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Cationic Cyclodextrins as Potential DNA Delivery Agents

By

Qibing ZHANG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

In

Pharmaceutical Sciences

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Date: Jan. 16, 2002

Dedicated to with love:

My son: Alex

My husband: Li Chen

My parents: Zhenzhu ZHANG & Kaijun LIU

Abstract

Cyclodextrins (CyDs) are widely used in many fields, including pharmaceutics and chemical analysis. More recently, CyDs have received more attention in the pharmaceutical sciences as drug delivery systems. Contemporary DNA delivery systems, especially synthetic DNA delivery systems, have become increasingly desirable in both basic research laboratories and clinical settings.

The goals of this project are, therefore, to synthesize new cationic cyclodextrin derivatives, and to explore their applications in cell biology – to see if they can serve as gene vectors, thereby providing a novel approach to gene transfer. Four cationic cyclodextrin derivatives (**6**, **9**, **17**, and **18**) were synthesized. One of them is a new compound and has not been reported previously. The four cationic CyDs were used to prepared complexes with pcDNA3.1/*LacZ* at five different concentrations, and were investigated for their capability to deliver genes. Three of the complexes showed some transfection efficiency, and one of these showed promising efficiency. All three of the CyDs that showed positive results in transfection have 2,3-ether methoxy groups instead of the normal 2,3-hydroxyl substituents. Although these three CyDs have the capability of transfecting pcDNA3.1/*LacZ* into COS-1 cells, their efficiency still requires optimization. It is anticipated that further modification will lead to useful non-viral vectors for effective gene delivery.

Key words: cyclodextrins, synthetic vector, gene therapy, transfection

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List of Abbreviations

AAV	adeno-associated viruses
CE	capillary electrophoresis
CLs	cationic liposomes
CM-β-CyD	carboxymethyl-β-cyclodextrin
CME-β-CyD	carboxymethyl-ethyl-β-cyclodextrin
CyDs	cyclodextrins
DDAB	dimethyldioctadecylammonium bromide, lipofectase
DEAE/Dextran	diethylaminoethyldextran
DE-β-CyD	diethyl-β-cyclodextrin
DM-β-CyD	dimethyl-β-cyclodextrin
DMEM	Dulbecco's Modified Eagle Medium
DMF	*N, N*-dimethylformamide
DMRIE	1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)propane
DOTMA	1,2-dioleyloxypropyl-3-trimethylammonium bromide, lipofectin
FBS	fetal bovine serum
FDA	US Food and Drug Administration
G_1- CyD	glucosyl-β-cyclodextrin
G_2-β-CyD	maltosyl-β-cyclodextrin
GI	gastrointestinal
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HP-β-CyD	2-hydroxypropyl-β-cyclodextrin
IP	intraperitoneal
KbCWL	*K. Bulgaricus* cell wall lectin
kbp	kilobase pair genome
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration

MCV	mean cell volume
NCBI	National Center for Biotechnology Information
PBS	phosphate buffered saline
pEI	polyethylenimine
pLL	polylysine
PPh_3	triphenylphosphine
RBC	erythrocyte count
R_f	retardation factor (rate of flow)
RM-β-CyD	randomly methylated β-cyclodextrin
SBE-β-CyD	sulfobutylether-β-cyclodextrin
TBDMSCl	*tert*-butyldimethylsilyl chloride
TE-β-CyD	heptakis(2,3,6-tri-O-ethyl)-β-cyclodextrin
THF	tetrahydrofuran
TLC	thin-layer chromatography
TM-β-CyD	heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin
TMS	tetramethylsilane
WBC	leukocyte count
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Introduction

1.1. Cyclodextrins

1.1.1. General review of cyclodextrins

The history of cyclodextrins can be traced back to one hundred years ago. The discovery of cyclodextrins (CyDs) is attributed to Villier, who isolated them as degradation products of starch (Uekama and Otagiri, 1987). The CyDs were then characterized by Schardinger in 1904 as cyclic oligosaccharides (*see* Cramer, 1981). The foundations of cyclodextrin chemistry, as well as the definition of CyD inclusion complexes, were established in the first part of the last century. However, until 1970, only small amounts of CyDs could be produced and the costs of production were very high, which prevented their comprehensive utilization and development. After the 1970's, the availability of highly purified CyDs and CyD derivatives resulted from biotechnological advancements, which then led to dramatic improvements in CyD production.

Over the past 30 years, the production and the applications, especially the complexing properties of cyclodextrins, have been of interest to scientists in many fields. More than one thousand articles on CyD related patents have been issued since the mid-1970's. Indeed, in the year 2000 alone, more than 400 papers were reported through a "cyclodextrins" keyword search using the National Center for Biotechnology Information's (NCBI) PubMed database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi, June, 2001). CyDs are mainly used as drug carriers to improve the properties of drugs (Zuo *et al.*, 2000; Shigeyama *et al.*, 2000). Ruebner *et al.* (1999) and Schaschke *et al.* (2000) have also considered using CyDs as possible carriers in tumor therapy. Cyclodextrins and their derivatives have been found to be remarkably active catalysts as well (Wenz, 1994). In the Kaukonen *et al.* (1997) study, it was observed that CyD derivatives showed an ability to catalyze the deacetylation of Spironolactone. Granaelos and de Rossi

(2001) also described their effect on the intramolecular catalysis of amide hydrolysis. The catalytic activity of CyDs is mostly a result of the state of ionization, site of binding, and the number of hydroxyl groups available for reaction (Jarho *et al.*, 2000). Recently, CyDs were the subject of many publications related to chemical analysis, especially in capillary electrophoresis (CE) (Clohs and McErlane, 2001; Sabbah and Scriba, 2001) and other chromatographic separations (Schneiderman and Stalcup, 2000). They were used as chiral buffer additives because of their structural properties, and they played an important role in these analysis methods. Today, the utilization of CyDs is widespread in many fields, including pharmaceutical preparations (Uekama *et al.*, 1998), chemical analysis (Rundlett and Armstrong, 2001; Clohs and McErlane, 2001), food, cosmetics (Irie and Uekama, 1997) and many other industries (Harada, 2001).

1.1.2. Structure and properties

Cyclodextrins (CyDs) are a class of cup-shaped molecules, which consist of cyclic α-(1,4)-linked glucose units (see Figure 1.1.2.). The most common natural cyclodextrins are called the α-, β-, and γ-cyclodextrins, which contain 6, 7, and 8 glucose units, respectively (Arima *et al.*, 2001). These parent cyclodextrins have interesting properties that can be explored for either physical or chemical uses. For many reasons, such as the separation, availability and cost, and also because of general nontoxicity and nonimmunogenicity (Roessler *et al.*, 2001), β-cyclodextrins are the most commonly used CyDs.

As a consequence of the C-1 chair conformation adopted by the α-1,4 linked D-glucose units in the CyD structure, the glucopyranose ring secondary hydroxyl groups at the 2- and 3- positions are on one side, and all the primary hydroxyl groups at C-6 are located on the other side. This three-dimensional structure has the overall shape of a truncated cone with the broader side formed by secondary hydroxyls and the narrower side formed by primary hydroxyls. The whole molecule assumes a rigid structure because of the formation of a belt of intra-molecular hydrogen bonds

between adjacent glucose units. The rotation of the secondary hydroxyl groups is thus restricted, whereas rotation of the primary hydroxyl groups is free.

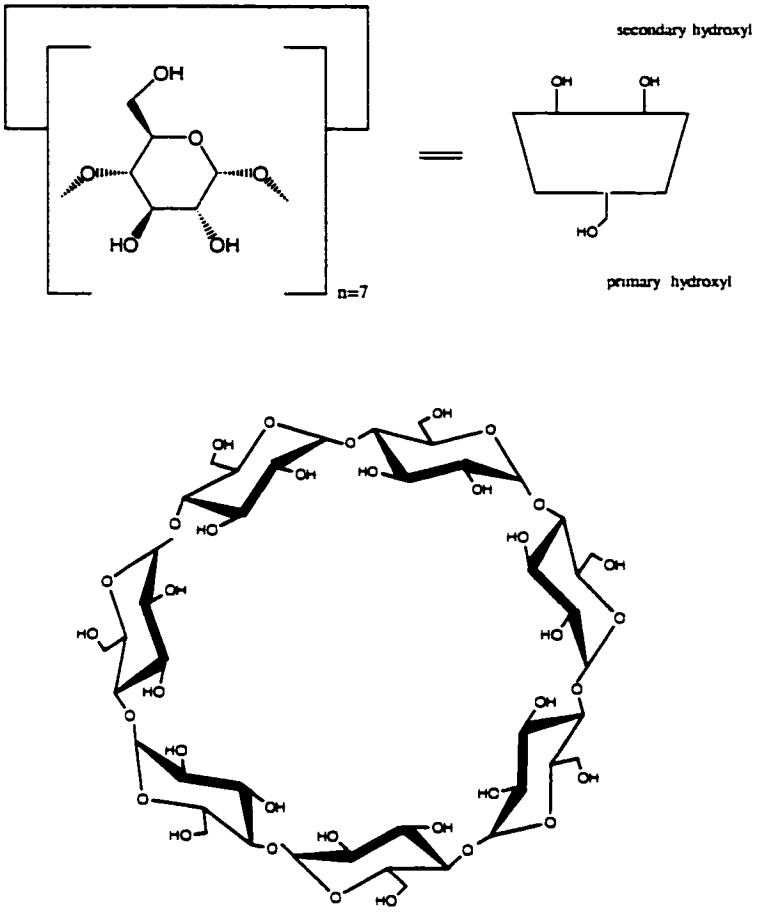
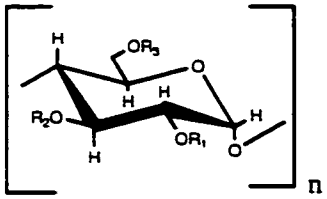


Figure 1.1.2. Structure of β-cyclodextrin

CyDs are of current pharmaceutical interest. The chemical structures of commonly used cyclodextrins and their derivatives mentioned in this thesis are shown in Table 1.1.2. (modified from Uekama *et al.*, 1998).

Table 1.1.2. Chemical structures of commonly used cyclodextrin derivatives



Compound	R_1	R_2	R_3	abbreviation
Hydrophilic derivatives				
methylated cyclodextrins				
3-mono-*O*-methylcyclodextrins	H	CH_3	H	M-CyDs
2,6-di-*O*-methylcyclodextrins	CH_3	H	CH_3	DM-CyDs
2,3,6-tri-*O*-methylcyclodextrins	CH_3	CH_3	CH_3	TM-CyDs
randomly methylated cyclodextrins	R_1, R_2, R_3 = H or CH_3			RM-CyDs
hydroxylalkylated cyclodextrins				
2-hydroxyethylcyclodextrins	R_1, R_2, R_3 = H or CH_2CH_2OH			HE-CyDs
2-hydroxypropylcyclodextrins	R_1, R_2, R_3 = H or $CH_2CH(OH)CH_3$			HP-CyDs
3-hydroxypropylcyclodextrins	R_1, R_2, R_3 = H or $CH_2CH_2CH_2OH$			3-HP-CyDs
2,3-dihydroxypropylcyclodextrins	R_1, R_2, R_3 = H or $CH_2CH(OH)CH_2OH$			
branched cyclodextrins				
6-*O*-glucosylcyclodextrins	H	H	H or glucose	G1-CyDs
6-*O*-maltosylcyclodextrins	H	H	H or maltose	G2-CyDs
6-*O*-dimaltosylcyclodextrins	H	H	H or $(maltose)_2$	$(G2)_2$-CyDs
Hydrophobic derivatives				
alkylated cyclodextrins				
2,6-di-*O*-ethylcyclodextrins	C_2H_5	H	C_2H_5	DE-CyDs
2,3,6-tri-*O*-ethylcyclodextrins	C_2H_5	C_2H_5	C_2H_5	TE-CyDs
acylated cyclodextrins				
2,3-di-*O*-hexanoylcyclodextrins	COC_5H_{11}	COC_5H_{11}	H	
2,3,6-tri-*O*-acetylcyclodextrins	$COCH_3$	$COCH_3$	$COCH_3$	TA-CyDs
2,3,6-tri-*O*-propanoylcyclodextrins	COC_2H_5	COC_2H_5	COC_2H_5	
2,3,6-tri-*O*-butanoylcyclodextrins	COC_3H_7	COC_3H_7	COC_3H_7	TB-CyDs
2,3,6-tri-*O*-valerylcyclodextrins	COC_4H_9	COC_4H_9	COC_4H_9	
2,3,6-tri-*O*-hexanoylcyclodextrins	COC_5H_{11}	COC_5H_{11}	COC_5H_{11}	
2,3,6-tri-*O*-octanoylcyclodextrins	COC_7H_{15}	COC_7H_{15}	COC_7H_{15}	
Ionizable derivatives				
anionic cyclodextrins				
6-*O*-(carboxymethyl)cyclodextrins	H	H	H or CH_2COONa	CM-CyDs
6-O-(carboxymethyl)-*O*-ethylcyclodextrins	C_2H_5	C_2H_5	H, C_2H_5 or CH_2COONa	CME-CyDs
cyclodextrin sulfates	R_1, R_2, R_3 = H or SO_3Na			S-CyDs
sulfobutylcyclodextrins	R_1, R_2, R_3 = H or $(CH_2)_4SO_3Na$			SBE-CyDs

1.1.3. Stability and metabolism of CyDs

Metabolic and stability studies are very useful for understanding the functions and toxicity of CyDs in their uses, and for designing new CyD derivatives effectively.

The α-(1,4) glycosidic bonds of CyDs are quite stable in alkaline solution, but they can be hydrolyzed by strong acids, giving rise to a series of linear maltosaccharides (Szejtli, 1988). Because of the size of the macrocycle and the patterns of conformation, the ring-opening rate of CyDs is much lower than the cleavage (hydrolysis) of corresponding acyclic oligosaccharides. The apparent rates of acid hydrolysis of various CyDs fall under the following generalization: 1) the hydrolysis of CyDs increases with an increase in the size of the macrocycle, α-CyD < β-CyD < γ-CyD < δ-CyD (Miyazawa *et al.*, 1995); and 2) the rate of hydrolysis of alkylated β-CyDs decreases in the order: heptakis(2,3,6-tri-*O*-methyl)-β-CyD (TM-β-CyD) > β-CyD > heptakis(2,6-di-*O*-methyl)-β-CyD (DM-β-CyD) > heptakis(2,3,6-tri-*O*-ethyl)-β-CyD (TE-β-CyD) > heptakis(2,6-di-*O*-ethyl)-β-CyD (DE-β-CyD) (Uekama *et al.*, 1987). The chemical structures of cyclodextrins mentioned above are shown in Table 1.1.2. Permethylated CyDs are the most susceptible to ring opening because of the markedly distorted ring conformation (Uekama and Otagiri, 1987). A recent study (Irie and Uekama, 1997) has shown that the ring-opening rate of β-CyD was decelerated by the addition of guest molecules. The deceleration may be because the guest sterically restricts access to the catalytic oxonium ions of the glycosidic oxygen atoms from inside of the cavity difficult (Irie and Uekama, 1997).

To realize the potential of CyDs in pharmaceutical formulations, their relevant biological profiles including *in vivo* fate have to be clarified. Their metabolism, using ^{14}C-labeled β-CyD (Andersen *et al.*, 1963), has been shown to be very similar to that of starch. Certain starch-degrading enzymes cleave the glycosidic bonds of CyDs with the proper substrate specificity. The major difference is that cyclodextrins are metabolized far more slowly than starch (oral: 9 h and 1 h, respectively) because they are more resistant to cleavage by α-1,4-glucanohydrase than linear dextrans,

and are not hydrolyzed by enzymes that attack terminal monosaccharides (Uekama and Otagiri, 1987). Human salivary and pancreatic α-amylases are capable of hydrolyzing the parent CyDs, and the introduction of substituents on the hydroxyl groups generally slows down enzymatic hydrolyses by either lowering the affinity of the modified CyDs to these enzymes or by reducing the intrinsic reactivity of the enzymes involved (Irie and Uekama, 1997). In addition, many bacterial strains that can degrade CyDs have been isolated from the human colon. This finding indicates that CyDs might be useful for the site-specific delivery of drugs, and can serve as a source of colon-targeting prodrugs (Minami *et al.*, 1998). The metabolic fate of some modified CyDs has been summarized by Uekama *et al.* (1998), including 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) and 6-*O*-maltosyl-β-cyclodextrin (G_2-β-CyD). These results are directly connected with toxicity and safety of parent CyDs and CyD derivatives.

1.1.4. Toxicity and safety of CyDs

Commercial development of pharmaceutical CyDs products is only possible when the safety of the CyDs is reliable. Currently, there are 6 available CyDs for use in pharmaceutical products. They are the parent α-, β-, and γ-CyD, and the methyl (M), hydroxypropyl (HP), and sulfobutylether (SBE) derivatives of β-CyD. Their toxicity and safety depend on the route of administration.

1.1.4.1. Oral safety

All studies on orally administered CyDs have shown that CyDs do not cause any developmental toxicity, even at high dose or in long-term study on animals. For instance, no obvious toxic effects were observed in rats fed 10% β-CyD diet for 90 days (Olivier *et al.*, 1991). A 52-week toxicity study using dietary administration showed that β-CyD is nontoxic at a daily dose < 600 mg/kg in rats or < 1800 mg/kg in dogs (Bellringer *et al.*, 1995). The oral LD_{50} value for β-CyD are reported to be > 12.5, 18.8, and 5 g/kg for mice, rats, and dogs, respectively (Szejtli, 1988). No effects were observed in rats or dogs for hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean cell volume

(MCV), erythrocyte count (RBC), and leukocyte count (WBC) after oral administration of β-CyD (Thompson, 1997). All of these studies gave β-CyD more chances to be developed.

1.1.4.2. Parenteral safety

The most critical problem of natural CyDs for parenteral use is their toxic effects on the kidneys (Frank *et al.*, 1976). The kidney is the main organ for the removal of CyDs from systemic circulation. CyDs localize in the proximal convoluted tubule after glomerular filtration.

Why CyDs are orally safe but not parentrally safe? Toxicity tests have proved that oral administration of cyclodextrins is harmless because of lack of absorption through the gastrointestinal (GI) tract (Irie and Uekama, 1997). While, renal damage may result from CyD accumulation in the kidney and, since CyDs are not easily hydrolyzed, they eventually overload the system to the point of renal toxicity (Uekama and Otagiri, 1987). However, chemical modifications have been made to the CyD structure with the hope that renal toxicity would be eliminated. One example is the development of anionic CyDs. Rajewski *et al.* (1995) directly administered sulfonated, sulfopropyl, and sulfobutylether derivatives of β-CyD to mice by intraperitoneal (IP) injection and determined that >10 g/kg could be administered without toxic effect on the kidney and demonstrated no adverse histopathology. They also showed that increasing the molar degree of substitution of SBE-β-CyDs reduced their membrane destabilization and decreased hemolysis and tissue damage. Some of these anionic CyDs were even less toxic than HP-β-CyDs.

1.1.4.3. Other safety profiles

Toxicity studies in which CyDs were administered by ocular, nasal, rectal, and dermal routes, have demonstrated different toxicities at different levels. Thompson (1997) reviewed the mutagenicity, carcinogneicity, and reproductive safety of parent CyDs and some modified CyDs. Some studies have shown a significant safety margin for certain CyD derivatives, such as methylated CyDs and HP-β-CyDs (Uekama *et al.*, 1998; Thompson, 1997). It has been proven that introduction of the

hydroxypropyl substituent eliminates systemic toxicity of the parent CyDs. A broad spectrum of safety studies for HP-β-CyD given orally as well as intravenously has already been well documented, and the details have been reported in the clinical trial data. An interesting example is the potential for HP-β-CyD in dermal applications (Irie and Uekama, 1997). Studies on antigenicity, mutagenicity, and topical irritation have proven that HP-β-CyD is as safe as many materials currently being used in perfumes and cosmetics. HP-β-CyD has a significant advantage in solubilizing fragrance materials over the surfactants, retaining them at the skin surface, thus avoiding cutaneous irritation, cloudiness of the preparation, and foaming, which sometimes are caused by surfactants (Tanaka *et al.*, 1996).

Generally speaking, the CyDs, including many modified CyDs, are known to have low toxicity. This is why they were developed so quickly and used in many fields. This trend will be continued in the next few years, particularly if CyDs can be licensed for further applications in food and drug production. Actually, the first drug formulation to contain a CyD, Sporanox, has been approved by the US Food and Drug Administration (FDA) in 1997 (Gonzalez *et al.*, 1999). This might indicate that CyDs are accepted to have relatively low toxicity and low immunogenicity.

1.2. Cyclodextrin Inclusion Complexes

1.2.1. General review of cyclodextrin inclusion complexes

CyDs form inclusion complexes with a variety of guest molecules. The lipophilic cavity microenvironment allows suitably sized drug molecules entrance into the cavity and to be retained. The exact nature of the binding mechanism still remains controversial, but cyclodextrin complexes are usually thought to be stabilized by various intermolecular forces, such as through van der Waals forces (i.e. hydrophobic interactions. Tabushi *et al.*, 1978; Nishijo and Nagai, 1991), hydrogen bonding (Tong *et al.*, 1991), and others like electrostatic interactions, induction forces, and the London dispersion force (Connors, 1997). Generally during CyD-drug complex formation, no covalent bonds are formed or broken and in

aqueous solutions, the complexes are easily dissociated. Inclusion complexes display different behaviors in solution and in the solid state, but they can be characterized by various physical or physicochemical methods.

1.2.2. Application of inclusion complexes

Inclusion complex formation is a major application of cyclodextrins. Cyclodextrin inclusion compounds are generally prepared for the modification of physical or chemical properties of guest molecules, which can lead to considerable pharmaceutical potential. This kind of research was initially started by Pringsheim in 1911 (*see* Straighten, 1990), and the first patent on CyDs and their complexes was registered in 1953 (*see* Loftsson and Brewster, 1996). Comprehensive accounts of the progress toward characterization and improvements on CyDs inclusion complexes are given elsewhere (Uekama and Otagiri, 1987; Adachi *et al.*, 1992; Abe *et al.*, 1995; Latrofa *et al.*, 2001). The most significant aspect regarding the use of CyDs in pharmaceutics is their effectiveness in the improvement of drug properties (Kondo *et al.*, 1996; Uekama *et al.*, 1995, 1998).

1.2.2.1. Enhancement of solubility and dissolution rate

The most common pharmaceutical application of CyDs is to enhance drug solubility in aqueous solutions. Many examples have demonstrated this effect (Latrofa *et al.*, 2001; Pose-Vilarnovo *et al.*, 2001; Loftsson *et al.*, 2001; Cappello *et al.*, 2001). Recently developed CyD derivatives with high aqueous solubility can improve the solubility of poorly water-soluble drugs. Examples include hydroxyalkylated, sulfated, sulfoalkylated, and branched cyclodextrins (Uekama *et al.*, 1998).

(1) Hydroxyalkylated CyDs, such as 2-hydroxyethyl-β-cyclodextrin and 2- (or 3-) hydroxypropyl-β-cyclodextrin with different degrees of substitution, have different effects on poorly water-soluble drugs such as hydrocortisone, digitoxin, diazepam, and indomethacin (Muller and Branus, 1985). The most popular and widely used derivative is 2-hydroxypropyl-β-cyclodextrin (Cappello *et al.*, 2001), and two pharmaceutical products containing 2-hydroxypropyl-β-cyclodextrin are

currently marketed. One is an aqueous mouthwash solution containing 0.3% (w/v) hydrocortisone solubilized with 4% (w/v) 2-hydroxypropyl-β-cyclodextrin (trade name is DEXACORT, Reykjavik, Iceland), which is used for the treatment of recurrent ulceration and erosive lichen planus of the oral mucosa (Kristmundsdottir *et al.*, 1996). Another formulation is the itoraconazole liquid preparations (trade name is SPORANOX, Beerse, Belgium), which is used to treat esophageal candidiasis (Putteman *et al.*, 1997).

(2) Sulfated and sulfoalkylated cyclodextrins are the other group of CyD derivatives that were studied most for their ability to solubilize drugs. For example, the sulfobutylether β-cyclodextrin solubilization of the three benzodiazepines, alprazolam, midazolam and triazolam, can be enhanced through ring-opening of the benzodiazepine rings and ionization of the ring-open forms (Loftsson *et al.*, 2001). Another example is sulfobutyl-β-cyclodextrin, which is reported to be an excellent solubilizer for various poorly water-soluble drugs such as kynostatin, steroids, pilocarpine, cinnarizine, indomethacin, naproxen, warfarin, papaverin, thiabendazole, micronazole, *etc.* (Thompson, 1997).

(3) Branched CyDs show higher affinity for drugs having steroid skeletons, such as progesterone, testosterone, dehydrocholic acid, difitoxigenin, and digitoxin (Yamamoto *et al.*, 1989; Okada *et al.*, 1990). Branched cyclodextrins, such as 6-*O*-glucosyl-β-cyclodextrin, 6-*O*-maltosyl-β-cyclodextrin and 6^A, 6^D-di-*O*-α-maltosyl-β-cyclodextrin, may be useful as solubilizers for parenteral preparations because of their solubilizing ability, weak hemolytic activity, and high bioadaptability (Uekama *et al.*, 1998).

