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ABSTRACT

CONSTRUCTION OF ACCURATE MOLECULAR MODELS USING LASER STEREOLITHOGRAPHY AND DETERMINATION OF NMR SPECTRA FOR AMILORIDE HYDROCHLORIDE AND ITS FREE BASE

by
Ana Deborah Ofsievich

Accurate molecular models were constructed by a rapid prototyping process, called stereolithography. This process uses a computer-controlled laser to cure and solidify a photosensitive liquid polymer. Using a computer-aided design (CAD) program, spheres of appropriate van der Waals or CPK radii were drawn and placed in the three dimensional CAD space, in accordance with the atomic coordinates obtained from quantum mechanical calculations and from neutron diffraction data. These design data were used to drive the stereolithography system where the models were built in the same shape as the CAD image. The models built for the purpose of this work consisted of three amino acids and two structural analogs used in the study of the L-alanine taste receptor of the channel catfish, as well as the enzyme mimic β-cyclodextrin along with the transition state for the cleavage of phenyl acetate by the 2' and 3' hydroxyl oxygens of β-cyclodextrin. Models of the drug amiloride, as well as two analogs of this compound were also constructed. These compounds were used in the study of the epithelial Na^+ channel.

Nuclear Magnetic Resonance spectroscopy was applied to the compounds amiloride hydrochloride and amiloride free base. Data generated with this methodology is useful in determining the conformations of these compounds in solution and for comparison with the results of theoretical calculations done in this laboratory. Models of the structures determined in this way can give a better approximation of the electrostatic and steric requirements necessary for the drug to bind with the receptor.

CONSTRUCTION OF ACCURATE MOLECULAR MODELS USING LASER STEREOLITHOGRAPHY AND DETERMINATION OF NMR SPECTRA FOR AMILORIDE HYDROCHLORIDE AND ITS FREE BASE

by
Ana Deborah Ofsievich

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Skawinski, W.J, Busanic, T.J, Ofsievich, A.D., Venanzi, T.J, Luzhkov, V.B, and Venanzi, C.A.
"The Application of Stereolithography to the Fabrication of Accurate Molecular Models", Journal of Molecular Graphics, 13, 126-135, 1995

Skawinski, W.J, Busanic, T.J, Ofsievich, A.D., Venanzi, T.J, Luzhkov, V.B, and Venanzi, C.A.
"The Use of Laser Stereolithography to Produce Three-Dimensional Tactile Models for Blind and Visually Impaired Scientists and Students", Information Technology and Disabilities, 1 (No 4, Article 6), 1994

This thesis is dedicated to my parents,
and to my beloved husband

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CHAPTER 1

INTRODUCTION

1.1 Objectives

The objectives of this study are twofold. The first objective is to construct accurate physical molecular models using laser stereolithography of molecules that have been studied in this laboratory: amino acids and analogs of amino acids, cyclodextrins and amiloride and its analogs. Another objective is to perform and analyze Nuclear Magnetic Resonance (NMR) spectra of the drugs amiloride hydrochloride (amiloride HCl), and its free base at low temperatures. In order to accomplish this objective, it is necessary to find a mixture of solvents that can be brought down to very low temperatures, and still remain liquid, in which the drug amiloride hydrochloride and its free base can be soluble. In the NMR spectra, the goal is to try to identify predominant low energy conformers of the drugs in solution. Once the conformers have been identified, it is necessary to calculate the rotational barrier between the conformers. In this way, these results can be compared with theoretical calculations performed in this laboratory, which will lead to the construction of the models of these molecules.

1.2 Introduction to Stereolithography

Visualization is the process of making visible that which is difficult or impossible to see in the physical world. As Rene Descartes said, in 1637, "imagination or visualization, and in particular the use of diagrams, has a a crucial part to play in scientific investigation"[1]. With the development of computer graphics, scientists have found that visualization is a very powerful tool in the understanding of large quantities of complex data. In the field of chemistry, scientists have been searching for different ways to depict geometries of molecules. There are many molecular graphics programs that allow one to construct the

geometry of a molecule on the screen of a computer. However, with these programs it is sometimes difficult to get the impression of a 3-dimensional model on the two-dimensional screen. In addition, a visually impaired person cannot see the screen of the computer and therefore will never know how a molecule looks. In this respect, a real physical model could be the solution[2]. There are many commercial kits to model molecules, such as the CPK and the Dreiding models. These models have been used for many years in basic organic courses to teach structures of different molecules. These models have some limitations. For example, with these models the bond lengths and atomic radii cannot be varied, because they are built with standard components. Furthermore, these models are unable to represent accurate torsional relationships, transition states or molecular properties such as the molecular electrostatic potential. The models presented here do not have these limitations.

