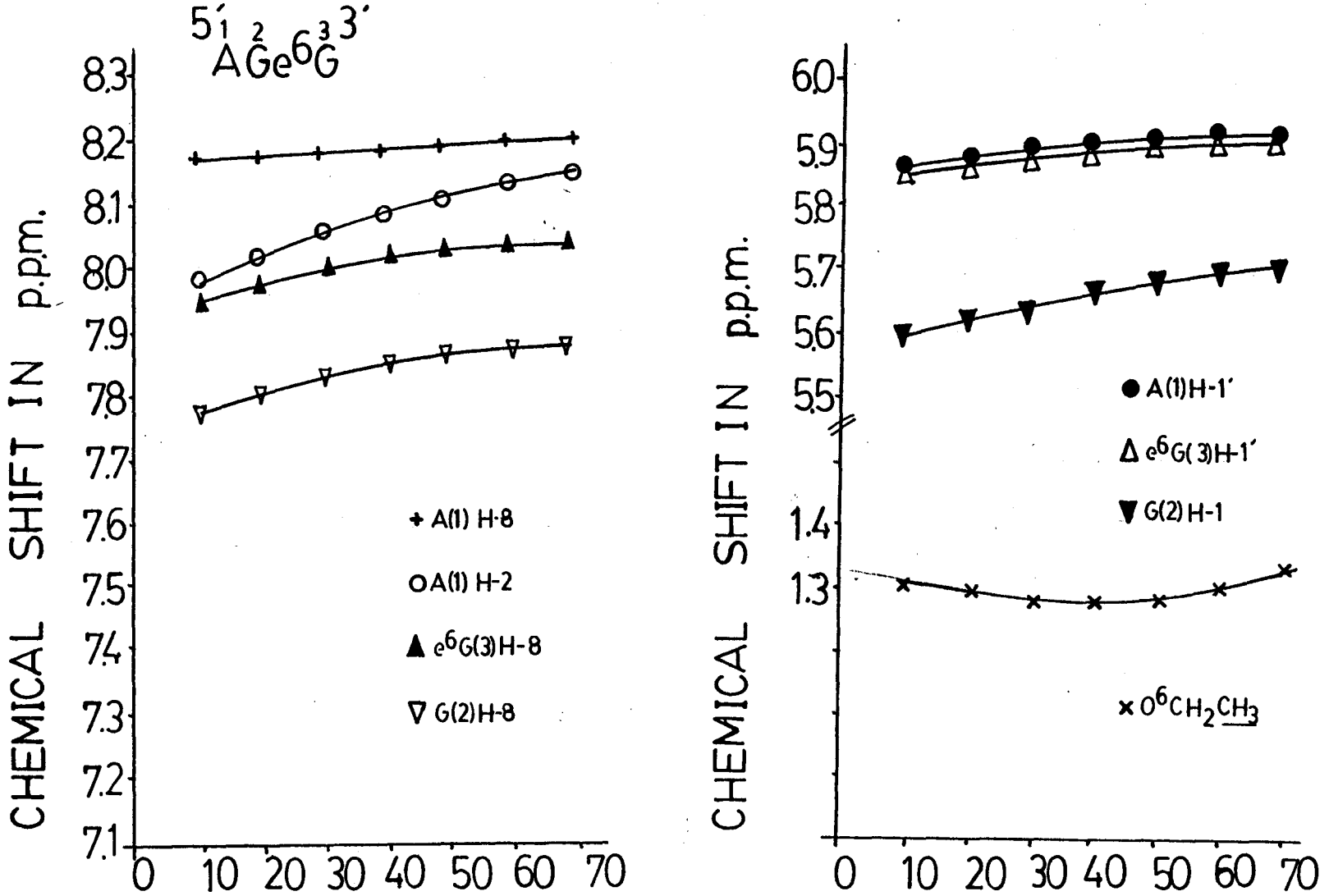


Figure 19. Variable Temperature Study of AGe6G

Table 23. ^{1}H NMR Chemical Shift Assignments for AGm^{2} m^{6}G over the Temperature Range 70°-0°C (68 OD's)

Resonance	70.5°	60.8°	49.9°	40.0°	30.0°	20.4°	10.2°
A(1)H-8	8.191	8.186	8.179	8.171	8.161	8.150	8.133
A(1)H-2	8.144	8.126	8.100	8.071	8.035	7.994	7.939
G(2)H-8	7.855	7.846	7.833	7.820	7.803	7.785	7.761
G(3)H-8	7.966	7.959	7.949	7.936	7.919	7.900	7.874
A(1)H-1'	5.928	5.922	5.913	5.903	5.892	5.880	5.863
G(2)H-1'	5.671	5.654	5.636	5.618	5.599	5.580	5.558
G(3)H-1'	5.913	5.903	5.892	5.880	5.867	5.853	5.834
O^{6}CH$_3$	3.920	3.903	3.885	3.868	3.849	3.831	3.806
N^{2}CH$_3$	2.879	2.872	2.863	2.853	2.841	2.826	2.802
$J_{1',2'}$ Coupling Constants in Hz							
A(1)H-1'	4.67	4.51	4.81	4.79	4.49	4.38	4.21
G(2)H-1'	5.32	5.13	4.96	4.72	4.45	4.17	3.87
G(3)H-1'	5.38	5.18	5.58	5.51	5.42	5.25	5.07

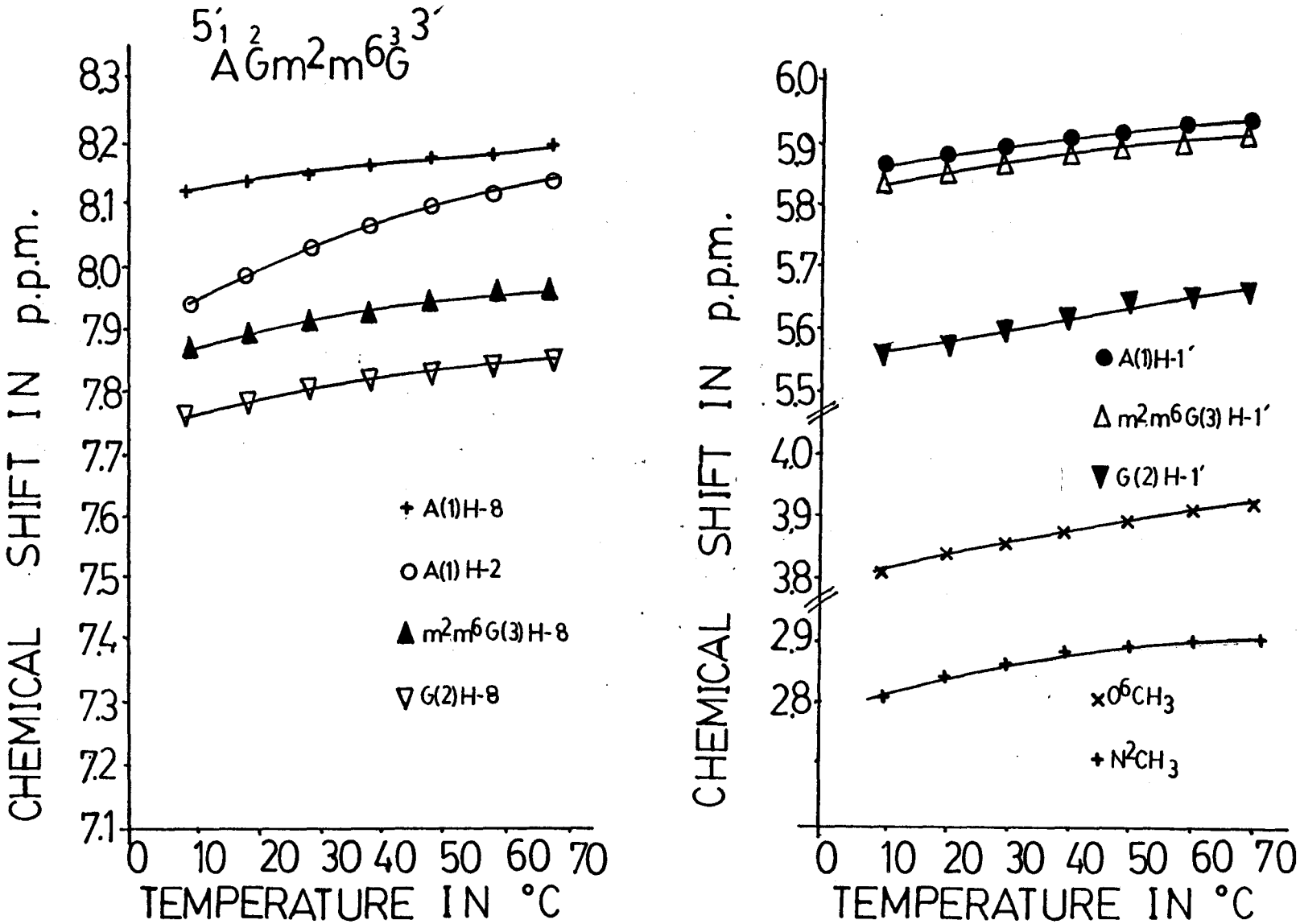


Figure 20. Variable Temperature Study of AGm^2m^6G

Table 24. ^{1}H NMR Chemical Shift Assignments for AGm^{2} m^{6}GC over the Temperature Range 70°-0°C (200 OD's)

Resonance	70.5°	60.8°	49.9°	40.0°	30.0°	20.4°	10.2°
A(1)H-8	8.186	8.179	8.169	8.159	8.149	8.140	8.128
A(1)H-2	8.140	8.116	8.086	8.054	8.016	7.977	7.927
G(2)H-8	7.875	7.871	7.866	7.857	7.843	7.823	7.796
G(3)H-8	7.966	7.955	7.940	7.922	7.899	7.874	7.845
C(4)H-6	7.685	7.668	7.646	7.624	7.600	7.578	7.559
C(4)H-5	5.795	5.751	5.697	5.645	5.592	5.543	5.504
A(1)H-1'	5.914	5.900	5.885	5.856	5.847	5.831	5.812
G(2)H-1'	5.685	5.679	5.672	5.663	5.651	5.636	5.614
G(3)H-1'	5.911	5.898	5.883	5.868	5.852	5.837	5.821
C(4)H-1'	5.818	5.797	5.768	5.738	5.705	5.673	5.641
O^{6}CH$_3$	3.930	3.920	3.912	3.906	3.902	3.898	3.887
N^{2}CH$_3$	2.870	2.862	2.853	2.843	2.833	2.823	2.807
$J_{1',2'}$ Coupling Constants in Hz							
AH-1'	3.98	3.95	4.22	3.53	2.71	1.89	0
GH-1'	5.37	5.21	5.04	4.77	4.46	4.19	3.47
G(3)H-1'	5.29	5.19	4.94	5.05	5.06	5.20	4.32
CH-1'	3.80	3.36	3.22	3.04	2.85	2.53	1.96
CH-6	7.49	7.57	7.57	7.60	7.57	7.58	7.57
CH-5	7.79	7.54	5.60	7.55	7.55	7.57	7.50

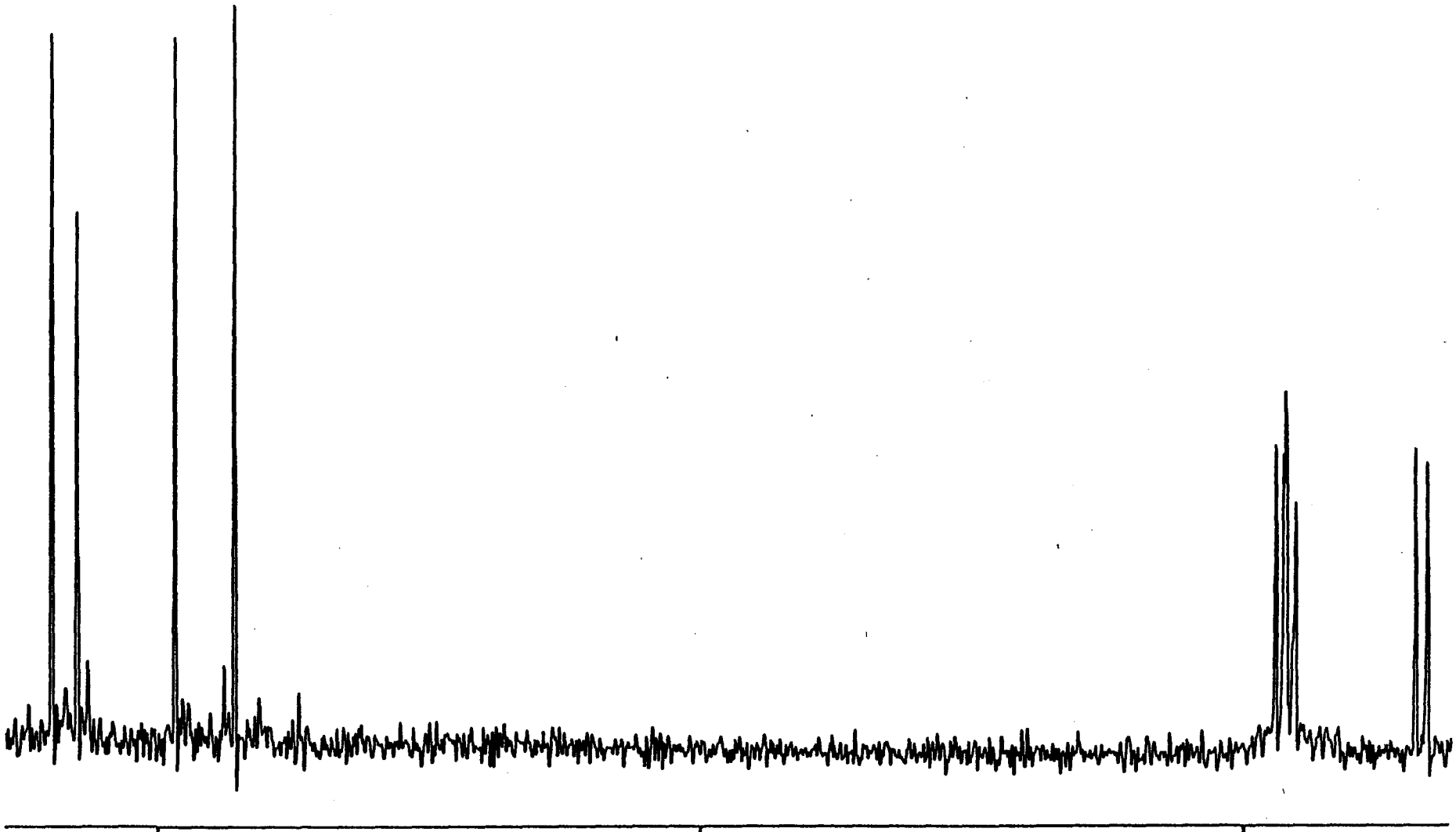


Figure 21. 70°C ^{1}H-NMR Spectrum of AGm^{2} m^{6}G

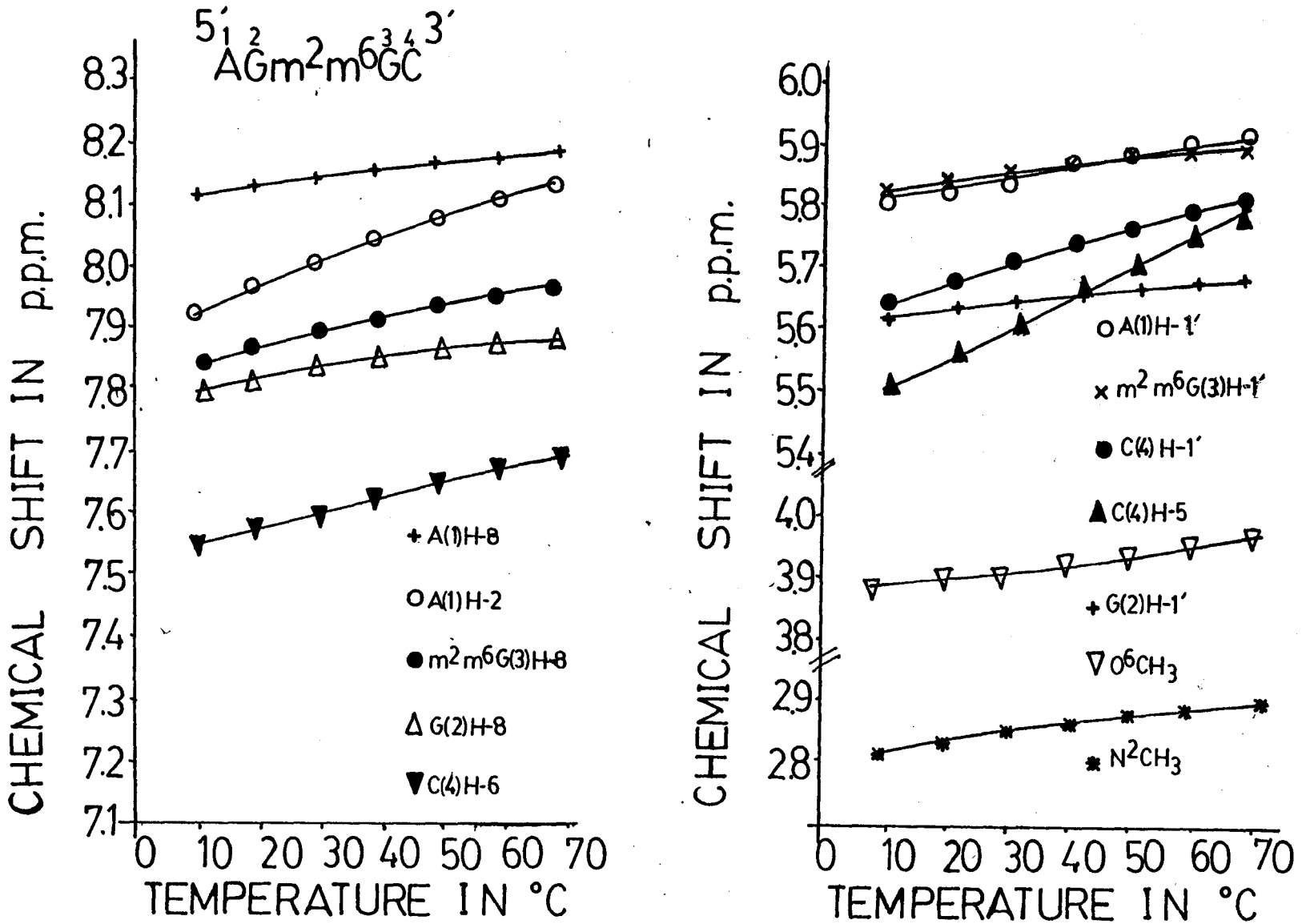


Figure 22. Variable Temperature Study of AGm^{2}m^{6}GC

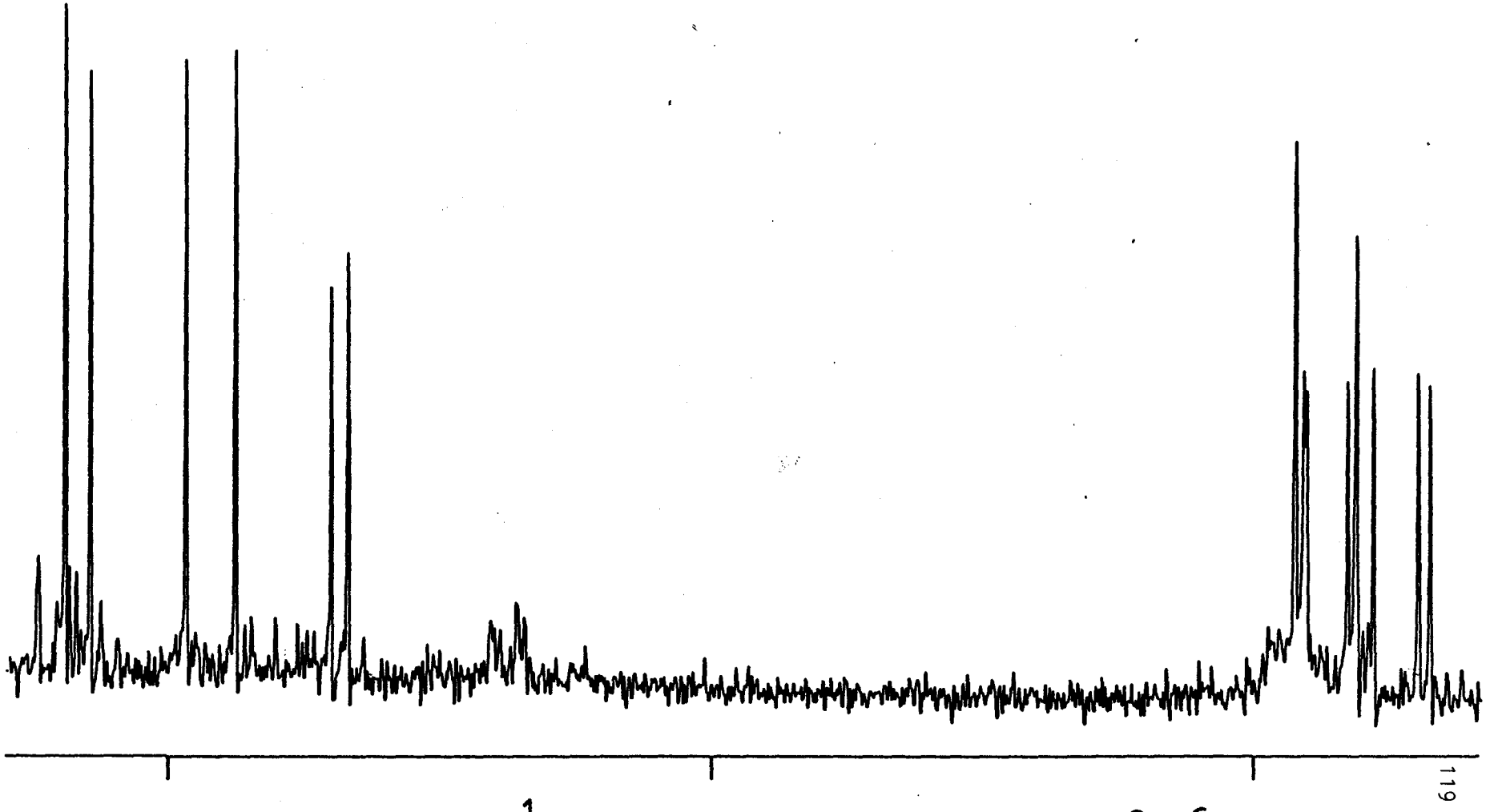


Figure 23. 70°C ^{1}H-NMR Spectrum of AGm^{2}m^{6}GC

relative to the G(2)H-8 resonance for all three AGN sequences (Table 6), in contrast to the situation for AGG, in which the G(3)H-8 signal is found upfield from that of G(2)H-8. Clearly, alkylation of the 3'-terminal G residue alters the local environment resulting in deshielding of the G(3)H-8 proton, relative to that in the nonmodified trimer. The expected alkyl signals appear upfield (from 2.5 - 4.0 ppm) in each AGN spectrum.