Loftsson and his colleagues (1996) made several general statements regarding drug solubility: 1. The lower the aqueous solubility of the pure drug, the greater the relative solubility increased through CyD complexation. 2. Lower molar substitution of CyD derivatives is generally better than the same type of derivatives of higher molar substitution. One example is that randomly methylated β-cyclodextrins with molar substitution 0.6 provides for better solubilization than with molar substitution 1.8, and permethylated derivatives can possess a lower complexing potential than the

parent CyD (Botsi *et al.*, 1995). 3. Charged CyDs can be powerful reagents to enhance solubility, but this property seems to rely on the relative proximity of the charge to the CyD cavity. For example, sulfobutyl ether β-cyclodextrin, where the anion has been moved away from the cavity by butyl ether spacer group, is an excellent solubilizer (Gorecka *et al.*, 1995). It is expected that the preparation of CyD-drug complexes may bring further improvements to dissolution properties.

1.2.2.2. Stabilization

The effect of CyDs on the chemical stability of drugs is another valuable property of these excipients that has been extensively examined (Connors, 1997; Thompson, 1997; Uekama *et al.*, 1998). CyDs interact with labile compounds and can accelerate or decelerate various kinds of reactions depending on the nature of the complexes formed. From the viewpoint of drug stabilization, the deceleration effect of CyDs is rather important in the pharmaceutical field because the drug must remain sufficiently stable, not only during storage but also in the GI fluids. Reactions which result in a product that is pharmacologically inactive or less active will reduce the therapeutic effectiveness of the drugs (Uekama *et al.*, 1998). Complexation with CyDs can improve stability, reduce or even prevent drug hydrolysis, oxidation, steric rearangement, and other forms of isomerization, polymerization, and even the enzymatic decomposition of drugs (Loftsson and Brewster, 1996). The stabilization effects of CyDs can occur in solution and in the solid states.

(1) The improvement of chemical stability of drugs in solution by CyDs is one of the most important applications of CyDs. For example, the potent cardiac glycoside digoxin is susceptible to hydrolysis in acidic media (Figure 1.2.2.2.).

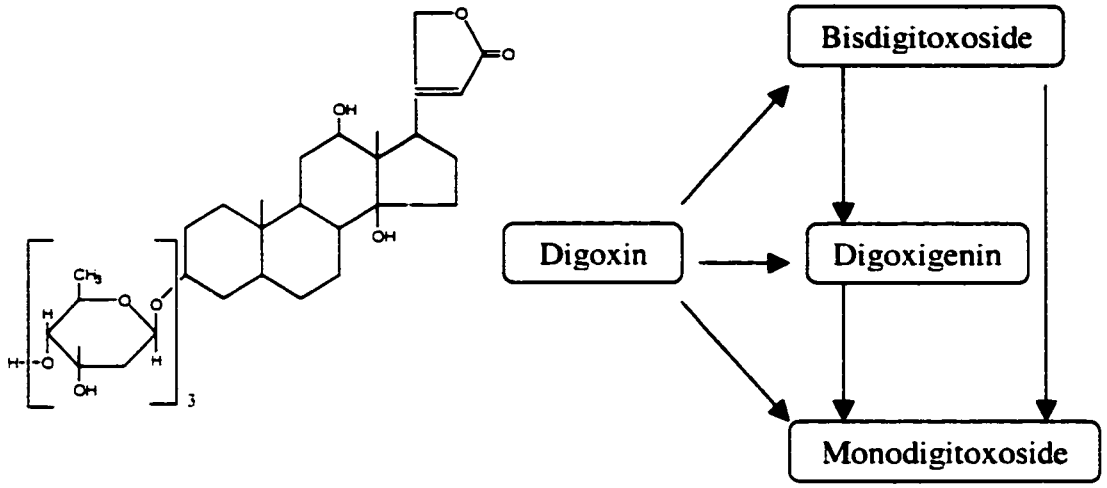


Figure 1.2.2.2. Structure of digoxin and acid-catalyzed hydrolysis of the glycoside bonds in digoxin (Uekama *et al.*, 1998)

In the degradation pathway shown in Figure 1.2.2.2, prevention of the appearance of digoxigenin might be clinically important because the cardioactivity of digoxigenin is about one-tenth of that of digoxin, whereas, mono- and bis-digoxosides posses approximately the same activity. The acid-catalyzed hydrolysis of the glycoside bonds in digoxin can be decelerated by the addition of cyclodextrins (Uekama *et al.*, 1982). In particular, the hydrolysis of the glycosidic linkage connecting the A-ring of digoxin and the sugar is completely inhibited by β-cyclodextrin (Uekama *et al*, 1998). Moreover, when the hydroxyl groups of CyDs are blocked by substituents, their stabilizing ability is enhanced. A typical example of stabilization is the well-known effects of methylated β-cyclodextrins on the degradation of E-type prostaglandins (Hirayama *et al.*, 1986). Connors (1997) has reviewed the stability of cyclodextrin complexes in solution. Detailed studies on the strength, structure, binding equilibria and kinetics of cyclodextrin complexes, have led to a greater understanding on the formation of drug-CyD complexes.

(2) Cyclodextrin complexation is also useful for the stabilization of drugs in the solid state. Solid compounds exist in a variety of crystalline forms such as amorphous, crystalline, or solvated forms. Among these solid states, amorphous forms are of pharmaceutical interest because they give a significant increase in dissolution and bioavailability of drugs (Uekama *et al.*, 1998). However, amorphous

forms easily transform to a stable crystalline form during the handling and storage of drugs. Therefore, the effect of CyDs on the physical stability of drugs in the solid state has received much attention in recent years. For example, crystalline nifedipine (a potent calcium-channel antagonist) can be converted to an amorphous state by spray-drying with amorphous 2-hydroxypropyl-β-cyclodextrin. The oral bioavailability of this drug is significantly improved (Hirayama *et al.*, 1994). The chemical properties of 2-hydroxypropyl-β-cyclodextrin make this CyD suitable for preventing the crystal growth of amorphous nifedipine. Such inhibition of crystallizaton and polymorphic transition are also reported for chloramphenicol palmitate (Hirayama *et al.*, 1997), metronidazole benzoate (Andersen and Bundgaard, 1984), and tolbutamide (Veiga *et al.*, 1996). The complex of spironolactone with 2-hydroxypropyl-β-cyclodextrin can be kept in an amorphous state for over two months at 75 % relative humidity and 60 °C (Soliman *et al.*, 1997).

1.2.2.3. Improvement of drug absorption

Bioavailability, which means the extent and rate of absorption of a drug from its dosage form into the systemic circulation, is an important index of a drug's efficacy. Many physicochemical factors may affect the bioavailability of a drug, such as solubility, stability, particle size, pKa, polymorphism, and partition coefficient (Uekama and Otagiri, 1987). CyDs can enhance drug absorption via different administered routes related to these factors.

Oral administration: For reasons of convenience, most drugs are administered orally, therefore drug stability in the GI fluids is very important in the efficiency of absorption. CyDs have already been shown to improve the oral bioavailability of poorly soluble drugs (Nambu *et al.*, 1978; Seo *et al.*, 1983; Nicolazzi *et al.*, 2001). It appears that the improvement of bioavailability by means of CyD-complexation is largely dependent on the magnitude of the stability constant and the increase in the dissolution rate of the drug that is finely dispersed in the GI fluids.

Rectal delivery: The application of CyDs for the rectal administration of drugs is another important example of drug delivery. One of the most striking effects was

found with 2-hydroxypropyl-β-cyclodextrin, which greatly enhanced the rectal absorption of ethyl 4-biphenylacetate, a lipophilic prodrug of the anti-inflammatory agent biphenylacetic acid (Arima *et al.*, 1992, 1989). Other applications of CyD derivatives include the methylated cyclodextrins, which can significantly enhance the rectal absorption of hydrophobic drugs such as flurbiprofen, also an anti-inflammatory agent (Uekama *et al.*, 1985), and the fluorourocil prodrug (Kikuchi *et al.*, 1987).

Nasal delivery: It is reported (Hermens *et al.*, 1990; Schipper *et al.*, 1990) that pulsatile release of steroids can be imitated by the nasal administration of water-soluble cyclodextrin complexes, such as steroid-2-hydroxypropyl-β-cyclodextrin complexes. Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin was reported to significantly enhance the rate of nasal absorption of morphine by facilitating the nasal epithelial permeability and consequently increased the entry of the opioid into the cerebrospinal fluid.

Ocular delivery: Possible advantages for the ophthalmic use of CyDs include increased solubility and stability, and the avoidance of irritation and discomfort caused by current drugs. Some hydrophilic CyDs can enhance the ocular boavailability of lipophilic drugs by keeping the drugs in solution and increasing their availability at the surface of the corneal barrier (Usayapant *et al.*, 1991; Jarho *et al.*, 1996). For instance, the inclusion complex of dexamethasone acetate with 2-hydroxypropyl-β-cyclodextrin can be prepared as an ophthalmic solution, and 2-hydroxypropyl-β-cyclodextrin may enhance the ocular bioavailability of dexamethasone acetate by inhibiting its bioconversion to dexamethasone. The latter shows lower corneal permeability than the parent drug.

Dermal delivery: Cyclodextrins have a significant safety margin in dermal applications, and can be applied to the transdermal delivery of drugs either for local or systemic use (Uekama *et al.*, 1998). Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin can enhance the release of ethyl 4-biphenylylacetate from the ointment into the skin as shown in studies with rats (Arime *et al.*, 1990; 1996). The enhancement of drug absorption is due to CyD-induced changes in

barrier function of the skin because CyDs may interact directly with some components of the skin (Vollmer *et al.*, 1994; Vitoria *et al.*, 1997).

In addition, Kaji *et al.* (1985) also proved that CyDs play a role in selective absorption using β-CyD containing polymer complex as a new delivery system. It is desirable to increase the selectivity of a drug to a target site because the drug can be used more effectively while minimizing its toxicity at other sites.

1.2.2.4. Reduction of side effects

By taking advantage of CyD-complexation, attempts have been made to reduce the side effects associated with certain drugs (Nicolazzi *et al.*, 2001; Pitha and Szente, 1983). Sometimes the detoxification of drugs is inevitably linked with the loss of some therapeutic efficiency because beneficial effects and toxic responses of drugs usually arise from similar pharmacological actions in tissues. Fortunately, CyD drug carriers may reduce drug toxicity without the drastic loss of therapeutic benefits since the complex always dissociates before or after reaching the action site (Uekama and Otagiri, 1987). The protective effects of CyDs relate to their ability to reduce either systemic toxicity or local toxicity. This reduction of local irritancy shown by CyD-drug complexes is probably due to the decrease in the binding ability of drugs to the erythrocyte membrane, which may prevent drug entry into the cells of nontargeted tissues. Local irritation is thus decreased (Uekama *et al.*, 1998). For example, CyDs can diminish the ulcerogenic potency of several acidic anti-inflammatory drugs (Puglisi *et al.*, 1995; Otero *et al.*, 1991) and mask their disgusting odor and taste (Andersen *et al.*, 1984; Reer *et al.*, 1994). Additional effects of CyDs in decreasing ophthalmic irritation has been reported recently (Suhonen *et al.*, 1995; Jarho *et al.*, 1996). Cyclodextrins also can be used in systemic detoxication. A retinal-dextran conjugate solubilized by β-CyD was reported to be less cytotoxic and the conjugate alone while still retaining the ability to inhibit the growth of cancer cells. 2-Hydroxypropyl-β-cyclodextrin can prolong the duration of analgesia and reduce the supraspinal side efffects when opioids are administered intrathecally in rats (Uekama *et al.*, 1998).

In summary, CyDs are capable of forming inclusion complexes with several important drugs. These non-covalent inclusion complexes can have physical, chemical, and biological properties that are dramatically different from those of either the parent CyDs or drugs. Studies in these areas will without doubt, promote the universal utilization of these valuable materials.

1.3. The Development of CyDs as Delivery Systems

1.3.1. CyDs as drug delivery systems

On the basis of the multifunctional properties of cyclodextrins, applications of CyDs in oral drug delivery, peptide and protein delivery, and site-specific delivery are of current interest. Recent reviews (Uekama *et al.*, 1998; Rajewski and Stella 1996) have summarized applications of both unmodified and modified CyDs for *in vivo* drug delivery. It seems that as more products containing CyDs move toward regulatory approval in the U.S. and elsewhere, general acceptance by researchers and the pharmaceutical industry of specific CyDs as enabling excipients is likely to increase. Obviously, as effective and valuable carriers, CyDs will play an important role in the improvement of drug formulations for various routes of administration.

1.3.1.1. Oral applications

The appropriate drug release from the dosage form is very important for realizing the drug's therapeutic efficacy. The growing interest in the use of CyDs in oral delivery is related to their ability to control drug release. Various cyclodextrin derivatives have been used to enhance the function of the host molecule through developing rate- or time-controlled release properties. Examples include: (1) Immediate release. Hydrophilic cyclodextrin derivatives, such as 2-hydroxypropyl-β-cyclodextrin (Betlach *et al.*, 1993), 6-*O*-maltosyl-β-cyclodextrin (Yamamoto *et al.*, 1992), and sulfobutyl-β-cyclodextrin (Jarvinen *et al.*, 1995) have been extensively used for immediate release of drug. They can provide an immediate release formulation, which is readily dissolved in the gastrointestinal tract, providing an increase of oral bioavailability of poorly water-soluble drugs. These drugs include

steroids, cardiac glycosides, nonsteroidal anti-inflammatory drugs, barbiturates, antiepileptics, benzodiazepines, antidiabetics, and vasodilators (Uekama *et al.*, 1998). Another approach is the use of amphiphilic CyDs (Skiba *et al.*, 1995). These formulations include β- or γ-CyD esterified on the secondary hydroxyl groups with alkyl chains. These CyDs can form self-assembled nanospheres that can be loaded with high amounts of poorly water-soluble drugs. The drug, being molecularly dispersed in the nanospheres, can be very rapidly released in aqueous medium. (2) Prolonged release. Many slow-release drug formulations have been prepared to maintain a constant blood level for a long period of time. This type of formulation can reduce the frequency of dosing, prolong drug efficacy, and avoid the toxicity associated with the administration of simple tablets (Uekama *et al.*, 1998). For this purpose, some hydrophobic CyDs are used as slow-release carriers for water-soluble drugs. For example, alkylated CyDs such as heptakis(2,6-di-*O*-ethyl)-β-cyclodextrin and heptakis(2,3,6-tri-*O*-ethyl)-β-cyclodextrin were the first slow-release carriers to be used in conjunction with diltiazem (Horiuchi *et al.*, 1990) and isosorbide dinitrate (Hirayama *et al.*, 1988). Peracylated β-cyclodextrins can also be used in prolonging release rate (Uekama *et al.*, 1994). (3) Modified release. Wang *et al.* (1993) developed a double-layer tablet using 2-hydroxypropyl-β-cyclodextrin along with other pharmaceutical excipients. Changing the mixing ratio of a fast-release and a slow-release portion can provide the optimal formulation of a double-layer tablet, which can increase the oral bioavailability of drugs. (4) Delayed release. This type of release is classified as time-controlled release and is mostly under the control of pH. As the delayed release dosage form passes from the stomach into the higher pH environment of the upper small intestine, it experiences increased drug release. For this purpose, 6-*O*-(carboxymethyl)-*O*-ethyl-β-cyclodextrin was developed to show pH dependent solubility for use in selective dissolution of the drug cyclodextrin complex (Uekama *et al.*, 1989). Other advantages of CyDs in oral applications include decreasing local tissue irritation and masking objectionable taste (Rajewski and Stella, 1996).

1.3.1.2. Peptide and protein delivery

CyDs can be used not only as enabling excipients in pharmaceutical formulations, but also as artificial carriers for either exogenous or endogenous lipophiles in the body (Pitha and Szente, 1983). CyDs may interact with accessible hydrophobic amino acid residues in peptides and proteins (Rozema and Gellman, 1996) because CyDs can recognize the size, shape and chirality of amino acids (Liu *et al.*, 1997). For example, the synthetic nonapeptide buserelin acetate, defined as pyroGlu-His-Trp-D-Ser(*tert*-butyl)-Leu-Arg-Pro-ethylamide, is a highly potent agonist of luteinizing hormone-releasing hormone. Spectroscopic studies (^{1}H and ^{13}C-NMR, Uekama *et al.*, 1998) indicated that the aromatic side chains of buserelin acetate, L-tryptophan and L-tyrosine residues, are incorporated into the hydrophobic environment of the heptakis(2,6-di-*O*-methyl)-β-cyclodextrin cavity. The tertiary butyl D-serine residue is inserted into the heptakis(2,6-di-*O*-methyl)-β-cyclodextrin cavity from the secondary hydroxyl side. Such interaction possibly affect the three-dimensional structure of peptides and proteins or inhibits their intermolecular association and thus changes their chemical and biological properties (Cooper, 1992). Hydrophilic cyclodextrins can be used to solubilize and stabilize various important peptides and proteins including growth hormones (Brewster *et al.*, 1991; Charman *et al.*, 1993), interleukin-2 (Brewster *et al.*, 1991), monoclonal antibody MN12 (Ressing *et al.*, 1992), aspartame (Prankerd *et al.*, 1992), tumor necrosis factor (Hora *et al.*, 1992), lactate dehydrogenase (Izutsu *et al.*, 1995), albumin and γ-globulin (Katakam and Banga, 1995). Absorption enhancement afforded by hydrophilic cyclodextrins, especially the methylated CyDs as nasal absorption enhancers for luteinizing hormone-releasimg hormone agonists (Matsubara *et al.*, 1995), insulin (Shao *et al.*, 1992; Watanabe *et al.*, 1992), and adrenocorticotropic hormone analogue (Schipper *et al.*, 1993), has been observed. Other derivatives of CyDs, such as hydrophobic cyclodextrin and sulfated cyclodextrins, can be used as sustained-released carriers of proteins and heparinoids (Uekama *et al.*, 1998).

1.3.1.3. Site-specific delivery

In recent years, intensive efforts have been made to design delivery systems able to deliver drugs more efficiently to specific organs, tissues, and cells. CyD-drug complexes are in equilibrium with free guest and host molecules in water with the degree of the dissociation being dependent on the magnitude of the stability constant. This property of CyDs is very desirable because the complex can dissociate to free cyclodextrin and drug at the absorption site, and only the drug in free form enters into the systemic circulation. A typical example is the application of 2-hydroxypropyl-β-cyclodextrin for the chemical delivery of testosterone (Brewster *et al.*, 1988), dexamethasone (Siegal *et al.*, 1997), and benzyl penicillin (Pop *et al.*, 1991). Some results on site-specific delivery using cyclodextrins are summarized below.

(1) Colon-targeting is essentially classified as a delayed release with fairly long lag time because the time required to reach the colon after oral administration is expected to be about 8 h in man (Friend, 1992). In anticipating a new candidate for a colon targeting prodrug, Uekama and co-workers have designed and studied the amide and ester type conjugates of the anti-inflammatory drug, biphenylylacetic acid, with three natural cyclodextrins (Uekama *et al.*, 1997; Hirayama *et al.*, 1996). They concluded that the ester type conjugate, in which drug release is triggered by the ring-opening of cyclodextrins, is preferable as a delayed-release prodrug that can release a parent drug selectively in the cecum and colon.

(2) In brain targeting, the β-cyclodextrin conjugate with *N*-Leucine-enkephalin is of interest because it has a vacant cavity with the appropriate dimensions to include the neurotropic drug dothiepine (Uekama *et al.*, 1998). The specific delivery of potential neuropharmaceuticals to the brain is obstructed by the presence of the blood-brain barrier, which is characterized by the endothelial cells of cerebral capillaries that have tight continuous circumferential junctions, thus restricting the passage of polar drugs to the brain (Begley, 1996). One of the strategies to overcome this transport problem is to prepare prodrugs with high lipophilicity that pass through

the blood-brain barrier, and this methodology can be applied to some neuropharmaceuticals such as morphine (Uekama *et al.*, 1998).

(3) Cell surface targeting is a third field of interest for cyclodextrin use. Intercellular recognition events are fundamental to many biological processes in which oligosaccharides on cell surface glycoproteins or lectins have been reponsible for cell-cell recognition and adhesion (Alberts *et al.*, 1994). Therefore, cyclodextrin derivatives bearing small saccharides may be useful as carriers for transporting active drugs to sugar receptors such as lectins located on the cell surface. β-Cyclodextrin conjugates with galactose were reported (Uekama *et al.*, 1998) to show higher recognition by the galactose specific *K. Bulgaricus* cell wall lectin (KbCWL). These CyDs inhibited flocculation of *K. Bulgaricus* cells induced by the isolated KbCWL lectin. Additionally their inhibition activity was found to be higher than that of galactose alone.

1.3.1.4. Other delivery applications

In addition to the above applications, CyDs can also be used in parenteral, ophthalmic, and nasal drug delivery.

(1) *Parenteral applications*. The expected uses of CyDs in parenteral drug delivery include solubilization of drugs, allowing for rapid and quantitative delivery of poorly water-soluble drugs for intravenous and intramuscular dosing, decreasing irritation at the site of administration, and stabilization of drugs unstable in an aqueous environment. Because the safety record of CyDs in parenteral applications is a more critical issue than for other routes of administration, a careful review of CyDs to effect parenteral delivery of drugs has been given much recent attention (Carpenter *et al.*, 1995; Irie *et al.*, 1992; Pitha *et al.*, 1994). Many CyDs, modified or unmodified, have been studied for parenteral use. It was found that some of them are unacceptable because of systemic toxicity, while some CyDs appear more promising, such as HP-β-CyDs (Rajewski and Stella, 1996). It has been noted that most drugs are rapidly and quantitatively released from CyD-solutions when these solutions are administered parenterally.

(2) *Ophthalmic applications*: Rajewski and Stella (1996) have reviewed the interactions between cyclodextrins and ophthalmic drugs. CyDs may increase the ophthalmic delivery of drugs through multiple routes including alteration corneal permeability and increasing the solubility of poorly water soluble (Reer *et al.*, 1994). Also, CyDs can help to decrease irritation typically associated with ophthalmic drugs (Jarho *et al.*, 1996).

(3) *Nasal applications*: The use of CyDs to improve the delivery of drugs after nasal administration is similar to the principles applied to ophthalmic drug delivery. Improved nasal delivery can be attributed to CyD-induced alterations in nasal mucosa permeability that affect drug absorption, changes in drug solubility, and in the case of some prodrugs and peptides, changes the metabolism rate of the drugs at the site of delivery (Rajewski and Stella, 1996).

(4) Other uses of CyDs in drug delivery include dermal (Larrucea *et al.*, 2001), rectal (Arima *et al.*, 1992), and pulmonary (Cabral Marques *et al.*, 1991a, 1991b) drug delivery, which generally improve local action either through increased solubility and stability of the drugs, or systemic delivery through a potential combination of these factors with a CyD-associated increase in membrane permeability.

In conclusion, the principal advantages of cyclodextrins as drug carriers include the following (Uekama *et al.*, 1998): (1) well-defined chemical structure, providing many potential sites for chemical modification or conjugation; (2) availability of different cavity sizes; (3) low toxicity and low pharmacological activity; (4) reasonable water solubility; and (5) protection of included/conjugated drugs from biodegration. These studies prompted us to start to think about the possibility of CyDs for gene delivery.

1.3.2. CyDs in gene delivery

1.3.2.1. An overview of gene therapy and gene transfection

Gene Therapy: From the second half of the 20th century, with the rise of the standard of living, people have longer and longer lives than ever before. But, at the same time, humans are threatened and bothered by more and more complicated diseases. Fortunately, because of recent developments in molecular biology techniques, most diseases have been found to be linked to a gene problem, gene defect or gene lesion. Now, there are more than four thousand known diseases that can be classified as genetic in origin. It is not surprisingly that gene therapy has become one of the most interesting new fields in pharmaceutical research. This is related to satisfying the hopes and dreams in understanding the root of life, at the molecular level. Gene therapy also has the potential to provide more effective treatment for many severe and debilitating diseases (e.g., diabetes, hemophilia, cystic fibrosis), chronic diseases (i.e., hypertension, asthma, Parkinson's disease, motor neuron disease, multiple sclerosis) (Dalgleish, 1997), cancer (Felgner *et al.*, 1995; Chong and Vile, 1997; Niculescu-Duvaz, 1998; Gomez-Navarro *et al.*, 1999), AIDS (Garnett, 1999) and other diseases, including heart disease (Smith, 1995), vascular disease (Svensson and Schwartz, 1998) and kidney disease (Imai and Isaka, 2000). Dramatic developments in molecular biology have made gene therapy more possible. The history of gene therapy is not very long, but it is leading to a new era of the therapy for many human diseases.