A very common method of quickly producing prototypes (real physical models) is by use of rapid prototyping systems[3]. Rapid prototyping systems, which were first introduced in the late 1980's, are completing the revolution initiated by computer-aided design (CAD) by bringing the drawing of parts into real life. Since the commercial introduction of the first rapid prototyping process, laser stereolithography, several different technologies have been applied. These technologies fall into five major categories: laser stereolithography, selective laser sintering, fused deposition modeling, laminated object manufacturing, and ballistic particle manufacturing. Stereolithography[4-11] is the process which creates three-dimensional plastic models directly from CAD data, through the process of photopolymerization. This process transforms a photosensitive liquid resin into a solid polymer by exposing the resin to ultraviolet light. Essential parts of the stereolithography apparatus include a vat of liquid photopolymer, a laser generator which creates a small, intense spot of UV radiation, a galvanometer mirror X-Y scanner, an elevating platform, and a computer with its driving software for control. Usually the input data to the stereolithography system comes from a computer-aided design system.

One of the CAD programs useful for this purpose is I-DEAS (SDRC Inc., Milford, OH). Additional software must serve as an interface to convert these input data to the proper format for the stereolithography system. This software reconfigures the entire model into a series of layers, which are the actual layers that are going to be built by the stereolithography apparatus. This software is referred to as the Slice program (3D Systems Inc., Valencia, CA).

The models constructed for this work are of molecules of special research interest in this laboratory and represent examples of various areas such as: chemoreception, biomimetic chemistry and host-guest interactions (molecular shape and molecular electrostatics). Following is a description of some studies made that describe each one of these areas.

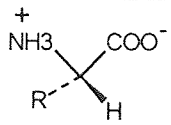
1.2.1 Chemoreception : Amino Acids

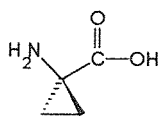
Taste receptors are necessary to perceive taste. Taste identification involves many signal transduction mechanisms. In order to trigger transduction of the stimulus, binding of stimulus molecules into a receptor must occur. However, to understand how this mechanism is triggered it is necessary to understand the specificity of individual classes of taste receptors. Structure-activity studies contribute to this end by defining receptor specificity in terms of molecular geometrical parameters and molecular properties[12].

Venanzi et al.[13] have been studying the binding of different molecules at the L-alanine receptor in the channel catfish. From experimental data it has been discovered that L-alanine binds to and activates specific taste receptors in the channel catfish[12]. Furthermore, it was observed that some analogs of L-alanine have a high affinity for the L-alanine receptor, but produce a lower neural response than L-alanine[12]. Some of these analogs are: glycine, L-serine, β-chloro-L-alanine, and 1-amino-1-cyclopropane carboxylic acid. All of these molecules have a carboxylate and an ammonium group, but they differ in the side chain, giving a specific volume to each one of the molecules. The IC_{50} value

indicates the ability of an analog to reduce specific binding of the ligand (L-alanine in this case) by 50%. A small number indicates that the molecule has a high affinity for the L-alanine receptor. Neural response is the measurement of the response of specific nerve fibers to a stimulus. Measurements of neural response are done by exposing the nerve to a stimulus and recording response amplitudes. These IC_{50} and neural response measurements allow one to define a relationship between the molecular structure and properties of a stimulus and receptor binding and subsequent activation. IC_{50} and neural response data for these molecules, as well as their chemical structure, are presented in Table 1.

Table 1. IC_{50} and neural response data for amino acids and amino acids analogs[12]



Name	R	IC_{50} (M)	Neural Response(%)
L-alanine	$-CH_3$	3.5	100
β-chloro-L-alanine	$-CH_2Cl$	2.5	88.6 ± 13.7
L-serine	$-CH_2OH$	2	58.6 ± 15.6
Glycine	-H	3	62.3 ± 9.4
1-Amino-cyclopropane-1-carboxylic acid		3.7	78.3 ± 9.3

Venanzi et al.[13] have developed a model of the steric and electrostatic features required in order to permit molecules to bind in the L-alanine receptor of the catfish. Using a

dielectric constant which approximates the receptor environment they determined the global minimum energy conformations of all these molecules. Using such data, models of some of these molecules were constructed using stereolithography.

1.2.2 Biomimetic Chemistry: Cyclodextrins

Some pharmaceutical companies are interested in developing certain types of artificial enzymes. Artificial enzymes are relatively small compounds. Reactions carried out by artificial enzymes mimic those of real enzymes. In order to mimic the enzyme reaction, substrate specificity is required and enzymatic reaction rates must be reproduced.