The tetramers AGe^6GC and AGm^2m^6GC, were distinguished from their corresponding trimers by the appearance of doublets resulting from C(4)H-6 (7.702, 7.685 ppm), and C(4)H-5 (5.839, 5.796 ppm) respectively.

The low field nonexchangable proton chemical shifts of the three pentamers were determined by comparison with those of the appropriate AGN trimers, with AGGCU, and by incremental analysis (see Tables 25 - 27, Figures 24 - 28).

The 70°C spectrum for AGm^6GCU was distinguished from that of AGm^6G by the appearance of two doublets at 7.687 and 7.749 ppm. Through examination of the $J_{1',2'}$ coupling constants (7.499, 8.161 Hz), and by looking at the relative trend of the C(4)H-6 and U(5)H-6 signal of AGGCU, these doublets were assigned to the C(4)H-6 and U(5)H-6 protons, respectively. Doublets present at 5.829 and 5.797

Table 25. ^{1}H NMR Chemical Shift Assignments for AGm^{6}GCU over the Temperature Range 70°-0°C (40 OD's)

Resonance	70.5°	60.5°	49.7°	40.0°	30.0°	20.4°	10.2°
A(1)H-8	8.199	8.195	8.192	8.191	8.192	8.195	8.201
A(1)H-2	8.153	8.135	8.111	8.086	8.057	8.026	7.991
G(2)H-8	7.886	7.882	7.873	7.859	7.836	7.802	7.756
G(3)H-8	8.016	8.000	7.977	7.950	7.914	7.873	7.826
C(4)H-6	7.686	7.663	7.630	7.594	7.552	7.508	7.462
U(5)H-6	7.748	7.750	7.752	7.755	7.758	7.763	7.770
C(4)H-5	5.829	5.751	5.689	5.626	5.558	5.493	5.431
U(5)H-5	5.797	5.780	5.758	5.736	5.713	5.689	5.664
A(1)H-1'	5.926	5.917	5.908	5.902	5.895	5.892	5.892
G(2)H-1'	5.709	5.702	5.692	5.677	5.656	5.628	5.597
G(3)H-1'	5.868	5.853	5.852	5.852	5.848	5.844	5.837
C(4)H-1'	5.853	5.824	5.787	5.748	5.703	5.661	5.618
U(5)H-1'	5.868	5.853	5.830	5.811	5.790	5.772	5.759
O^{6}CH$_3$	3.959	3.954	3.952	3.953	3.954	3.956	3.864
$J_{1',2'}$ Coupling Constants in Hz							
AH-1'	5.41	5.33	5.39	5.32	4.87	4.34	3.78
GH-1'	5.35	5.07	5.45	4.45	3.90	3.29	1.83
G(3)H-1'	3.25	4.33	4.22	3.99	4.48	4.10	4.64
CH-1'	4.14	3.90	3.30	2.67	3.01	1.93	1.09
UH-1'	3.25	4.33	3.50	2.96	2.57	2.03	0
CH-5	7.77	7.43	7.19	7.57	7.52	7.53	7.57
CH-6	7.49	7.61	7.54	7.53	7.70	7.69	7.44
UH-5	8.29	7.91	8.09	8.22	7.92	8.25	7.57
UH-6	8.16	8.11	8.12	8.09	8.34	8.23	6.59

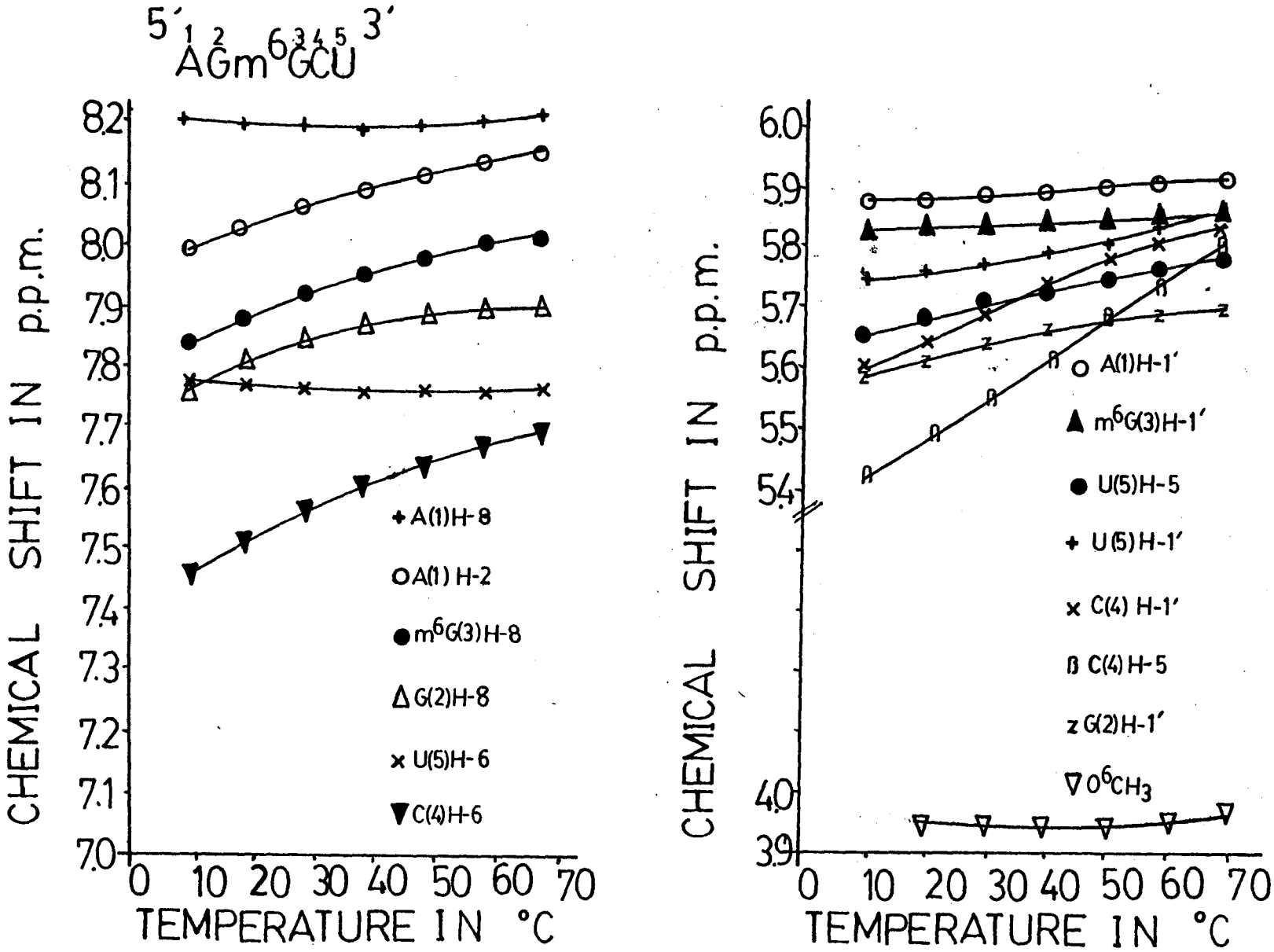


Figure 24. Variable Temperature Study of AGm^{6}GCU

Table 26. ^{1}H NMR Chemical Shift Assignments for AGe^{6}GCU over the Temperature Range 70°-0°C (37 OD's)

Resonance	70.5°	60.5°	49.7°	40.0°	30.0°	20.4°	10.2°
A(1)H-8	8.198	8.194	8.190	8.186	8.182	8.183	8.174
A(1)H-2	8.152	8.140	8.118	8.094	8.066	8.035	8.000
G(2)H-8	7.895	7.893	7.889	7.880	7.864	7.839	7.802
G(3)H-8	8.028	8.016	7.998	7.975	7.945	7.908	7.865
C(4)H-6	7.699	7.676	7.645	7.609	7.566	7.518	7.469
U(5)H-6	7.750	7.752	7.755	7.757	7.760	7.764	7.771
C(4)H-5	5.821	5.775	5.715	5.652	5.581	5.511	5.443
U(5)H-5	5.801	5.785	5.764	5.743	5.719	5.693	5.666
A(1)H-1'	5.929	5.920	5.910	5.902	5.893	5.887	5.884
G(2)H-1'	5.709	5.705	5.699	5.692	5.681	5.667	5.649
G(3)H-1'	5.880	5.865	5.854	5.851	5.847	5.843	5.837
C(4)H-1'	5.856	5.844	5.811	5.774	5.731	5.688	5.649
U(5)H-1'	5.871	5.858	5.848	5.829	5.808	5.787	5.772
$O^{6}CH_2\underline{CH_3}$	1.322	1.326	1.335	1.346	1.357	1.369	
$J_{1',2'}$ Coupling Constants in Hz							
AH-1'	5.54	5.44	5.55	5.39	5.39	5.17	4.64
GH-1'	5.67	5.34	5.24	5.03	4.39	5.00	0
G(3)H-1'	4.18	3.86	3.91	4.32	4.19	4.37	4.03
CH-1'	3.43	3.85	3.56	2.98	2.25	2.11	0
UH-1'	4.09	2.98	3.32	3.45	2.72	2.43	0
CH-5	7.64	7.63	7.49	7.45	7.53	7.51	6.96
CH-6	7.63	7.48	7.57	7.69	7.67	7.39	7.45
UH-5	8.14	7.99	8.11	8.04	8.23	8.12	8.91
UH-6	8.09	8.16	8.12	8.08	8.12	8.14	8.18

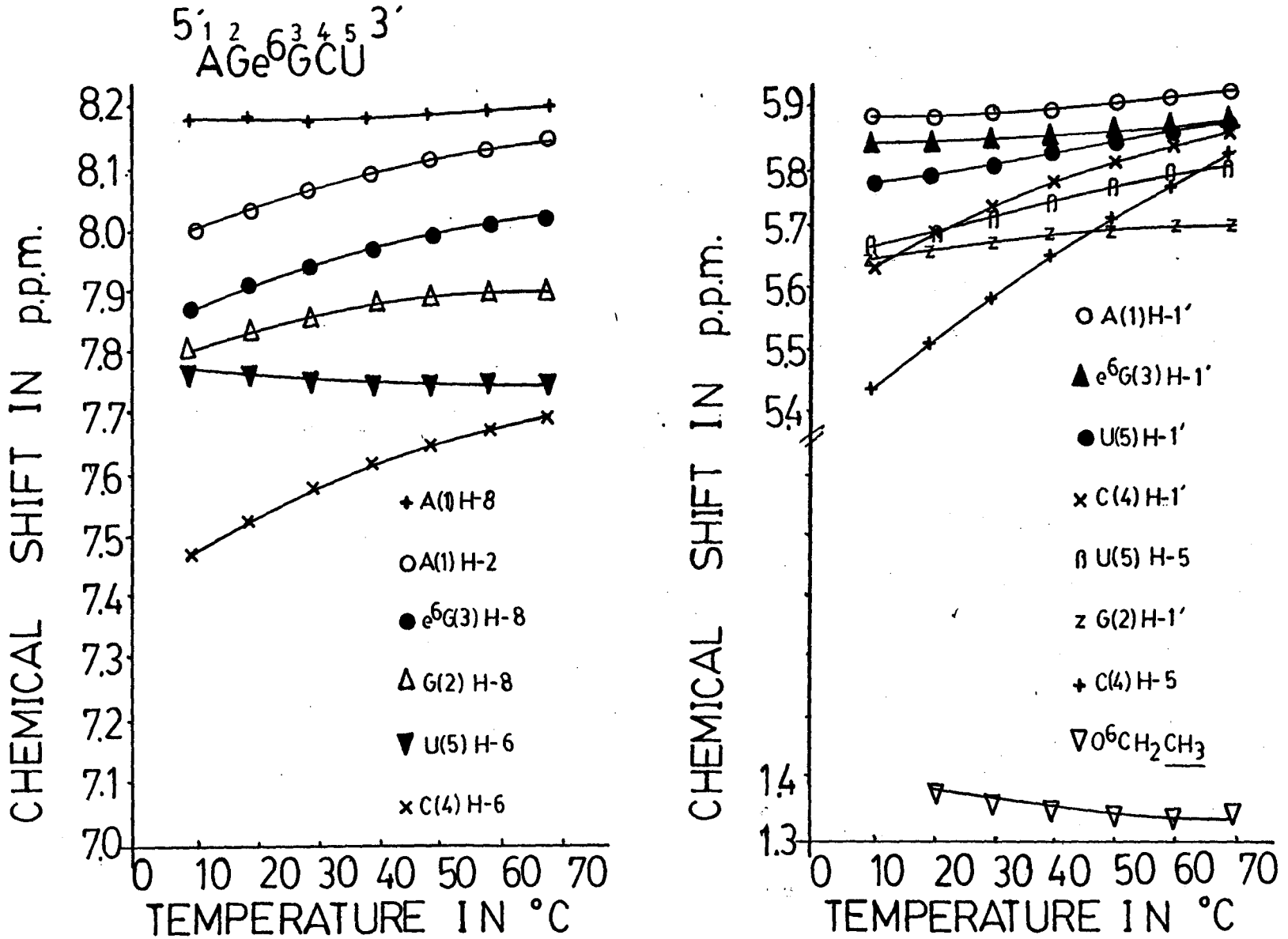


Figure 25. Variable Temperature Study of AGe6GCU

Table 27. ^{1}H NMR Chemical Shift Assignments for AGm^{2}m^{6}GCU over the Temperature Range 70° - 0° C (40 OD's)

Resonance	70.2°	60.4°	50.9°	40.2°	30.1°	20.3°	10.4°
A(1)H-8	8.189	8.181	8.175	8.169	8.165	8.164	8.167
A(1)H-2	8.156	8.137	8.116	8.809	8.061	8.030	7.996
G(2)H-8	7.877	7.877	7.876	7.872	7.861	7.841	7.809
G(3)H-8	7.960	7.949	7.937	7.917	7.894	7.865	7.830
C(4)H-6	7.670	7.643	7.613	7.574	7.533	7.490	7.448
U(5)H-6	7.740	7.740	7.743	7.745	7.750	7.754	7.760
C(4)H-5	5.754	5.705	5.646	5.571	5.500	5.431	5.369
U(5)H-5	5.791	5.771	5.751	5.727	5.707	5.680	5.657
A(1)H-1'	5.909	5.894	5.880	5.864	5.852	5.843	5.837
G(2)H-1'	5.689	5.688	5.689	5.688	5.684	5.672	5.649
G(3)H-1'	5.891	5.876	5.861	5.838	5.821	5.804	5.791
C(4)H-1'	5.816	5.779	5.739	5.684	5.632	5.579	5.529
U(5)H-1'	5.840	5.838	5.836	5.834	5.833	5.831	5.827
O^6CH_3	3.933	3.927	3.926	3.928	3.931	3.934	3.935
N^2CH_3	2.869	2.863	2.859	2.854	2.850	2.846	2.842
$J_{1',2'}$ Coupling Constants in Hz							
A(1)H-1'	5.04	5.41	5.59	5.15	5.19	5.03	4.81
G(2)H-1'	5.50	5.25	5.11	4.82	4.51	3.79	3.72
G(3)H-1'	3.85	3.43	3.94	2.34	1.99	1.99	1.08
C(4)H-1'	4.44	3.85	2.17	2.99	2.38	1.92	1.17
U(5)H-1'	3.42	4.14	4.21	4.66	4.13	4.18	4.06
C(4)H-5	7.43	7.53	7.48	7.54	7.48	7.59	7.56
C(4)H-6	7.59	7.62	7.77	7.58	7.43	7.80	7.70
U(5)H-5	8.14	7.76	8.18	8.09	8.05	8.10	7.84
U(5)H-6	8.06	8.12	8.21	8.10	8.17	8.20	8.01