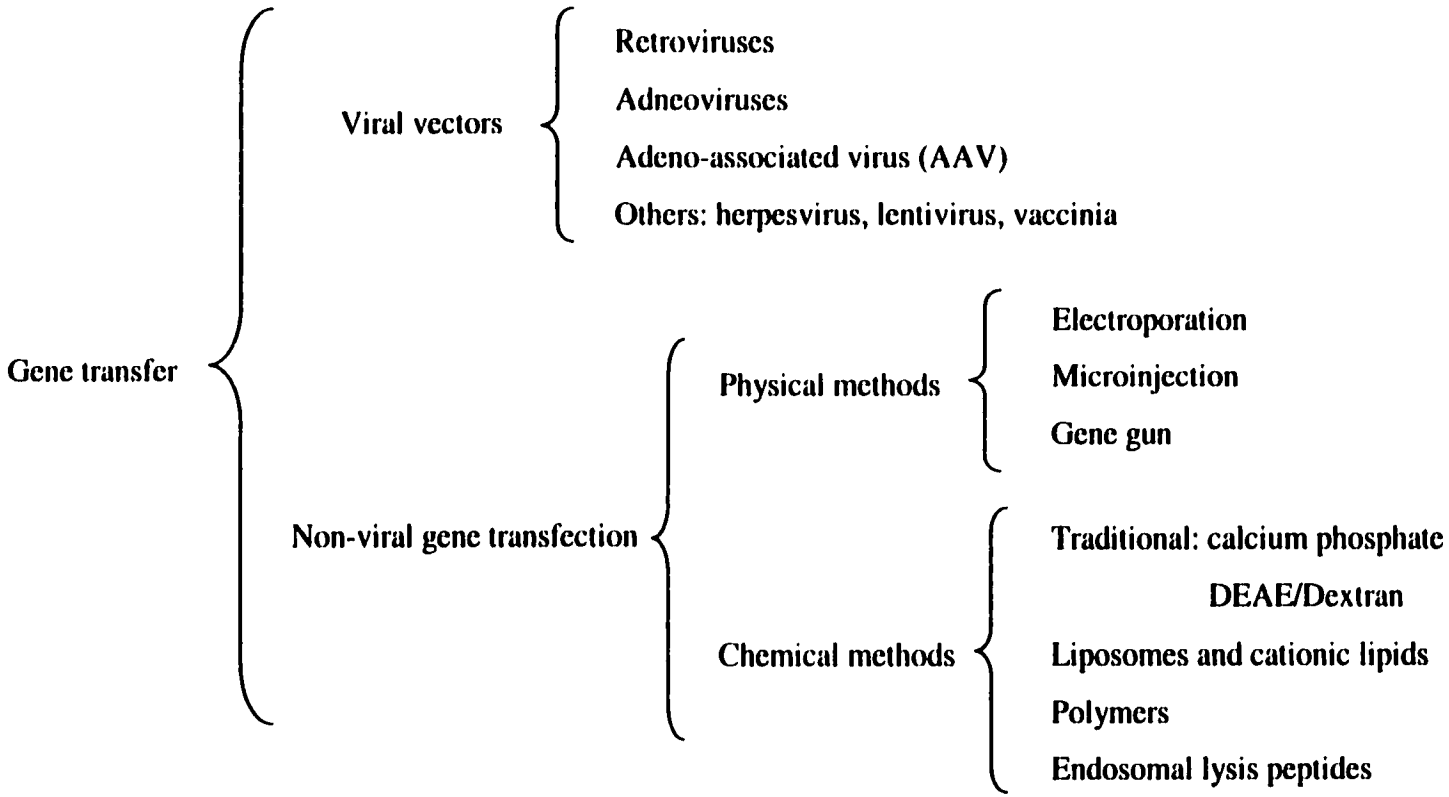
Broadly speaking, gene therapy can be defined as a genetic technology aimed at changing cellular function or structure at a molecular level for therapeutic purposes. The basis of gene therapy is that the DNA encoding known gene functions is inserted into cells and expected to express and develop its function. Generally, gene therapy includes several approaches which include homologous recombination to replace a mutated gene, insertion of a new gene that can synthesize a therapeutic protein, or using drugs to modulate endogenous cellular gene expression. Almost all of these methods rely on a single technology, gene transfer, i.e. transfection, which is

currently the most important technique in molecular biology for the introduction of a select gene into living cells.

Gene Transfer: Gene transfer is a major technique for the introduction of genes into cells of various origins. Indeed, it is not only the most straightforward way to study gene and protein function and regulation, but may also have important economic implications through the genetic engineering of microorganisms, plants, and animals (Behr, 1994). The diversity of gene transfer applications has resulted in the development of a variety of artificial techniques which have aided the development of gene therapy.

At present, there are two primary strategies for gene transfer (Anwer *et al.*, 2000; Salyapongse *et al.*, 1999; Ledley, 1995) (See Scheme 1.3.2.1). One is the virus-based method, including retroviruses and adenoviruses, etc. The second is based on non-viral methodology, which includes both physical and chemical methods. Each approach has its own advantages, unique clinical applications and associated risks.

Figure 1.3.2.1. Gene Transfer Protocols (Anwer *et al.*, 2000; Salyapongse *et al.*, 1999; Ledley, 1995)



1.3.2.1.1. Viral vectors

Viral vectors are among the earliest vectors used in gene transfer. For several years, gene therapy almost exclusively relied on recombinant viruses because they are far more efficient at transfer and expression than artificial vectors (Friedmann, 1997; Crystal, 1995; Mulligan, 1993). Viral transfection mainly relies on the mechanisms specific to each virus to cross the cell membrane. Once the virus enters the cell, translocation of its genetic information to the nucleus leads to expression of its genetic message in host cell. At present, there are several types of viral vectors (Salyapongse *et al.*, 1999) used in clinical trails including:

Retroviruses wer the first vectors to be introduced for gene therapy in 1980s, and are single-stranded RNA viruses that have the ability to insert a copy of their genes permanently into host cell chromosomes (Friedmann, 1997). Most current gene therapy clinic trials use retroviral vectors since much is known about their stucture and function. Studies show that retroviruses can mediate stable gene transfer with low potential immunogenicity (Anwer *et al.*, 2000; Fong *et al.*, 2000). Even so, retroviruses still have many limitations which restrict their clinic use. Limitations include the fact that only dividing cells can be infected by the random insertion of the sequence into the host cell chromosome, which may lead to insertional mutagenesis and oncogenesis (Rolland, 1998). Also, recombinant viruses are not easily purified to homogeneity after production (Miller, 1992).

Adenoviruses are double-stranded DNA viruses that can infect dividing and non-dividing cells in many tissues. Adenoviruses have the capability of carrying large segments of DNA (36 kbp), and can be prepared in high titer (10^{11} mL^{-1}) (Miller, 1992) as compared to the 10 kbp limit, and difficulty in producing high titer (10^{6-7} mL^{-1}) found with retroviruses (Behr, 1993; Niculescu-Duvvaz, 1998). Their ability to infect multiple cell types in many tissues, including infecting tissues *in situ*, make this vector system an important tool in gene therapy (Anwer *et al.*, 2000). The major disadvantage of adenovirus vectors is their toxicity. They are highly immunogenic (Rolland, 1998). Other adverse effects include potential instability of gene expression because adenoviral DNA does not integrate into host cell

chromosomal DNA. Additionally, adenoviruses may exist in the nucleus as an episome (i.e. extrachromosomal element), which may not be passed through later cellular divisions (Salyapongse *et al.*, 1999).

Adeno-associated Viruses (AAV) are small, single-stranded DNA viruses similar to retroviruses. An AAV can permanently integrate itself into chromosomes of host cells. The difference between a retrovirus and AAV is that AAV is capable of infecting some non-dividing cells. As a DNA virus, however, AAV may be less susceptible to recombination than retroviruses (Ledley, 1996). One advantage of recombinant AAV vectors is that they can avoid the immune response generated by adenoviral vectors because AAV vectors lack virally encoded genes. Another characteristic of AAV is that they have been shown to express target genes for months after transfection because of the absence in the infection of a host cell by a helper virus (e.g., adneovirus or herpesvirus). This makes AAV an attractive vector for conditions requiring more enduring therapy (Salyapongse *et al.*, 1999). The major limitation of AAV is related to helper virus. In the absence of a helper virus, AAV has difficulty in developing package cell lines that can produce sufficient titers of the virus for clinical use (Miller, 1992).

Other viral vectors, including herpesvirus, lentivirus, vaccinia virus and several RNA viruses, were recently reviewed by Salyapongse *et al.* (1999). Some of these have received more attention than others. Although advances in virus-mediated gene delivery technology have shown significant results of efficient gene delivery, there are still persistent concerns raised with this class of vectors, especially related to their safety. These concerns include the possibility of recombination with endogenous viruses, which could generate mutants and cause infection (Anwer *et al.*, 2000). Retrovirus-mediated gene transfer methods result in stable transformation of the target cells. This attribute, which is generally regarded as one of its most obvious advantages, may also be its biggest problem. Some virus-based vectors may induce an immune response, and in some cases these vectors have been shown to sensitize animals leading to a toxic inflammatory reaction to the viral vector (Smith *et al.*, 1993). *For all these reasons, it is appropriate to consider developing those gene transfer techniques that do not employ viruses.*

1.3.2.1.2. Non-viral gene transfection

Non-viral methods of transfection include direct microinjection of DNA, permeabilization that can be mediated either by chemical or physical methods, and the creation of complexes that can cross the cell membrane by means of fusion or receptor-mediated endocytosis. The physical methods are more dependent on approaches such as microinjection, electroporation, gene gun, etc.

Electroporation has proven to be a powerful means of transfection for cell cultures in suspension (Salyapongse *et al.*, 1999). This method is based on the finding that when a strong electric field (typically kilovolts/cm) is applied to a cell suspension for a few microseconds, some regions of the cytoplasmic membrane undergo a slow reversible breakdown, transiently allowing DNA to enter cells. The use of electroporation to deliver genes has become an area of greater research interest because this method is simple and easy, and gives stable transfection at a high rate when used for *in vitro* delivery (Zheng and Chang, 1991; Sukharev *et al.*, 1992). Even *in vivo*, the application of electric fields coupled with the successful delivery of many types of chemotherapeutic agents implies that electroporation may be an attractive alternative for site-specific gene delivery (Jaroszeski *et al.*, 1999). This method, however, needs special equipment that is very expensive.

Microinjection is conceptually the simplest method of introducing the gene directly into the nucleus of cultured cells leading to expression and occasional integration of recombinant genes into the chromosomes of the injected cells (Capecchi, 1980). The number of cells that can be transfected using this method is obviously very limited, and it will be hard to apply this technique to *in vivo* gene therapy.

Particle-mediated gene transfer (gene gun) represents the most recent physical transfection technology. The particles (e.g., gold or tungsten) are accelerated by helium pressure or a high voltage electrical discharge and are capable of penetrating the cell membrane. The advantages of this method include the ability to introduce large DNA constructs or to deliver multiple genes simultaneously. Also, this method has proved successful in both *in vitro* and *in vivo* models and results in

minimal toxicity (Salyapongse *et al.*, 1999). Unfortunately, the gene gun is not capable of penetrating deeply into tissues, and effective gene delivery is observed only in superficial layers of the injected tissue (Ledley, 1994).

Calcium phosphate mediated transfection was the first chemical method used in gene transfer (Graham and Van Der Eb, 1973). By precipitating DNA with calcium phosphate and applying this precipitate to cells in culture, the calcium-DNA complex precipitates onto the surface of the cultured cell, leading to non-specific endocytosis. The method is simple, but it is not effective for *in vivo* gene transfer.

1.3.2.1.3. Synthetic gene delivery systems

Recently, chemical methods, especially synthetic vectors, have been the focus of gene delivery technology inspired by the initial report of Felgner *et al.* (1987). Several excellent reviews on the development of nonviral-synthetic delivery systems are available (Garnett, 1999; Rolland, 1998; Friedmann, 1997; Felgner, 1997; Mahato *et al.*, 1997; Marshall, 1995; Behr, 1994; Zhu *et al.*, 1993; Felgner and Rhodes, 1991). Even though the transfection rates of non-viral systems are significantly lower than those of viral vectors, these vectors still have many advantages over viral vectors. An important property of this kind of delivery system is that DNA only rarely integrates into the chromosomes of host cells. Because of this, the synthetic delivery systems do not display the serious mutagenic problems associated with viral vectors. Another one of the exciting advantages of non-viral delivery over viral gene delivery systems is their potential of transferring and expressing large pieces of DNA into cells. Compared with viral vectors which have a maximum gene carrying capacity of 40 kbp (Friedmann, 1997; Crystal, 1995), the non-viral systems may have large size capacity of 100 kbp to 1 Mbp of DNA segments (Lin *et al.*, 2000; Roush, 1997). These developments will be very important for gene therapy applications. At present, most of synthetic gene delivery systems available are cationic agents (Garnett, 1999; Rolland, 1998).

1.3.2.2. Cationic delivery systems

The technical challenge of delivering DNA into cells is that plasmid DNA has a size similar to that of the potential recipient cell. Particles of this size (>100 nm) will not be allowed to spontaneously cross an intact lipid membrane or other biological barriers. Also, because it is negatively charged, DNA cannot bind to the negatively charged cell surface. This macromolecular polyanion (DNA) requires specific delivery systems or specialized methods to enter cells in order to achieve the goal of gene transfer and gene therapy. At present, condensation of DNA is easily achieved by a number of agents including neutral compounds such as polyethylene glycol, polyvinyl pyrrolidone, and anionic compounds such as polyacrylate (Lerman, 1971). The most efficient and useful condensing agents are cationic compounds. The cationic systems, because of their positive charges, bind the negative charges of DNA in a strong but non-damaging electrostatic interaction, yielding a soluble polycation-DNA complex. The studies using synthetic gene delivery systems are therefore mainly focused on cationic lipids, polymers, and endosomal lysis peptides.

Liposomes and cationic lipids: After the initial report by Felgner *et al.* (1987), there have been a large number of reports focused on cationic liposome reagents for the delivery of DNA, mRNA (Weiss *et al.*, 1989), antisense oligomers (Chiang *et al.*, 1991; Bennett *et al.*, 1992), and proteins (Debs *et al.*, 1990) into living cells. This class of delivery agent has been studied the most, and is thought to be the most efficient type of synthetic delivery system. Many different liposome forming cationic lipid molecules have now been identified, and have been shown to enhance gene delivery *in vitro* and *in vivo*. Also, many derivatives of these lipids are available commercially today for *in vitro* transfection applications; examples include DOTMA (Lipofectin) (Felgner *et al.*, 1994), DDAB (Lipofectase) (Rose *et al.*, 1991), DOTAP (Felgner *et al.*, 1995), and DMRIE (Felgner *et al.*, 1994). The latter was developed at Vical, and has already been administered to patients in human gene therapy clinical trails for the treatment of cancer. The structures of these compounds are shown in Figure 1.3.2.2.

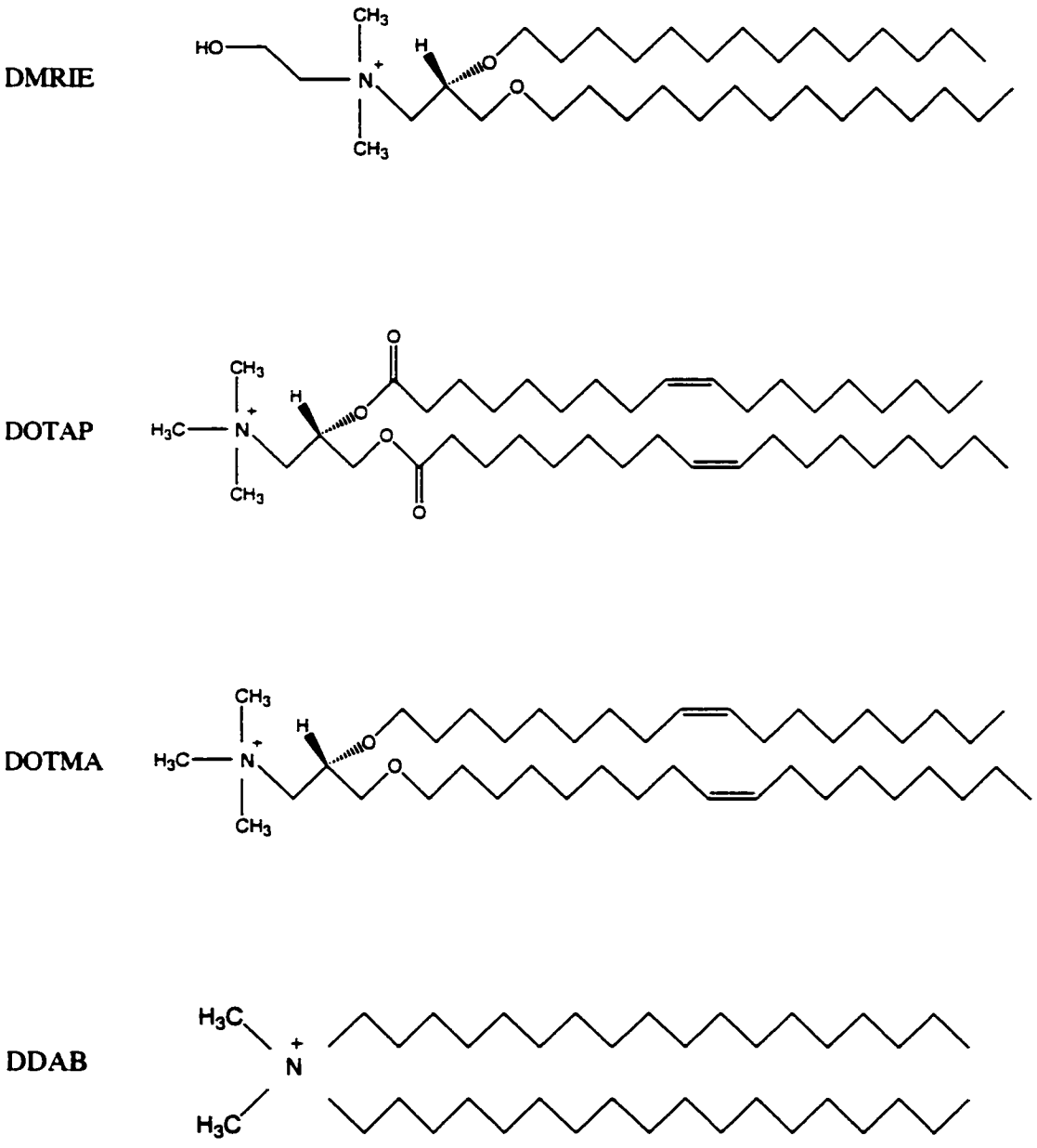


Figure 1.3.2.2. Structure of cationic lipids used in gene transfer

Cationic liposome transfer systems generally exhibit low toxicity, non-immunogenicity, and are easily produced. The mechanism of transfection via cationic liposomes (CLs) is not yet totally understood. Recently several reports on self-assembled stuctures of CL-DNA complexes have appeared (Lasic *et al.*, 1997; Raedler *et al.*, 1997; Koltover *et al.*, 1998; 1999; Lin *et al.*, 2000). Even though the mechanism of the action remains unclear, without doubt, these structure-based studies will be very helpful not only for increasing the efficiency of CL-DNA complexes, but also for understanding and rational design of other synthetic non-viral gene delivery systems.

Polymers: Another method for gene delivery utilizes cationic polymers. Several different classes of cationic polymers have been developed to enhance the uptake of DNA into cells (Figure 1.3.2.3). Polyamidoamine dendrimers are a class of polymers in which an amine starting material is repeatedly substituted at its amino termini to provide a branched structure. These polymers have high cationic charge density of primary amine groups on their surface. Their unique and well-defined properties are suitable for application as DNA delivery agents. Application of these novel polymers for gene delivery was first reported by Haensler and Szoka (1993). Since then, a number of articles along this line have been reported (Kukowska-Latallo *et al.*, 1996; Tang *et al.*, 1996). Transfection has also been reported using a synthetic polyamino polymer with a glucose backbone (Goldman *et al.*, 1997), and with polyethylenimine (Boussif *et al.*, 1995; Lemkine and Demeneix, 2001). Other polymers, including polyamino acids have also been studied (Garnett, 1999). All of these polymers have a high cationic charge density that can bind DNA electrostatically and form different charge ratios of polymer to DNA, resulting in increased flexibility.

Endosomal lysis peptides – receptor-mediated endocytosis: Most agents of this class are semi-synthetic polypeptides (Niidome *et al.*, 2000). The representative polylysine carrier was pioneered by Wu *et al.* (1987, 1988) (Figure 1.3.2.3). Receptor-mediated gene delivery then followed (Schwarzenberger *et al.*, 2001; Uherek and Wels, 2000; Trubets *et al.*, 1992; Gottschalk *et al.*, 1993). DNA-binding

polycationic proteins, such as polylysine and protamin, can be chemically linked to cell binding ligands, such as asialoglycoprotein or galactose for hepatocytes, anti-CD3 and anti-CD5 for T-cells, and transferrin (Wagner *et al.*, 1998), ferritin or insulin (Behr, 1994; Cotten *et al.*, 1993). These ligands can be recognized by a specific cell surface receptor, leading to active endocytosis (Singh *et al.*, 2001). This ligand-mediated gene delivery is an effective method for transferring genes into a variety of cells *in vitro* (Gaucheron *et al.*, 2001; Han and Il Yeom, 2000). Even *in vivo*, studies have proven the successful delivery of genes to the liver (Wu *et al.*, 1991; Chowdhury *et al.*, 1993; Tarakura *et al.*, 2001) and lung (Wagner *et al.*, 1998) using this technique.

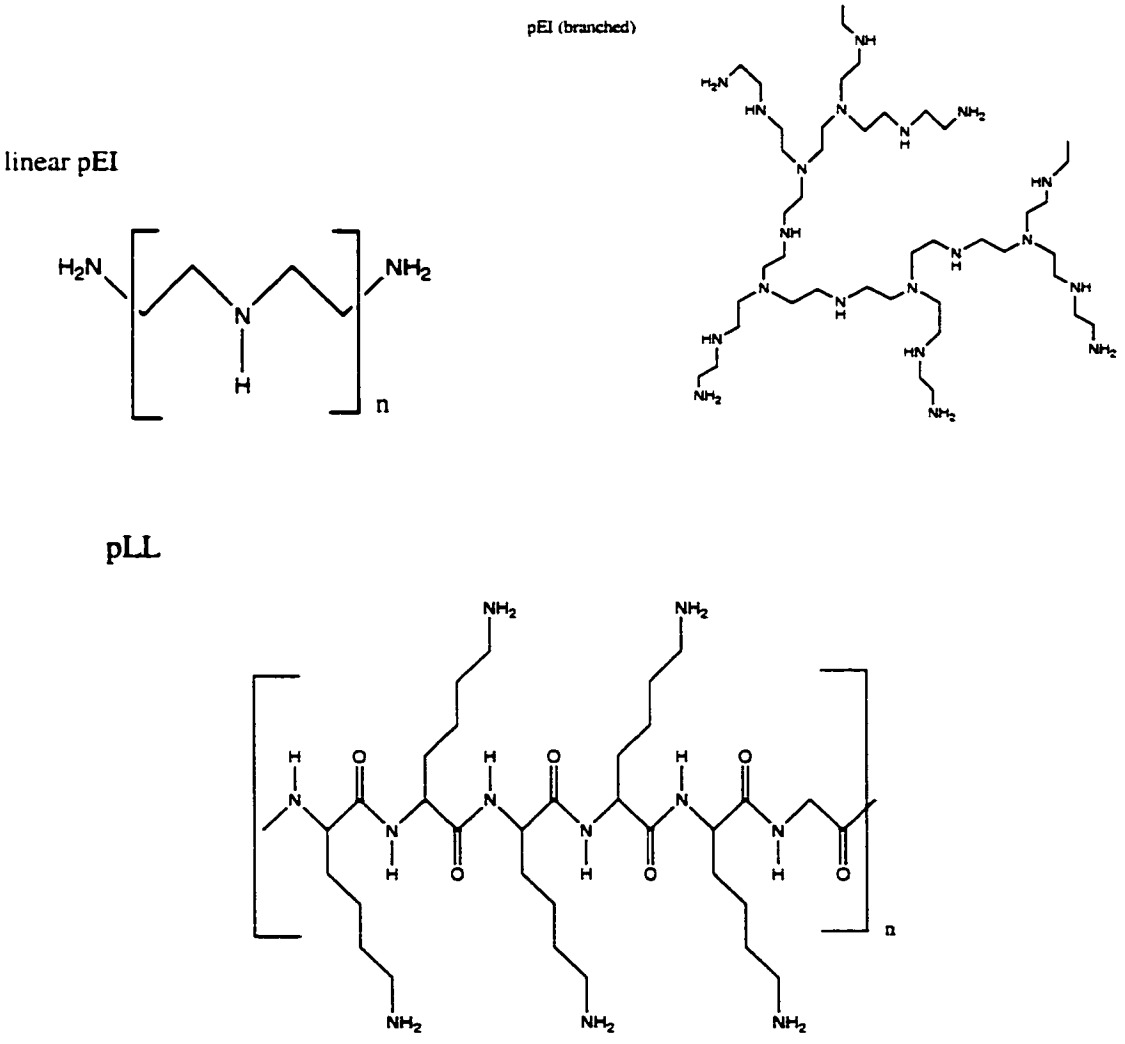


Figure 1.3.2.3. Structure of polymers and endosomal lysis peptides

1.3.2.3. The application of linear chain dextran in gene delivery

Looking back over the developments of gene transfection, it can be seen that linear dextrans were used very early in this field. From the start in 1965 (Vaheri and Pagano, 1965), DEAE/Dextran transfection is one of the oldest chemical methods. DEAE/Dextran is a cationic polymer that can associate tightly with the negatively charged nucleic acids. An excess of positive charge, contributed by the polymer in the DEAE/Dextran-DNA complexes, allows the complex to come into close association with the negatively charged cell membrane. After the complex is deposited onto the cell surface, uptake of the complex is presumably internalized by endocytosis. The precise mechanism of the action of DEAE/Dextran remains unclear, but the proposed mechanism suggests that the polymer may bind to DNA and inhibit the action of nucleases, or bind to cells and promote endocytosis of DNA (Yang and Yang, 1997). This method is successful for delivery of nucleic acids into cells for transient expression; that is, for short-term expression of a few days in duration. As one of the first chemical reagents used for gene transfer, this method is relatively inexpensive and can provide high efficiency of transfer in some cell types. DEAE/Dextran as a gene facilitator has many limitations, such as cytotoxicity and the difficulty of application to *in vivo* studies. Also, it cannot be used with serum in culture medium, nor for stable transfection. However, as a simple and effective method, DEAE/Dextran is still widely used in the laboratory for *in vitro* transfection (Schenborn and Goiffon, 2000; Mack *et al.*, 1998; Pari and Keown, 1997).