The Breslow[14] and Bender groups[15-19], have studied compounds that contain functional groups able to imitate the action of the Serine-195, Histidine-57, and the Aspartate-102 residues of α-chymotrypsin. One of these molecules is β-cyclodextrin and its structure is shown in Figure 1. The Breslow[14] and Bender groups[15-19] have independently studied the reaction between cyclodextrins and esters simulating models of the enzyme-substrate complex formed during the acyltransfer step, initiated by the Ser-195 of chymotrypsin. This model has been shown to catalyze the hydrolysis of esters twice as fast as chymotrypsin[15]. From experimental data it can be shown that cyclodextrins react with phenolic esters via an alkoxide ion, formed from the secondary hydroxyl groups, resulting in the formation of a covalent intermediate and in the subsequent release of corresponding phenols. However, it is not clear whether the reaction occurs at the 2' [20] or 3'-hydroxyl group[21].

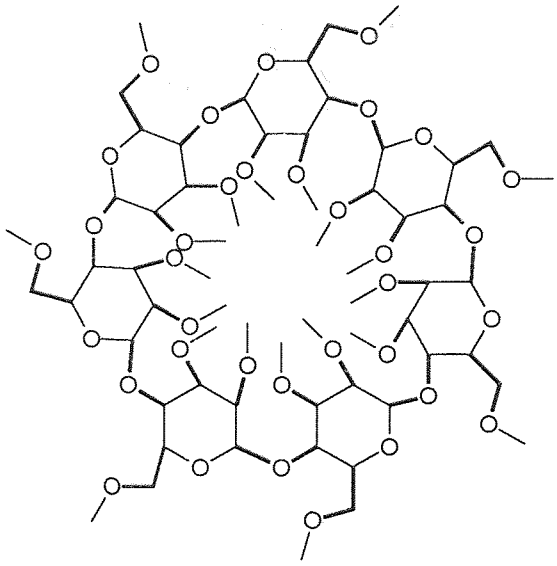
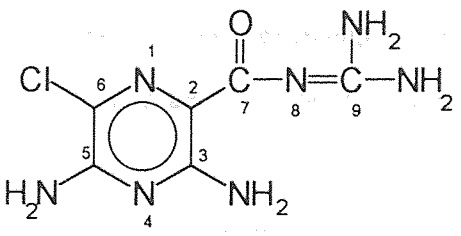


Figure 1. Molecular structure of β-cyclodextrin.

Venanzi et al.[22] have studied the reaction path of ester hydrolysis by the hydroxide ion of cyclodextrins, using the semiempirical AM1 method and the Langevine dipole solvent model, in order to determine if there is a difference in the reactivity of the secondary 2' and 3' hydroxyl oxygens of β-cyclodextrin. One of the results of the study is that acylation at the 3'-hydroxyl position was favored over the 2'-position by about 15 Kcal/mol, presenting less structural reorganization of the macrocycle during hydrolysis at the 3' site. Having a solid model of the calculated transition state would be useful in interpreting the data.

1.2.3 Host Guest Interactions: Amiloride

The compound amiloride, **1**, 3,5-diamino-6-chloro-N-(diaminomethylene)pyrazine carboxamide, is a potassium-sparing acylguanidine diuretic.



1

Usually, this drug is used as a companion to the potassium ion-losing diuretics such as the thiazide diuretics. The thiazide diuretics cause hypokalemia, secretion of potassium ion, which leads to deficiency related physiological effects. Among these effects are cardiac disturbances, anorexia, muscle weakness and lethargy[23].

Ion channels permit movements of ions across cellular membranes, both intra- and extracellularly. Amiloride is known to be a potent inhibitor of Na^+ transport in a variety of cellular and epithelial transport systems. These systems include: conductive Na^+ channel, the electroneutral Na^+/H^+ exchange system, and the electrogenic Na^+/Ca^{+2} antiporter[24].

1.2.3.1 Conductive Na^+ Channel. Amiloride blocks the passive Na^+ reabsorption, by interacting with and blocking the epithelial Na^+ channel. This phenomenon causes less Na^+ ion to be exchanged with K^+ ion, and eventually, the interruption of the electrogenic Na^+ transport. Such reaction leads to secretion of K^+ ions and this effect permits the use of this drug as a diuretic and antihypertensive agent.

There are three major regions of interest in the molecule: the guanidino group, the 5-amino group and the 6-position substituent. These groups can be substituted by different groups resulting in different responses. For example, the substitution of one of the terminal guanidinio groups by hydrophobic groups makes the drug more active. Other important features for inhibition of the Na^+ channel include having an unsubstituted 5-amino group and a chlorine atom in the 6-position[24].