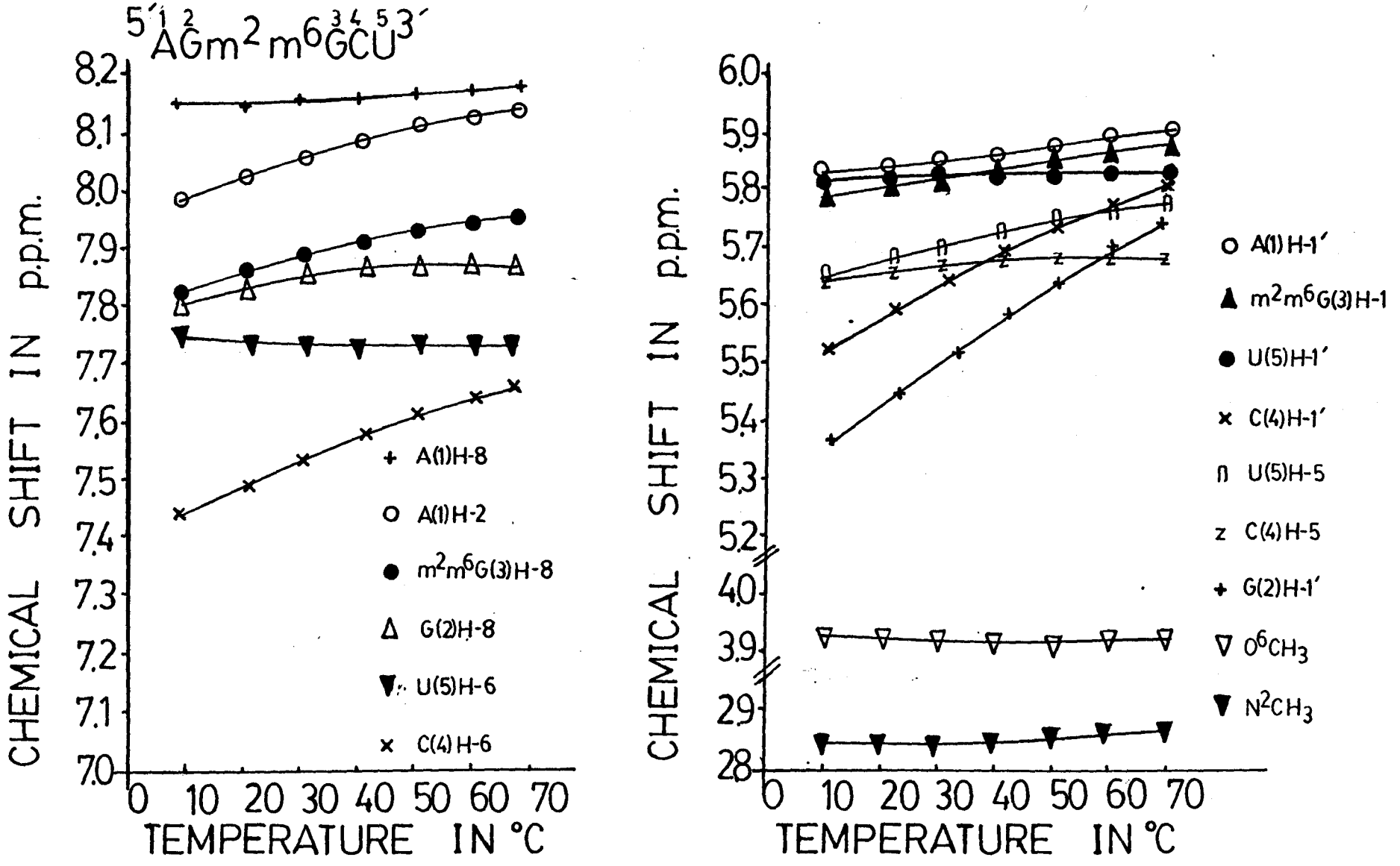


Figure 26. Variable Temperature Study of AGm2m6GCU

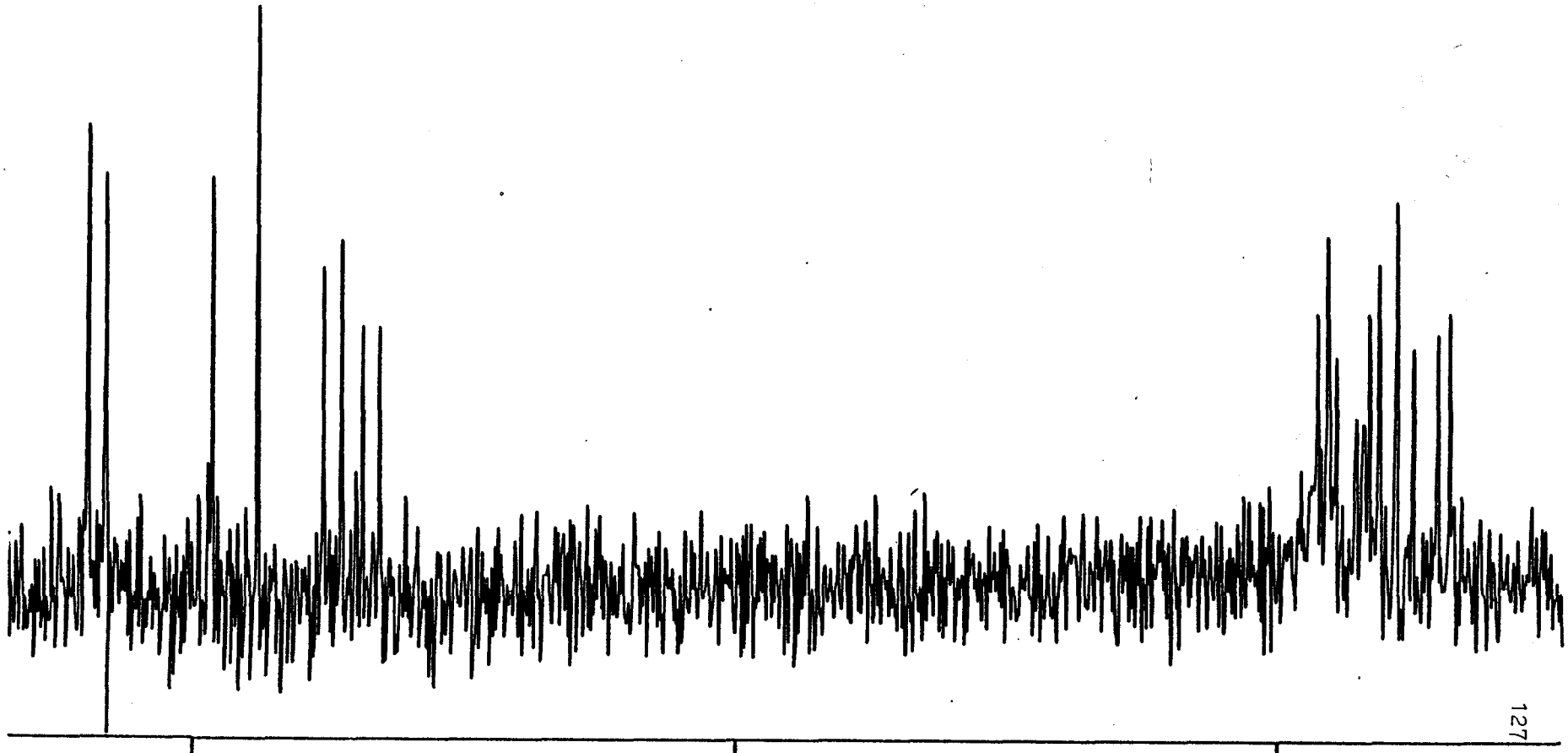


Figure 27. ^{1}H-NMR Spectrum of AGm^{2}m^{6}GCU

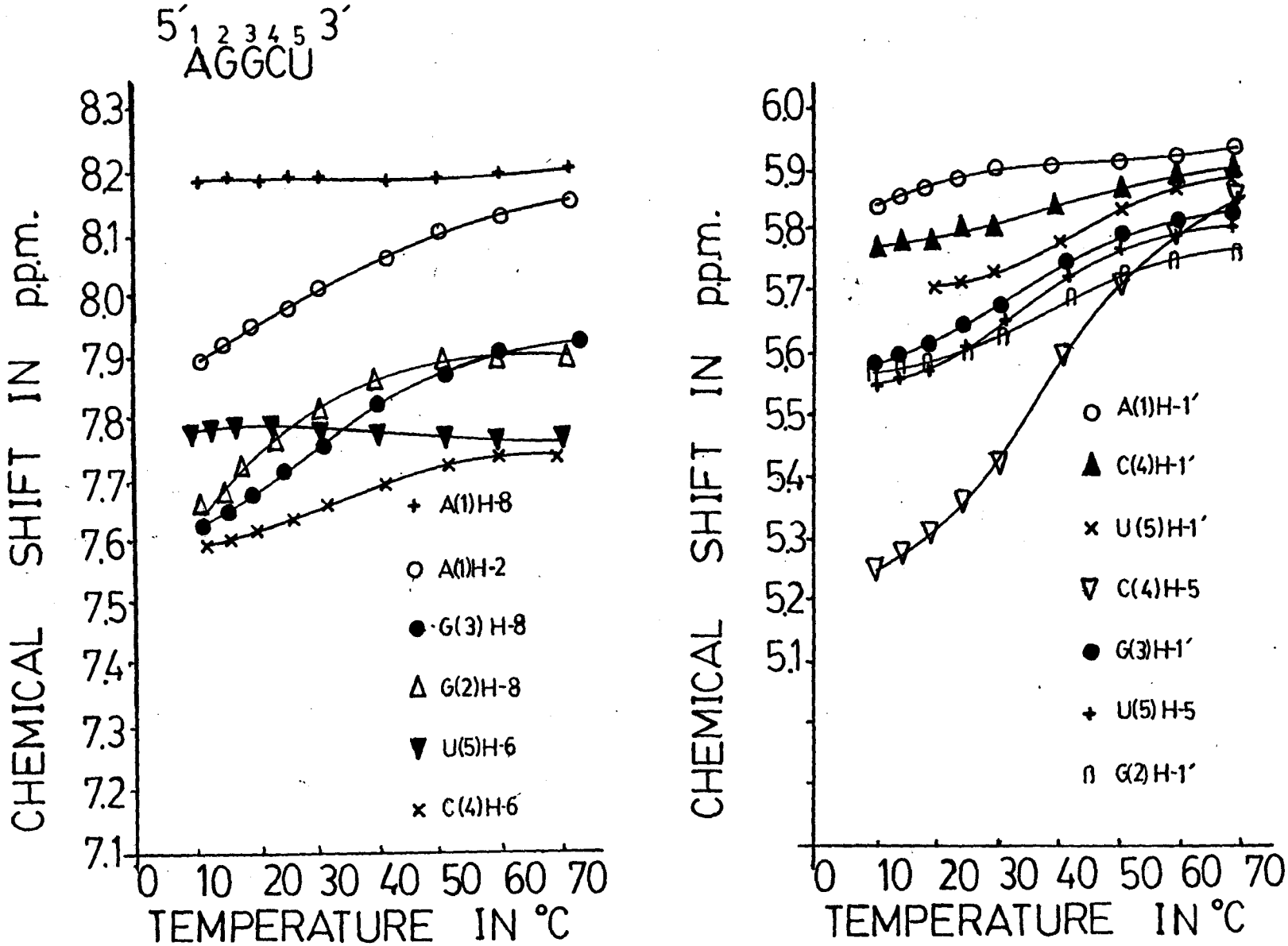


Figure 28. Variable Temperature Study of AGGCU

ppm, were similarly assigned to the C(4)H-5 and U(5)H-5 resonances, respectively.

In the case of AGe^{6}GCU, comparison with AGe^{6}GC reveals a doublet at 7.751 ppm, and at 5.802 ppm, corresponding to the U(5)H-6 and U(5)H-5 signals. Similarly, for AGm^{2}m^{6}GCU, the U(5)H-6 and U(5)H-5 doublets, absent in the AGm^{2}m^{6}GC spectrum, are assigned at 7.740 and 5.791 ppm, respectively.

The trend of the C(4)H-6 and U(5)H-6 chemical shifts for the alkylated pentamers is the same as that in AGGCU (ie. U(5)H-6 is downfield of C(4)H-6). A corresponding pattern is also seen in the C(4)H-5 and U(5)H-5 signals (ie. U(5)H-5 is further upfield), for AGm^{6}GCU and AGe^{6}GCU; however, this order is reversed in the case of AGm^{2}m^{6}GCU.

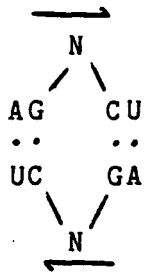
Mixing experiment assignments of AGNCU : AGUCU and AGNCU : AGCCU duplexes were made by comparison with those of the corresponding single strands. The reference compounds, AGGCU : AGUCU and AGGCU : AGCCU, were assigned by comparison with appropriate values of similar mixing experiments at higher concentrations (Alkema, et al, 1982) (see Tables 28 - 33, Figures 29 - 36).

3.3.1.1 Effect of a Non-Complementary Base Opposition on the Stability of a Short Duplex

Theoretically, a pentaribonucleotide AGNCU, can form a slipped duplex

$$\overrightarrow{\text{AGNCU}}$$
$$\cdots\cdot$$
$$\overleftarrow{\text{UCNGA}}$$

or a duplex containing a central looped-out region as shown:

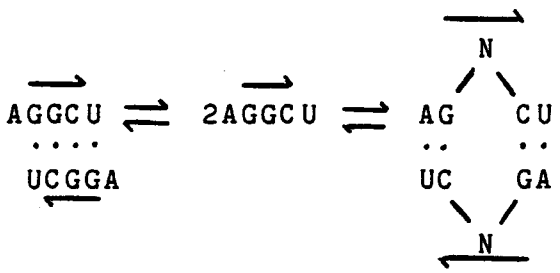


(N = m^6G, e^6G, m^2,m^6G)

A duplex with such a structure would provide information on the effects of single-base bulge loops on duplex stability. A previous study was carried out using AGNCU pentamers (N = A,G,C,U) (Alkema, et al., 1982). Of particular interest from this study is the AGGCU case, as it differs from the pentamers examined only by the lack of G(3) modification.

AGGCU undergoes duplex formation with decreasing temperature, as indicated by the sigmoidal upfield shifts of its melting curves. The duplex formed may not be unique however. Alkema (Alkema, et al., 1982) postulates the existence of two possible

structures :



Unlike AGGCU, the chemical shifts the alkylated pentamers experience only small upfield shifts with decreasing temperature, implying that no duplex formation occurs.

3.3.1.2 Studies of the Stabilities of Short Duplexes Containing Central Modified G : U and G : C Base Pairs

Mixing of two pentaribonucleotide sequences, AGNCU (N=m^6G, e^6G, m^2m^6G) and AGPCU (P=U,C) could potentially form a duplex of the type :

AGNCU
UCPGA

containing a central modified G : pyrimidine base pair.

By comparison with reference duplexes AGGCU : AGUCU and AGGCU : AGCCU, such studies would provide insight into the effect of G alkylation on the strength of Wobble (G : U) and Watson-Crick (G : C)-type base pairs, and thus on the stability of the

Table 28. ^{1}H NMR Chemical Shift Assignments for $\overset{AGm^6GCU}{UC\ U\ GA}$ over the Temperature Range 70° - 5°C (1.8 mM)

Resonance	70.9°	61.0°	50.0°	45.0°	40.0°	35.0°	29.9°	25.0°	20.0°	15.0°	9.9°	5.2°
A(5)H-8	8.257	8.258	8.260	8.261	8.262	8.264	8.266	8.271	8.274	8.274	8.270	
A(1)H-8	8.216	8.213	8.210	8.207	8.208	8.207	8.207	8.210	8.211	8.219	8.228	8.236
A(5)H-2	8.207	8.194	8.178	8.169	8.159	8.146	8.129	8.107	8.078	8.004	7.948	
A(1)H-2	8.173	8.156	8.133	8.122	8.110	8.095	8.080	8.064	8.042	8.004	7.948	7.868
G(3)H-8	8.035	8.021	8.001	7.990	7.978	7.963	7.946	7.928	7.902	7.869	7.805	
G(4)H-8	7.963	7.954	7.941	7.932	7.922	7.908	7.890	7.865	7.824	7.762	7.723	
G(2)H-8	7.899	7.895	7.888	7.884	7.878	7.869	7.860	7.843	7.824	7.793	7.705	
C(2)H-6	7.865	7.868	7.872	7.871	7.874	7.876	7.875	7.880	7.885	7.885	7.887	7.899
U(1)H-6	7.803	7.808	7.814	7.816	7.819	7.821	7.823	7.826	7.824	7.823	7.857	7.868
U(3)H-6	7.766	7.763	7.755	7.750	7.745	7.738	7.733	7.727	7.718	7.704	7.708	
U(5)H-6	7.766	7.763	7.766	7.766	7.768	7.770	7.770	7.773	7.774	7.778	7.788	7.775
C(4)H-6	7.762	7.738	7.711	7.696	7.681	7.663	7.644	7.625	7.602	7.575	7.539	7.494
C(2)H-5	6.066	6.054	6.038	6.030	6.020	6.008	5.992	5.973	5.939	5.902	5.841	
A(5)H-1'	5.979	5.975	5.971	5.969	5.967	5.965	5.963	5.959	5.951	5.911	5.867	
A(1)H-1'	5.938	5.929	5.922	5.916	5.915	5.912	5.909	5.906	5.900	5.897	5.867	
U(1)H-1'	5.929	5.919	5.903	5.898	5.892	5.880	5.869	5.854	5.852	5.850		
C(2)H-1'	5.897	5.880	5.864	5.860	5.843							
U(3)H-1'	5.880	5.862	5.843	5.818	5.776							
G(3)H-1'	5.864	5.845	5.843	5.845	5.843	5.842	5.835	5.802	5.784	5.775		
U(5)H-1'	5.864	5.845	5.818	5.786								
U(1)H-5	5.864	5.832	5.811	5.795	5.755	5.727	5.705	5.687	5.673	5.649		
C(4)H-1'	5.856	5.826	5.803	5.786	5.766							

Table 28 con't. ^{1}H NMR Chemical Shift Assignments for $\overset{AGm^6GCU}{UC\ U\ GA}$ over the Temperature Range 70° - 5° C (1.8mM)

Resonance	70.9°	61.0°	50.0°	45.0°	40.0°	35.0°	29.9°	25.0°	20.0°	15.0°	9.9°	5.2°
C(4)H-5	5.841	5.832	5.774	5.753	5.725	5.696	5.666	5.635	5.599	5.552	5.503	5.408
U(5)H-5	5.809	5.793	5.779	5.764	5.755	5.742	5.731	5.718	5.704	5.682	5.632	
U(3)H-5	5.794	5.773	5.747	5.732	5.716	5.696	5.673	5.648	5.618	5.579	5.503	
G(2)H-1'	5.718	5.711	5.702	5.697	5.691	5.686	5.662	5.664	5.651	5.637	5.632	
O^6CH_3	3.964	3.958	3.955	3.954	3.954	3.953	3.954	3.955	3.956	3.959	3.965	3.971

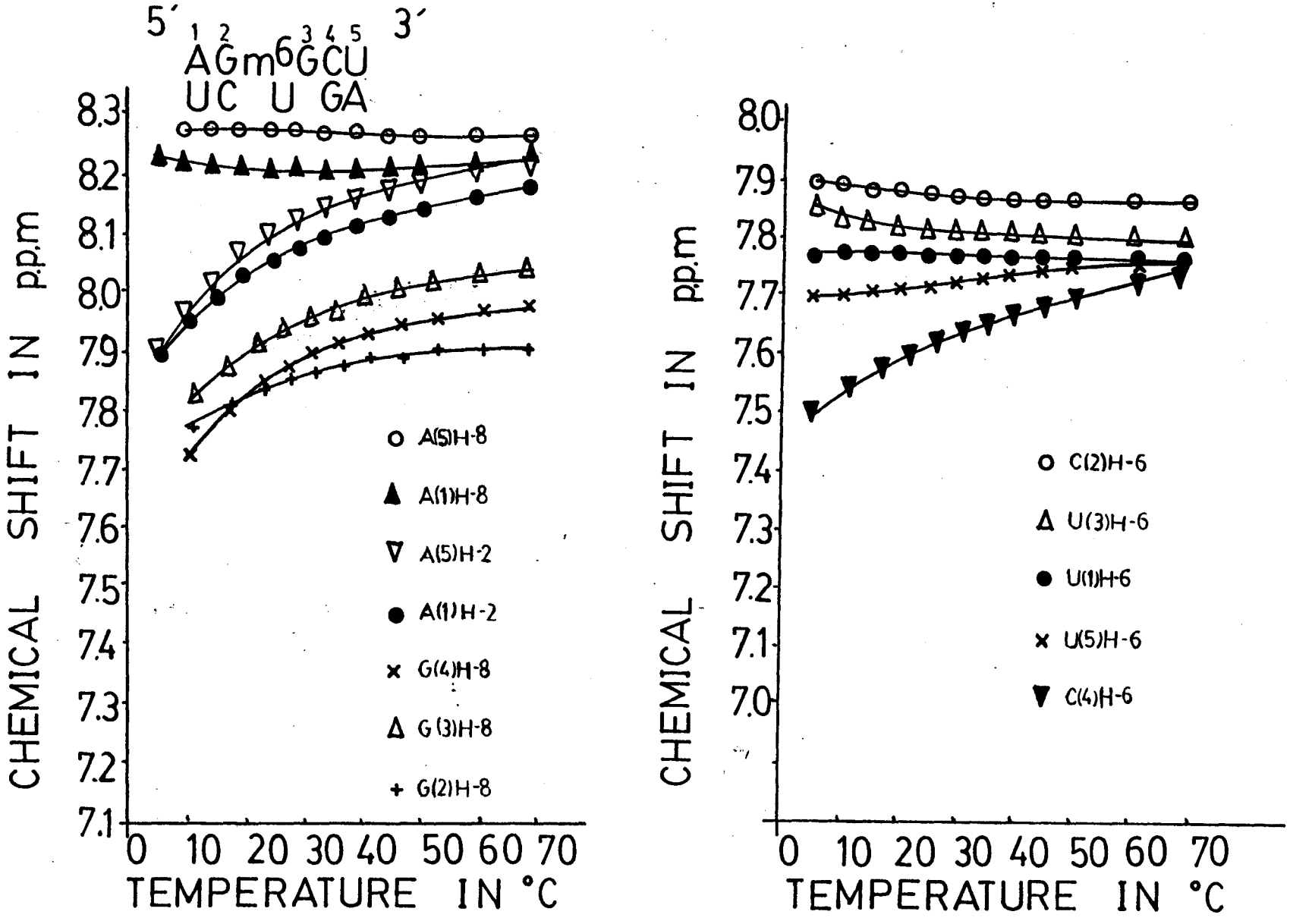
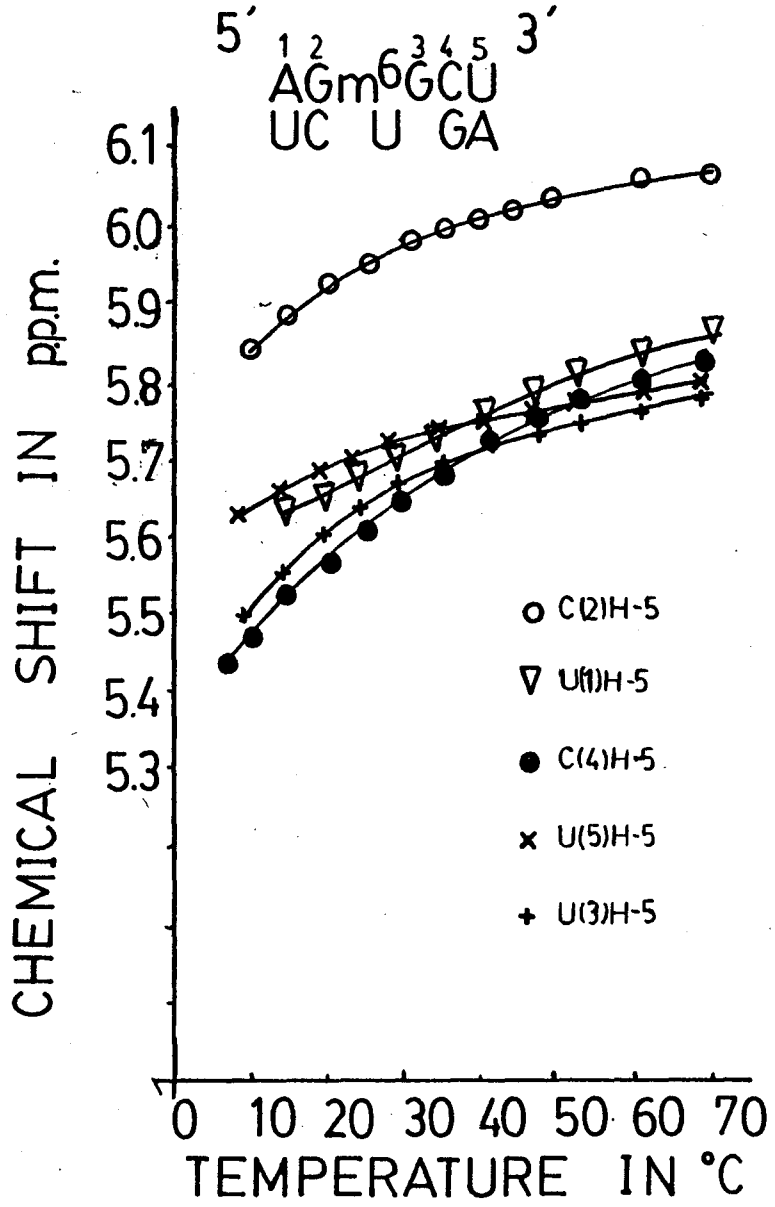