1.3.2.4. Suitability of CyD for gene delivery

Compared with dextrans, CyDs have very similar composition as both are based on glucose linkages. The polycation DEAE/Dextran reportedly stimulates cellular endocytosis (Ryser, 1967) and may also provide a positively charged anchor between the negatively charged cell membrane and the polyanionic DNA (Sompayrac and Danna, 1981). Such considerations raise the possibility that the DNA binding and entry may be significantly enhanced if CyDs could be used to concentrate DNA on the target cell surface. Recently, studies on the use of

cyclodextrins and their derivatives in gene transfer (Hwang *et al.*, 2001; Roessler *et al.*, 2001; Croyle *et al.*, 1998; Abdou *et al.*, 1997; Freean and Niven, 1996) and oligonucleotide delivery (Zhao *et al.*, 1995) have been reported. Preliminary studies involving: (1) The transfection of a firefly luciferase reporter gene into cells has shown that a cationic amphipathic cyclodextrin can mediate detectable levels of gene transfer (Woltjen *et al.*, 1997). (2) Cationic β-CyDs such as tertiary amino-β-CyDs, act as a viral dispersant, which results in an increase in adenoviral transduction in human colon adenocarcinoma Caco-2 cells by enhancing both viral binding and internalization (Croyle *et al.*, 1998). (3) β-CyD can increase gene transfer in the lungs of rats (Freean and Niven, 1996). (4) 2-Hydroxypropyl-β-CyD (HP-β-CyD) and 2-hydroxyethyl-β-CyD (HE-β-CyD) can increase the uptake of the antisense phosphorothioate oligodeoxynucleotides in the human T cell leukemia H9 cell line without cytotoxicity (Zhao *et al.*, 1995). (5) 6-Deoxy-6-*S*-β-D-galactopyranosyl-6-thio-CyD can improve the antiviral activity of the antisense phosphodiester oligonucleotide in human adenocarcinoma cells (Abdou *et al.*, 1997). The interest in this class of compounds for use in gene delivery is related to the availability of materials, potential for structural diversity, and particularly its low toxicity.

Cationic CyDs: As described in section 1.3.2.2, cationic compounds are the most efficient and useful condensing agents currently known. We choose cationic CyDs as our target, not only because DNA bears negative charges that come from its phosphate groups, but also because the cell membrane has negative charges. Electrostatic interactions of CyDs with plasmid DNA are critical for both forming suitable particle size and interaction of the cationic complexes with the cell membrane. The later is thought to help to concentrate DNA on the target cell surface and to stimulate endocytosis of the DNA-polycation complexes (Woltjen *et al.*, 1997). Cationic CyDs can be obtained through chemical modification of the large number of hydroxyl groups in parent CyDs.

Carrier/Delivery systems: Advanced drug delivery, particularly the development of particulate drug delivery systems, represents an important experimental and theoretical foundation for developing non-viral gene therapies.

Non-viral delivery methods, especially synthetic systems, make genes more like a "drug" or "medicine". Compounds can be designed to complex with DNA and then be directly administered to patients by conventional routes. Expression of those therapeutic products over a finite period of time in a manner similar to conventional medicines is possible, such as the half-life in the body. Some initial preclinical and clinical studies have indicated that non-viral gene delivery methods exhibit safety profiles similar to conventional pharmaceutical or biological products, which may soon allow gene therapy to be applied in clinical practice alongside traditional medicines for the treatment of common diseases (Ledley, 1994).

Many methods used for non-viral gene delivery were developed originally for controlled delivery of conventional drugs and biological products. Ledley (1996), advanced the theory and methods for controlled delivery of small-molecule drugs using particulate drug carriers. These techniques have proven to be useful for delivering genes to cells *in vitro* or *in vivo*. As soluble carriers, the CyDs have been studied for many years as drug delivery systems and have proven to be very useful carriers in drug delivery. This is why we put our attention on the cyclodextrins. We hypothesized that their application could be extended to gene delivery if the appropriate modifications were made.

In gene transfer, the first and the most important step is introducing DNA into the cells. Most DNA delivery systems operate at one of three general levels (Luo and Saltzman, 2000a): DNA condensation and complexation, endocytosis, and nuclear targeting. Negatively charged DNA molecules are usually complexed with cationic transfection reagents before delivery. As far as CyDs, they are known to form inclusion complexes with a variety of guest molecules. Also, they can be modified by incorporation of cationic site suitable for binding DNA molecules. Some studies have already shown that there is an interaction between the cyclodextrin and DNA (Gonzales *et al.*, 1999) or oligonucleotides (Zhao *et al.*, 1995). These studies indicate that such properties would help CyDs in forming complexes with DNA strands for the delivery of the genetic material into the cells.

Protection and release of DNA: The more efficient methods for DNA gene delivery involve entry of DNA into the cell by endocytosis. One of the factors that limit the efficiency of gene delivery to the nucleus after endocytosis is the rapid degradation of DNA within the endosome. Many studies (Rsjewski and Stella, 1996; Connors, 1997) have shown that cyclodextrins can be used for both increasing and decreasing the availability of an active substance and for protecting it from degradation. Drug-CyD complexation can be regarded as molecular encapsulation, that is, encapsulation of drug at the molecular level. The cyclodextrin molecule shields, at least partly, the drug molecule from attack in a potential corrosive environment. This is one premise for the choice of CyDs as a carrier. It is our hope that the CyD can prevent a labile compound, such as DNA, from enzymatic degradation.

Since most DNA delivery systems rely on complex formation, release of DNA molecules from a macro-molecular assembly must occur before transcription can proceed. The stability of complex formation, i.e. the rate of DNA released from complexes, will affect the efficiency of gene expression. If DNA can be protected by complex formation with CyDs, because there are no covalent bonds formed or broken during the CyD-drug complex formation, the complexes are easily dissociated. On the other hand, because transfection efficiency may be limited by a simple physical barrier, such as low DNA concentration at the cell surface (Luo and Saltzman, 2000b), new methods designed to increase transfection efficiency should aim to increase DNA concentration at the target cell surface without adding to the toxicity. Roessler *et al.* (2001) have demonstrated that the presence of modified β-CyDs can improve surface distribution of Dendrimer/DNA complexes. Also, some results obtained by Hwang *et al*, (2001) indicate that the incorporation of β-cyclodextrin in the cationic polymers can significantly decrease polymer toxicity. This lower *in vitro* and *in vivo* toxicity make them promising and attractive agents for *in vivo* gene delivery applications. At the same time, many CyD derivatives are available right now that can serve as carriers to study this kind of delivery application and their ability of improving the DNA uptake.

Modifiable properties: Synthetic delivery systems can often be improved by chemical modification. This is proved by several liposomes, including DDAB, DOTAP, and DMRIE, etc., which used DOTMA as a prototype (Felgner *et al.*, 1995). This approach has also been demonstrated in the development of several polymers, including polyethylenimine (pEI) (Diebold *et al.*, 1999), gelatin (Truong-Le *et al.*, 1998), methacrylate/methacrylamide polymers (Van de Wetering *et al.*, 1999), and pyridinium surfactants (Van de Woude *et al.*, 1997).

One reason that CyDs are used extensively is that the modification of cyclodextrins offers enormous opportunities for chemists. Because of their specific structure, CyDs can be modified in a variety of ways. The strategy for modification can be designed for many different uses of the final products. In their native state, cyclodextrins are rigid molecules and offer limited utility in terms of size, shape, and availability of chemically useful functional groups. The three types of hydroxyl groups available on two different sides of CyDs however, make them versatile with respect to modification. Even though these hydroxyl groups provide challenges in terms of selective modification (Khan *et al.*, 1998), advanced organic chemistry can now make cyclodextrin derivatives of any size, shape, and most importantly containing any functional groups. There are many reviews on chemically modified CyDs (Croft and Bartsch, 1983; Wenz, 1994) and some of them are specially focused on the selective modifications of CyDs (Khan *et al.*, 1998). CyDs attracted chemists not only because they are chemically stable and can be modified in a regioselective manner, but also because they can form a homologous series of water-soluble host molecules that can be used as models for studying weak interactions.

Interaction with lipophiles and biological membranes: One of the most essential requirements for potential drug carriers is that they have low levels of intrinsic cytotoxicity. The CyDs are known to induce shape changes (e.g., membrane invagination) in human erythrocytes leading to lysis at high concentrations (Ohtani *et al.*, 1989). This may result from the fact that CyDs can extract cholesterol or remove proteins from biological membranes. However, these induced cell changes may also lead to an increase in membrane fluidity and permeability, which would help drug or

DNA macromolecules to pass through the biological membrane (Masson *et al.*, 1999). On the other hand, when the character of the lipophilic cavity of CyDs is changed by chemical modification, the effects on cell membranes can dramatically change (Attioui *et al.*, 1994). Even though the process of the absorption of CyDs is not known in great detail, as drug carriers and absorption enhancers, they are known to be able to keep drug molecules in solution and deliver them to the surface of the biological membrane. Previous studies in our laboratory (Woltjen *et al.*, 1997) suggested that β-CyD with a cationic moiety may result in its ability to deliver DNA efficiently to cells. This process may in part be related to the unique interaction of β-CyD with cell membranes.

Renewable resource and other factors: Finally, the CyDs are of interest because they can be obtained easily from a renewable resource – starch. Their low price provides motivation and possibilities for the discovery of new applications. At the same time, developments in this field are large and many detailed and basic studies have been carried out on the applications of CyDs, the formation of inclusion complexes, and even on the toxicity, mutagenicity, and cancenogenicity of cyclodextrins that have established the foundation for further novel developments.

Until 2000, there was only one paper reporting the beneficial use of CyD conjugates for gene transfer *in vitro* (Gonzalez *et al.*, 1999). Even though there are no details about the function of cyclodextrins in this article, the authors indicated the application of CyDs in this area has already started. In 2001, several papers on gene delivery related to cyclodextrins have been published (Arima *et al.*, 2001; Hwang *et al.*, 2001; Roessler *et al.*, 2001). All of these studies are focused on defining potential interactions between β-CyDs and polymer-based transfection systems. To our knowledge, besides the initial reports from our laboratory (Morin *et al.*, 1996), there are no reports that CyDs or their derivatives alone can display transfection activity, or the enhancing effects of the cellular association of DNA, or the protecting ability of DNA against DNase. But no doubt, many advantages of CyDs, as well as a large number of existing results of CyD studies, will allow this research to continue.

Chapter 2

Hypothesis and Objectives

According to the previous discussion, the following hypothesis was established:

- Cationic CyDs may be potential DNA delivery systems

To test this hypothesis, a number of specific experimental objectives were identified:

- To synthesize several cationic cyclodextrin derivatives
- To investigate the capabilities of these CyD derivatives to deliver reporter genes *in vitro*

Chapter 3

Experimental

3.1. Chemistry

3.1.1. General methods and materials

β-Cyclodextrin (β-CyD) was purchased from Sigma-Aldrich Chemical Company Inc., and was dried over P_2O_5 at room temperature for 24 – 48 h before use. Other chemicals were purchased from Sigma-Aldrich and Fisher. NaH was washed with hexane and dried in vacuum. Solvents were purchased from Sigma-Aldrich and dried according to literature procedures. The solvents used for synthesis were reagent grade unless specified, and other chemicals and solvents were of analytical grade.

Thin-layer chromatography (TLC) was carried out on Kieselgel 60 F254 (Merck) and visualization was accomplished by charring with 5% methanolic sulfuric acid. Column chromatography was performed using silica gel 60 (70-230 Mesh ASTM, 0.063-0.2 mm, Rose Scientific Ltd.). A Labconco Freeze Dryer 3 was used for lyophilization. IR spectra were recorded with a Perkin-Elmer 700 spectrometer. ^{1}H-NMR spectra and ^{13}C-NMR spectra were recorded at Bruker AM-300 (282.35 MHz for carbon) or 500 MHz Varian VXR-500 spectrometer, and operated by Dr. Somayaji or Dr. Diakur, respectively. All chemical shifts are quoted in ppm, referenced to residual $CHCl_3$ at δ 7.27 for $CDCl_3$ solutions or HOD at δ 4.82 (25 °C) for D_2O solutions. Coupling constants (*J*) are reported in Hertz. ^{13}C-NMR spectral assignments were aided by the J-MOD technique.

3.1.2. Synthesis of compounds 1 - 18

Heptakis(6-bromo-6-deoxy)-β-cyclodextrin (1). A solution of triphenylphosphine (42.5 g, 162 mmol) and DMF (125 mL) was cooled (ice-bath) and then a solution of bromine (8.35 mL, 162 mmol) and DMF (35 mL) was added slowly. After addition was complete, the reaction was stirred for 30 min. Dry β-

cyclodextrin (8.75 g, 7.7 mmol) was then added and the solution was stirred at 80 °C for 15 h. The mixture was concentrated to half the volume under high vacuum. The pH was adjusted to 9-10 by the addition of fresh CH_3ONa/CH_3OH (3 M, 130 mL) with simultaneous cooling. The solution was kept at room temperature to destroy the formate esters formed in the reaction, and after 30 min, the mixture was poured into ice water. The precipitate was collected by filtration and washed sequentially with water, ether and dichloromethane and dried over night in vacuo. Yield 11.98 g (quantitative).

Crude Heptakis(6-azido-6-deoxy)-β-cyclodextrin (2). A solution of compound **1** (11.98 g, 7.6 mmol) and NaN_3 (10.38 g, 160 mmol) in DMF (140 mL) was stirred at 90 °C for 60 h. After cooling, the reaction mixture was diluted with DMF, then evaporated until 25% of the solution remained. Water (200 mL) was added, the suspension filtered, and the solid was washed with water (60 mL x 5) and then ether (60 mL x 4). The residual brown solid was dried overnight.

Heptakis(2,3-di-*O*-acetyl-6-azido-6-deoxy)-*β*-cyclodextrin (3). Pyridine (150 mL) and acetic anhydride (75 mL) were added to a flask that contained crude compound **2**. The mixture was stirred at room temperature for 24 h. The reaction was then evaporated using toluene (100 mL x 2). The residue was dissolved in CH_2Cl_2 (300 mL), then successively washed with 1% H_2SO_4 (300 mL), H_2O (300 mL), saturated aqueous $NaHCO_3$ (300 mL), and saturated aqueous NaCl (300 mL). This solution was dried (Na_2SO_4) and the solvent was evaporated. Column chromatography of the residue with hexane-ethyl acetate-ethanol (5:5:1) as the eluant gave 8.15 g of pure **3** as white solid. IR: ν_{max} 1750 (C=O), 2106 (N_3). ^{1}H-NMR data ($CDCl_3$): δ 5.32 (t, 1H), 5.11 (d, 1H), 4.84 (t, 1H), 4.01 (m, 2H), 3.79-3.60 (m, 2H), 2.12-2.04 (2s, 6H); ^{13}C-NMR data ($CDCl_3$): δ 170.3, 169.3 (C=O), 96.7 (C-1), 77.2 (C-4), 71.0, 70.7, 70.4 (C-2, 3, 5), 51.6 (C-6), 20.8, 20.7 (CH_3).

Purified Heptakis(6-azido-6-deoxy)-*β*-cyclodextrin (4). Freshly prepared MeONa/MeOH (1.5 g sodium + 100 mL methanol) was added to a solution of compound **3** (8.15 g, 4.3 mmol) in chloroform (20 mL), and the reaction mixture was

stirred at room temperature overnight. The solution pH was adjusted to 4-5 using acetic acid, and the mixture evaporated to dryness. Water and methanol were added to the residue. The light yellow solid was collected by filtration and the solid was dried in vacuo to yield 4.34 g (77 %) of pure **4**. ^{1}H-NMR data (DMSO-d_6): δ 5.74 (s, 1H), 4.88-4.87 (d, 4H), 3.76-3.68 (m, 2H), 3.37-3.29 (m, 2H); ^{13}C-NMR data (DMSO-d_6): δ 102.1 (C-1), 83.2 (C-4), 72.6, 72.1, 70.4 (C-2, 3, 5), 51.4 (C-6).

Heptakis(6-amino-6-deoxy)-β-cyclodextrin (5). Compound **4** (390 mg, 0.3 mmol) and 1.64 g (6.3 mmol) of triphenylphosphine were dissolved in DMF (3 mL) and the mixture was stirred at room temperature for 30 min. Aqueous ammonia (3 mL) was added and the mixture was then stirred at room temperature for 48 h. The mixture was diluted with methanol and evaporated under high vacuum. The residue was diluted with 50 mL of water, then washed with chloroform until no UV active material was present in the organic layer. The aqueous layer was evaporated to dryness and the resulting solid dissolved in water, filtered and lyophylized from water to give 336 mg (80 %) of **5** as a white solid.

Heptakis(6-amino-6-deoxy)-β-cyclodextrin, hydrochloride salt (6). Compound **5** (104 mg, 0.09 mmol) was treated with aqueous HCl (0.113 M, 5.7 mL) and the solution lyophilized to yield 120 mg (96 %) of the title compound. ^{1}H-NMR data (D_2O, *see Appendix*): δ 5.17 (d, 1H), 4.16-4.14 (m, 2H), 4.00 (t, 1H), 3.69 (dd, 1H), 3.58 (t, 1H), 3.44-3.40 (d, 2H), 3.24 (q, 1H), 2.99 (s, 1H), 2.84 (s, 1H); ^{13}C-NMR data (D_2O, *see Appendix*): δ 104.0 (C-1), 84.8 (C-4), 74.8, 74.2, 70.8 (C-2, 3, 5), 42.9 (C-6).

Heptakis(6-azido-6-deoxy-2,3-di-*O*-methyl)-β-cyclodextrin (7). Sodium hydride (272 mg, 11.3 mmol) was added to a solution of compound **4** (350 mg, 0.27 mmol) in dry DMF (5 mL). After stirring for 15 min, the mixture was cooled to 0 °C using an ice water bath. Methyl iodide (0.7 mL, 11.3 mmol) was added slowly to the stirred mixture. After the addition was complete, the mixture was allowed to warm to room temperature and then stirred for 2 h. The reaction then was cooled to 0 °C, and excess methanol was added to decompose the sodium hydride. The solvents were

evaporated and the residue was diluted with dichloromethane (100 mL), washed with water (50 mL x 4) and saturated aqueous sodium chloride (NaCl), dried (Na_2SO_4) and evaporated. Column chromatography of the residue with hexane-ethyl acetate-ethanol (20:10:1) as eluant gave 290 mg (72 %) of **7** as a white solid. ^{1}H-NMR data ($CDCl_3$): δ 5.11 (d, 1H, H-1), 3.78-3.17 (m, 6H, ring protons), 3.62 (s, 3H, OCH_3) and 3.53 (s, 3H, OCH_3).

Heptakis(6-amino-6-deoxy-2,3-di-*O*-methyl)-β-cyclodextrin (8). Compound **7** (84 mg, 0.06 mmol) and 293 mg (1.12 mmol) of triphenylphosphine were dissolved in methanol (15 mL) and stirred at room temperature for 30 min. Then, 5 mL of aqueous ammonia was added dropwise, and the mixture was stirred at room temperature for 48 h. Purification by column chromatography using chloroform-methanol-ammonia (60:40:10) as eluant yielded **8** (72.8 mg, 98.6 %) as a light yellow solid.

Heptakis(6-amino-6-deoxy-2,3-di-*O*-methyl)-β-cyclodextrin, hydrochloride salt (9). Compound **8** (32 mg, 0.024 mmol) was treated with aqueous HCl (0.113 M, 1.4 mL) then lyophilized to yield the title compound 37 mg (99 %). ^{1}H-NMR data (D_2O, *see Appendix*): δ 5.40 (d, 1H), 3.83-3.33 (m, 14H), 3.67 and 3.57 (2s, 6H, 2×OMe); ^{13}C-NMR data (D_2O, *see Appendix*): δ 100.6 (C-1), 82.6 (C-4), 82.4, 82.2 (C-2, 3), 70.8 (C-5), 62.8, 61.1 (C-CH_3), 43.0 (C-6).

TsO-CH_2-CH_2-O-CH_2-CH_2-OTs (10). *p*-Toluenesulfonyl chloride (33.8 g) was dissolved in CH_2Cl_2 (75 mL) and added dropwise over a period of 30 min to a flask containing 9 mL (95 mmol) of di(ethylene glycol), pyridine (76 mL) and CH_2Cl_2 (85 mL), and the reaction was stirred at room temperature for 5 h. The mixture was then diluted with CH_2Cl_2 and washed with H_2O (150 mL x 2), dried (Na_2SO_4) and evaporated to dryness. The residue was treated with ether to give 21.3 g (54 %) colorless needles: R_f 0.52 (hexane-ethyl acetate 1:1); ^{1}H-NMR data ($CDCl_3$): δ 7.7 (d, 2H, aromatic), 7.28 (d, 2H, aromatic), 4.03-4.00 (t, 2H), 3.55-3.52 (t, 2H), 2.38 (s, 3H).

TsO-CH_2-CH_2-O-CH_2-CH_2-N_3 (11). NaN_3 (2.6 g, 40 mmol) was added into a solution of compound **10** (10 g, 24 mmol) in DMSO (70 mL), and the mixture was stirred at room temperature overnight. The mixture was diluted with H_2O (150 mL) and CH_2Cl_2 (200 mL) and stirred. The organic layer was separated and further washed with H_2O (100 mL x 3), dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography to give 4.24 g (62 %) of **11** as a colorless oil: R_f 0.34 (hexane-ethyl acetate 2:1); ^{1}H-NMR data ($CDCl_3$): δ 7.74 (d, 2H, aromatic), 7.28 (d, 2H, aromatic), 4.12-4.08 (t, 2H), 3.64-3.61 (t, 2H), 3.55-3.52 (t, 2H), 3.26-3.23 (t, 2H), 2.38 (s, 3H).

I-CH_2-CH_2-O-CH_2-CH_2-N_3 (12). Compound **11** (2.0 g, 7.0 mmol) and KI (1.4 g, 8.4 mmol) were dissolved in DMSO (35 mL) and stirred at room temperature for 16 h. Two more charges of KI were added at 24 h intervals. Then, H_2O (80 mL) and CH_2Cl_2 (120 mL) were added, and the mixture was stirred 30 min. The separated CH_2Cl_2 layer was further washed with H_2O (100 mL x 3) and aqueous sodium thiosulfate, dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography over silica gel to yield 1.44 g (85 %) of **12** as a yellow oil: R_f 0.51 (hexane-ethyl acetate 5:1); ^{1}H-NMR data ($CDCl_3$): δ 3.80-3.75 (t, 2H), 3.70-3.67 (t, 2H), 3.43-3.39 (t, 2H), 3.29-3.25 (t, 2H).

Heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (13). A mixture of heptakis(6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (1.52 g, 0.79 mmol) and sodium hydride (1.32 g, 55 mmol) in dry THF (16 mL) was stirred for 20 min under argon. Methyl iodide (4.9 mL, 50 mmol) was added to the stirred mixture and reaction was allowed to proceed in the dark and under an atmosphere of argon for 48 h. 2-Propanol (5 mL) was added, followed by stirring at room temperature for 1 h. The solvents were then removed and the residue was dissolved in CH_2Cl_2 (100 mL). The organic solution was washed with 10 % sodium thiosulfate (75 mL x 2), and then by water (100 mL x 2). The organic layer was then dried over Na_2SO_4 and the solvent was evaporated. Purification on a column of silica gel (hexane-ethyl acetate 4:1 to 3:1) afforded 963 mg (58 %) of **13** as a white solid. ^{1}H-NMR data ($CDCl_3$, *see Appendix*): δ 5.20-5.18 (d, 2H), 4.13-4.09 (d, 2H), 3.75-3.51 (m, 10H),

3.06 (dd, 1H), 2.04 (s, 1H), 0.87 (s, 9H), 0.02 (s, 6H); ^{13}C-NMR data ($CDCl_3$, *see Appendix*): δ 98.1, 82.3, 82.1, 78.7, 72.2, 62.4, 61.4, 58.6, 36.7, 26.0, 24.7, 18.3, -4.8, -5.1.