1.2.3.2 Na^+/H^+ Exchange. The Na^+/H^+ exchange contributes to maintaining intracellular pH homeostasis, solute uptake and cell volume regulation. Amiloride blocks this exchange, leading to changes in cellular function. As a diuretic this reaction leads to an alkanization of the urine, attributable to inhibition of hydrogen ion secretion in the distal nephron. Substitution of the 6-chloro by -Br or -I results an enhancement of potency. In addition, substitutions of the 5-amino by ethyl, butyl hexyl or phenyl groups also result in an increment in the drug potency.

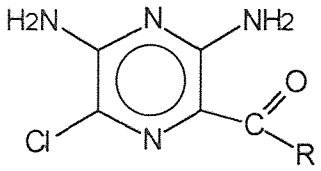
1.2.3.3 Na^+/Ca^{+2} Exchange. Ca^{+2} is a very important ion for the intracellular signal. This Na^+/Ca^{+2} exchange also helps to maintain a low cytosolic Ca^{+2} level. Amiloride is a weak inhibitor of this pump. The substitution of the guanidinio group by phenyl or benzyl groups gives the drug more potency.

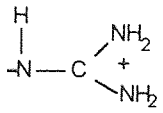
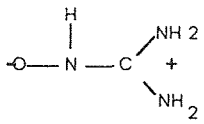
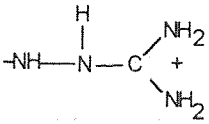
1.2.3.4 Some Studies on Amiloride and its Analogs. More than 1000 amiloride analogs have been synthesized. One of the reasons for studying amiloride has been to try to understand the characteristics of the binding site on ion channel proteins. For this purpose, different structure-activity studies on amiloride analogs have been carried out.

Li et al.[25,26] made electrophysical studies on the apical channels of the abdominal skin of *Rana ridibunda*. From these studies information about the rate constant for the binding of different analogs was obtained. Some of this information is shown in Table 2. K_{on} is the microscopic association constant and K_{off} is the dissociation constant. Analogs **18** and **19** differ from amiloride in their side chain which is elongated. Both analogs retain the ability to bind and block the Na^+ channel, with analog **19** being a slightly better blocker than amiloride as indicated by the K_{off} values[25,26]. From these studies, Li et al. proposed a model for the analog-channel interaction. He suggested a two-step model. In the first step, the guanidinium sidechain enters into the channel and interacts with an anionic site to form an encounter complex. In the second step either there is no blocking and the

molecule is released, or the substituent at the 6-position binds to an electropositive site on the channel, resulting in a stable complex.

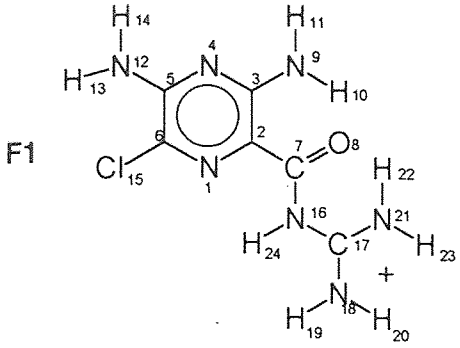
Table 2. Structure-activity relationships for selected amiloride analogs[25,26]



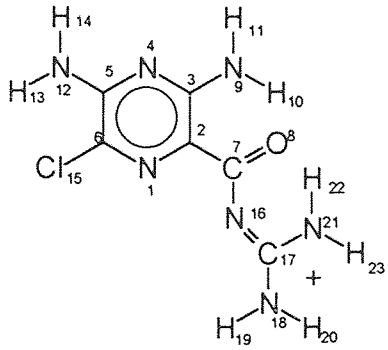
Analog Number	R	pK_a	K_{on} ($s^{-1}\mu M^{-1}$)	K_{off} (s^{-1})
1		8.67	13.17 ± 0.25	3.93 ± 0.19
18		4.50	1.22 ± 0.07	20.67 ± 3.72
19		9.00	2.16 ± 0.11	3.41 ± 0.55

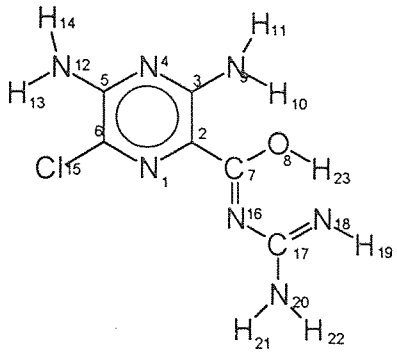
Using ^{1}H and ^{13}C NMR techniques and CNDO/2 theoretical calculations, Smith et al.[27] found amiloride HCl to exist as conformer F1 and amiloride free base as conformers A1 and/or A4. From theoretical calculations these authors also found that conformer A1 is more stable in vacuum than conformer A4 by 0.9 Kcal/mol. They were unable to identify from NMR data which of these conformers was preferred in solution. From NMR data[27], tautomer A was more stable in solution than tautomer E. On the other hand,

theoretical calculations in vacuum predict that tautomer E is more stable. Molecular structures of conformers F1, A1, A4, and E are shown in Figure 2.