Figure 29. Variable Temperature Study of AGm^6GCU
UC U GA



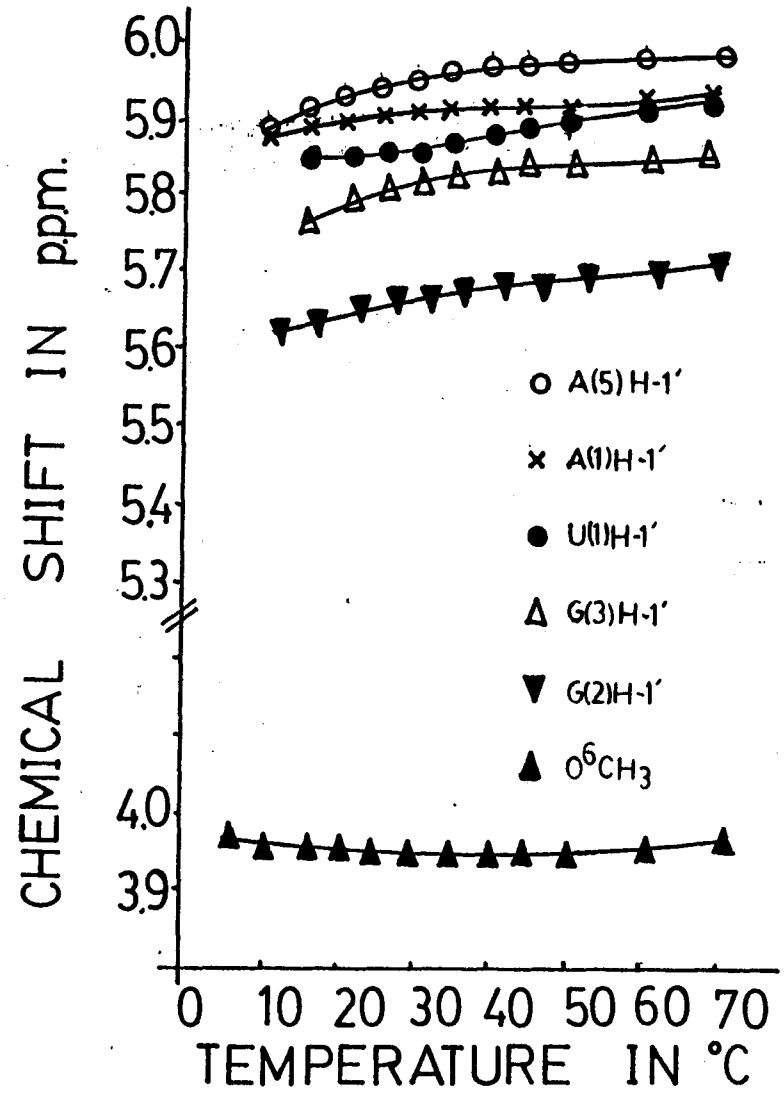


Figure 29 con't. Variable Temperature Study of AGm6GCU (135) UC U GA

Table 29. ^{1}H NMR Chemical Shift Assignments for $\begin{matrix} AGe^6GCU \\ UC\ U\ GA \end{matrix}$ over the Temperature Range 70°- 5°C (1.8mM)

Resonance	70.8°	60.5°	50.7°	45.7°	41.1°	35.2°	30.1°	25.2°	20.3°	15.4°	9.9°	5.2°
A(5)H-8	8.239	8.241	8.242	8.244	8.245	8.247	8.248	8.250	8.257	8.262	8.268	8.272
A(1)H-8	8.198	8.194	8.189	8.188	8.183	8.183	8.182	8.182	8.185	8.192	8.205	8.214
A(5)H-2	8.188	8.175	8.159	8.149	8.140	8.124	8.110	8.092	8.066	8.034		
A(1)H-2	8.159	8.142	8.121	8.110	8.099	8.081	8.066	8.050	8.029	7.997	7.938	7.864
G(4)H-8	8.033	8.021	8.005	7.996	7.985	7.967	7.951	7.933	7.907	7.878	7.839	7.809
G(3)H-8	7.954	7.944	7.930	7.921	7.911	7.892	7.871	7.849	7.806	7.768	7.777	
G(2)H-8	7.899	7.897	7.893	7.891	7.887	7.878	7.871	7.856	7.837	7.813	7.805	
C(2)H-6	7.811	7.815	7.818	7.821	7.824	7.826	7.829	7.833	7.838	7.845	7.855	
U(1)H-6	7.797	7.803	7.808	7.811	7.815	7.816	7.818	7.821	7.822	7.823	7.822	
U(3)H-6	7.755	7.758	7.760	7.761	7.764	7.765	7.766	7.768	7.771	7.775	7.788	7.775
U(5)H-6	7.755	7.750	7.742	7.737	7.732	7.721	7.712	7.703	7.691	7.673	7.647	7.620
C(4)H-6	7.706	7.684	7.657	7.640	7.623	7.597	7.574	7.551	7.524	7.491	7.447	7.411
C(2)H-5	6.013	6.000	5.983	5.974	5.964	5.948	5.933	5.917	5.895	5.879	5.865	5.850
A(5)H-1'	5.972	5.968	5.964	5.962	5.961	5.959	5.957	5.956	5.952	5.942	5.900	
A(1)H-1'	5.930	5.922	5.914	5.912	5.904	5.898	5.893	5.892	5.888	5.882	5.879	
U(1)H-1'	5.930	5.919	5.904	5.896	5.888	5.877	5.877	5.848	5.831	5.806	5.784	
U(1)H-5	5.856	5.846	5.835	5.828	5.807	5.787	5.760	5.740	5.714	5.702	5.684	
C(4)H-5	5.824	5.779	5.729	5.696	5.664	5.622	5.586	5.550	5.509	5.461	5.396	5.388
U(5)H-5	5.804	5.790	5.770	5.760	5.749	5.736	5.721	5.707	5.691	5.679	5.652	5.628
U(3)H-5	5.780	5.757	5.729	5.712	5.695	5.667	5.642	5.615	5.582	5.539	5.487	
G(2)H-1'	5.712	5.708	5.703	5.701	5.699	5.692	5.685	5.678	5.668	5.658	5.638	
$O^6CH_2CH_3$	1.322	1.329	1.336	1.341	1.346	1.353	1.358	1.365	1.370	1.376	1.378	1.376

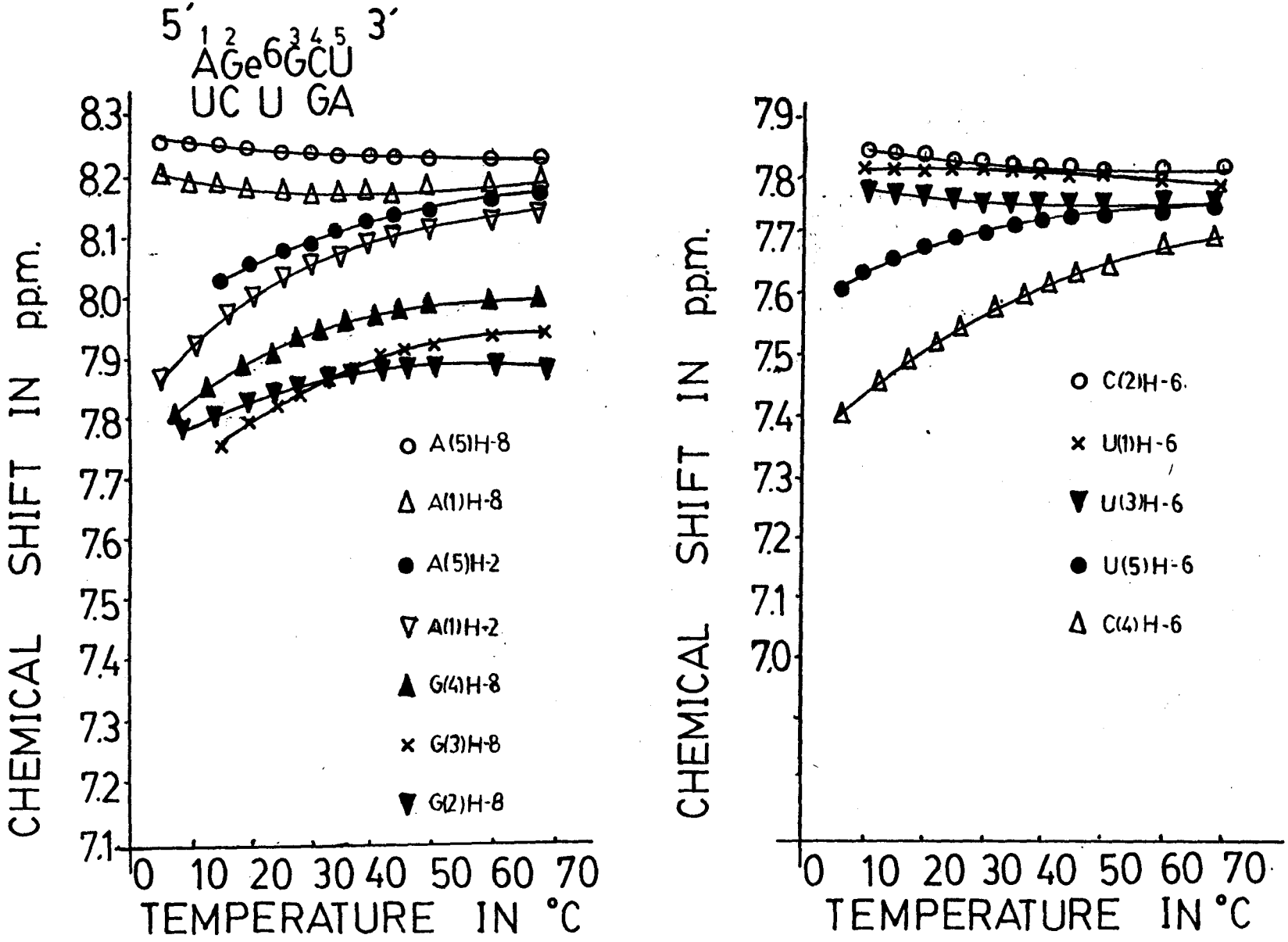
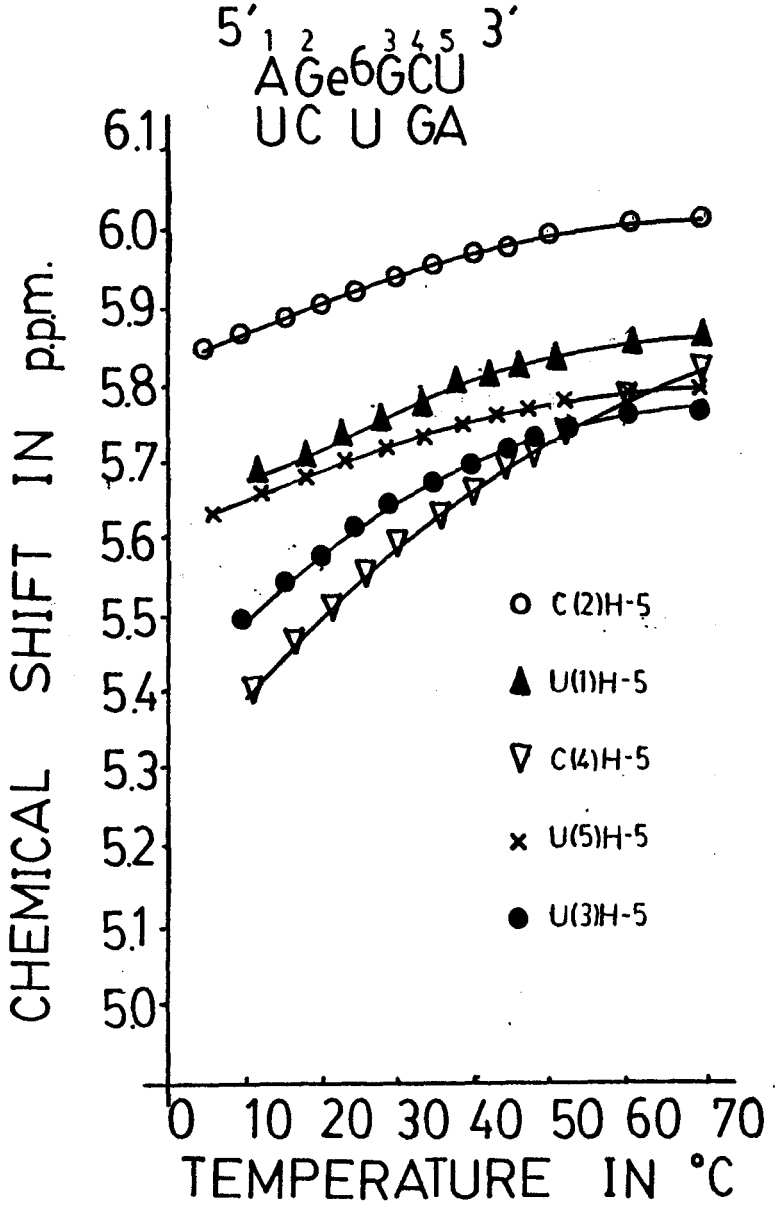


Figure 30. Variable Temperature Study of AGe6GCU / UC U GA



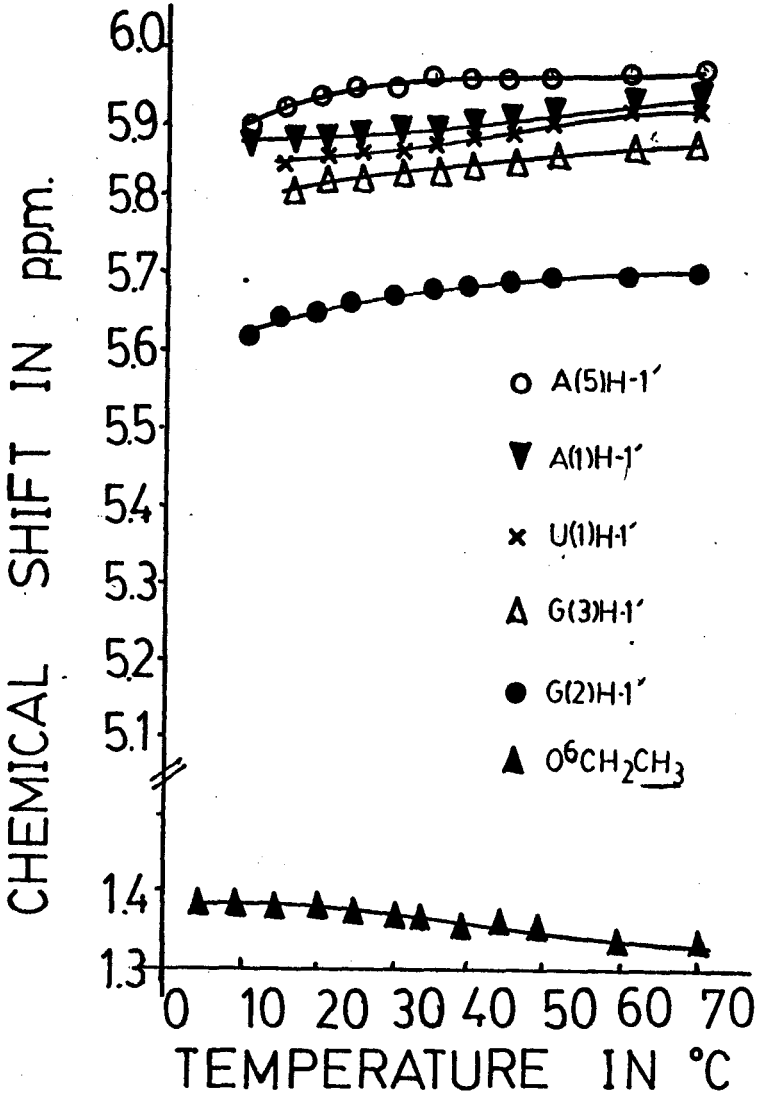


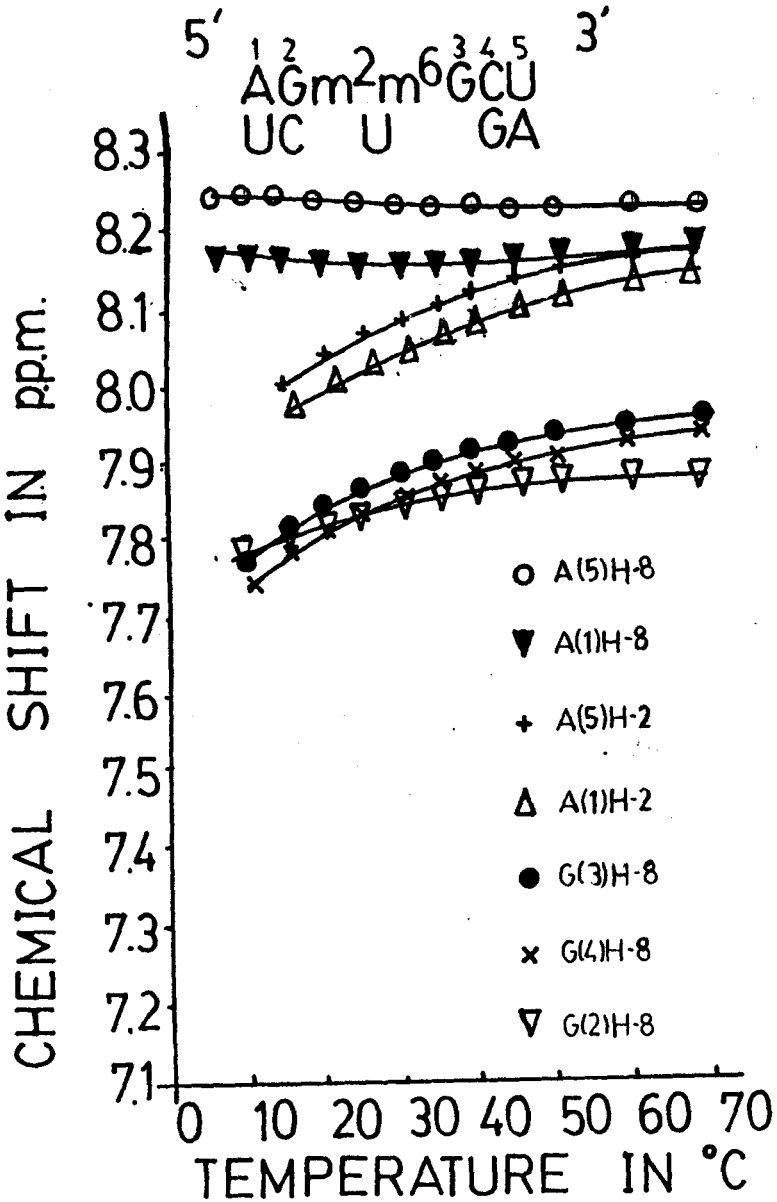
Figure 30 cont. Variable Temperature Study of AGe6GCU / UC U GA