Heptakis(2,3-di-*O*-methyl)-*β*-cyclodextrin (14). Compound **13** (730 mg, 0.34 mmol) was dissolved in chloroform (3.4 mL) then BF_3-Et_2O (2.1 mL, 16.8 mmol) was added and the mixture was stirred at room temperature for 20 min. Saturated aqueous $NaHCO_3$ was added dropwise to the mixture until the pH remained at 7-8. The mixture was then stirred at room temperature for 2 h to ensure the solution remained weakly basic. The solvent was evaporated, and the resulting white solid was extracted with chloroform-methanol (3:1), filtered and washed several times with this solvent. The filtrate was evaporated and dried. Column chromatography over silica gel using chloroform-methanol 5:1 and then 3:1 gave 317 mg (70 %) of compound **14**. ^{1}H-NMR data ($CDCl_3$, *see Appendix*): δ 5.07 (d, 1H), 3.76-3.48 (m, 13H), 3.63 and 3.51 are O-CH_3 (2s, 6H); ^{13}C-NMR data ($CDCl_3$, *see Appendix*): δ 98.8, 82.0, 81.6, 80.2, 72.4, 61.5, 61.3, 58.6.

Heptakis(2,3-di-*O*-methyl-6-*O*-(2-(2-azidoethoxy)ethyl))-*β*-cyclodextrin (15). Compound **14** (170 mg, 0.13 mmol) and NaH (64 mg, 2.68 mmol) were dissolved in dry DMF (2 mL) and then stirred at room temperature for 20 min. The mixture was then cooled to 0 °C using an ice-bath. Compound **12** (646 mg, 2.68 mmol) was added dropwise into the mixture, and the reaction was allowed to warm to room temperature and stirred overnight. Fresh NaH (21 mg, 0.89 mmol) and compound **12** (215 mg, 0.89 mmol) were added daily for three days. After this time methanol was added to stop the reaction, and after 30 min the solution was diluted with CH_2Cl_2 (70 mL). The organic layer was washed with water (80 mL x 3) and saturated aqueous sodium chloride. The CH_2Cl_2 layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The resulting yellow solid was purified by column chromagraphy over silica gel using hexane-ethyl acetate-ethanol 5:5:1 as eluant to yield 68 mg (25 %) of **15** as a white solid. ^{1}H-NMR data ($CDCl_3$, *see Appendix*): δ 5.16 (d, 1H), 3.97-3.15 (m, 20H).

Heptakis(2,3-di-*O*-methyl-6-*O*-(2-(2-aminoethoxy)ethyl))-*β*-cyclodextrin (16). Compound **15** (46 mg, 0.022 mmol) and triphenylphosphine (119 mg, 0.46 mmol) were dissolved in methanol (8.4 mL) and stirred at room temperature for 30 min. Then aqueous ammonia (2.8 mL) was added dropwise and the mixture stirred at room temperature for 48 h. Evaporation of solvents and then purification by column chromatography using chloroform-methanol-ammonium 65:35:8 as eluant yielded 17 mg (41 %) of **16** as a light yellow solid. ^{1}H-NMR data (D_2O, *see Appendix*): δ 5.32 (brs, 1H), 3.85-3.31 (m, 20H), 2.89-2.88 (s, 2H); ^{13}C-NMR data (D_2O, *see Appendix*): δ 100.0, 83.7, 82.9, 80.1, 73.9, 73.4, 72.8, 72.2, 71.9, 62.5, 60.8, 43.5.

Heptakis(2,3-di-*O*-methyl-6-*O*-(2-(2-aminoethoxy)ethyl))-*β*-cyclodextrin, hydrochloride salt (17). Compound **16** (16 mg, 8 μM) was treated with aqueous HCl (0.106 M, 0.54 mL) and lyophilized to provide the title compound (18 mg).

Heptakis(tetramethylammonium-6-deoxy-2,3-di-*O*-methyl)-*β*-cyclodextrin iodide (18). Compound **8** (33 mg, 0.025 mmol) and NaOH (100 mg, 2.5 mmol) were dissolved in MeOH (2.5 mL). The mixture was cooled to 0-5 °C in an ice bath and then CH_3I (75 μL) was added. This mixture was then stirred overnight, after which time a second portion of NaOH and CH_3I was added. The mixture was again stirred overnight. The reaction mixture evaporated and the resulting crude solid was separated on a column of Sephadex G-10 (HPLC grade water packed gel; water - 0.5 % methanol as eluant) to give **18** as a white solid (52 mg, 83 %).

3.2. Cell Biology

3.2.1. General methods and materials

Plasmid. The DNA-pcDNA3.1/*LacZ*, which was used to drive the expression of β-galactosidase from SV40 promoter, was obtained from Weili Duan. DNA was stored at – 20 °C and was serially diluted with water to provide a final concentration from 3.9 μg/μL to 0.39 μg/μL prior to use.

Cells. The fibroblast-like cell line COS-1 (monkey kidney) was obtained from Weili Duan. The cells were kept in Dulbecco's minimum essential medium (DMEM) containing 10 % fetal bovine serum (FBS), cultured at 37 °C in a 5 % CO_2 humidified atmosphere and passaged every 4-5 days.

Controls. To produce the positive controls, DEAE/Dextran (Sigma-Aldrich Co.) and DOTAP (Boehringer Mannheim), were used according to the manufacturer's recommended protocol.

Solution of buffers used in transfection were as follows:

PBS (Phosphate Buffered Saline): The solids NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.15 g), and KH_2PO_4 (0.2 g) were dissolved in water (~100 mL), and then diluted to final volume of 1 L with water. The final pH was mantained at 7.4 and this solution was autoclaved and stored at room temperature.

Trypsin solution: The trypsin solution was prepared by diluting from *10 × Trypsin-EDTA* with PBS buffer. *10 × Trypsin-EDTA* was purchased from Gibco BRL, and contains 0.5 % trypsin and 5.3 mM EDTA-4Na [prepared with 5.0 g of trypsin (1:250), 2.0 g of EDTA-4Na and 8.5 g of NaCl/L]. The trypsin solution was stored at – 5 to – 20 °C.

DMEM (Dulbecco's Modified Eagle Medium) was purchased from Gibco BRL. The powder had a net weight 13.4 g/pkg, and was stored at 2 to 8 °C in the dark and kept dry. This medium is high in glucose content, with L-glutamine, pyridoxine hydrochloride, and without sodium pyruvate and sodium bicarbonate.

To prepare liquid medium, distilled water was measured out using a mixing container that is as close to the final volume as possible (~950 mL). The powdered medium was added to water with gentle stirring, and the inside of package was rinsed to remove all traces of powder. Then $NaHCO_3$ (3.7 g per liter of medium) was added to the solution. The solution was diluted to the desired volume (1 L) with water and was stirred until all the solid had dissolved. The pH of medium was adjusted to 0.2-0.3 pH units below desired final working pH* (*pH will usually rise

0.1-0.3 units upon filtration) using 1N NaOH or 1N HCl (added slowly with stirring). After the pH had been adjusted, the container was kept closed until the medium was filtered. The medium was sterilized immediately by membrane filtration, and this medium was stored at 4 °C until use.

C-DMEM medium was used for normal cell cultures. *DMEM liquid medium* contained 10 % (v/v) FBS (sterile Fetal Bovine Serum).

HEPES buffer solution (1M) was purchased from Sigma-Aldrich, which contains HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid 238 g/L) prepared with distilled water. The solution has pH 7.2 to 7.5 and pKa 7.3 at 37 °C. The recommended storage condition is 2 °C to 8 °C. The solution of H-DMEM (HEPES-DMEM) used in transfection was *DMEM liquid medium* containing 20 mM *HEPES buffer solution.*

X-gal used in the β-galactosidase assay was purchased from Gibco BRL. The X-gal powder was dissolved in *X-gal buffer* when used for stain the cells. Standard solutions of *X-gal buffer* were prepared as shown in Table 3.2.1.

Table 3.2.1. Composition of X-gal buffer

	Molecule weight	Total 100 mL needs (mL)	Final concentration
1 M Na_2HPO_4 (pH 7.0)	110.99	1	10 mM
1 M $MgCl_2$	95.21	0.1	1 mM
1 M NaCl	58.45	15	150 mM
100 mM $K_4Fe(CN)_6 \cdot 3H_2O$	422.39	3.3	3.3 mM
100 mM $K_3Fe(CN)_6$	329.25	3.3	3.3 mM
distilled water (H_2O)	/	77.24	/

3.2.2. Preparation of cells for transfection

Trypsinizing cells for subculturing or cell counting is an important technique that is critical to successful cell transfection studies. First, the medium was removed from the tissue culture dish, and enough PBS solution was added to cover the cell monolayer. The plate was swirled to distribute the solution evenly, then the buffer was removed and tube wash was repeated once more. After the final wash, enough sterile trypsin solution (~10 mL) was added to cover the cell monolayer. The plates were placed in a 37 °C incubator until the cells just began to detach (usually 1-2 min). The adherent cells were loosened, and the cells were viewed under a microscope to check whether all cells had detached from the growth surface. When all cells had detached, medium-containing serum (C-DMEM, 10 mL) was added to the cells to inactivate the trypsin. The cells were gently pipeted to make sure cell clumps were broken up. The mixture was then centrifuged at 1,000-x *g* for 10 min and the supernatant was discarded. The pellet was suspended in 10 mL of C-DMEM until cell clumps were broken up. The cells were then counted using a hemacytometer and distributed to fresh plates for subculturing. (Solution compositions and preparations are provided in the section of 3.2.1.)

3.2.3. Preparation of cationic CyDs for transfection studies

Compounds **6**, **9**, **17**, and **18** were selected for evaluation as potential vectors for transfecting COS-1 cells with the pcDNA3.1/*LacZ* gene. Their structures are shown in Figure 3.2.3.

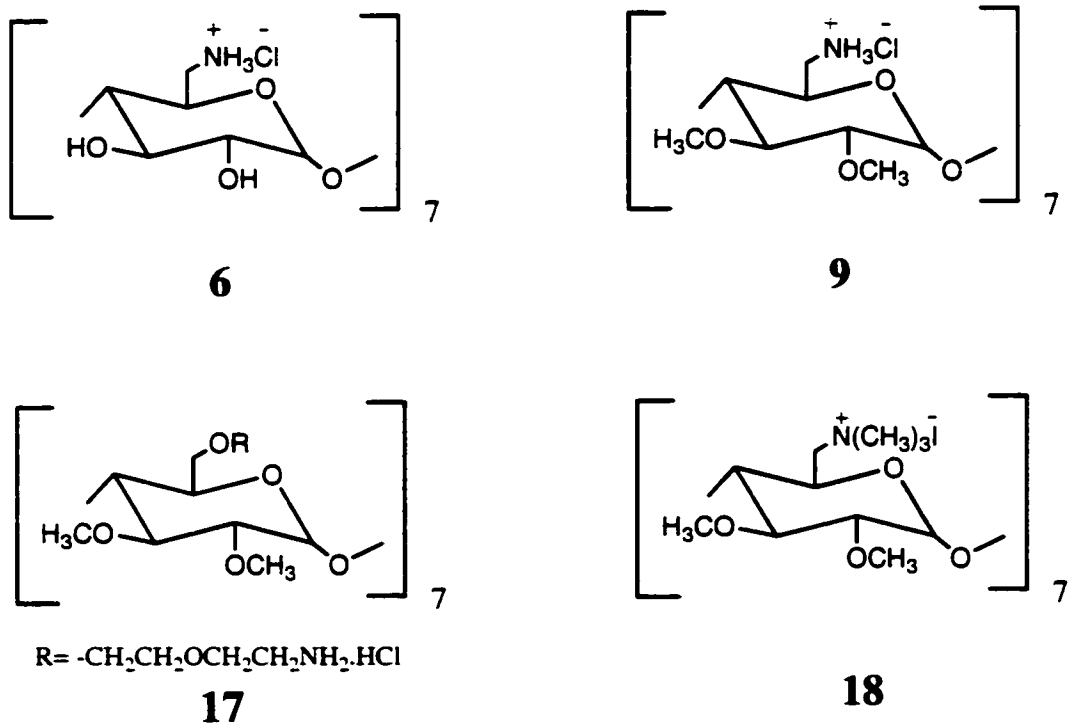


Figure 3.2.3. Structure of cationic CyDs used in transfection studies

Each compound was prepared at five different concentrations (see Table 3.2.3.) to determine which ratio of DNA to compound was best for transfection. First, the highest concentration of 100 μg/μL was prepared. This solution was diluted using sterile PBS solution to provide final concentrations of 50, 25, 10, 1 μg/μL.

Table 3.2.3. The cationic CyD solutions used in transfection

Sample No.	100 μg/μL	50 μg/μL	25 μg/μL	10 μg/μL	1 μg/μL
6	6-1	6-2	6-3	6-4	6-5
9	9-1	9-2	9-3	9-4	9-5
17	17-1	17-2	17-3	17-4	17-5
18	18-1	18-2	18-3	18-4	18-5

The positive control vectors were DEAE/Dextran and DOTAP. The respective solid crystals were dissolved in sterile PBS solution to prepare concentrations of 10 μg/μL or 60 μg/μL for each vector.

3.2.4. Preparation of DNA-CyD complexes and DNA-control complexes

The following experiments were performed duplicate in 24-well plates. Every well received a total of 100 μL (or 50 μL) of transfection solution containing 1.5 μg of DNA.

Cyclodextrin complexes with plasmid DNA were prepared in microfuge tubes by mixing 1.5 μg (3.85 μL, 0.39 μg/μL in distilled water) of pcDNA3.1/*LacZ* with 20 μL of serially diluted solutions of the CyD vectors, using HEPES-DMEM to dilute to a final volume of 100 μL.

Positive control-1 consisted of DNA-DEAE/Dextran complexes that were prepared in the same manner as the DNA-CyDs, but using 20 μL (10 μg/μL) of DEAE/Dextran instead of the CyDs.

Positive control-2 consisted of DOTAP as carrier. DNA (1.5 μg; 3.85 μL, 0.39 μg/μL in distilled water) was mixed with 10 μL (60 μg/μL) of DOTAP solution, using HEPES-H_2O (HEPES buffer, 20 mM) to dilute to a final volume of 50 μL.

The negative control contained 1.5 μg (3.85 μL) of DNA only, (no CyD, DOTAP or DEAE/Dextran in the control). The control was diluted to final volume of 100 μL using HEPES-H_2O.

All samples were kept at room temperature for 20 min, and then used as transfection solutions.

3.2.5. Transfection studies with the *LacZ* gene

The following procedure was adopted from protocols recommended for DOTAP and DEAE/Dextran. Transfection of pcDNA3.1/*LacZ* using CyD derivatives and the commercial transfection reagents DOTAP and DEAE/Dextran were performed and analyzed using the expression of pcDNA3.1/*LacZ* containing *β*-galactosidase under the control of SV40 promoter. The transfection solutions (DNA-CyDs or DNA-controls) were prepared as outlined above (see section 3.2.4.) and then allowed to stand for 20 min. COS-1 cells were sub-cultured at a density of 10,000 cells per well in 24-well plates the day before transfection. Cells were rinsed twice with wash solution (PBS) using 100 μL per well, and the DNA-compound mixtures (100 μL per well) were dispersed evenly over each well. The plates were incubated at 37 °C for 30 min with gentle swirling to keep the cells moist. After 30 min, 900 μL of H-DMEM medium was added to each well and the cells were incubated for up to 2.5 h at 37 °C. After washing once with PBS, the C-DMEM growth medium containing 10% FBS (1 mL) was added to each well. The cells were further incubated at 37 °C for 48 h after transfection. The positive control performed with DEAE/Dextran was prepared in the same manner as described above. The DOTAP positive control was performed by first washing, then adding 500 μL of H-DMEM, following by 50 μL of DNA-DOTAP solution to each well and incubating at 37 °C for 6 h. C-DMEM (10 % FBS, 1 mL) was added to each well, followed by incubation at 37 °C for 48 h.

For *in situ* *β*-galactosidase activity determination, cells were washed two times with PBS and then fixed with 0.25 % glutaraldehyde (0.5 mL per well) for 15 min at

room temperature. After fixation, the cells were washed twice with PBS and incubated with X-gal in X-gal buffer at 37 °C for 1-4 h. Each well needed 500 µL of a 0.2% X-gal stock solution. This was checked every half an hour to avoid over-staining. Once stained, the cells were rinsed twice with PBS, and then kept in PBS. To determine the percentage of transfected cells, the blue stained cells were counted in several randomly chosen visual fields in the microscope and the mean was calculated.

Chapter 4

Results and discussion

4.1. Chemical Synthesis

As discussed previously, modified CyDs are of current interest for potential application as DNA delivery systems. In order to investigate this possibility, several cationic CyD's derivatives were designed and synthesized.

4.1.1. Synthesis of heptakis(6-azido-6-deoxy)-β-cyclodextrin (compound 4)

The synthesis of heptakis(6-azido-6-deoxy)-β-cyclodextrin (**4**) was carried out by the selective halogenation at primary positions of the parent β-CyD, followed by substitution of the C-6-halogen by azide (Scheme 4.1.1).

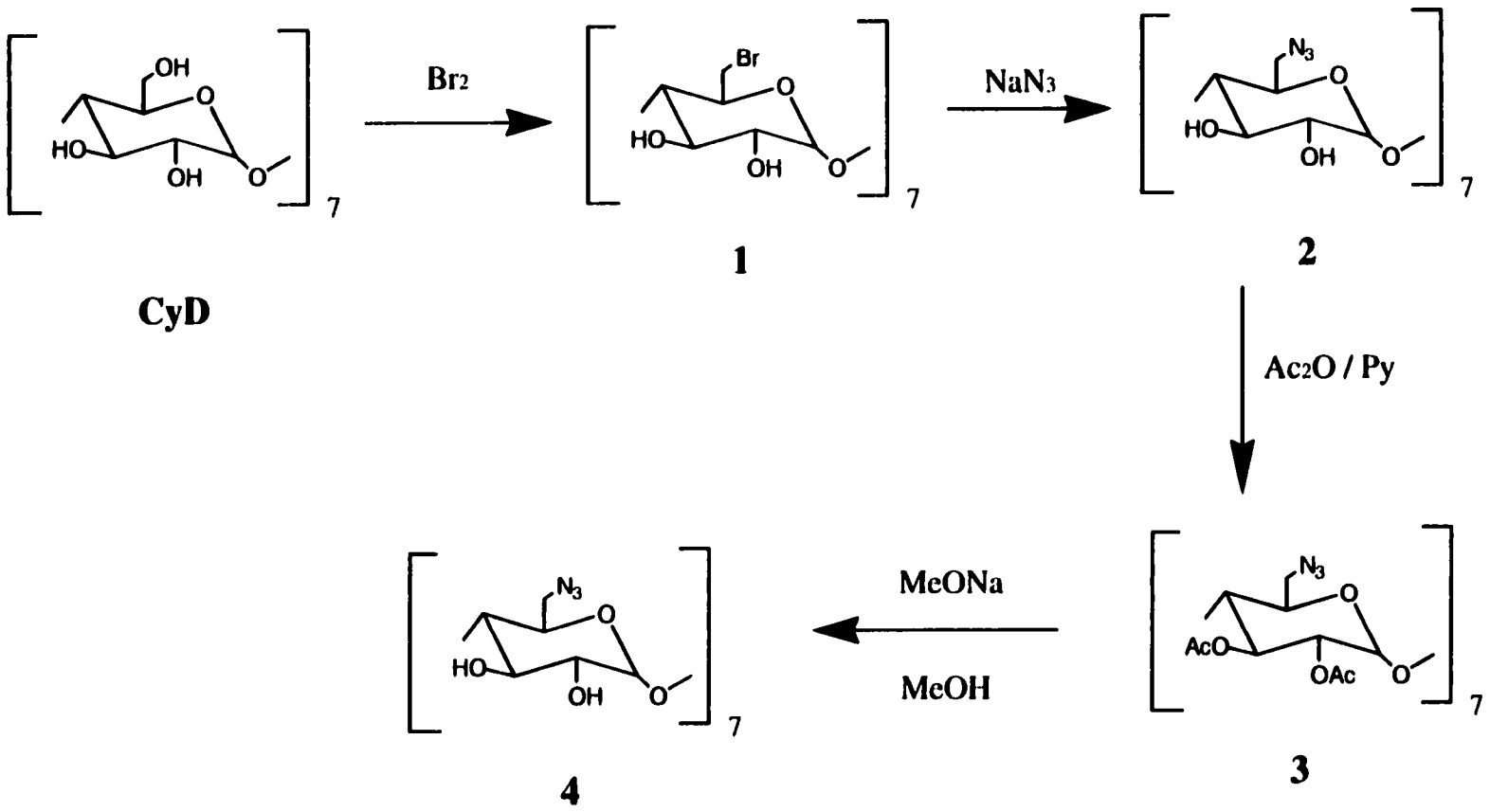
The CyD ring contains primary hydroxyl groups at the 6-position and secondary hydroxyl groups at the 2- and 3-position. Among these three types of hydroxyl groups, those at the 6-position are the most reactive, and those at the 3-position are the most inaccessible (Hybl *et al.*, 1965). Electrophilic reagents generally attack the 6-position under the normal conditions. More reactive reagents will not only react with hydroxyl groups at the 6-positon but also react with hydroxyl on the secondary side, while less reactive reagents will react more selectively with the 6-position hydroxyl groups. At room temperature the less reactive reagent *tert*-butyldimethylsilyl chloride (TBDMSCl) (Takeo *et al.*, 1988) reacts selectively with 6-position hydroxyl groups, and can be used as a blocking group for the synthesis of compound **13**.

Perbromo- at the 6-positions of CyDs is of interest as reactive intermediates for the further modification of CyDs (Croft and Bartsch, 1983). One route to these derivatives involves direct sulfonylation at primary positions, followed by displacement of the 6-sulfonate by a halide salt. However, this method, although well documented, does not usually proceed with high yield and selectivity (Boger *et al.*, 1978). Takeo *et al.* (1974) introduced an improvement, in which the per-6-bromo-6-

deoxy-CyDs were obtained by the reaction of the CyD with methanesulfonyl bromide in DMF at elevated temperature. We chose to use the efficient method reported by Gadella and Defaye (1991). Compound **1** was synthesized by the reaction of β-CyD with three equivalents of bromine per OH in dry DMF and triphenylphosphine. The solution was stirred at 80 °C for 15 h, then concentrated and the pH was adjusted to 9-10. The mixture was poured into ice water and was kept at room temperature for 30 min. The precipitate was collected by filtration to yield heptakis(6-bromo-6-deoxy)-*β*-cyclodextrin (**1**).

Perazidocyclodextrins are useful because they can be selectively alkylated or acylated on the secondary side (Khan *et al.*, 1998). They can be obtained easily from per-6-mesitylated CyDs (Tabushi *et al.*, 1977), 6-chloro-6-deoxy CyDs and 6-bromo-6-deoxy CyDs (Parrot-Lopez *et al.*, 1992) by displacement with sodium azide in DMF. The bromo groups of compound **1** were easily displaced in DMF at 90 °C for 60 h by treatment with sodium azide (3 equiv. per OH) (Szurmai *et al.*, 1990). The crude mixture then was acetylated in pyridine-acetic anhydride (2:1) to provide the acetylated compound **3**, which was easily purified by column chromatography over silica gel. Pure **3** was deacetylated in methanolic sodium methoxide solution to give pure heptakis(6-azido-6-deoxy)-*β*-cyclodextrin (**4**) in 77 % yield. The strong band at 2106 cm^{-1} in the IR spectrum indicated the presence of the N_3 group. The structure of **3** was confirmed by ^{13}C-NMR spectroscopy. Its spectrum showed ten carbon signals, six belonging to the ring carbons and the other four to the acetyl groups. Deacetylation of **3** gave the crystalline heptakis(6-azido-6-deoxy)-*β*-cyclodextrin (**4**), and its symmetry was again verified by ^{13}C-NMR. Removal of the acetyl groups caused strong downfield shifts at C-1 carbons (~5.39 ppm) and C-4 carbons (~6.05 ppm). The other carbon signals showed only limited changes.