F1





E

A1 A1 $O_8 C_7C_2N_1 = 180$

A4 A4 $O_8 C_7C_2N_1 = 0$

Figure 2. Molecular structures of conformers: F1, A1, A4 and E

In order to interpret the structure-activity data of Li et al.[25,26], Venanzi and coworkers have carried out conformational analysis[28-30], molecular electrostatic potential analysis[31,32], and molecular dynamics and static solvation studies of amiloride and its analogs [29,33]. Calculations of the minimum energy conformers for amiloride and analogs **18** and **19** showed that amiloride has a planar conformation while **18** and **19** have nonplanar conformations. In **18** the ring is approximately 30° out of the plane of the side chain, whereas that of **19** is closer to 90°. These data could explain results obtained by Li et al.[25,26] that these compounds form a stable blocking complex with the ion channel. Venanzi et al. [28,29] carried out molecular orbital calculations with geometry optimization

using the 3-21G* basis set, and molecular dynamics and static solvation studies for different conformers for amiloride HCl and its free base[29]. The results of these calculations identified the A tautomer as more stable than the E tautomer[28]. Furthermore, conformer A1 was found to be more stable than A4 conformer[28,29].

1.2.3.5 Description of Nuclear Magnetic Resonance Spectroscopy. Nuclear magnetic resonance[34-36] is based on the fact that nuclei of certain elements have a spin, a spin angular momentum and an associated magnetic moment. When no magnetic field is applied, these nuclei can spin at random in their atomic or molecular environment. When placed in a strong magnetic field, these nuclei can adopt one of a number of quantized orientations, each orientation corresponding to a particular energy level. These nuclei will adopt one of several possible 2I+1 orientations with respect to the external magnetic field, where I is the nuclear spin, and is given by the magnetic quantum number m_I. The orientation with the lowest energy is the one in which the nuclear magnetic moment is most closely aligned with the external magnetic field, while the orientation with the highest energy is the one in which the nuclear magnetic moment is least closely aligned with the magnetic field. Nuclear magnetic resonance involves transitions between these energy levels with respect to the external magnetic field by absorption of electromagnetic radiation of the correct frequency. The relationship between the electromagnetic frequency ν and the magnetic field strength B_0 is governed by the Larmor equation (equation **(1)**).

$$\nu = \gamma B_0 / 2\pi \qquad \textbf{(1)}$$

Where γ is the magnetogyric ratio. When a nucleus of magnetogyric ratio γ is placed in a magnetic field B_0, the resonant condition is satisfied when the frequency of the applied radiation ν is given by equation **(1)**.

One of the variables that affects chemical shifts is temperature. Depending on the temperature at which the experiment is carried out, not only could the chemical shifts of a specific compound be different, but also the environment of each atom in the molecule could differ. Rotations about single bonds occur in a molecule and these rotations are very fast. For example, the three hydrogens of a methyl group would appear to be equivalent in a proton NMR. When the temperature is lowered, these rotations about single bonds could be reduced. As a result the NMR spectrometer may see those hydrogens as not being equivalent, because other parts of the molecule may now influence these chemical shifts. In the present work, ^{1}H and ^{13}C NMR spectra of amiloride HCl and its free base were performed and analyzed at different low temperatures, in order to determine the solution structures of these compounds and for comparison with quantum mechanical calculations carried out in this laboratory.

CHAPTER 2

MATERIALS AND EXPERIMENTAL METHODS

2.1 Description of the Construction of the Models

The first step in the construction of a model is to obtain the coordinates for each atom in the molecule. These coordinates can be obtained from neutron diffraction data or from quantum mechanics calculations. The coordinates referenced in this work for all the models were provided by Dr William Skawinski, Dr Carol Venanzi, and collaborators. The global minimum energy coordinates of the amino acids and amino acid analogs[13] were determined by the self-consistent reaction field (SCRF) method[37-41] using the 6-31G* basis set. The coordinates of the neutron diffraction structure of β-cyclodextrin undecahydrate[42] were obtained from the Cambridge Structural Database[43]. All the water molecules were removed from the structure. The transition state of the reaction of β-cyclodextrin with phenyl acetate[22,33] was determined using the AM1 method in the MOPAC-93 program[44]. Two different positions of the phenyl acetate with β-cyclodextrin were calculated[22]. The minimum energy conformers of water, methane and phenyl acetate[22] were also calculated using the AM1 method in the MOPAC-93 program. The coordinates for amiloride[28] and its analogs[30] were those of the global minimum energy conformations determined using the 6-31G* basis set. The values for methyl chloride and cyclohexyl chloride were calculated using the AM1 method in the Quanta program[45]. From these calculations, the Cartesian coordinates of the center of each atom was computed in Angstroms. These coordinates were used in the construction of the models.