Table 30. ^{1}H NMR Chemical Shift Assignments for $^{AGm^2m^6GCU}_{UC\ U\ GA}$ over the Temperature Range 70°- 5°C (1.8 mM)

Resonance	70.2°	60.4°	50.9°	45.7°	40.2°	35.2°	30.1°	25.2°	20.3°	15.4°	9.9°	5.2°
A(5)H-8	8.238	8.239	8.240	8.240	8.241	8.241	8.243	8.245	8.250	8.254	8.259	8.250
A(1)H-5	8.187	8.180	8.174	8.170	8.166	8.164	8.162	8.162	8.164	8.169	8.179	8.186
A(5)H-2	8.184	8.168	8.151	8.141	8.125	8.112	8.096	8.077	8.046	7.968		
A(1)H-2	8.152	8.133	8.111	8.100	8.082	8.067	8.051	8.032	8.008	7.968		
G(3)H-8	7.959	7.949	7.937	7.929	7.915	7.904	7.890	7.874	7.853	7.822	7.750	
G(4)H-8	7.948	7.937	7.921	7.911	7.893	7.877	7.857	7.830	7.797	7.785	7.750	
G(2)H-8	7.876	7.877	7.876	7.875	7.869	7.865	7.857	7.844	7.818	7.792	7.783	7.789
C(2)H-6	7.801	7.805	7.810	7.813	7.815	7.818	7.822	7.828	7.845	7.844		
U(1)H-6	7.791	7.796	7.802	7.805	7.807	7.809	7.811	7.814	7.813	7.813		
C(4)H-6	7.668	7.643	7.614	7.597	7.574	7.553	7.532	7.511	7.489	7.465	7.444	
U(3)H-6	7.745	7.740	7.731	7.725	7.715	7.707	7.698	7.688	7.676	7.657	7.657	7.624
U(5)H-6	7.739	7.740	7.743	7.745	7.746	7.747	7.749	7.752	7.754	7.758		
C(2)H-5	6.011	5.996	5.979	5.969	5.953	5.943	5.928	5.911	5.890	5.852	5.833	
A(5)H-1'	5.969	5.965	5.961	5.960	5.957	5.954	5.952	5.949	5.944	5.924		
A(1)H-1'	5.907	5.893	5.878	5.872	5.863	5.850	5.833	5.831	5.820	5.813	5.817	
U(1)H-1'	5.922	5.910	5.897	5.890	5.892	5.891	5.890	5.873	5.867	5.829	5.817	
G(3)H-1'	5.891	5.876	5.861	5.853	5.837	5.831	5.813	5.802		5.779		
U(3)H-1'	5.891	5.853	5.837	5.810	5.791	5.778	5.760	5.755	5.732	5.704	5.656	
U(1)H-5	5.853	5.844	5.831	5.827	5.818	5.806	5.793	5.778	5.757	5.721	5.656	
U(5)H-1'	5.840	5.837	5.837	5.836	5.834	5.833	5.833	5.831	5.830	5.829	5.830	
U(5)H-5	5.790	5.771	5.752	5.741	5.727	5.715	5.703	5.690	5.674	5.661	5.656	
U(3)H-5	5.771	5.746	5.715	5.697	5.672	5.648	5.621	5.593	5.560	5.503	5.415	

Table 30 con't. ^{1}H NMR Chemical Shift Assignments for $^{AGm^2m^6GCU}_{UC\ U\ GA}$ over the Temperature Range 70° - 5°C (1.8 mM)

Resonance	70.2°	60.4°	50.9°	45.7°	40.2°	35.2°	30.1°	25.2°	20.3°	15.4°	9.9°	5.2°
C(4)H-5	5.760	5.706	5.647	5.614	5.572	5.536	5.499	5.464	5.424	5.375	5.317	5.314
G(2)H-1'	5.689	5.688	5.689	5.690	5.687	5.687	5.680	5.678	5.666	5.664	5.656	5.653
O^6CH_3	3.931	3.925	3.924	3.923	3.923	3.924	3.926	3.927	3.928	3.930	3.931	
N^2CH_3	2.867	2.862	2.857	2.855	2.851	2.847	2.844	2.840	2.835	2.823	2.800	



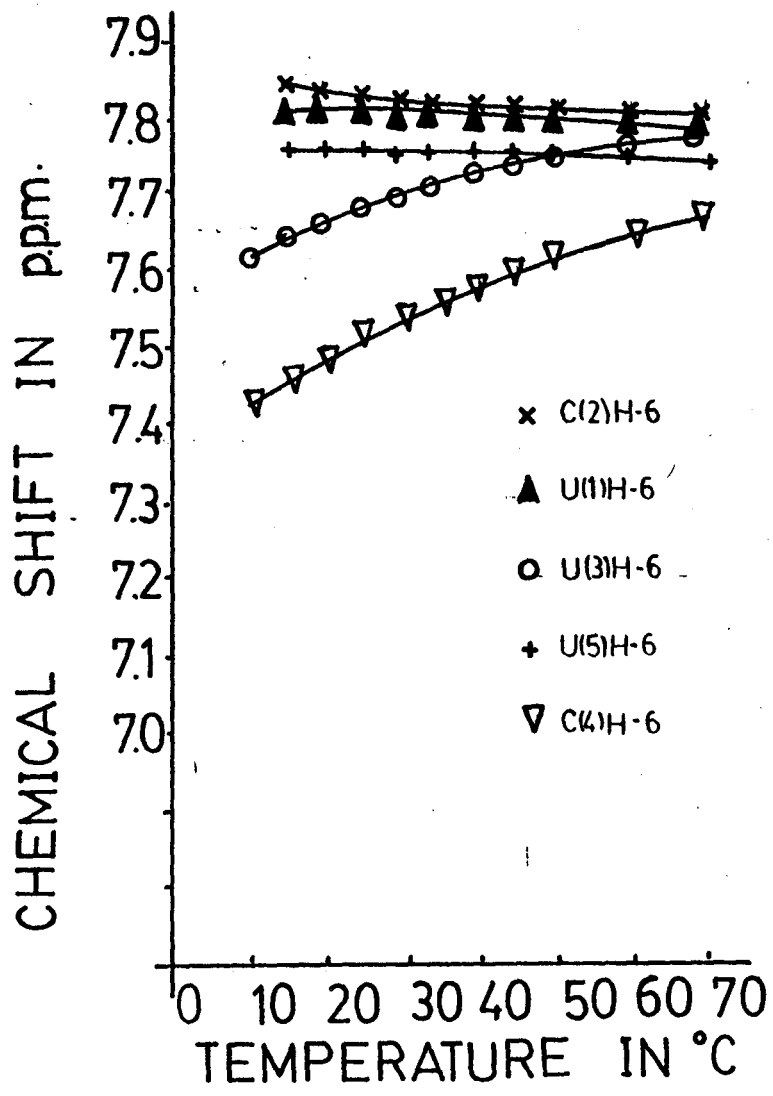
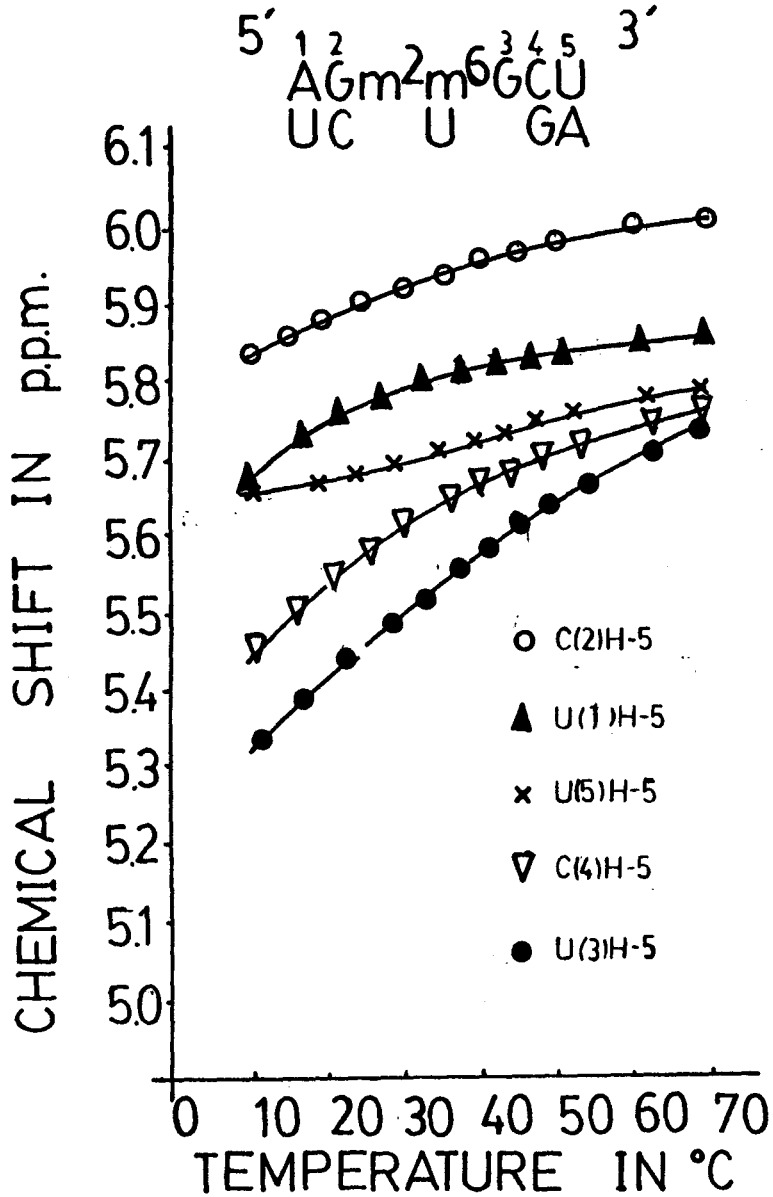


Figure 31. Variable Temperature Study of AGm2m6GCU / UC U GA



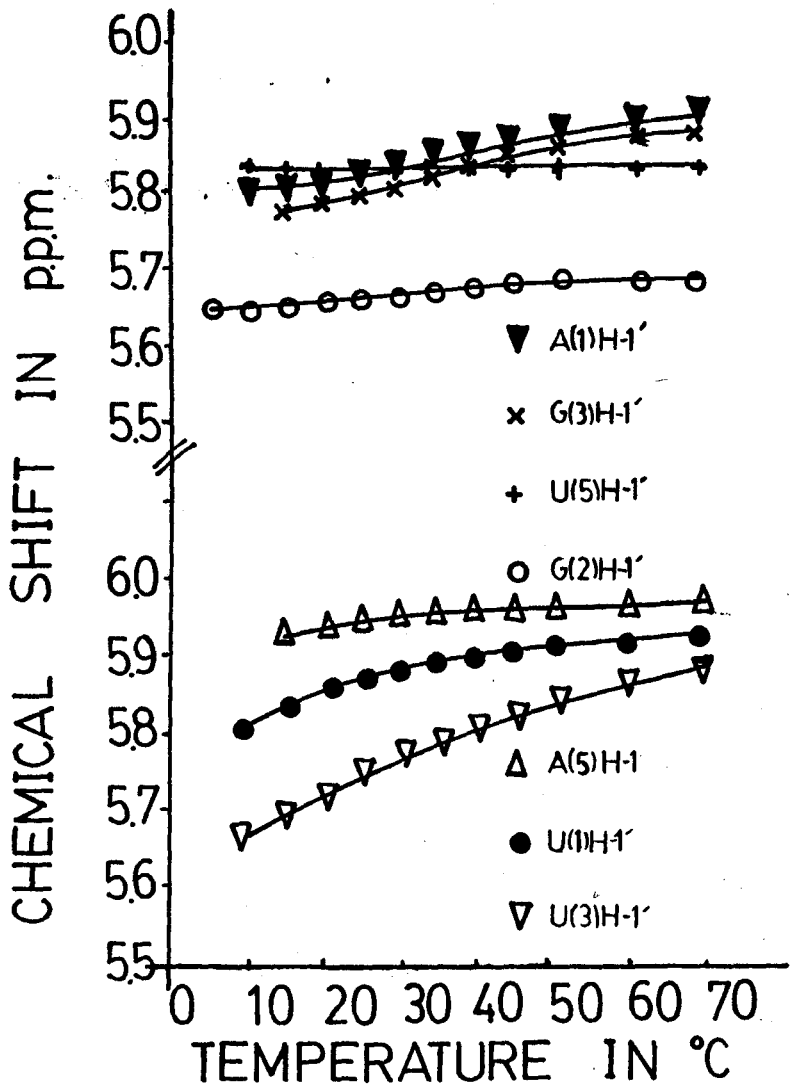


Figure 31 con't. Variable Temperature Study of AGm2m6GCU / UC U GA

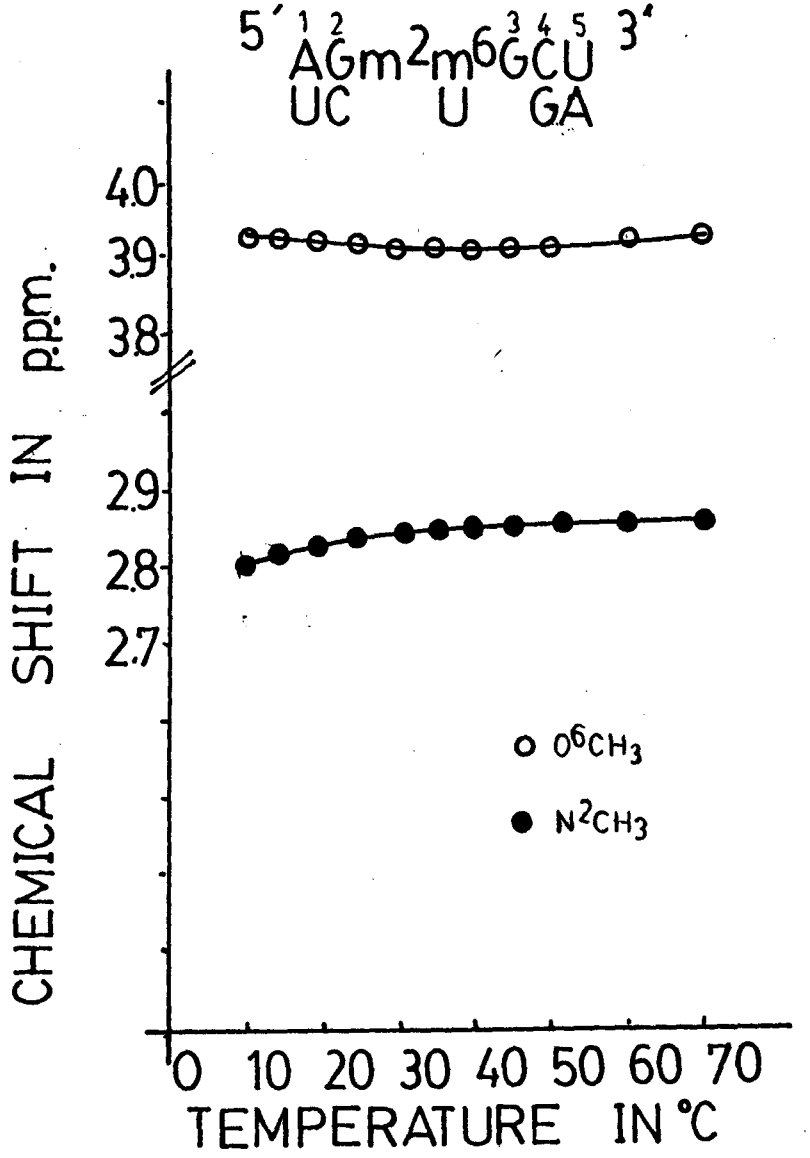
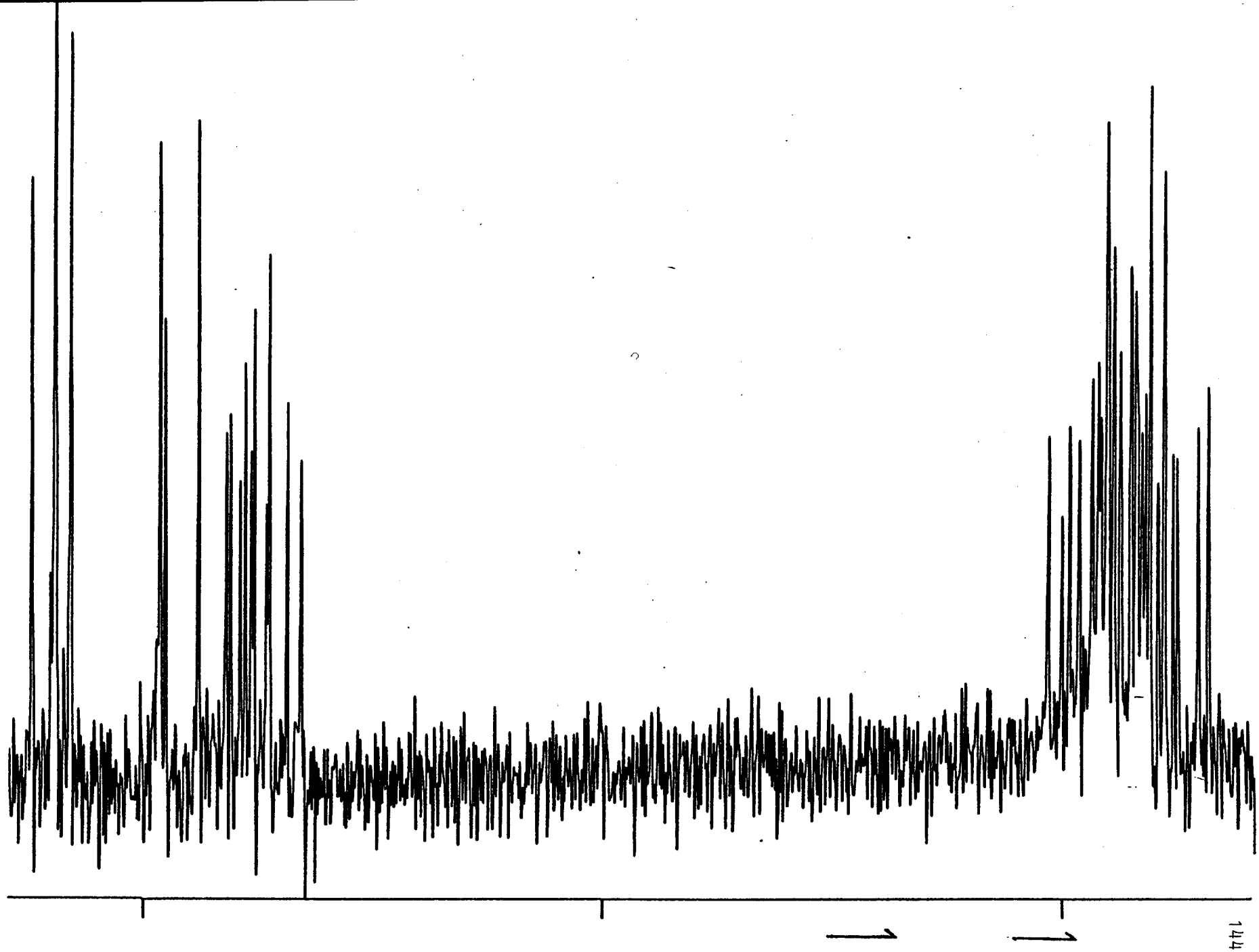