Figure 4.1.1. Synthesis of Heptakis(6-azido-6-deoxy)-β-cyclodextrin (compound **4**)



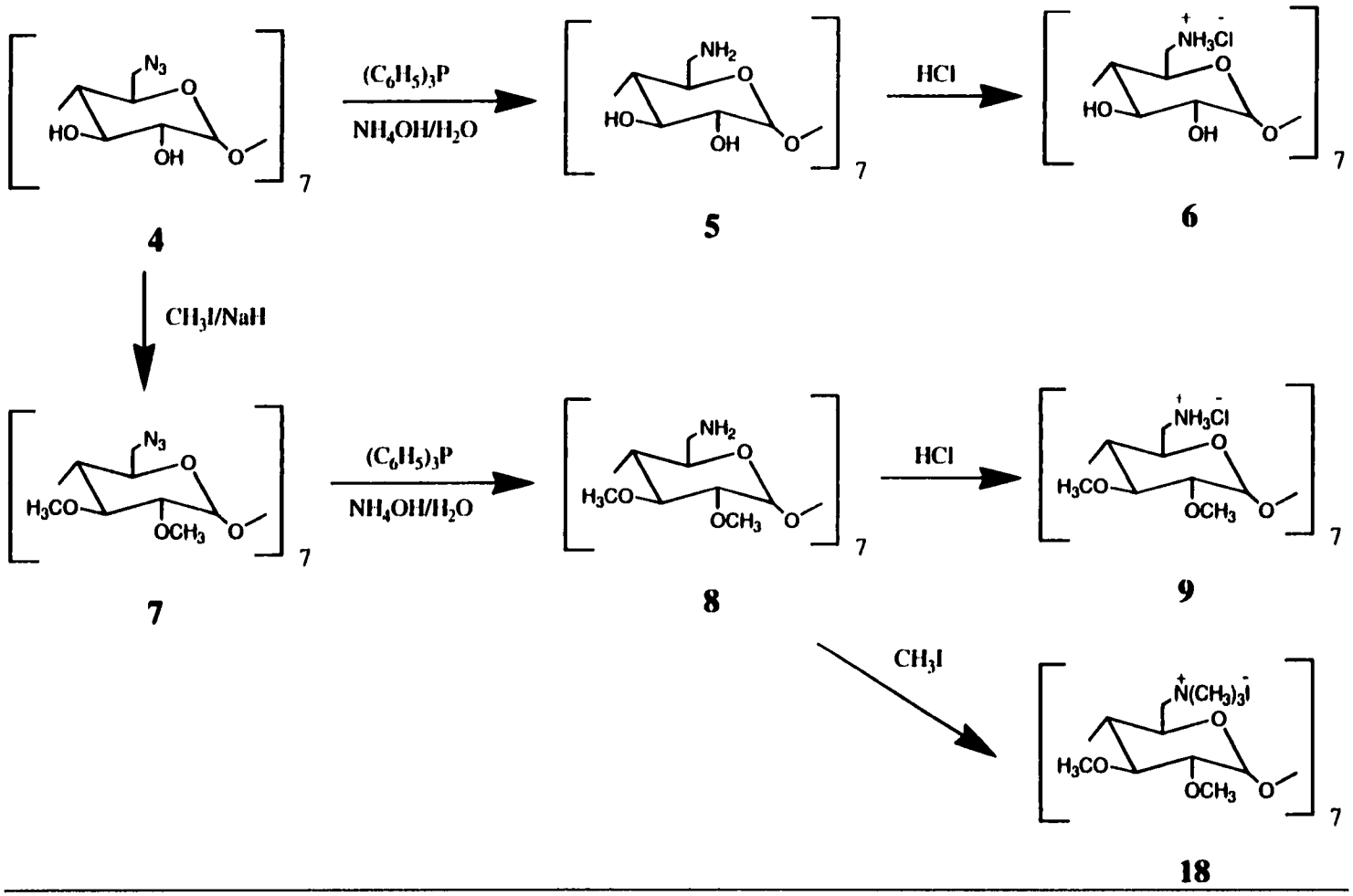
4.1.2. Synthesis of 6, 9, 17, and 18

Reduction of the azido group with triphenylphosphine in aqueous ammonia solution followed by treatment with dilute hydrochloric acid yielded the corresponding amine salts (compound **6**, **9**, and **17**). This approach gives aminocyclodextrins as their salts in good yield. A major disadvantage of this method is that it can be difficult to remove Ph_3P/Ph_3PO from the final product (Khan *et al.*, 1998) without chromatography. This is due to the formation of a complex between Ph_3P/Ph_3PO and the newly formed aminocyclodextrin. Complete removal of triphenylphosphine from an acidic solution of aminocyclodextrin **6** was not achieved even after stirring in this medium for a long time, but the amount can be reduced to a negligible level. The synthesis of **6**, **9**, and **17** are all prepared using the series of reactions shown in Scheme 4.1.2.

Compound **6** was synthesized by the reaction of compound **4** with triphenylphosphine in DMF for 30 min. Aqueous ammonia was then added and the mixture stirred at room temperature for 24-48 h. The mixture was washed with methanol and evaporated under high vacuum, and the residue was diluted with water then washed with chloroform until no UV activity could be detected and gave a white solid (80 % yield). Compound **5** was then treated with seven equialents aqueous HCl then lyophylized to give the title compound.

Methylation of compound **4** in dry DMF was carried out by treatment with sodium hydride and methyl iodide. The addition of methyl iodide at 0 °C gave heptakis(6-azido-6-deoxy-2,3-di-*O*-methyl)-β-cyclodextrin **7** in 72 % yield after column chromatography using hexane-ethyl acetate-ethanol (20:10:1) as eluant. Compound **7** was dissolved in DMF and reacted with triphenylphosphine and aqueous ammonia as before. Evaporation and column chromatography over silica gel using $CHCl_3/MeOH/NH_4OH$ (60:40:10) gave amino-CyD **8**. Treatment with aqueous HCl gave the title compound **9**. The structure of **9** was confirmed by ^{13}C-NMR spectroscopy. Methylation of the OH groups caused downfield shifts at C-2s and C-3s (> 7 ppm). The spectroscopic data are reported in the Experimental and are in good agreement with the literature.

Figure 4.1.2. Synthesis of CyDs **6**, **9**, and **18**



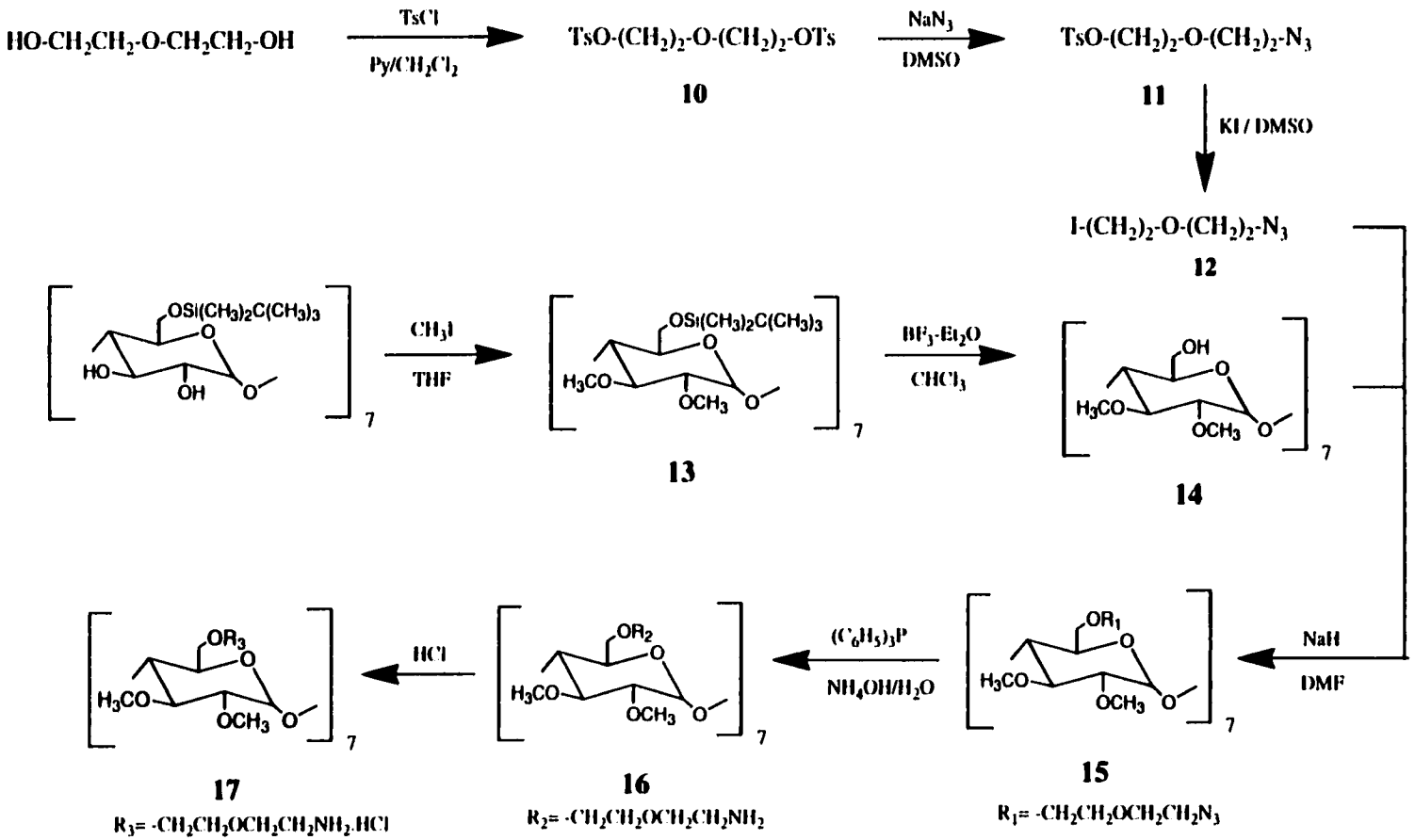
Compound **18** was synthesized directly from compound **8** using six equivalents of methyl iodide in the presence of NaOH. The product was separated by a Sephadex G-10 gel to give a white amorphous solid after lyophilization. Compound **18** is not very stable, After a few days in room temperature, a significant color change from white to light pink was observed, implying that a trace amount of iodine was probably present. Good NMR spectral data was hard to obtain.

Compound **17** was designed to provide a longer separation between cationic charges. Synthesis of this cyclodextrin is shown in Scheme 4.1.3.

Starting material **12** was prepared by dissolving di(ethylene glycol) in pyridine:CH_2Cl_2 solution (1:1) as described by Damani *et al.* *p*-Toluenesulfonyl chloride was added dropwise over 30 min at 0-5 °C. Compound **10** was obtained as colorless needles in 54 % yield from ether after work-up. Compound **10** was then dissolved in DMSO and NaN_3 (1.67 equiv.) was added. The reaction was carried out at room temperature overnight and provided a 62 % yield of compound **11** as an oil after column chromatography over silica gel. The remaining *p*-toluenesulfonyl group of compound **11** was displaced with iodide using NaI in DMSO to give **12** as a yellow oil in 85 % yield.

The synthesis of the hydrochloride salt of heptakis(2,3-di-*O*-methyl-6-*O*-(2-(2-aminoethoxy)ethyl))-*β*-cyclodextrin (**17**) was carried out in two steps starting from heptakis(6-*O*-*tert*-butyldimethyl-silyl)-*β*-cyclodextrin. Treatment with sodium hydride in dry THF for 20 min under argon followed by the addition of methyl iodide and reaction for 48 h in the dark under an atmosphere of argon was carried out as the first step. Purification of compound **13** was carried out on silica gel, using hexane-ethyl acetate 4:1 then switching to 3:1 to give **13** as a white solid in 58 % yield. Compound **13** was dissolved in chloroform and then de-silylated with BF_3-Et_2O (7 equiv.). Then reaction mixture was neutralized by the addition of saturated aqueous $NaHCO_3$ solution. Column chromatography over silica gel using chloroform-methanol 5:1 then 3:1 gave compound **14** in 70 % yield. Compound **14** was treated first with NaH in dry DMF, and after 20 min at room temperature, the mixture was cooled to 0 °C. Compound **12** was added dropwise and the reaction was

Figure 4.1.3. Synthesis of CyD **17**



stirred at room temperature overnight. An additional portion of NaH and compound **12** was added daily for three days. Crude **15** was purified by column chromatography over silica gel using hexane-ethyl acetate-ethanol (5:5:1) to give compound **15** in 25 % yield as a white solid. This compound **15** was then reduced to the peramino CyD **16** by treatment with $PPh_3/NH_3.H_2O$ in 41 % yield. Treatment with aqueous HCl yielded the title ammonium CyD derivative **17**.

Methylated cyclodextrins have received considerable attention because their physicochemical properties and inclusion behavior are different from natural cyclodextrins (Pitha, 1981; Nakai *et al.*, 1982; Uekama *et al.*, 1985). When methyl groups are introduced onto the hydroxyls at C-2, C-3, or C-6, hydrogen bonding is no longer possible and the physicochemical properties of cyclodextrins are significantly altered.

4.2. Cell Biology

4.2.1. Gene transfer study

Cationic β-cyclodextrin derivatives **6**, **9**, **17**, and **18** were synthesized in an effort to develop a delivery vehicle that would be nontoxic and would condense DNA to particles to enable efficient transfection. Plasmid pcDNA3.1/*LacZ*, which contains the β-galactosidase reporter gene (LacZ), was used in the transfection assay. Transfection with DEAE/Dextran was used as a positive control and was performed by mixing DEAE/Dextran (10 mg/mL) in H-DMEM (without serum) with plasmid DNA (1.5 μg). The mixture (100 μL per well) was kept at room temperature for 20 min then added to COS-1 cells (1×10^4) for 30 min incubation (37 °C). H-DMEM (900 μL per well) was added and the cells were incubated for 2.5 h. The solution was removed and 1 mL of growth media (with serum) was added. The cells were then incubated for 48 h before analysis. Transfection with synthesized compounds was performed in a similar manner using varying amount solutions of compounds **6**, **9**, **17**, and **18** in H-DMEM. These cationic cyclodextrins were mixed with plasmid DNA (1.5 μg) and added to COS-1 cells (1×10^4). After 30 min, H-DMEM (900 μL per well) was added and the cells were incubated for 2.5 h. Removal of the solution, addition of 1 mL of growth media (with serum) and incubation of the cells for 48 h was carried out prior to staining with X-Gal. Cationic liposome-mediated transfection was performed with DOTAP (Boehringer Mannheim). Plasmid DNA (1.5 μg) in HEPES buffer (20 mM) was mixed with DOTAP in HEPES buffer and incubated at room temperature for 20 min. This liposome mixture (50 μL per well) was added to adherant COS-1 cells (1×10^4) in serum-free DMEM and incubated for 6 h before replacement with growth media (C-DMEM, containing 10% FBS), and the cells incubated for 48 h before analysis. Cells were fixed with glutaraldehyde 48 h post-transfection and stained for β-galactosidase using standard techniques. The cells expressing β-galactosidase were stained blue. The results were collected and the efficiency of transfection was calculated.

4.2.2. Transfection results

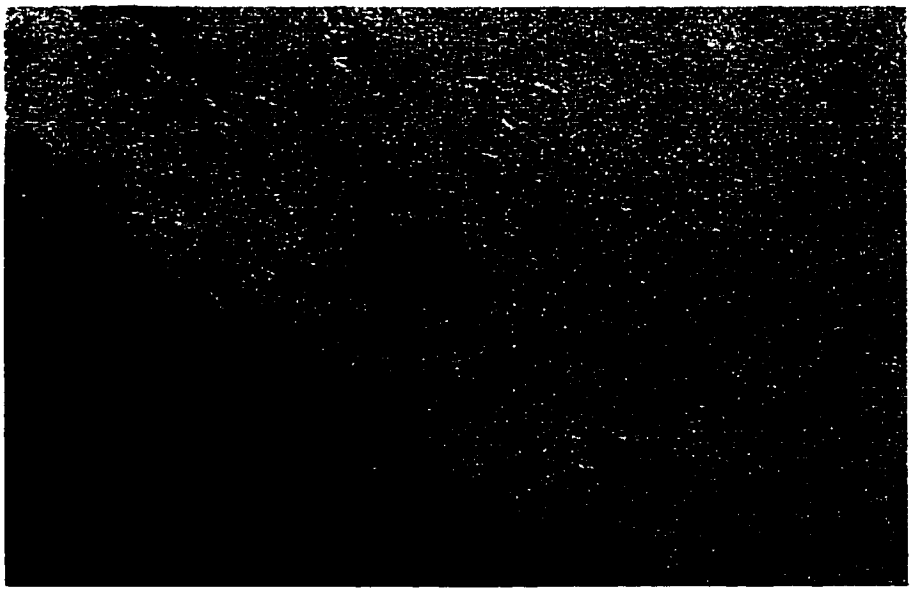
In the past few years, a number of cDNAs have been cloned in COS cells for the identification of cDNAs encoding functional or immuno-reactive recombinant proteins (Kluxen and Lubbert, 1993). Transient expression of heterologous reporter genes in COS monkey kidney cells are considered to be a convenient system for studies of enhancer and promoter function, translational control, and mRNA stability (Puchalski and Fahl, 1992). In some articles (Kluxen and Lubbert, 1993), COS-1 was pointed out to have more efficient protein synthesis, which is probably related to the fact that COS-1 cells grow faster than other COS cells. We chose COS-1 cells to investigate the transfection and expression of a selected gene. COS-1 is a fibroblast-like cell line established from CV-1 simian cells (ATCC CCL 70) which were transformed by an origin-defective mutant of SV40 that codes for wild-type T antigen. This line contains T antigen, retains complete permissiveness for lytic growth of SV40, supports the replication of ts A209 virus at 40 °C and supports the replication of pure populations of SV40 mutants with deletions in the early region. This line contains a single integrated copy of the complete early region of SV40 DNA. This is a suitable host for transfection, especially for vectors requiring expression of SV40 T antigen (Gluzman, 1981).

4.2.2.1. Transfection results of positive and negative controls

Figure 4.2.2.1. shows the results collected from the the positive controls. Figure 4.2.2.1. (a) shows the result with DEAE/Dextran at a concentration of 10 μg/μL. This compound was the most efficient cationic cyclodextrin. Transfection with DOTAP at concentration of 60 μg/μL is shown in Figure 4.2.2.1. (b).

The negative controls did not stain, which indicated that the cells could not incorporate the DNA alone. This result implied that, if there were some transfected cells in the wells to which compounds **6**, **9**, **17**, and **18** were added, gene transfer with cationic CyD derivatives would be possible.

(a) control-1, transfected with reagent DEAE/Dextran



(b) control-2, transfected with DOTAP

⟶ : **shows transfected cells**

Figure 4.2.2.1. Histochemical staining of COS-1 cells for β-galactosidase. (a) control-1, transfected with reagent DEAE/Dextran, and (b) control-2, transfected with DOTAP

*4.2.2.2. Transfection with compounds **6**, **9**, **17**, and **18***

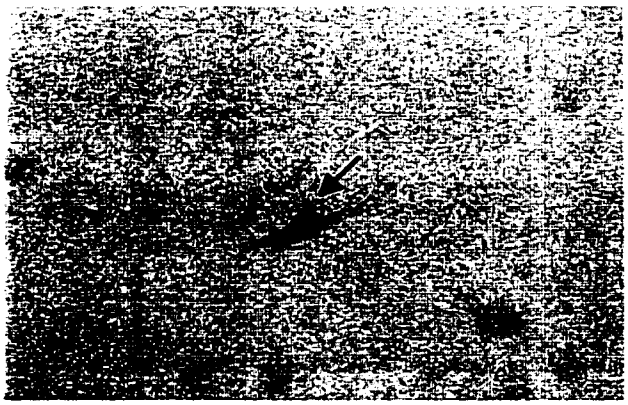
Of the four cationic CyDs prepared herein for the study of the transfection of COS-1 cells with pcDNA3.1/*LacZ*, three were found to transfer the LacZ gene leading to the expression of β-galactosidase as was seen by the blue color when treated with X-Gal. Figure 4.2.2.2. shows the results collected from the wells 9-2, 17-4, and 18-2 (compound **9**, **17**, and **18**, respectively).

Figure 4.2.2.2. (a) shows the results with compound **9** at a concentration of 50 μg/μL. This compound was the most efficient cationic cyclodextrin. Interestingly, cells were not stained at other concentrations with this compound. Wells 17-4 (10 μg/μL of compound **17**, Figure 4.2.2.2. (b)) and 18-2 (50 μg/μL of compound **18**, Figure 4.2.2.2. (c)) show marginal transfection efficiency cell. No concentration of compound **6** was found to transfect COS cells.

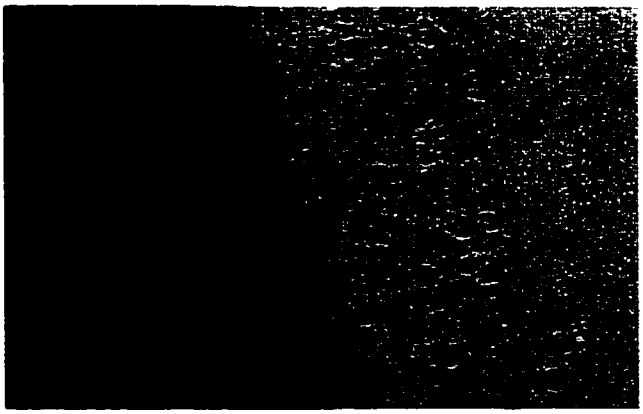
The transfection was performed in the absence of serum. It was observed that the presence of 10 % serum decreased transfection efficiencies (data not shown). Similar results were obtained by Gonzalez *et al.* (1999) and Woltjen *et al.* (1997).

(a)

(b)



(c)



⟶ : shows transfected cells

Figure 4.2.2.2. Staining of COS-1 cells for β-galactosidase. (a) 9-2, transfected with compound **9** at a concentration of 50 μg/μL, (b) 17-4, transfected with compound **17** (10 μg/μL), and (c) 18-2, transfected with compound **18** (50 μg/μL).

4.2.3. Discussion - Compounds have positive transfection results

Table 4.2.3. shows the results of transfection with the four compounds, **6**, **9**, **17**, and **18** (refer to Table 3.2.3.), and the results with DEAE/Dextran and DOTAP.

Table 4.2.3. Transfection rate of compounds

Sample's No.	100 μg/μL	50 μg/μL	25 μg/μL	10 μg/μL	1 μg/μL
6	-	-	-	-	-
9	-	+ (~10%)	-	-	-
17	-	-	-	+	-
18	-	+	-	-	-
Control-1	+ (~ 40%, DEAE/Dextran, 10 μg/μL)				
Control-2	+ (~50%, DOTAP, 60 μg/μL)				

As shown in Table 4.2.3., the positive controls both revealed the results as expected. From Figure 4.2.2.1, we can see that the efficiencies of transfection with DEAE/Dextran and DOTAP are both around 30-50%. Most standard protocols for DEAE/Dextran transfection can get efficiency up to 30 %, such as the introduction of pSV2neo DNA into Fisher-rat 3T3 (FR3T3) cells (Yang and Yang, 1997), pCH110 was introduced into COS-1 cells by DEAE/Dextran mediated DNA uptake (Kluxen and Lubbert, 1993). The transfection with the plasmid pRSV-IL2R by incubation of Hela cells with DEAE/Dextran – DNA complexes even achieved to 60 % (Holter *et al.*, 1989). Lipid formulations, particularly cationic lipids, have been important molecules for mediating gene transfer. Some of them have been reported may mediate > 50 % transfection efficiency *in vitro* under optimal condition (Ledley, 1996). Transfection performed with DOTAP (Boehringer Mannheim) was up to 30 % when transfected with the plasmid, pCMVLacZll and pSluc2, into KBALB cells (Woltjen *et al.*, 1997). This research also indicated that one of cationic CyD derivatives is capable of delivery gene to cells, although it is significantly less effective than the commercial cationic lipid preparation (DOTAP).

Among the four compounds studied in this thesis, compound **6** did not facilitate transfection while the other three (**9**, **17**, and **18**) were able to transfect the COS-1 cells with LacZ gene. Interestingly, these threeCyDs have methyl groups in 2,3-hydroxyl positions, while compound **6** does not (see Figure 3.2.3.). Our results imply that the introduction of methyl groups may be lead to higher activity in gene transfer. However, this is currently a postulation, as the mechanisms responsible for gene transfection using this novel type of cationic reagent are still not understood.

Another possible functional group for β-CyDs is the amines. The importance of a spacer arm between the cyclodextrin and its amine functional groups has been discussed in some literature (Hwang *et al.*, 2001; Gonzalez *et al.*, 1999). Gonzalez *et al.* (1999) reported that moving the amine further from the CyD could dramatically increase the binding of CyD-containing monomer to DNA from 0 % to 88 %. According to this, we designed and synthesized compound **17**. However, low transfection occurred only at 10 μg/μL. The reasons for this low transfection efficiency could include non-optimal spacer chain length for efficient binding. Plank *et al.* (1999) found that DNA binding affinity was mainly a function of the number of cationic residues, and the transfection efficiency was influenced by the type of cationic residues used. Also, the amine functional groups in these reports were always on the primary side. More study will be required to resolve this problem.

We obtained some positive results, although the transfection efficiencies of these compounds are much lower than the commercial cationic compounds. There could be many reasons. Generally, the major barriers to cationic-mediated transfection include: (1) interaction between complexes and cell surfaces, (2) the endosomal release of DNA or complexes, and (3) entry of DNA into the nucleus (de Smedt *et al*, 2000). A number of literature reports may provide useful insights for future work.

(1) DEAE/Dextran can mediate effective transfection. One reason may be because this vector is linear Dextran, in which the cationic changes are more dispersed. This might be beneficial to the binding of DEAE/Dextran with DNA and DEAE/Detran-DNA complexes with cell membrane. While the binding of CyDs to

DNA may be poor, which is possibly due to the steric hindrance of the bulky cyclodextrin cups and the proximity of the positive charges to each other. The same reasons may affect complexes binding to cell membranes too.