The next step was to design the molecular model in a CAD program. There are several CAD programs that translate images into a format that can be read by the stereolithography apparatus. The program used in this study for the models of amino

acids and analogs of amino acids described above was I-DEAS version 6, installed on a Sun SparcStation 10, model 41 (one processor), 96 MB RAM. The rest of the models were designed with the I-DEAS master series, version 1.3c, installed on a Personal Iris Silicon Graphics workstation. The first step in the CAD design was to draw a sphere with a specific radius. The radii chosen for the construction of some of the models are the standard van der Waals radii: H (1.2 Å), N (1.5 Å), O (1.4 Å), Cl (1.8 Å), and aliphatic C (2.0 Å), aromatic and carbonyl C (1.85Å). Other models were constructed with CPK radii: H (1.0 Å), N (1.7 Å), O (1.35 Å), Cl (1.8 Å), and aliphatic C (1.5 Å), aromatic and carbonyl C (1.7 Å). In order to position a sphere in space, the previously calculated coordinate was specifically assigned for that atom. Then, another sphere was drawn, with the specific radius for that atom type, and positioned in space at the calculated coordinates. At this point two spheres can be seen overlapping with each other, but the CAD program recognizes the two spheres as individual objects. In order to merge these two spheres the JOIN command is invoked so that the program can recognize the overlapping spheres as a single object. This procedure was repeated until the entire model was finished. This procedure could lead to a series of errors such as misplaced signs and numbers when the data is input. In order to avoid these kinds of mistakes, an input program was written within I-DEAS to allow automatic input of the structural data from a text file. The input data consisted of five parameters for each atom: the atomic symbol, the van der Waals radius, and the x, y, z coordinates in Angstroms. An example of the input data is shown in Table 3.

Many CAD and solid modeling software packages, and especially I-DEAS, represent surfaces of parts as facets. These are planar faces in the form of polyhedrons. The number of facets can be changed. The larger the number of facets, the smoother the sphere will be, but this could lead to a longer building process and a larger file size. Changing the number of facets may be an advantage in certain cases. Once the model is constructed, it is possible to detect the different number of facets, by touching or seeing

each atom type. This is very beneficial when the users are visually impaired. Actually, these persons can identify each atom type in accordance with a classification made in advance.

Table 3. Example of input data for I-DEAS. These coordinates represent amiloride coordinates

Atomic Symbol	Radius(Å)	X Coordinate	Y Coordinate	Z Coordinate
N	1.7	-0.587328	-0.691711	0.000000
C	1.7	0.000000	0.511604	0.000000
C	1.7	-0.809879	1.658474	0.000000
N	1.7	-2.142612	1.529082	0.000000
C	1.7	-2.701087	0.333044	0.000000
C	1.7	-1.866531	-0.819538	0.000000
C	1.7	1.463724	0.599433	0.000000
O	1.35	2.020381	1.722582	0.000000
N	1.48	-0.333854	2.904784	0.000000
H	1.0	0.657720	3.036832	0.000000
H	1.0	-0.985609	3.657401	0.000000
N	1.48	-4.038984	0.257809	0.000000
H	1.0	-4.521056	-0.612114	0.000000
H	1.0	-4.553748	1.110992	0.000000
Cl	1.8	-2.579962	-2.409841	0.000000
N	1.48	2.118578	-0.580042	0.000000
C	1.5	3.426054	-0.656657	0.000000
N	1.48	3.955953	-1.893663	0.000000
H	1.0	3.312465	-2.654996	0.000000
H	1.0	4.934293	-2.071495	0.000000
N	1.48	4.268435	0.386732	0.000000
H	1.0	3.839945	1.294778	0.000000
H	1.0	5.257307	0.276162	0.000000

The older version of I-DEAS (version 6) used for some of our models has a command which specifically asked for the number of facets. This feature, however, was not available in the new version (master series 1.3), and the number of facets was input by

default. For the models that were constructed with I-DEAS version 6, the numbers of facets used were: H (8x8), C (8x8), O (12x12), N (16x16), Cl (20x20).