Figure 31 con't. Variable Temperature Study of AGm2m6GCU / UC U GA



144

Figure 32. ^{1}H-NMR Spectrum of AGm^{2}m^{6}GCU : AGUCU

Table 31. ^{1}H NMR Chemical Shift Assignments for $^{AGm^2m^6GCU}_{UC\ U\ GA}$ over the Temperature Range 70° - 10° C (1.8 mM)

Resonance	70.2°	60.4°	50.9°	45.7°	40.2°	35.2°	30.1°	25.2°	20.3°	15.4°	9.9°
A(5)H-8	8.247	8.252	8.258	8.263	8.271	8.280	8.293	8.308	8.317	8.308	8.270
A(1)H-8	8.189	8.183	8.175	8.172	8.169	8.168	8.169	8.173	8.178	8.183	8.174
A(5)H-2	8.171	8.152	8.128	8.113	8.093	8.074	8.049	8.013	7.936	7.801	7.676
A(1)H-2	8.153	8.133	8.110	8.098	8.080	8.062	8.039	8.005	7.936	7.789	7.676
G(3)H-8	7.960	7.951	7.936	7.928	7.915	7.902	7.886	7.867	7.839	7.801	7.791
G(4)H-8	7.922	7.898	7.864	7.841	7.807	7.771	7.725	7.675	7.615	7.557	7.513
G(2)H-8	7.878	7.879	7.877	7.874	7.869	7.860	7.844	7.816	7.780	7.755	
C(2)H-6	7.803	7.807	7.810	7.811	7.811	7.811	7.807	7.798		7.789	7.763
U(1)H-6	7.788	7.793	7.797	7.798	7.799	7.799	7.798	7.798	7.797	7.789	7.791
U(5)H-6	7.740	7.742	7.744	7.746	7.748	7.750	7.753	7.756	7.763	7.755	
C(3)H-6	7.705	7.686	7.661	7.645	7.625	7.608	7.591	7.582	7.469	7.432	7.383
C(4)H-6	7.669	7.645	7.614	7.597	7.573	7.552	7.527	7.502	7.469	7.432	7.383
C(2)H-5	5.975	5.920	5.920	5.900	5.839	5.753	5.728	5.695	5.682		
A(5)H-1'	5.979	5.979	5.979	5.980	5.982	5.984	5.985	5.980	5.950	5.877	5.809
A(1)H-1'	5.909	5.892	5.890	5.888	5.883	5.875	5.856	5.849	5.826	5.813	
U(1)H-1'	5.909	5.877	5.880	5.873	5.865	5.856	5.831	5.812	5.797	5.790	
C(2)H-1'	5.891	5.877	5.861	5.851	5.848	5.834	5.827	5.824	5.818	5.813	5.809
G(3)H-1'	5.891	5.877	5.861	5.851	5.832	5.831	5.815	5.812	5.797	5.790	
U(1)H-5	5.802	5.773	5.742	5.717	5.681	5.652	5.628	5.615			
U(5)H-5	5.790	5.773	5.753	5.741	5.727	5.713	5.697	5.678	5.651	5.635	5.611
C(3)H-5	5.790	5.734	5.660	5.613	5.557	5.503	5.442	5.381	5.310	5.229	
C(4)H-5	5.760	5.707	5.646	5.613	5.568	5.527	5.481	5.430	5.359	5.247	

Table 31 con't. ^{1}H NMR Chemical Shift Assignments for $^{AGm^2m^6GCU}_{UC\ U\ GA}$ over the Temperature Range 70° - 10° C (1.8 mM)

Resonance	70.2°	60.4°	50.9°	45.7°	40.2°	35.2°	30.1°	25.2°	20.3°	15.4°	9.9°
G(2)H-1'	5.692	5.691	5.691	5.691	5.685	5.666	5.660	5.670	5.657	5.635	5.611
O^6CH_3	3.933	3.927	3.926	3.928	3.931	3.934	3.935	3.916	3.901	3.869	
N^2CH_3	2.869	2.863	2.859	2.854	2.850	2.846	2.842	2.833	2.818	2.791	

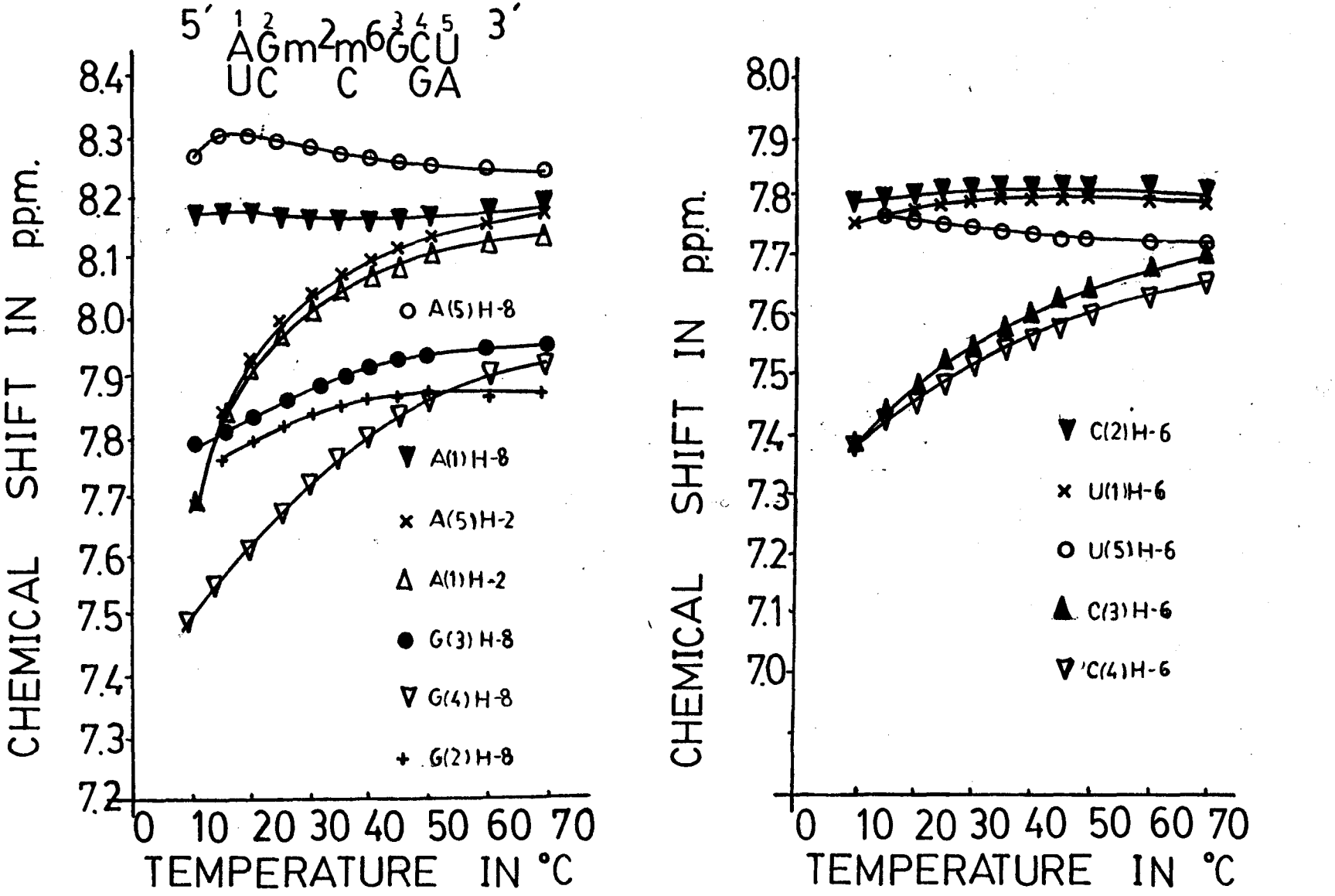


Figure 33. Variable Temperature Study of AGm2m6GCU / UC C GA

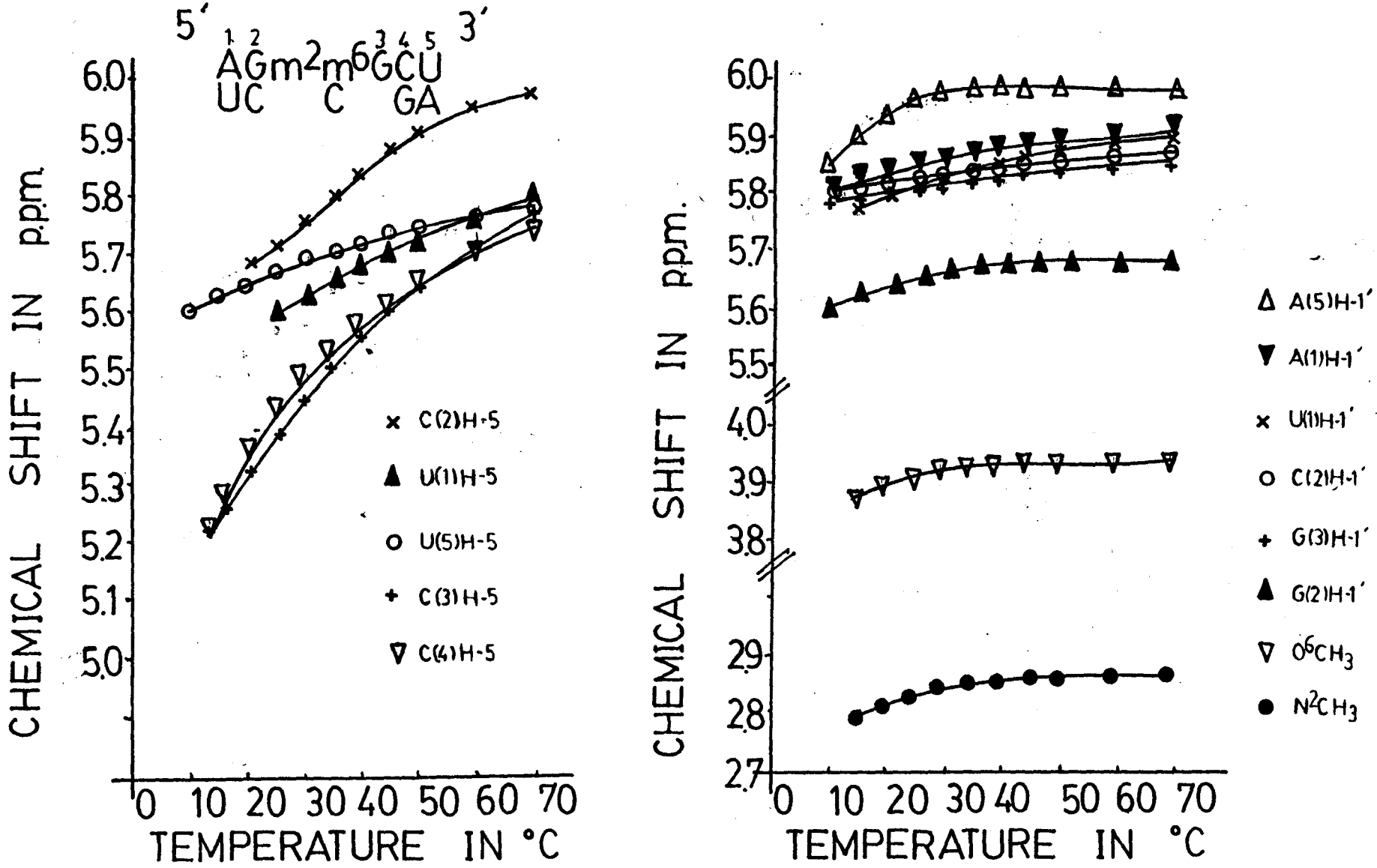


Figure 33 con't. Variable Temperature Study of $\overset{1\ 2}{AGm^2}\overset{3}{m^6}\overset{4\ 5}{GCU}$ (UC C GA)

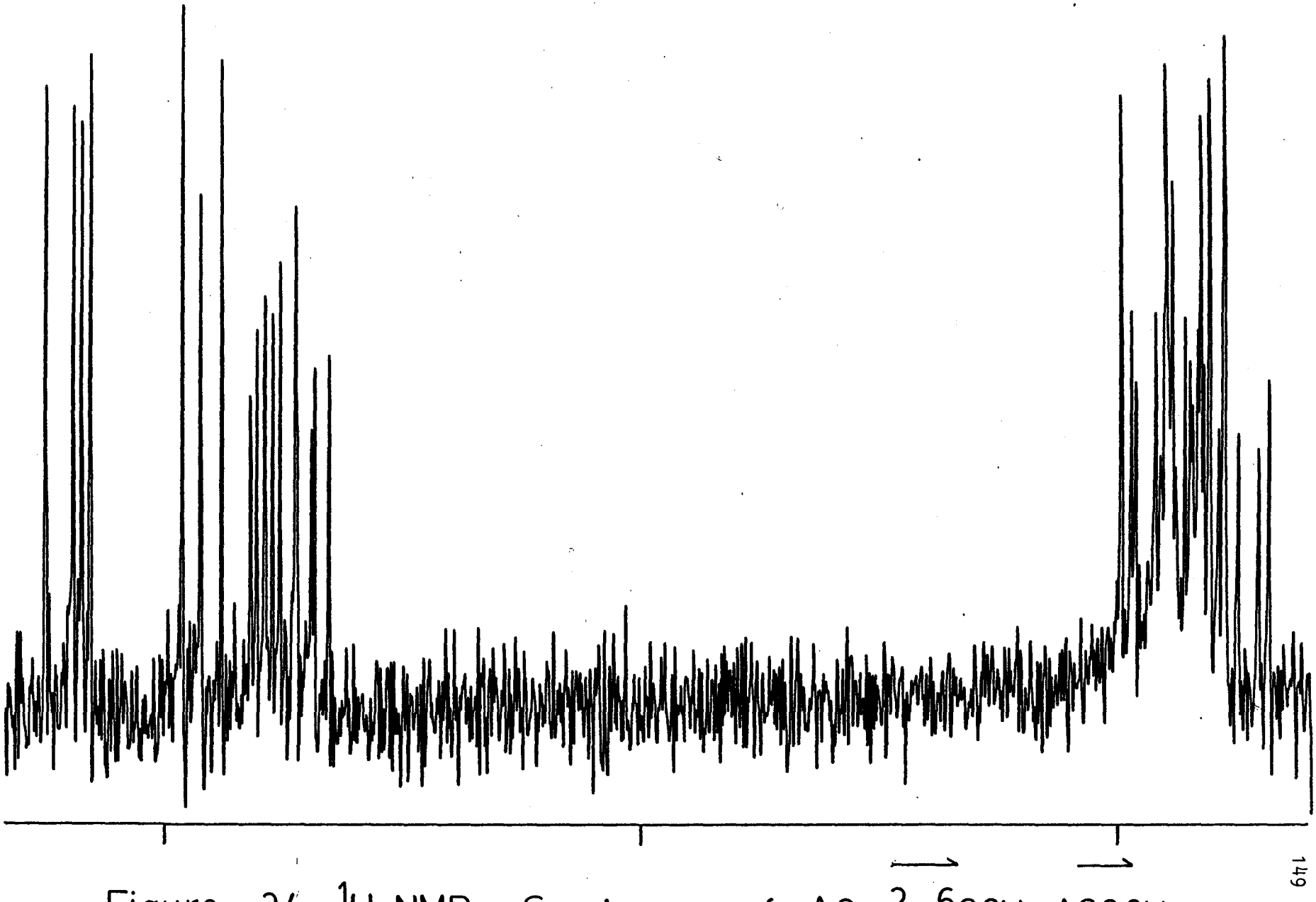


Figure 34. ^{1}H-NMR Spectrum of $AGm^{2}m^{6}GCU : AGCCU$

Table 32. ^{1}H NMR Chemical Shift Assignments for $\frac{\text{AGGCU}}{\text{UCUGA}}$ over the Temperature Range 70°- 20°C (1.8 mM)

Resonance	70.2	60.4	50.9	45.0	39.8	34.9	30.8	25.8	20.0	Tm, °C
A(5)H-8	8.239	8.240	8.241	8.245	8.252	8.261	8.260	8.251	8.230	*
A(1)H-8	8.210	8.207	8.205	8.207	8.215	8.231	8.247	8.251	8.248	34.6°
A(5)H-2	8.183	8.167	8.146	8.126	8.095	8.020				*
A(1)H-2	8.161	8.142	8.116	8.094	8.062	8.000	7.864	7.819	7.806	30.9°
G(4)H-8	7.947	7.935	7.914	7.892	7.855	7.785	7.700			*
G(3)H-8	7.922	7.906	7.878	7.846	7.795	7.702	7.574			*
G(2)H-8	7.906	7.897	7.882	7.867	7.840	7.785	7.700	7.646		32.3°
C(2)H-6	7.801	7.806	7.812	7.817	7.826	7.842	7.865	7.894	7.900	29.6°
U(1)H-6	7.790	7.795	7.799	7.802	7.803	7.801	7.796	7.804	7.806	*
U(5)H-6	7.767	7.770	7.773	7.776	7.778	7.784	7.796	7.804	7.806	*
U(3)H-6	7.744	7.738	7.730	7.725	7.721	7.718	7.716	7.713	7.721	*
C(4)H-6	7.744	7.738	7.724	7.712	7.697	7.682	7.668	7.646	7.506	*
C(2)H-5	6.013	5.998	5.977	5.957	5.929	5.884	5.845	5.758		*
A(5)H-1'	5.970	5.965	5.961	5.959	5.953	5.920	5.870	5.888	5.874	*
A(1)H-1'	5.938	5.928	5.921	5.919	5.917	5.910	5.863	5.823	5.804	*
U(1)H-1'	5.919	5.908	5.891	5.879	5.863	5.848	5.806	5.758	5.719	27.7°
C(4)H-1'	5.891	5.888	5.891	5.889	5.884	5.874	5.854	5.823	5.804	27.9°
C(2)H-1'	5.891	5.869	5.866	5.863	5.856	5.836	5.806	5.758	5.719	*
U(5)H-1'	5.874	5.856	5.837	5.812	5.771	5.726	5.714	5.689		*
U(3)H-1'	5.856	5.823	5.822	5.798	5.771	5.718	5.714	5.660		*
U(1)H-5	5.851	5.844	5.833	5.813	5.791	5.751	5.698	5.674	5.676	32.8°
C(4)H-5	5.851	5.812	5.748	5.695	5.652	5.544	5.467	5.399	5.375	32.6°

*non sigmoidal behavior

Table 32 con't. ^{1}H NMR Chemical Shift Assignments for $\frac{AGGCU}{UCUGA}$ over the Temperature Range 70°- 20°C (1.8 mM)

Resonance	70.2	60.4	50.9	45.0	39.8	34.9	30.8	25.8	20.0	Tm, °C
G(3)H-1'	5.824	5.792	5.788	5.773	5.754	5.709	5.667			*
U(5)H-5	5.819	5.802	5.780	5.757	5.728	5.682	5.631	5.612	5.562	35.2°
U(3)H-5	5.769	5.744	5.711	5.684	5.626	5.607	5.553	5.490	5.461	30.5°
G(2)H-1'	5.769	5.756	5.741	5.728	5.712	5.693	5.667			*
								avg Tm		31.4°C