(2) For effective gene delivery, the polycation must protect the DNA from nuclease activity. CyDs have been proved to be able to protect certain small molecules (*see section 1.3.2.4*). There are also some reports (Hwang *et al.*, 2001; Arima *et al.*, 2001) that some β-cyclodextrin-containing polymers could completely or partial protect plasmid DNA from degradation by DNase. At the current stage we still cannot conclude that CyDs may protect DNA effectively.

(3) Different cationic CyDs were previously evaluated in our laboratory. Positive results, repeating 5-10 % efficiency, were obtained by Kavadas *et al.* (1998) and Woltjen *et al.* (1997). These transfections were performed with different cell lines and different plasmid DNAs. These results suggest that transfection efficiency would be dependent on a variety of factors. Concentration of reagents, amount of input DNA, time of exposure and cell types are all factors that can affect efficiency of gene transfer.

Based on this information, further experimentation is still required to find more effective compounds, to fully characterize and quantify the formation of cationic CyD-DNA complexes, and to study the toxicity and mechanism of this cation-mediated gene transfection.

Chapter 5

Conclusions

The objective of this thesis was to prepare and investigate a series of cationic cyclodextrins for gene transfection. To achieve this, four cationic cyclodextrin derivatives were synthesized. These four compounds are the hydrochloride salts of heptakis(6-amino-6-deoxy)-*β*-cyclodextrin (compound **6**), heptakis(6-amino-6-deoxy-2, 3-di-*O*-methyl)-*β*-cyclodextrin (compound **9**), heptakis(2,3-di-*O*-methyl-6-*O*-(2-(2-azidoethoxy)ethyl))-*β*-cyclodextrin (compound **17**), and heptakis(tetramethyl-ammonium-6-deoxy-2, 3-di-*O*-methyl)-*β*-cyclodextrin iodide (compound **18**). One of these compounds, the hydrochloride salt heptakis(2,3-di-*O*-methyl-6-*O*-(2-(2-azidoethoxy)ethyl))-*β*-cyclodextrin (**17**), is a new compound and has not been previous reported. The structures of these compounds were confirmed by spectroscopic studies (^{1}H and ^{13}C-NMR, see *Appendix*).

The four cationic CyDs were used to prepare complexes with pcDNA3.1/*LacZ* at five different concentrations, and were evaluated on their efficiency in transfecting COS-1 cells. Three of the complexes show different levels of transfecting efficiency; compound **9** at a concentration of 50 μg/μL, **17** at a concentration of 10 μg/μL, and **18** at a concentration of 50 μg/μL. The three CyDs that show positive results in transfection have methyl groups in 2,3-hydroxyl positions. Among these three compounds, compound **9** showed the highest efficiency in transfecting COS-1 cells with the pcDNA/*LacZ* gene.

The study reported in this thesis is preliminary, and the mechanisms responsible for gene transfer using this kind of cationic reagent are still not understood. However, the results obtained encourage further study into the use of cationic CyDs for gene transfer.

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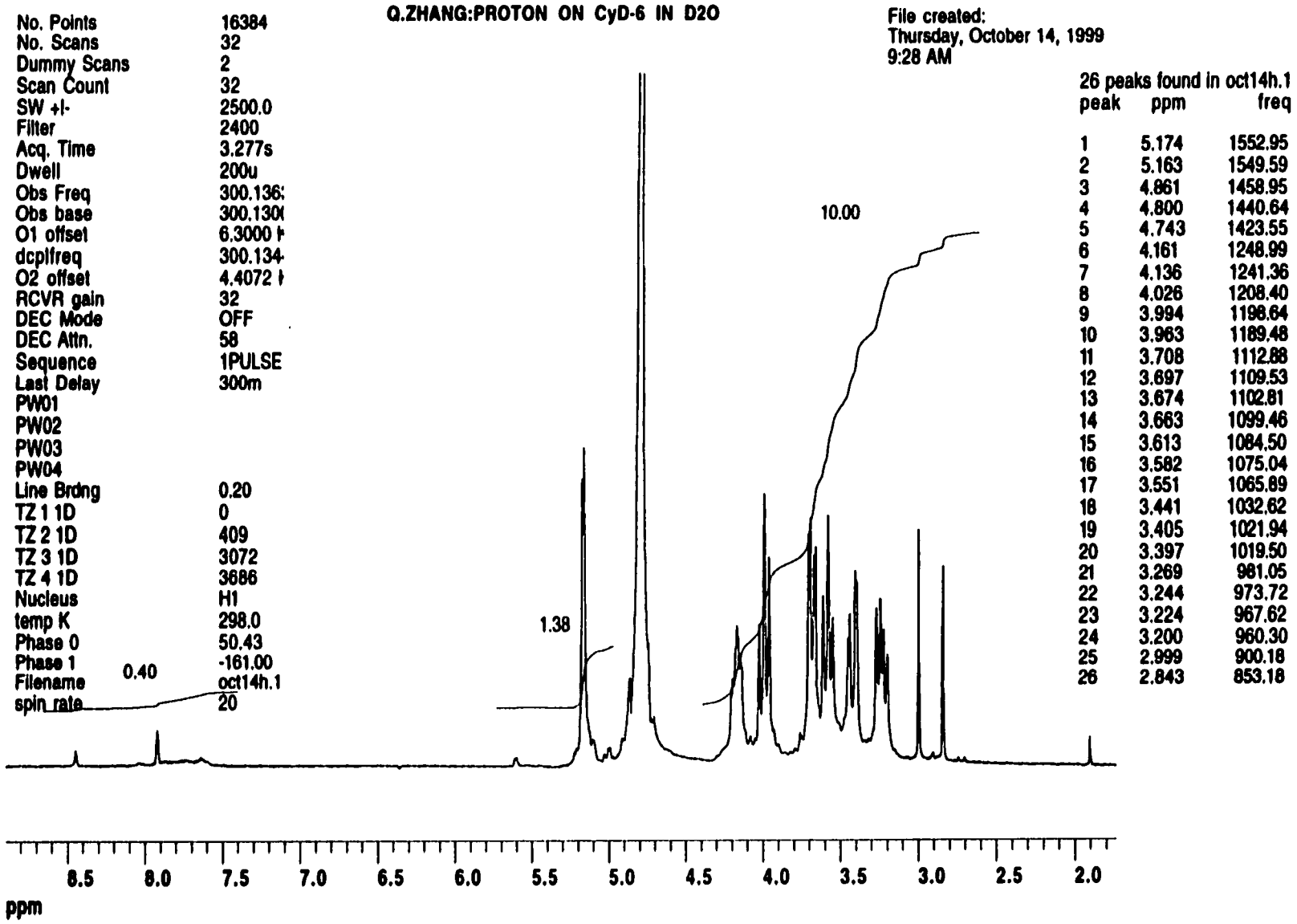
Appendix

(^{1}H-NMR and ^{13}C-NMR spectra)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2 5.163 1549.59
3 4.861 1458.95
4 4.800 1440.64
5 4.743 1423.55
6 4.161 1248.99
7 4.136 1241.36
8 4.026 1208.40
9 3.994 1198.64
10 3.963 1189.48
11 3.708 1112.88
12 3.697 1109.53
13 3.674 1102.81
14 3.663 1099.46
15 3.613 1084.50
16 3.582 1075.04
17 3.551 1065.89
18 3.441 1032.62
19 3.405 1021.94
20 3.397 1019.50
21 3.269 981.05
22 3.244 973.72
23 3.224 967.62
24 3.200 960.30
25 2.999 900.18
26 2.843 853.18
0.40
1.38
10.00
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
ppm

Q.ZHANG:APT CARBON ON CyD-6 IN D20

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O2 offset	5.0000 k
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DEC Attn.	112
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PW02	
PW03	
PW04	
Line Brdng	1.50
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TZ 2 1D	409
TZ 3 1D	3072
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Nucleus	H1
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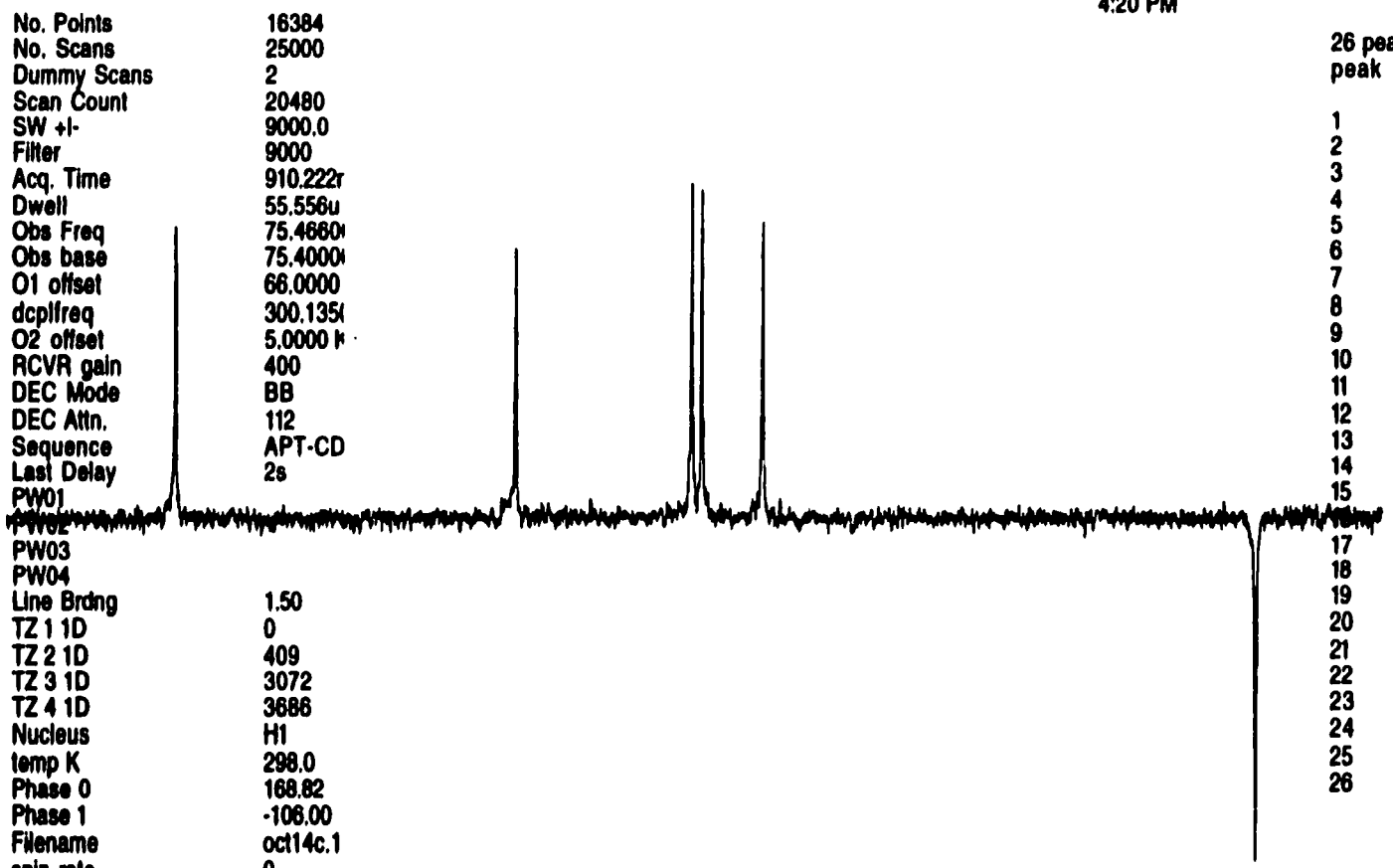

26 peaks found in oct14c.1

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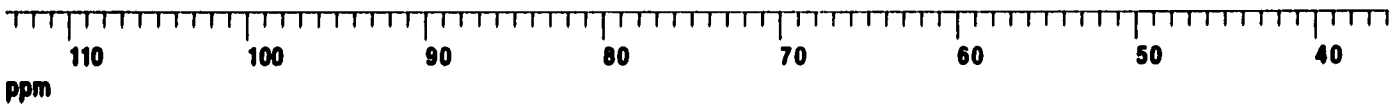
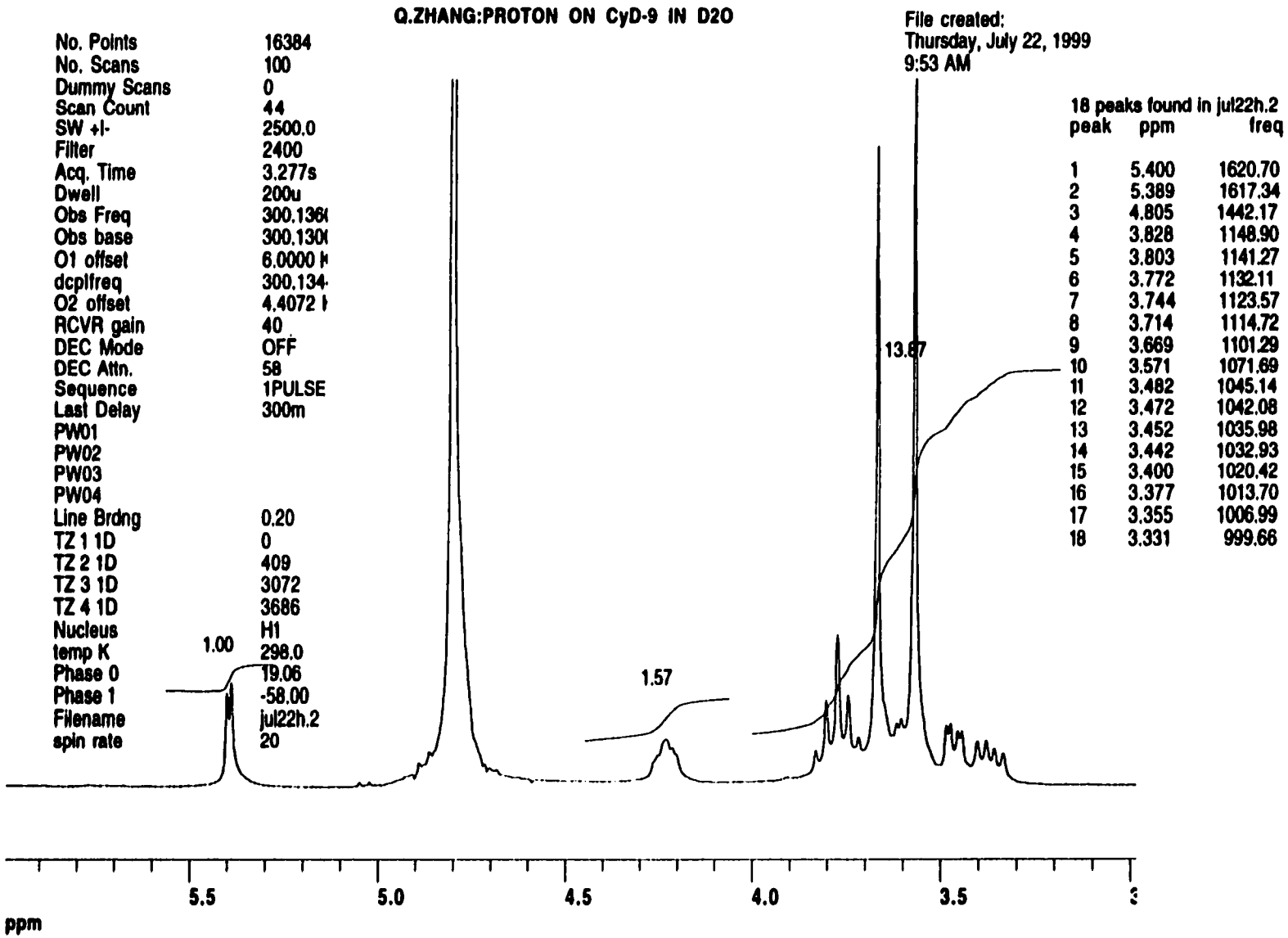
2	104.231	7865.1:
3	104.187	7861.8
4	103.998	7847.5
5	103.852	7836.5
6	85.056	6418.2
7	85.013	6414.9
8	84.794	6398.4
9	75.010	5660.1
10	74.937	5654.6
11	74.792	5643.6
12	74.661	5633.8
13	74.399	5614.0
14	74.370	5611.8
15	74.224	5600.8
16	74.064	5588.7
17	74.020	5585.4
18	73.889	5575.5
19	71.050	5361.3
20	71.021	5359.1
21	70.759	5339.3
22	43.183	3258.5
23	43.154	3256.3
24	42.907	3237.6
25	42.761	3226.6
26	42.732	3224.5

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18 peaks found in jul22h.2
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1 5.400 1620.70
2 5.389 1617.34
3 4.805 1442.17
4 3.828 1148.90
5 3.803 1141.27
6 3.772 1132.11
7 3.744 1123.57
8 3.714 1114.72
9 3.669 1101.29
10 3.571 1071.69
11 3.482 1045.14
12 3.472 1042.08
13 3.452 1035.98
14 3.442 1032.93
15 3.400 1020.42
16 3.377 1013.70
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4.0
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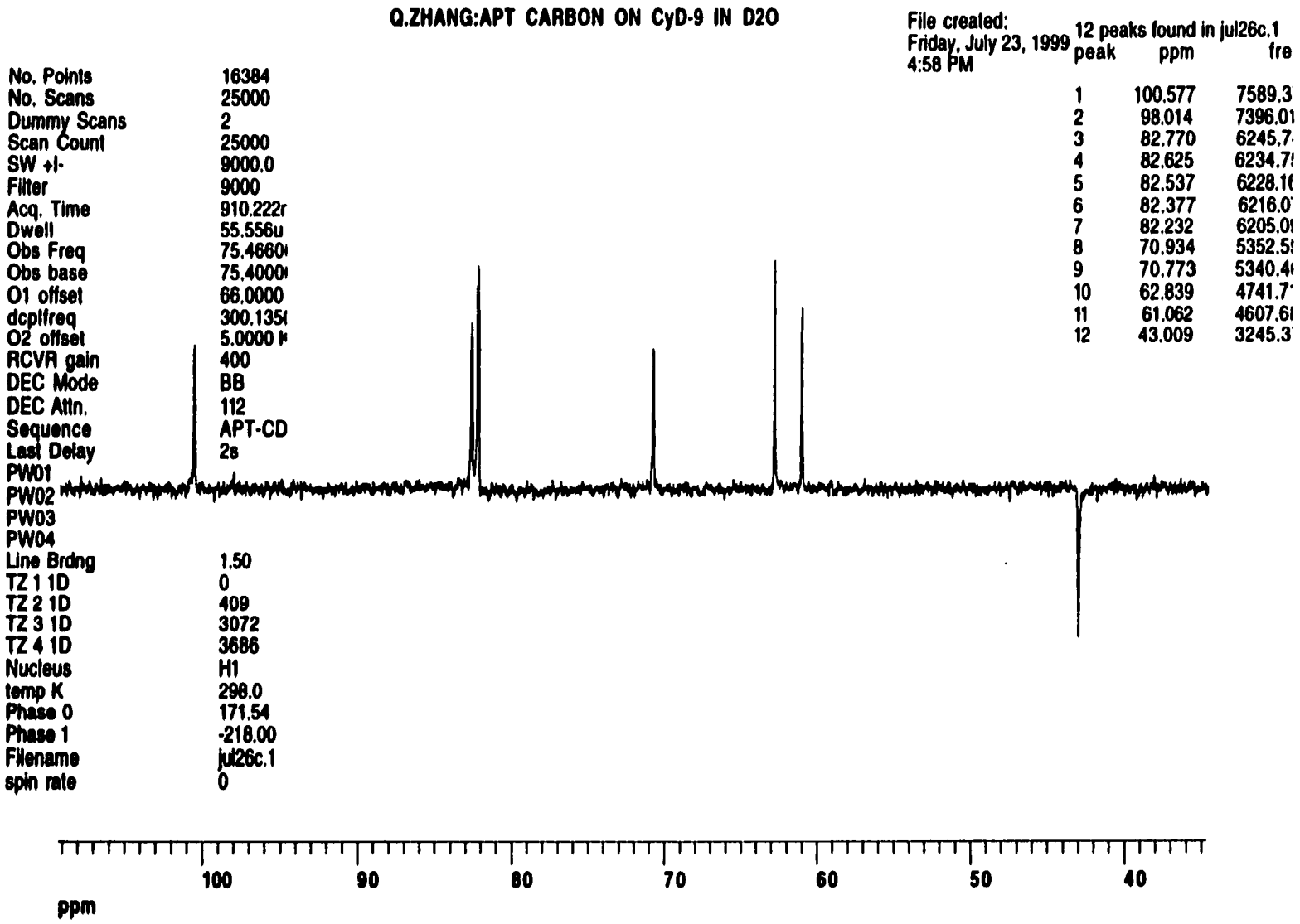
ppm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3 82.770 6245.7
4 82.625 6234.7
5 82.537 6228.1
6 82.377 6216.0
7 82.232 6205.0
8 70.934 5352.5
9 70.773 5340.4
10 62.839 4741.7
11 61.062 4607.6
12 43.009 3245.3
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PW02
PW03
PW04
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TZ 2 1D 409
TZ 3 1D 3072
TZ 4 1D 3686
Nucleus H1
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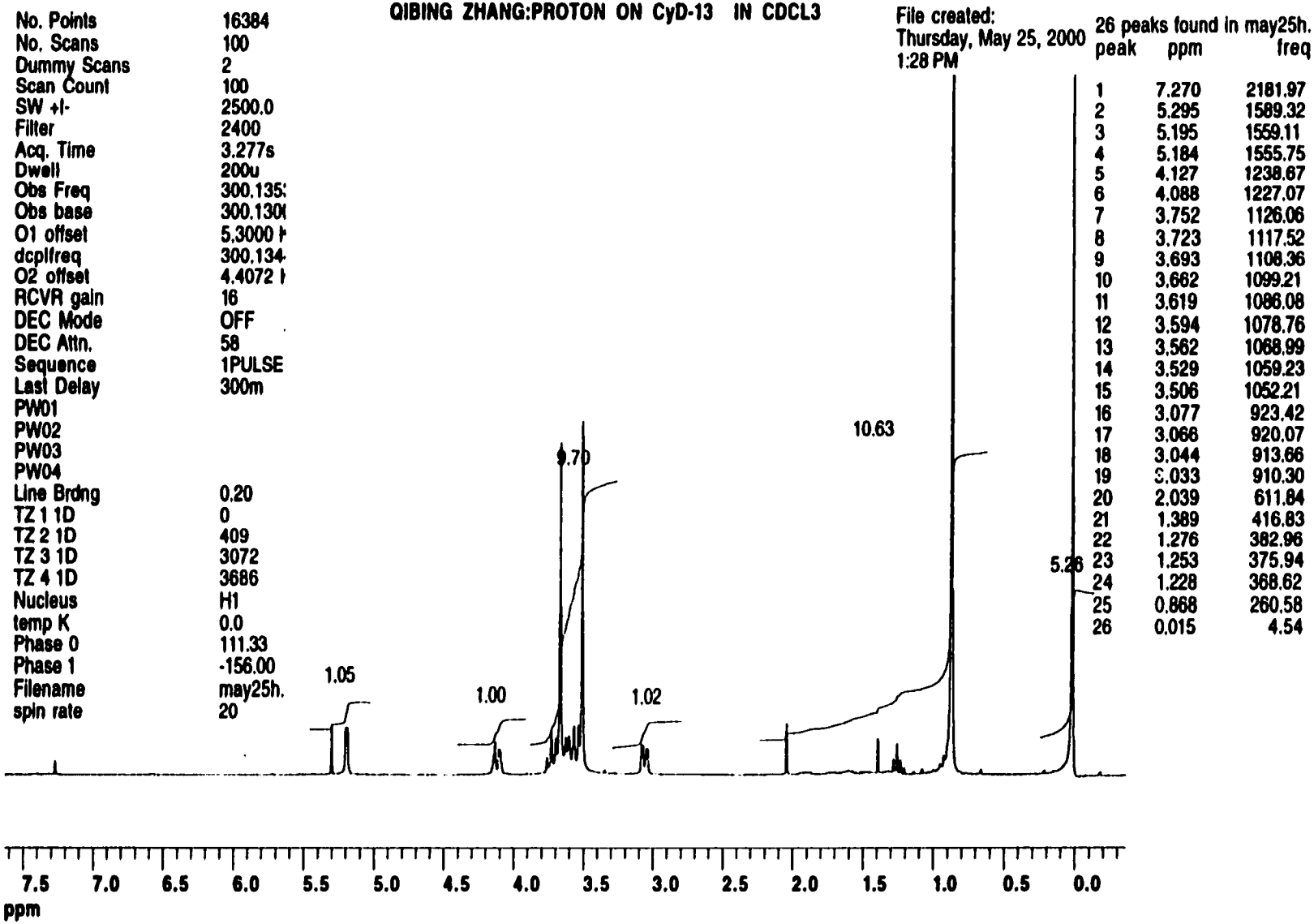
70
60
50
40
ppm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PW02
PW03
PW04
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TZ 2 1D 409
TZ 3 1D 3072
TZ 4 1D 3686
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26 peaks found in may25h.
peak ppm freq
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5 4.127 1238.67
6 4.088 1227.07
7 3.752 1126.06
8 3.723 1117.52
9 3.693 1108.36
10 3.662 1099.21
11 3.619 1086.08
12 3.594 1078.76
13 3.562 1068.99
14 3.529 1059.23
15 3.506 1052.21
16 3.077 923.42
17 3.066 920.07
18 3.044 913.66
19 3.033 910.30
20 2.039 611.84
21 1.389 416.83
22 1.276 382.96
23 1.253 375.94
24 1.228 368.62
25 0.868 260.58
26 0.015 4.54
1.05
1.00
9.70
1.02
10.63
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PW02	
PW03	
PW04	
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1	98.140	7405.5
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3	82.285	6209.12
4	82.052	6191.5
5	78.674	5936.6
6	77.422	5842.17
7	77.000	5810.31
8	76.578	5778.4
9	72.239	5451.0
10	62.353	4705.0
11	61.523	4642.4
12	61.421	4634.7
13	58.597	4421.6
14	36.714	2770.3
15	25.955	1958.51
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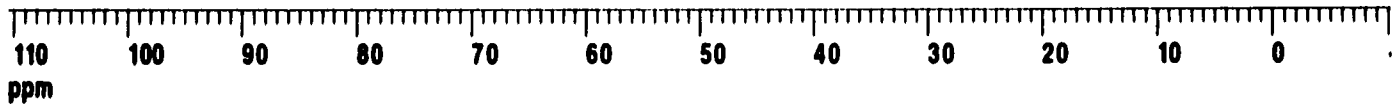
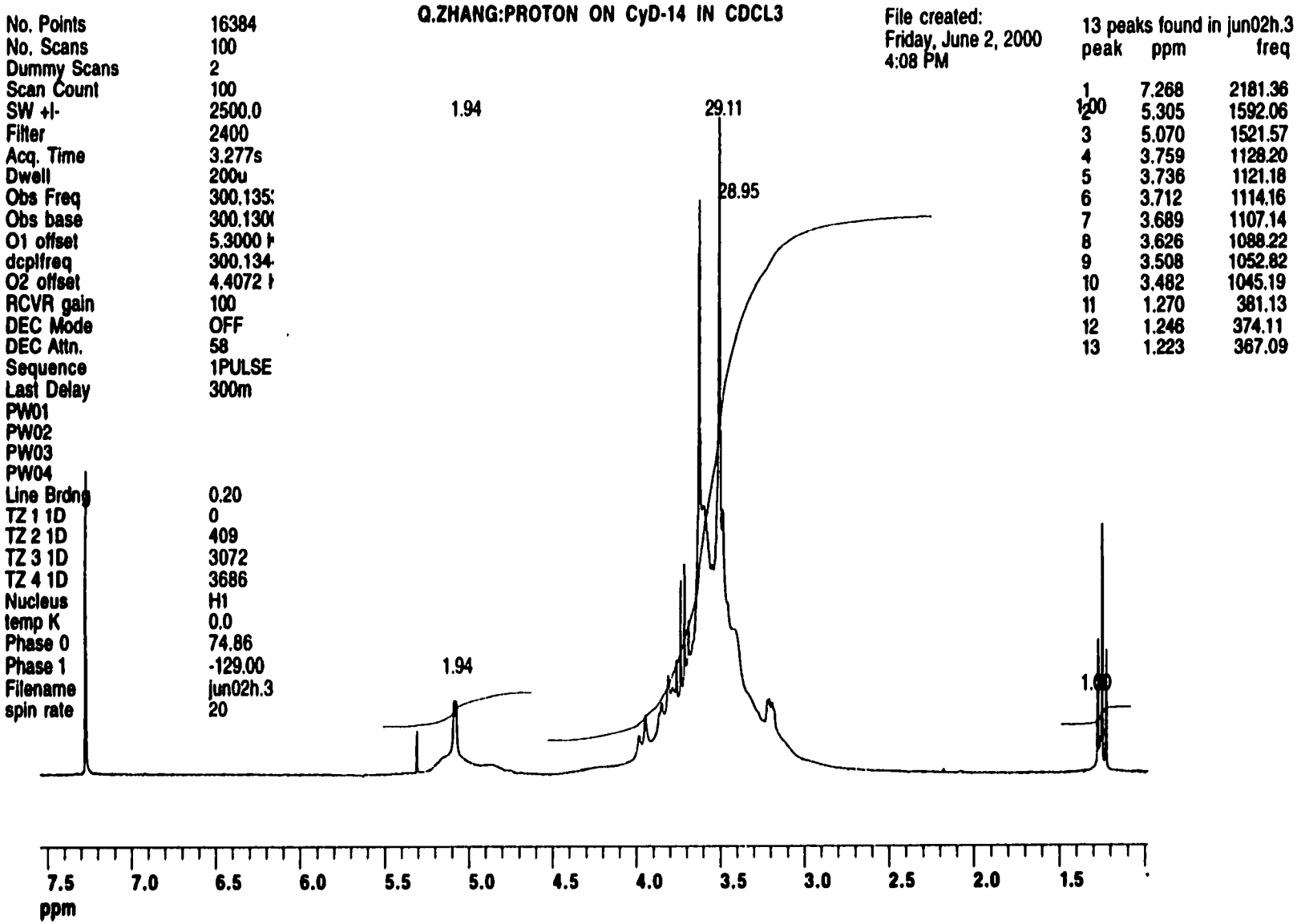
19	-5.130	-387.0