The next step in this procedure was to scale the model to an appropriate size to fit in the cubic vat of 10x10x10 inches of the stereolithography apparatus. The dimensions were set by taking the CPK or van der Waals radii and setting the model radius to that value in inches in the CAD program and then multiplying the result by different scale factors. Scale factors used for some of the models are shown in Table 4.

Table 4. Scales factors used to construct some of the models

Model	**Scale Factors**
Amino acids	0.3
Cyclodextrins	0.35
Amiloride	1.25

Once the desired size of the model has been obtained, it needs to be translated into a positive space, because the stereolithography system begins making the models starting from x, y, z equal to 0, 0 ,0. At this point the model was ready to be written as an STL format, which is the output format needed for the stereolithography system.

Some models of β-cyclodextrin and β-cyclodextrin with phenyl acetate as well as models of L-alanine, glycine, L-serine and β-chloro-Lalanine were made of solid spheres. However, the rest of the models were made hollow. In order to create the hollow cavities, the input program was modified to yield spheres in the same way as described before, but with a smaller radius. Then the two models were superimposed, with one model inside the other, and the CUT command was invoked. The result of this operation was to cut away the common volume of the two parts yielding a hollow model. In the case of these

models, additional small holes had to be cut to let the resin drain during the construction of the part in the stereolithography system.

The next step was the construction of temporary support structures. For that purpose, the software package Bridgeworks (Solid Concepts, Inc., Valencia, CA), installed on a 486 DX66 PC, with 16 MB RAM memory at Center for Manufacturing Systems, at New Jersey Institute of Technology was used. The Bridgeworks software package is the automatic support generator for rapid prototyping. As an automated program, this software applies a set of support design rules which produce enough supports to ensure proper building of the part. Depending on the different processes or resins being used, it is possible to vary different parameters. Bridgeworks reads a STL file, which defines the part geometry, analyzes the support requirements and generates the necessary supports to a separate STL file. Supports are required because the stereolithography system builds models layer by layer on the surface of a liquid resin. Each layer must be moved below the liquid level after being drawn. This requires that each layer be attached to the layer below and that the very first layer be attached to the moving platform of the stereolithography apparatus. Sometimes, the current layer is larger than the previous layer and without the supports it would collapse. Supports are also required to reduce curling during the building process and to some extent during post curing. These structures were removed after the model was built.

After all the STL files were created the Slice program was used to process these files. The Slice program, installed on a 486 DX66 PC, with 16MB RAM memory at Center for Manufacturing Systems was needed to modify the data by cutting the object into a series of horizontal layers. For this specific work different parameters must be given with the appropriate values. Layer Thickness is a Slice parameter that permits one to specify the distance between vertical layers. This determines the accuracy of the part, the vertical resolution, and the height of each step. A decrease in the thickness of the layer results in a smoother surface, since the height of each step is reduced. Another

important parameter is the Hatch Fill Spacing. This parameter determines how much resin is going to be cured by the laser light. Large values of this parameter indicate that less resin is going to be cured. For the support structures, this parameter is set at high values, resulting in very flexible parts. This is done so that the supports can be removed very easily after the building process. The values of the parameters used are shown in Table 5. The files resulting from the Slice program are called SLI files. The model and the supports files were merged using the Slice program. The MERGE command was invoked which generated four files: L, R, V and PRM. These four files contained all the information for the building process. These files were used as input to the computer of the stereolithography apparatus.

Table 5. Values of parameters used in Slice program

Parameter	Model	Support
Slice Output Scale	1.000	1.000
Resolution	5000	5000
Layer Thickness[a]	0.01	0.01
X Hatch[a]	0.01	0.15
Y Hatch[a]	0.01	0.15
X Skin Fill Spacing[a]	0.004	0
Y Skin Fill Spacing[a]	0	0

[a] Values in inches

2.2 Description of Stereolithography Methodology

The resin used to construct the models is composed of two materials. One is a photoinitiator which absorbs the laser energy and forms reactive radical species, which in turn initiate the polymerization process. The other is an acrylic functionalized monomer