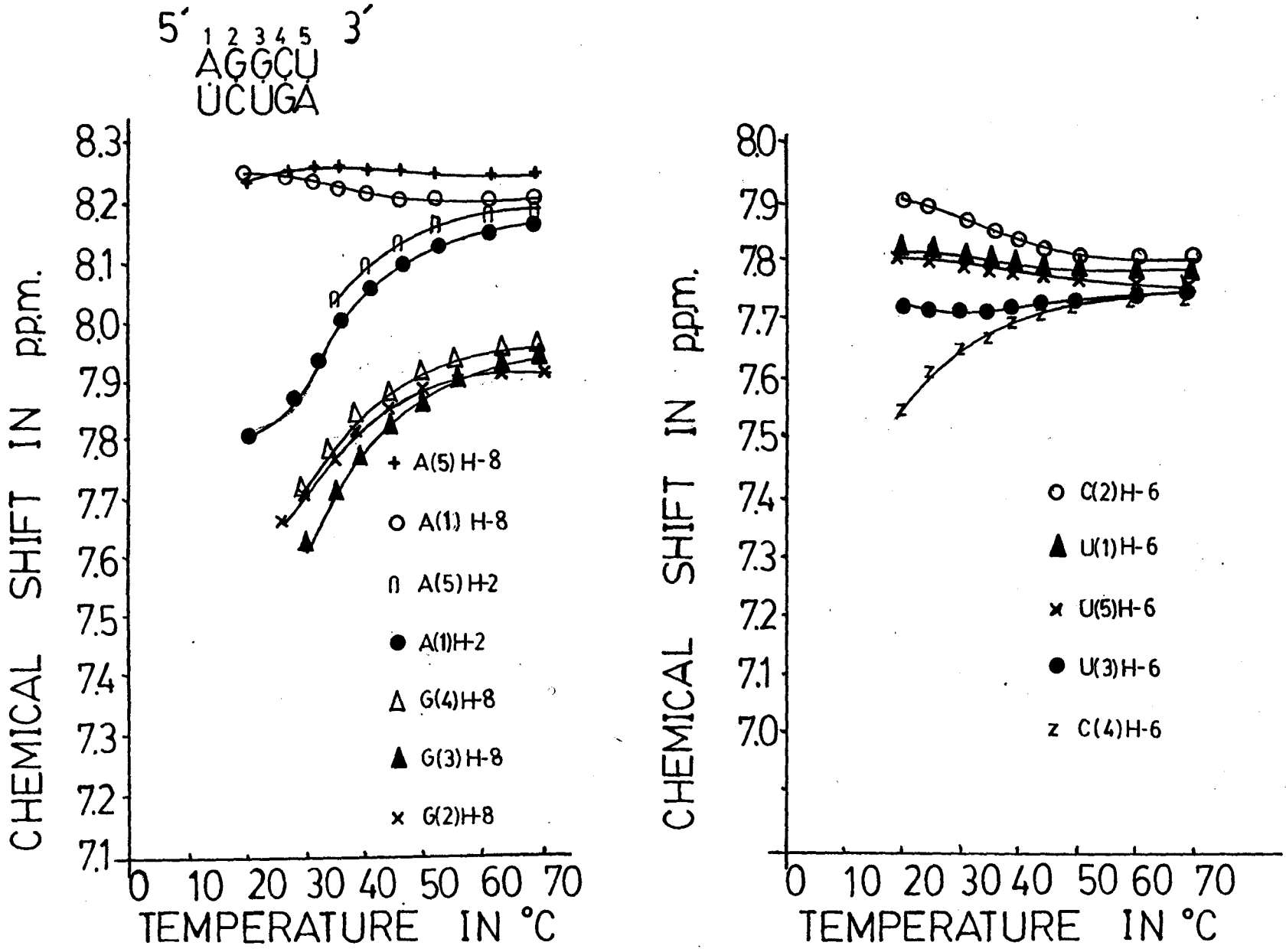


Figure 35. Variable Temperature Study of AGGCU UCUGA

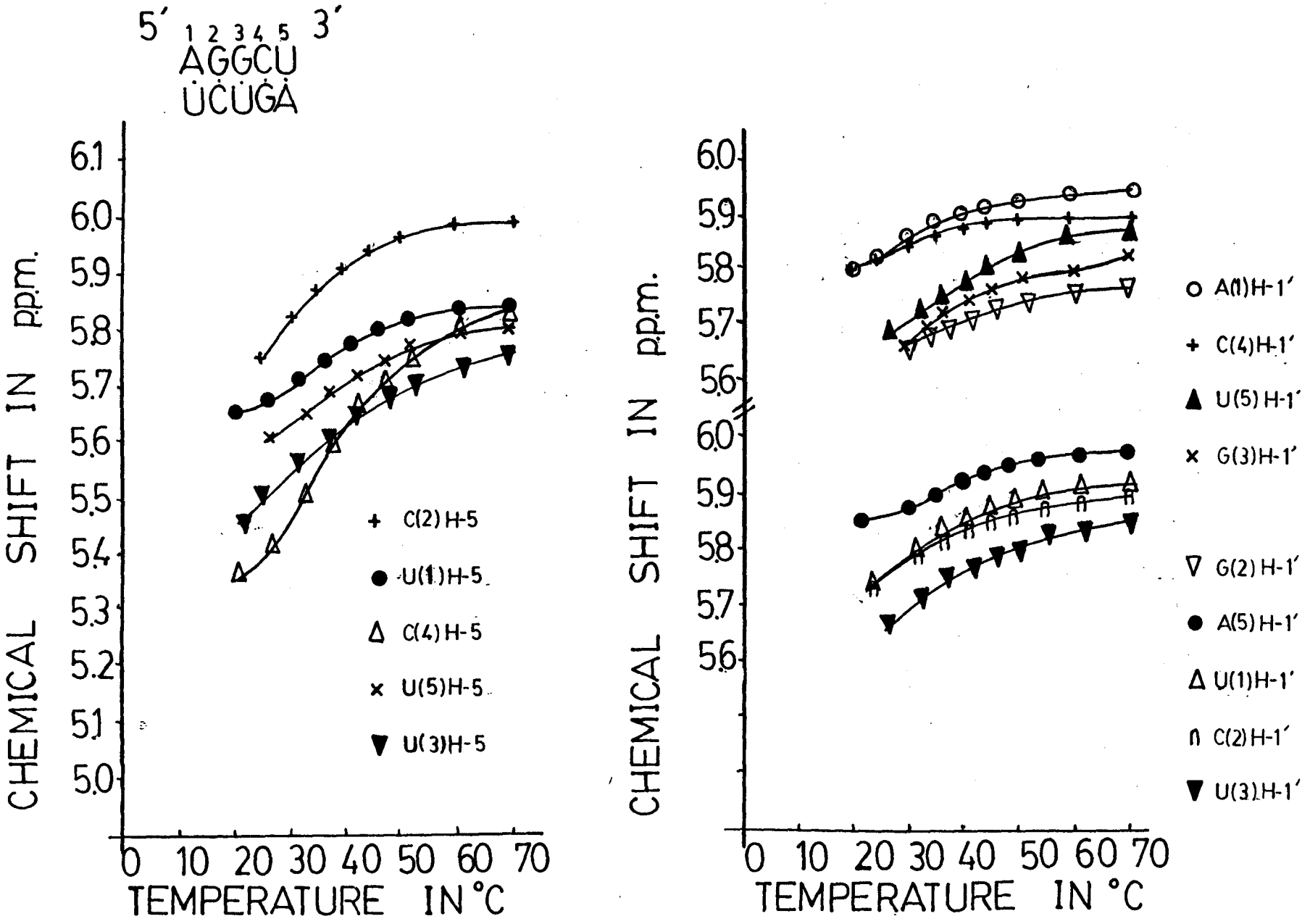


Figure 35 con't. Variable Temperature Study of AGGCU UCUGA

Table 33. ^{1}H NMR Chemical Shift Assignments for AGGCU / UCCGA over the Temperature Range 70° - 10°C (1.3 mM)

Resonance	70.9°	61.0°	54.9°	50.0°	45.0°	40.0°	35.0°	29.9°	25.0°	20.0°	15.0°	9.9°	Tm, °C
A(5)H-8	8.248	8.259	8.269	8.282	8.296	8.306	8.312	8.310	8.304	8.294	8.283	8.272	52.6°
A(1)H-8	8.212	8.218	8.230	8.251	8.279	8.306	8.312	8.310	8.304	8.294	8.283	8.272	51.1°
A(5)H-2	8.172	8.142	8.120	8.085	8.040	7.977	7.857						*
A(1)H-2	8.163	8.131	8.105	8.061	8.001	7.977	7.857						*
G(4)H-8	7.921	7.884	7.858	7.820	7.774	7.725	7.677	7.645	7.623				43.6°
G(3)H-8	7.916	7.862	7.807	7.775	7.659	7.508	7.401						41.2°
G(2)H-8	7.906	7.882	7.853	7.807	7.770	7.686	7.658	7.645					44.2°
C(2)H-6	7.804	7.802	7.798	7.791	7.784	7.776	7.769	7.764	7.767	7.765	7.763		45.1°
U(1)H-6	7.788	7.791	7.791	7.791	7.790	7.789	7.791	7.794	7.783	7.781	7.779	7.782	*
U(5)H-6	7.768	7.771	7.772	7.775	7.776	7.780	7.791	7.794	7.783	7.795	7.798	7.800	*
C(4)H-6	7.743	7.720	7.701	7.675	7.644	7.622	7.604	7.585	7.596				46.8°
C(3)H-6	7.706	7.688	7.680	7.675	7.675	7.671	7.673	7.673					*
A(5)H-1'	5.981	5.983	5.985	5.988	5.982	5.965	5.934	5.897	5.888	5.878	5.868	5.853	*
A(1)H-1'	5.941	5.938	5.942	5.950	5.952	5.942	5.907	5.879	5.868	5.860	5.848	5.834	*
C(2)H-5	5.968	5.911	5.877	5.824	5.711	5.660	5.626						*
U(1)H-1'	5.893	5.869	5.865	5.859	5.853		5.839	5.829	5.819	5.815	5.812	5.782	*
U(5)H-1'	5.889	5.860	5.824	5.789	5.772	5.776	5.780	5.777	5.777	5.768	5.765	5.751	*
C(2)H-1'	5.885	5.854	5.824	5.784	5.750	5.712	5.674	5.652	5.652	5.656	5.662	5.662	47.0°
C(4)H-1'	5.893	5.887	5.882	5.873	5.862		5.839	5.829	5.819	5.779	5.765	5.751	*
C(3)H-1'	5.865	5.813	5.808	5.784	5.741	5.676	5.659	5.630	5.597				*
C(4)H-5	5.839	5.775	5.742	5.696		5.428	5.372	5.359					*
G(3)H-1'	5.833	5.774	5.755	5.714	5.682	5.676	5.659	5.630	5.631	5.568			*

*non sigmoidal behavior

Table 33 con't. ^{1}H NMR Chemical Shift Assignments for $\frac{AGGCU}{UCCGA}$ over the Temperature Range 70°- 10°C (1.3 mM)

Resonance	70.9°	61.0°	54.9°	50.0°	45.0°	40.0°	35.0°	29.9°	25.0°	20.0°	15.0°	9.9°	Tm, °C
U(5)H-5	5.847	5.809	5.781	5.745	5.706	5.660	5.637	5.615					*
U(1)H-5	5.783	5.759	5.754	5.711	5.663	5.615	5.580						*
C(3)H-5	5.783	5.694	5.638	5.568	5.498	5.367		5.236					*
G(2)H-1'	5.791	5.754	5.755	5.714	5.675	5.643	5.636		5.572		5.559	5.549	*
G(4)H-1'	5.775	5.736	5.714	5.701	5.672	5.643	5.636	5.600	5.583	5.568	5.559	5.549	*
											avg Tm		47.0°C

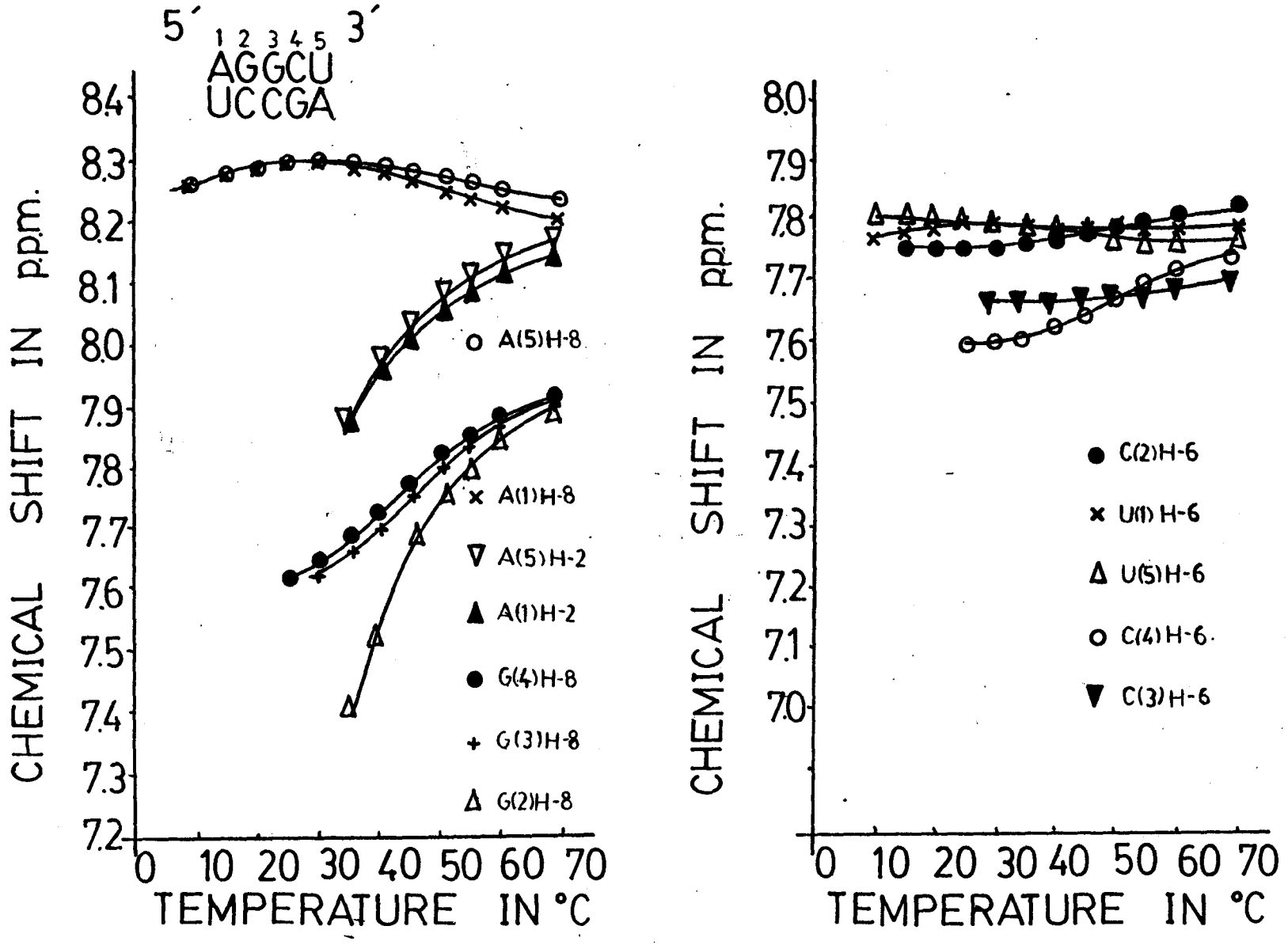


Figure 36. Variable Temperature Study of AGGCU UCCGA

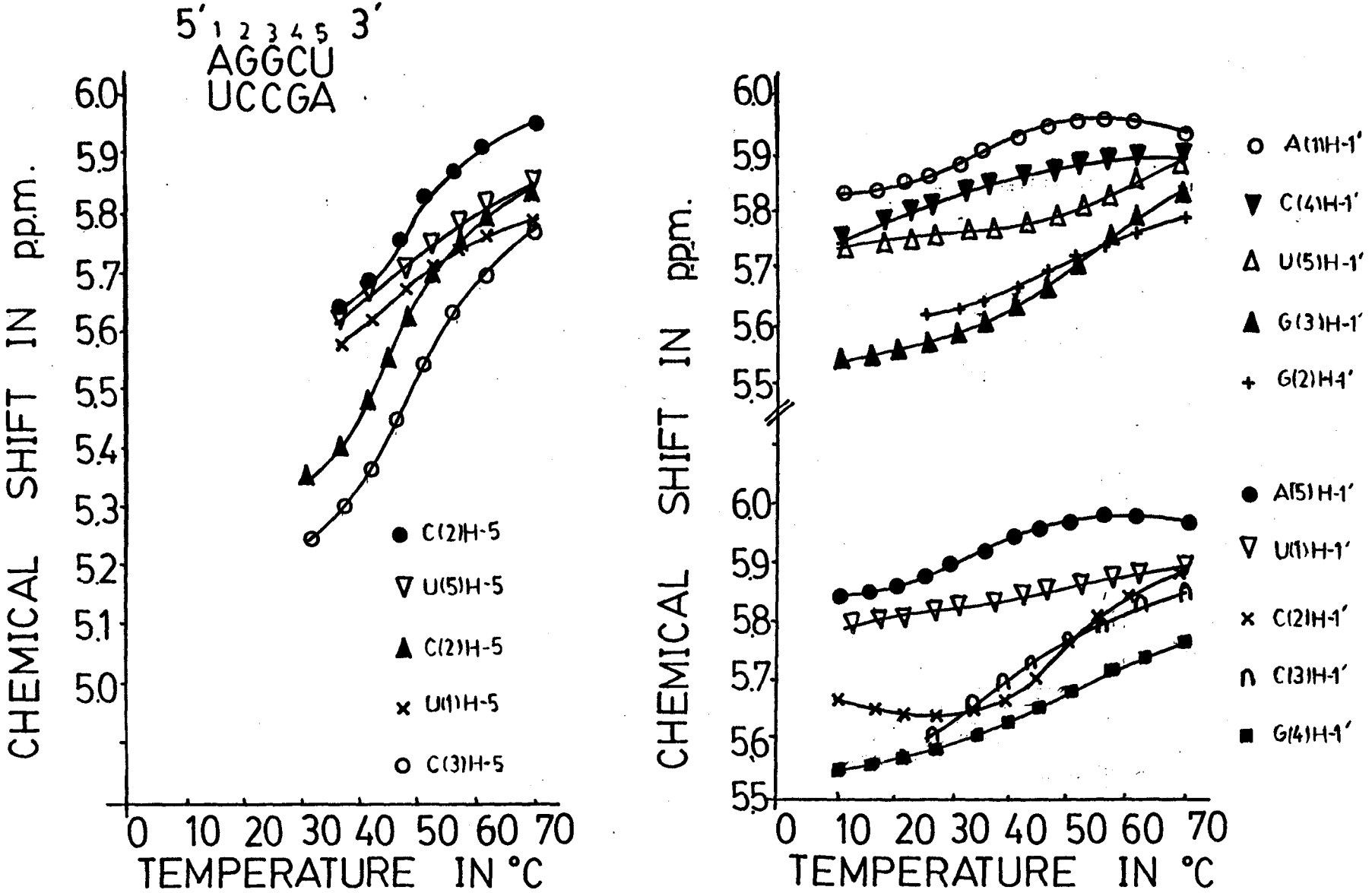


Figure 36 con't. Variable Temperature Study of AGGCU UCCGA

potential duplex formed.

Melting temperatures for the reference duplexes AGGCU : AGUCU (1.8 mM) amd AGGCU : AGCCU (1.3 mM) were obtained through computer analysis (see section 3.2.1) to be 31.4°C and 47.0°C, respectively. A Tm for the latter reference at 1.8 mM was estimated through extrapolation, using melting temperatures at two different concentrations (Tm 54.0°C, 6.3mM; Tm 47.0°C, 1.3 mM), to be 48.0°C.

Unlike the reference compounds, the modified pentamer mixing experiments revealed no Tm values. The nonexchangeable proton resonances tended to shift upfield with decreasing temperature, but showed no clear indication of sigmoidal behavior.

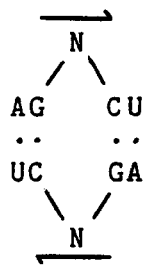
3.3.2 Discussion

This work is an extension of an earlier study which investigated the effect of G:C and G:U base pairs when flanked by two Watson-Crick G:C base pairs, in a short RNA duplex (Alkema, et al., 1982).

In Alkema's study, the self-complementary pentamer, AGGCU (6.25 mM), was found to form a stable duplex with Tm = 30°C. While not an absolute reference for the AGNCU pentamers investigated in this study (1.7-1.8 mM), AGGCU provides an indication of the types of duplexes which could potentially form.

Insertion of a G:C or G:U base pair into the self-complementary duplex AGCU : AGCU, was found to increase stability (Tm's : AGCU : AGCU, 34.0°, AGGCU : AGUCU, 44.0°, AGGCU : AGCCU, 54.0° C, concentrations of 6.25 mM) (Alkema, et al, 1982). As melting temperature is to some degree concentration dependent, it was necessary to determine the Tm's of these parent duplexes at concentrations near to those of the alkylated pentamers (i.e. 1.80 mM).