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PW02
PW03
PW04
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TZ 3 1D 3072
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3 5.070 1521.57
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6 3.712 1114.16
7 3.689 1107.14
8 3.626 1088.22
9 3.508 1052.82
10 3.482 1045.19
11 1.270 381.13
12 1.246 374.11
13 1.223 367.09
1.94
29.11
28.95
1.94
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ppm

Q.ZHANG:APT CARBON ON CyD-14 IN CDCL3

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Obs base	75.4000(
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O2 offset	5.0000 I
RCVR gain	400
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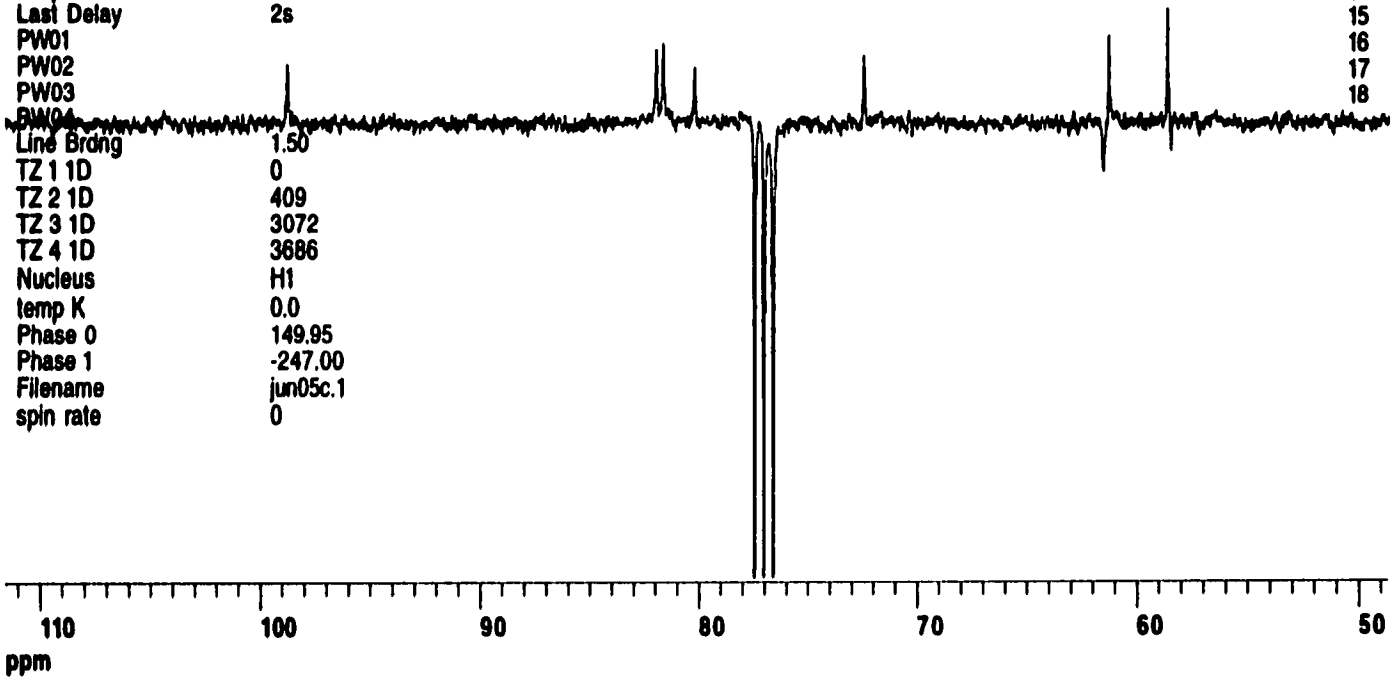
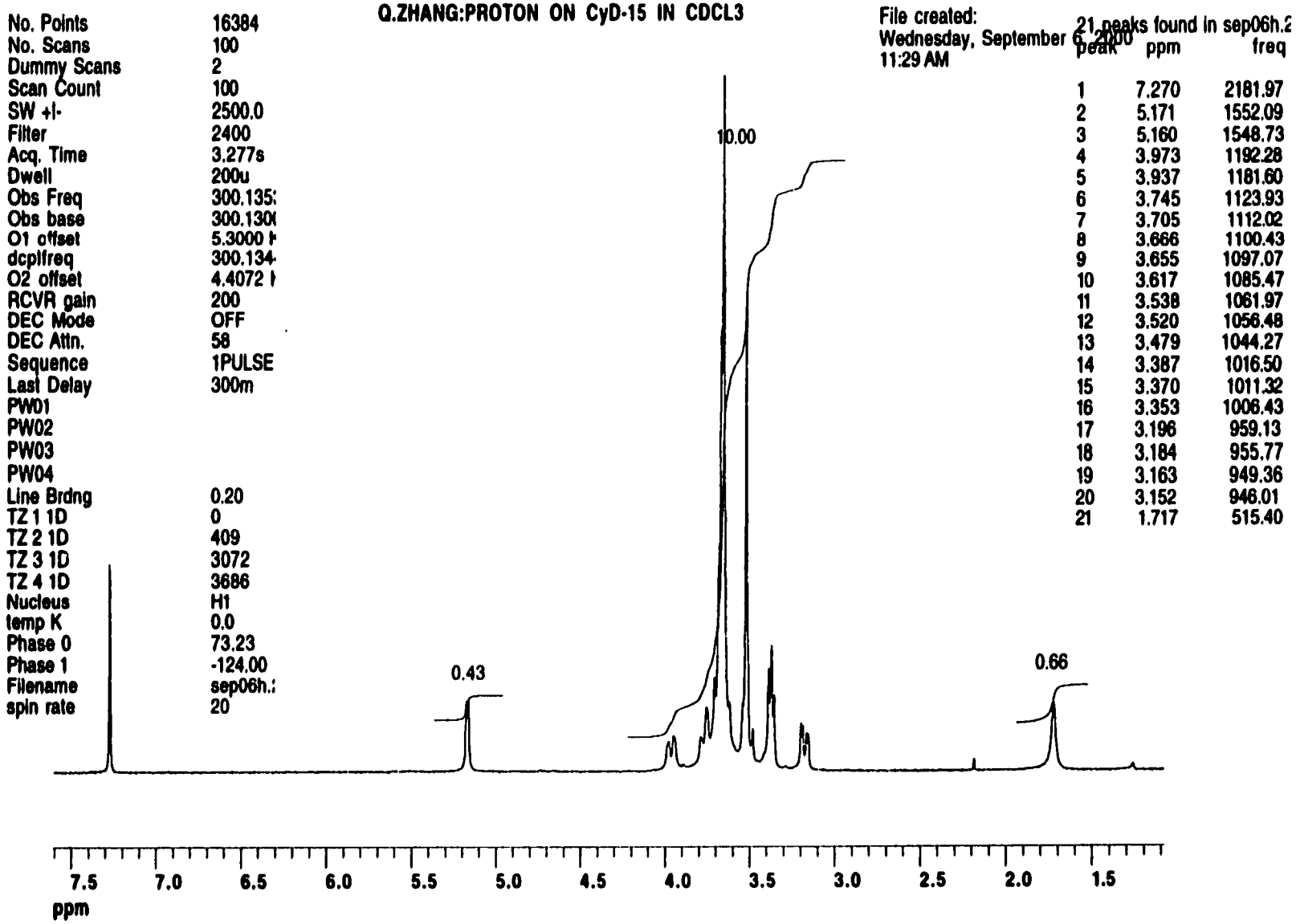
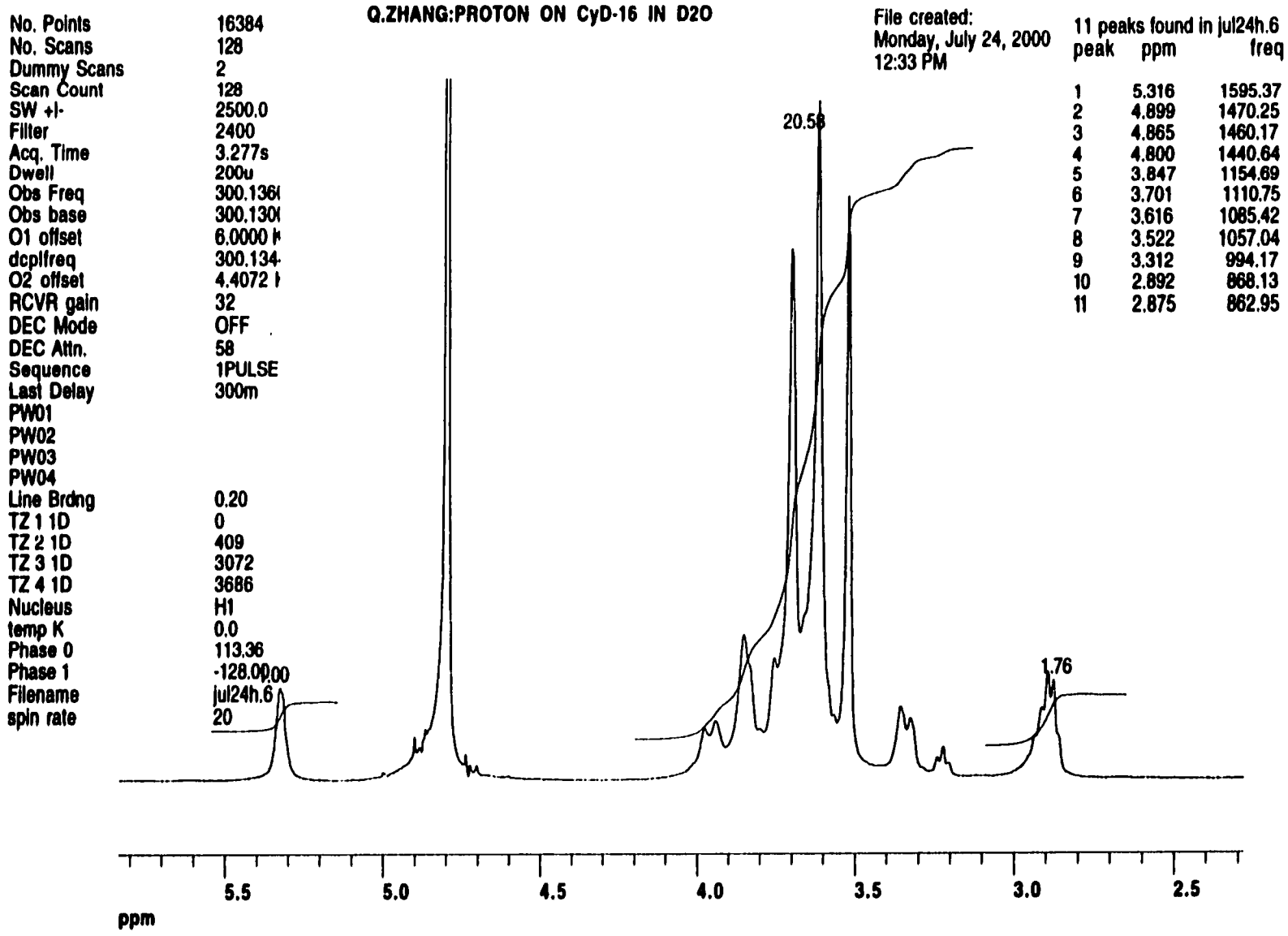
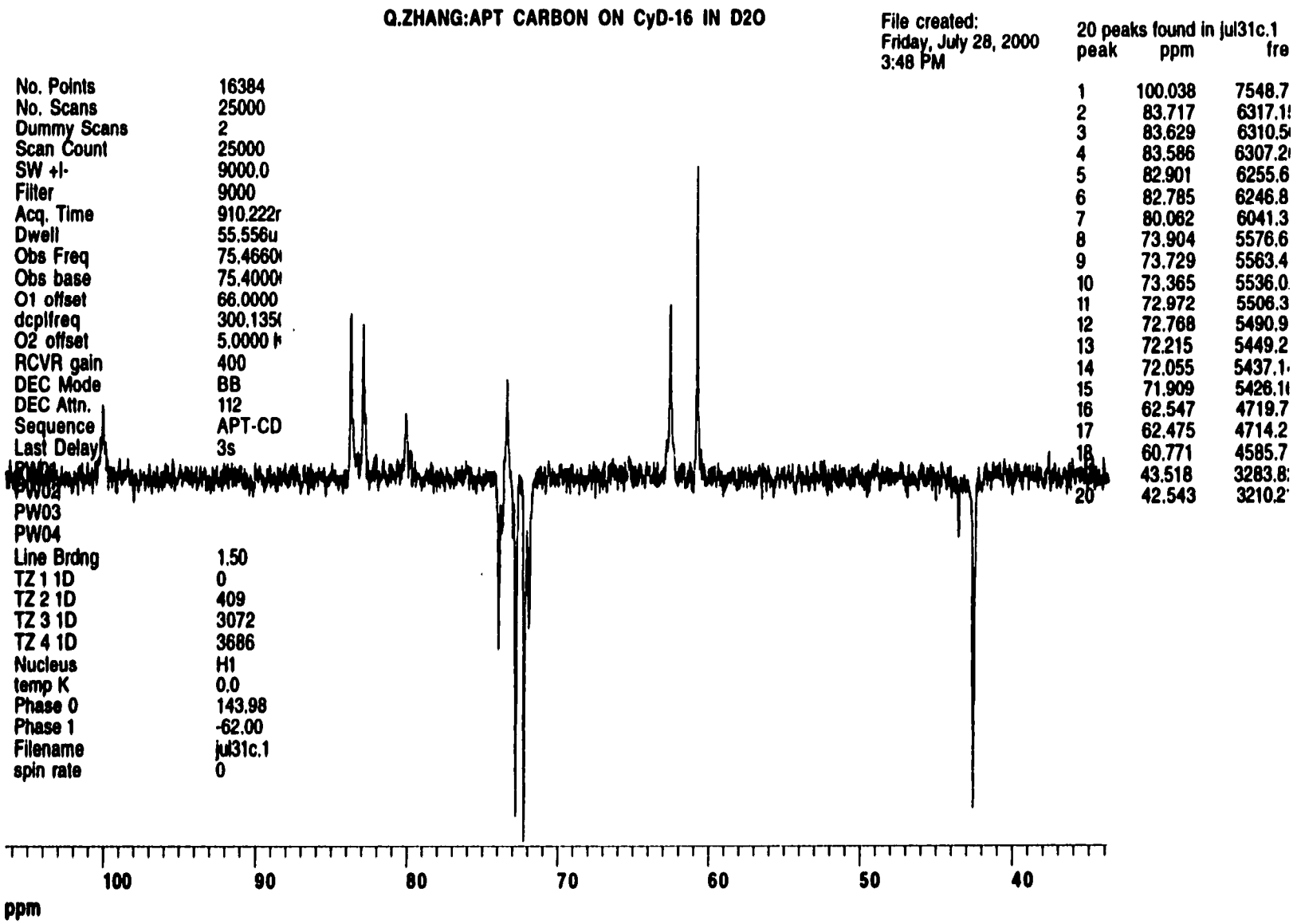
1	98.766	7452.7
2	82.008	6188.2
3	81.950	6183.8
4	81.630	6159.6
5	81.543	6153.0
6	80.189	6050.91
7	77.422	5842.17
8	77.000	5810.31
9	76.840	5798.2
10	76.811	5796.0
11	76.767	5792.7
12	76.578	5778.4
13	76.447	5768.5
14	72.428	5465.3
15	61.509	4641.3
16	61.261	4622.6
17	58.611	4422.7
18	58.451	4410.6




Q.ZHANG:PROTON ON CyD-15 IN CDCL3
No. Points 16384
No. Scans 100
Dummy Scans 2
Scan Count 100
SW +|- 2500.0
Filter 2400
Acq. Time 3.277s
Dwell 200u
Obs Freq 300.135
Obs base 300.130
O1 offset 5.3000 I
dcpIfreq 300.134
O2 offset 4.4072 I
RCVR gain 200
DEC Mode OFF
DEC Attn. 58
Sequence 1PULSE
Last Delay 300m
PW01
PW02
PW03
PW04
Line Brdng 0.20
TZ 1 1D 0
TZ 2 1D 409
TZ 3 1D 3072
TZ 4 1D 3686
Nucleus H1
temp K 0.0
Phase 0 73.23
Phase 1 -124.00
Filename sep06h.
spin rate 20
File created:
Wednesday, September 6, 2000
11:29 AM
21 peaks found in sep06h.
peak ppm freq
1 7.270 2181.97
2 5.171 1552.09
3 5.160 1548.73
4 3.973 1192.28
5 3.937 1181.60
6 3.745 1123.93
7 3.705 1112.02
8 3.666 1100.43
9 3.655 1097.07
10 3.617 1085.47
11 3.538 1061.97
12 3.520 1056.48
13 3.479 1044.27
14 3.387 1016.50
15 3.370 1011.32
16 3.353 1006.43
17 3.196 959.13
18 3.184 955.77
19 3.163 949.36
20 3.152 946.01
21 1.717 515.40
10.00
0.43
0.66
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ppm


Q.ZHANG:PROTON ON CyD-16 IN D2O
File created:
Monday, July 24, 2000
12:33 PM
No. Points 16384
No. Scans 128
Dummy Scans 2
Scan Count 128
SW +|- 2500.0
Filter 2400
Acq. Time 3.277s
Dwell 200u
Obs Freq 300.136
Obs base 300.130
O1 offset 6.0000
dcplfreq 300.134
O2 offset 4.4072
RCVR gain 32
DEC Mode OFF
DEC Attn. 58
Sequence 1PULSE
Last Delay 300m
PW01
PW02
PW03
PW04
Line Brdng 0.20
TZ 1 1D 0
TZ 2 1D 409
TZ 3 1D 3072
TZ 4 1D 3686
Nucleus H1
temp K 0.0
Phase 0 113.36
Phase 1 -128.00
Filename jul24h.6
spin rate 20
11 peaks found in jul24h.6
peak ppm freq
1 5.316 1595.37
2 4.899 1470.25
3 4.865 1460.17
4 4.800 1440.64
5 3.847 1154.69
6 3.701 1110.75
7 3.616 1085.42
8 3.522 1057.04
9 3.312 994.17
10 2.892 868.13
11 2.875 862.95
20.58
1.00
1.76
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm


Q.ZHANG:APT CARBON ON CyD-16 IN D2O
File created:
Friday, July 28, 2000
3:48 PM
No. Points 16384
No. Scans 25000
Dummy Scans 2
Scan Count 25000
SW +l- 9000.0
Filter 9000
Acq. Time 910.222r
Dwell 55.556u
Obs Freq 75.4660
Obs base 75.4000
O1 offset 66.0000
dcpIfreq 300.135
O2 offset 5.0000 h
RCVR gain 400
DEC Mode BB
DEC Attn. 112
Sequence APT-CD
Last Delay 3s
PW01
PW02
PW03
PW04
Line Brdng 1.50
TZ 1 1D 0
TZ 2 1D 409
TZ 3 1D 3072
TZ 4 1D 3686
Nucleus H1
temp K 0.0
Phase 0 143.98
Phase 1 -62.00
Filename jul31c.1
spin rate 0
20 peaks found in jul31c.1
peak ppm fre
1 100.038 7548.7
2 83.717 6317.1
3 83.629 6310.5
4 83.586 6307.2
5 82.901 6255.6
6 82.785 6246.8
7 80.062 6041.3
8 73.904 5576.6
9 73.729 5563.4
10 73.365 5536.0
11 72.972 5506.3
12 72.768 5490.9
13 72.215 5449.2
14 72.055 5437.1
15 71.909 5426.1
16 62.547 4719.7
17 62.475 4714.2
18 60.771 4585.7
19 43.518 3283.8
20 42.543 3210.2
100
90
80
70
60
50
40
ppm