Three pentaribonucleotide sequences, AGm^6GCU, AGe^6GCU, AGm^2m^6GCU, were synthesized. Each sequence could potentially form a duplex of the type :



with an internal non-complementary base pair opposition, and can be compared to the reference system AGGCU : AGGCU (Alkema, et al., 1982). Mixing of the modified pentamers with AGUCU or AGCCU, potentially provided a duplex with an internal potential modified G : U or modified G : C base pair. These may be compared with the parent systems, AGGCU : AGUCU, and AGGCU : AGCCU. By examining the various duplex systems over the temperature range 70° - 0°C, it

was possible to determine the effect of : (1). non-complementary, modified-base oppositions; and (2). modified G : pyrimidine base pairs, on duplex stability, when flanked by G : C base pairs.

Figures 24-26 illustrate the "Chemical Shift versus Temperature" plots for AGm^6GCU, AGe^6GCU, AGm^2m^6GCU, respectively. The melting curves show a general upfield shift with decreasing temperature, but show no sigmoidal behavior. In several instances, it appears that an inflection point might be detected somewhere between 0°-10°C, however. These result indicate that no duplex formation occurs in any of the pentamers, down to 10°C.

The O^6-methyl group of AGm^6GCU experiences shielding and then deshielding with decreasing temperature, while the methyl of the O^6-ethyl group of AGe^6GCU undergoes deshielding. The O^6-methyl and N^2-methyl groups of AGm^2m^6GCU experience shielding as the temperature is lowered.

The results above are in contrast with those obtained for AGGCU, in which sigmoidal behavior is seen in 8 of 13 melting curves, to give an average Tm of 30°C (6.25 mM) (Alkema, et al., 1982). Alkema has suggested that this Tm arises from the formation of a duplex of the type :

containing two 3'-terminal, non-base paired adenosine residues, two terminal G : U base pairs, and two internal G : C base pairs, as the related duplex GGCU : GGCU (Mizuno, et al., 1981) forms with a Tm of about 30°C (Alkema,D., Bell,R.A., Hader,P.A., Neilson,T., unpublished observations). As no absolute proof exists for the formation of this type of duplex uniquely, Alkema has not discounted the possibility that a central-bulge loop duplex forms competitively.

It is apparent that for the AGNCU pentamers examined in single-stranded studies, the presence of an alkyl group at the O^6 or N^2 and O^6 positions of the G(3) residue, prevents any duplex formation. Two possibilities exist here : (1). the presence of an alkyl group in these positions generates sufficient steric hindrance such that they cannot be easily accomodated within a duplex; or (2). the altered hydrogen-bonding potential of the modified G residues disrupts the stability of G : C bonds (in the second and fourth positions of the duplex) so much as to prevent formation of a slipped-type duplex. In fact, model building reveals that the spatial positions of the alkyl groups sterically interfere with hydrogen-bonding.

Mixing of the appropriate pentamers potentially allowed for formation of duplexes with a central modified G :U (N=m^6G, e^6G, m^2m^6G) or modified G : C (N=m^2m^6G) base pair. The effect of these G modifications may be determined through comparison with appropriate reference compounds.

Figures 29-31 and Tables 28-30, provide the "Chemical Shift versus Temperature" information for the AGm^6GCU : AGUCU, AGe^6GCU : AGUCU, AGm^2m^6GCU : AGUCU mixing experiments. Examination of the melting curves reveals general upfield shifts with decreasing temperature, but virtually no sigmoidal behavior. In several instances, as in the single strand experiments, inflection points might be found somewhere between 0-10°C; however, no Tm is evident in the temperature range studied. Comparison of many of the melting curves of the mixing experiments with their single stranded counterparts, reveals the same general shape. In some instances, especially for the aromatic resonances,the melting curves of the mixing studies begin to dip sooner than those of the single stranded studies. Due to the low temperatures, however, this cannot be taken as evidence of initial duplex formation; aggregation and slowing molecular motion could also be responsible. The alkyl groups of the mixing experiments experience the same shielding or deshielding pattern as do those of the single

stranded studies.

Due to the similar nature of the melting curves in the three modified G : U mixing experiments, and because no Tm's can be measured, statements regarding the relative effect of each modifiction cannot be made. It is evident however, that all alkylated guanosine residues have destabilized the duplex by at least 20°C, relative to the parent AGGCU : AGUCU (Tm 31.4°C), containing a normal Wobble base pair (Table 32, Figure 35).

At least two factors contribute to this destabilizing effect. First, aromatization of the guanosine ring upon O^6-alkylation alters the normal G : U hydrogen-bonding pattern (see Figures 2 and 4). In order for hydrogen bonding to occur, there must be a shift in the glycosyl torsion angle of the modified guanosine residue. This shift in turn, alters the normal geometry of the helix backbone. Second, and more important are the spatial positions of the O^6 and N^2 alkyl groups. Model building indicates that these alkyl groups interfere sterically with the modified G : U hydrogen-bonding sites. The O^6-alkyl cannot lie near the N^7 nitrogen, due to the latter's electronegativity and tendency to be associated with a water molecule (this is also seen in the crystal structure of m^2m^6G ,Allore *et al*, submitted). Shift of the N^2 alkyl group away from the hydrogen bonding

region, moves the alkyl group to a position outside the hydrophobic helix core, thus preventing it from participating in core extension.

In the case of the modified G : C mixing experiment (AGm^2m^6GCU : AGCCU) (Figure 33, Table 31), similar results are seen. However, the resonances of this mixing experiment broaden much earlier (below 25° C) than do those of the modified G : U mixing experiments (below 10°C). This result might be explained by formation of a competing duplex AGCCU : AGCCU. Alkema's study (Alkema, et al, 1982) found that at concentrations of 6.3 mM, AGCCU : AGCCU had a Tm of 25°C, while AGUCU : AGUCU had no Tm. If the former duplex forms detectably at a concentration of 1.8 mM, then it could compete with the AGm^2m^6GCU : AGCCU duplex , to create a greater than two-state system. Such a result, base on Alkema's work, would not be seen in the modified G : U mixing experiments.

Although the effect of G dimethylation on duplex stability (AGm^2m^6GCU : AGCCU) is clouded by the possibility of competing duplex formation, it is still evident from the non-sigmoidal melting curves, that G(3) modification has destabilized the AGGCU : AGCCU -type duplex (ie.no Tm can be found) relative to the reference AGGCU : AGCCU (Tm 48.0°C). Aromatization of the G(3) residue upon O^6-alkylation has resulted in loss of one of G : C hydrogen bonding sites

(ie N^1-H$\cdots$$N^3$) (see Figures 2 and 4). As well, the spatial position of the methyl groups, particularly the O^6-methyl, interferes extensively with the remaining hydrogen-bonding positions. The methyl groups in this experiment show the same pattern of shielding and deshielding as is evident in the AGm^2m^6GCU : AGUCU mixing experiment.

In summary, studies involving the AGNCU pentamers (N=m^6G, e^6G, m^2m^6G) indicate that these modified G residues have a considerable destabilizing effect when present in an internal non-bonded or bonded position. This is evidenced by the apparent lack of duplex formation in cases of non-bonded base oppositions, and in modified Wobble and modified Watson-Crick bonding situations. The destabilizing effect of the centrally-located modified G residues is in stark contrast to the stabilizing effect observed when these residues exist as 3'-terminal dangling bases, as in the GpCpN system (see section 3.2). This result, although initially surprising may in fact be quite reasonable, as modified residues in nature may serve different purposes, depending on their location within a duplex. Alkylated nucleosides present at junctions between duplexed and single-stranded regions (such as neck and stem regions of tRNA) may serve to guard against fraying (as in the GpCpN model system). Modified residues located internally, within a

double-stranded region (as in the ApGpNpCpU model system), are centres of instability, which may encourage small-loop formation, perhaps to relieve constraint in the local helix backbone, or to serve as recognition sites of some sort.

4. CONCLUSIONS

Several short oligoribonucleotide sequences containing modified purine residues of biological significance, were synthesized using a phosphotriester method, and studied by variable temperature proton nuclear magnetic resonance (NMR) spectroscopy. The experiments performed were extentions of those reported earlier : GpCpN studies (Alkema, et al, 1981(a); D'Andrea, et al, 1983); ApGpNpCpU studies (Alkema, et al, 1982).

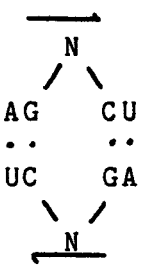
The triribonucleotide GpCpA, was the first trimer shown to form a stable duplex (Tm 33°C) containing two G : C Watson Crick base pairs and two 3-dangling adenosine residues (Alkema, et al, 1981). Studies involving GpCpN sequences (N=m^6A, $m^6{}_2A$, m^1G) examined the effect of N-alkylation on duplex stability (D'Andrea, et al, 1983). These studies indicated that methylation of the 3'-dangling residue enhanced base stacking and duplex stability, with site and degree of modification being significant factors (Tm's : N=m^6A, 34.7°, m^1G, 39.8°, $m^6{}_2A$, 41.5°C).

The present studies involving O-alkylated guanosine residues (N=m^6G, e^6G, m^2m^6G) support the conclusions of the N-alkylation studies and imply that size of the hydrophobic substituent may be of some importance (Tm's : N=m^6G, 36.2°, e^6G, 37.1°, m^2m^6G,

38.9°C). The similar Tm's of GpCpm^{2}m^{6}G and GpCpm^{1}G (38.9°, 39.8°C, respectively), imply that alkyl substitution in the N^1-N^2 spatial region of guanosine, has a more stabilizing effect than does alkylation in the O^6 region.

Studies in the ApGpNpCpU series (N=m^{6}G, e^{6}G, m^{2}m^{6}G) allowed for examination of the effect of G modification in an internal strand position. Studies involving the ApGpNpCpU single strands revealed no duplex formation. Theoretically, a slipped duplex of the type :

or a looped-out duplex having a non base-paired opposition :



could have formed, as in the case of ApGpGpCpU (6.25 mM). Clearly, G(3) modification has prevented such interstrand interactions.

Mixing experiments involving modified G : U (N=m^{6}G, e^{6}G, m^{2}m^{6}G) and modified G : C (N= m^{2}m^{6}G) base pairs, also revealed no duplex formation, in contrast with that seen for reference duplexes ApGpGpCpU : ApGpUpCpU (Tm 31.4°C) and ApGpGpCpU : ApGpCpCpU (Tm

47.0°C). Evidently, modification of the G(3) residue has a significant destabilizing effect on duplex stability. Two factors are thought to account for this result : (1). the altered hydrogen-bonding pattern of the modified G residue, and the resulting steric constraint, and more importantly, (2). the spatial locaation of the O^6 and N^2 groups (particularly the former), which block the remaining hydrogen-bonding positions.

Thus the effect of alkylated purine residues on duplex stability is a function of the position of these residues in the duplex strands : a terminal position is stabilizing, while an internal position is destabilizing. Perhaps such residues in nature have two-fold functions : to strengthen duplexes at junction points, and to induce loop formation (possible recognition sites) in double strands.

Bibliography

Abraham,R.J. & Loftus,P. (1979) "Proton and Carbon-13 NMR Spectroscopy", J.W. Arrowsmith, Bristol

Alkema,D., Bell,R.A., Hader,P.A., and Neilson,T. (1981) J. Am. Chem. Soc. 103, 2866-2868

Alkema,D., Bell,R.A., Hader, P.A., and Neilson,T.(1981) in "Biomolecular Stereodynamics", Sarma,R.H. ed., Adenine Press, N.Y.,417-428

Alkema,D. PhD. Thesis, McMaster University, 1982

Alkema,D., Hader,P.A., Bell,R.A., and Neilson,T. (1982) Biochemistry 21, 2109-2117

Altona,C. in "Structure and Conformation of Nucleic Acids and Protein-Nucleic Acid Interactions", Sundaralingham,M. and Rao,S.T. eds., Baltimore,University Park Press, 1975,613-629

Bamborschke,S., O'Conner,P.J., Margison,G.P., Kleihus,P., and Maru,G.B. (1983) Cancer Reasearch 43, 1306-1311

Bogden,J.M., Eastman,A., and Bresnick,E. (1981) Nucl. Acids . Res. 9, 3089-3103

Borer,P.N., Kan,L.S., and Ts'O,P.O.P. (1975) Biochemistry 14, 4847

Bridges,B. & Lehmann,A. (1982) Nature 298, 118-119

Cedergren,R.J., Sankoff,D., LaRue,B., and Grosjean,H. (1981) CRC Crit. Rev. Biochem. 11, 35-107

Cheng,D.M., Danyluk,S.S., Dhingra,M.M., Ezra,F.S., MacCross,M., Mitra,C.K., and Sarma,R.H. (1980) Biochemistry 19, 2491-2497

Clarke,B.F.C. (1977) Prog. Nucl. Acids Res. and Mol. Biol. 20 1 - 19

Crick,F.H.C. (1966) J. Mol. Biol. 19, 548

D'Andrea, P.L. 4th year thesis, (1982), McMaster University

D'Andrea,P.L., Alkema,D., Bell,R.A., Coddington,J.M., Hader,P.A., Hughes,D.W., and Neilson,T. (1983) J. Am. Chem. Soc. 105, 636-638

Ehrlich,M. & Wang,R.Y.H.(1981) Science 212, 1350-1357

England,T.E. & Neilson,T. (1976) Can. J. Chem. 54, 1714-1721

England,T.E. & Neilson,T. (1977) Can. J. Chem. 55, 365

Ezra,F.S., Lee, C.H., Kondo, N.S., Danyluk,S.S., and Sarma,R.H. (1977) Biochemistry 16, 1977-1987

Fieser,L.F. & Fieser,M. (1967) in Reagents for Organic Synthesis vol. 1, John Wiley & Sons, N.Y., p 192

Fowler, K.E., Buchi,G., and Essigman,J.M. (1982) J. Am. Chem. Soc. 104, 1050-1054

Gaffney,B.L. & Jones, R.A. (1982) Tet. Letts. 23, 2253,2256

Gauss,D.H. & Sprinzl,M. (1981) Nucl. Acids Res. 9, r1-r23

Gellert,M., Lipsett,M.N., and Davies,D.R. (1962) Proc. Natl. Acad. Sci. 48, 2013-2018

Gerchman,L.L. & Ludlum,D.B. (1973) Biochem. et Biophys. Acta. 308, 310-316

Gregoire,R.J. & Neilson,T. (1978) Can. J. Chem. 56, 487-490

Griffen,B.E., Jarman,M., Reese,C.B., Sulston,J.E., and

Trentham, D.R. (1966) Biochemistry 5, 3638

Grosjean,H., Soll,D.G., and Crothers,D.M. (1976) J. Mol. Biol. 103, 499-519

Guttenplan,J.B., & Milstein,S. (1982) Carcinogenesis 3, 327-331

Haasnoot,C.A.G., den Hartog,J.H.J., de Rooij,J.F.M., Van Boom,J.H., and Altona,C. (1979) Nature 281, 237

Haasnoot,C.A.G., den Hartog,J.H.J., de Rooij,J.F.M., Van Boom,J.H., and Altona,C. (1980) Nucl. Acids Res. 8,

Hora,J.F., Eastman,A., amd Bresnick,E. (1983) Biochemistry 22, 3759-3763

Hurd,R.E. & Reid,B.R. (1979) Biochemistry 18, 4017

Johnston,P.D. & Redfield,A.G. (1978) Nucl. Acids Res. 5, 3913

Johnston, P.D. & Redfield,A.G. (1981) Biochemistry 20, 3996 - 4006

Karran,P. & Marinus,M.G. (1982) Nature 296, 868-869

Kataguri,M., Ikatura,K., amd Narang, S.A. (1974)
J. Am. Chem. Soc. Comm. 355

Kuzmich,S., Marky.L.A, and Jones,R.A. (1983) Nucl. Acids
Res. 11 3393-3404

Lee,C.-H. & Tinoco, Jr., I. (1978) Nature 274, 609

Lomant,A.J. & Fresco, J.R. (1975) Prog. Nucl. Acids Res.
and Mol. Biol. 15, 185

Loveless,A. (1969) Nature 223, 206-207

Lutz,W.K. (1982) TIPS October,398-399

Markiewicz,W.T. & Wiewiorowski, M. (1978) Nucl.Acids Res.
Spec. Public. #4, 5185-5188

Markiewicz,W.T. (1979) J. Chem. Res. 5, 24-25

Martin,F.H., Uhlenbeck,O.C., and Doty,P. (1971) J. Mol. Biol.
57 201-215

McCloskey,V.A. & Nishimua,S. (1977) Acc. Chem. Res. 10,
403-410

Medcalf,A.S.C. & Lawley, P.D. (1981) Nature 289, 796-798

Mehta,J.R., Ludlum,D.B. Renard,A., and Verly,W.G. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6766-6770

Mizuno,H., Tomita,K.-I., Nakagawa,E., Ohtsaka,E., and Iketura,M. (1981) J. Mol. Biol. 148, 103

Muller,R. & Rajewsky, M.F. (1983) Cancer Research 43, 2897-2904

Neilson,T. & Werstiuk, E.S. (1971) Can. J. Chem. 49, 493

Neilson,T. Romaniuk,P.J., Alkema,D., Everett,J.R., Hughes,D.W., and Bell,R.A. (1980) Nucl. Acids Res. Spec. Publ. #7, 293-311

Ogilvie,K.K., Beaucase,L.L., Gillen,M.F., and Entwistle,D.W. (1978) Can. J. Chem. 56, 2768-2780

Olsthoorn,C.S.M., Haasnoot,C.A.G., and Altona,C. (1980) Eur. J. Biochem. 106, 85-95

Patel, D.J. & Hilbers, C.W. (1975) Biochemistry 14, 2651 - 2660

Pegg,A.E. & Perry, W. (1981) Carcinogenesis 2, 1195-1200

Romaniuk,P.J., Neilson,T., Hughes, D.W., and Bell,R.A. (1978) Can. J. Chem. 56, 2249 - 2251

Romaniuk,P.J., Hughes,D.W., Gregoire, R.J., Bell,R.A., and Neilson,T. (1979) Biochemistry 18, 5109 - 5116

Pereira,M.A. Lin,L.-H.C. and Herren,S.L. (1983) Chem.-Biol. Interactions 43, 313-322

Reddy,E.P., Reynolds, R.K., Santos,E., and Barbacid,M. (1982) Nature 300, 149-152

Reid,B.R., McCollum,L., Ribeira,M.S., Abbak,J., and Hurd,R.E. (1979) Biochemistry 18, 4017

Robillard,G.T., Hilbers,C.W., Reid, B.R., Gangloff,J., Dirheimer,G., and Shulman,R.G. (1976) Biochemistry 15, 1883

Romaniuk, P.J., Neilson,T., Hughes,D.W., and Bell,R.A. (1978) Can. J. Chem. 56, 2249-2251

Sedgewick,B. & Lindahl,T. (1982) J. Mol. Biol. 154, 169-175

Silverstein,R.M. & Bassler,G.C. in Spectrometric Identification of Organic Compounds, John Wiley & Sons, N.Y., 1967, 110-134

Singer,B. (1979) JNCl. 62 1329-1339

Tabin,C.J., Bradley,S.M., Bargmann,C.I., Weinberg,R.A., Papageorge,A.G., Scolnick,E.M., Dhar,R., Lowy,E.R., and Chang,E.H. (1982) Nature 300, 143-149

Tazawa,I., Koike,T., and Inoue,Y. (1980) Eur. J. Biochem. 109 33-38

Uhlenbeck,O.C., Martin,F.H., and Doty,P. (1971) J. Mol. Biol. 57, 217

Van Boom,J.H., Bungers, P.M.J., Van Deuran, P.H., Arentzen,R., and Reese,C.B. (1977) Tet.Letts. 3785

Van Charldorp,R., Verhoeven,J.J., Van Knippenberg,P.H., Haasnoot,C.A.G., and Hilbers,C.W. (1982) Nucl. Acids Res. 10, 4237-4245

Waldstein,E.A., Cao,E.H., and Setlow,R.B. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 5117-5121

Werstiuk,E.S. & Neilson,T. (1972) Can. J. Chem. 50, 1283-1291

Werstiuk,E.S. & Neilson,T. (1973) Can. J. Chem. 51, 1068-1074

Werstiuk, E.S. & Neilson,T. (1976) Can. J. Chem. 54, 2689-2692