which has the ability to polymerize when exposed to a free radical source. The overall dimensions of the solid polymer formed when a UV laser is focused on the surface of a photopolymer are controlled by the intensity of the laser beam and the period of exposure. A longer exposure time or increased laser energy or both will increase the depth and width of the solid region[46]. The resin is a toxic material and has to be handled appropriately. Acrylate resins may cause skin burns with prolonged contact and they also release toxic vapors. This means that safety gloves must be worn, and the laboratory where the stereolithography system is installed must have very good ventilation. The stereolithography apparatus used was the model SLA-250 manufactured by 3D Systems Inc. At the start of the building process[47], the elevating platform is positioned just below the surface of the liquid resin. The UV laser spot moves back and forth, causing the liquid to solidify whenever it is impinged upon, thereby forming a thin, solid cross section on the platform. When the first cross section is completed, the platform goes down a step and the solid layer is covered by another layer of liquid. Instructions for the formation of the next cross section are received, and the process is repeated. This procedure, which is automatic and can proceed without operators, is performed until the entire model is completed. After the part is finished, the elevator raises it out of the vat to allow excess liquid to drain. Although the part looks and feels solid, it still contains large amounts of uncured resin. Therefore, the model must undergo further treatment with intense UV radiation to complete the curing process. Before the final curing of the model, the support structure is trimmed away. Then, the part is cleaned with alcohol to remove the rest of the resin. Once the part is cleaned, it is ready for the final curing process. The duration of the curing and postcuring depends on the complexity of the part, its size and the type of polymer.

2.3 NMR Procedure

2.3.1 Preparation of Amiloride Free Base

Amiloride hydrochloride was purchased from the Sigma Chemical Company (St. Louis, MO). The first step was to find a way to obtain the free base of amiloride HCl. 500 mg of amiloride HCl were weighed and diluted with 115 ml of water until all the compound was dissolved. Next, a few drops of 0.5 M bicarbonate solution was added until the pH was raised to 8.5, measuring the pH with a ionalyzer specific ion meter (Orion Research, Inc., Cambridge, MA) model 407A, with a gel-filled combination electrode. Then, a few drops of 1M NaOH were introduced until pH = 10. The solution was left for a few minutes in a cold bath with mechanical stirring until the free base precipitated. The solution was filtered through a funnel using vacuum, with a filter paper (Whatman 1 qualitative) at the bottom. The residue was recovered and placed in a desiccator for two days. The walls of the desiccator were covered with paper to prevent exposure of the sample to light. In order to identify the compounds, the melting points of the amiloride free base and amiloride HCl were measured. Other tests, such as differences in the solubility of various solvents, were performed to identify amiloride free base. The solvents utilized were: water, chloroform, ethyl ether, ethanol, acetone, dimethylformamide (DMF), toluene, 2-propanol, and butyl ethyl ketone.

Some other tests carried out were Infrared Spectrometry and Mass Spectrometry. The latter was carried out with a INCOS 50 Mass Spectrometer interfaced with a Hewlett Packard 5890 Gas Chromatograph (located at the Chemistry Department at Rutgers University, Newark, NJ) with a DB-5 column. A Bio Rad Fourier Transform Infrared Spectrometer (Cambridge, MA) model FTS 40, was used to perform the infrared spectra. To run the mass spectra, different solutions of amiloride HCl and amiloride free base were prepared. A few milligrams of amiloride HCl and amiloride free base were dissolved in a few millilitres of different solvents and these solutions were then input into the mass spectrometer. The solvents used were ethanol, dimethylsulfoxide (DMSO), DMF, and

toluene. For the infrared spectra, a few milligrams of amiloride HCl or amiloride free base were added to three drops of mineral oil and mixed. Then, each mixture was put on a KCl plate and spectra run for both compounds.

The next step was to identify an appropriate mixture of solvents that can be cooled to -50°C without freezing. For this purpose, different mixtures of solvents were introduced in a cooler at -50°C for half an hour. A flexi-cool U159 cooler manufactured by FTS Systems, Inc. (Stone Ridge, NY) with a thermo container filled with 2-propanol was used to cool the solutions to -50°C. The solvent mixtures examined are shown in Tables 6 and 7.

Table 6. Cryosolvent systems examined (v/v) of dimethylsulfoxide (DMSO) and methylene chloride

Mixture Number	DMSO	Methylene Chloride
1	1	1
2	4	3
3	1	2
4	1	1.5
5	1	1.2

Table 7. Mixtures (v/v) of DMF and methylene chloride examined

Mixture Number	DMF	Methylene Chloride
6	1	2
7	1	1

Next, the solubility of the samples had to be tested in the appropriate mixtures of solvents. In this case, the solubility of amiloride HCl and amiloride free base were evaluated for the mixtures of solvents that did not freeze at -50°C. Then, the solutions

were introduced into the cooler for half an hour to see if the compound was still soluble at this low temperature.

2.3.2 NMR spectra

Solutions for the NMR spectra were prepared with deuterated solvents, purchased from Aldrich Chemical Co., Inc., (Milwakee, WI). The mixture of solvents chosen for these spectra was 1/1.2 (v/v) DMSO-6d/deuterated methylene chloride. Solutions for the NMR spectroscopy were prepared with this mixture of solvents, with 30 mg of amiloride HCl added to one, and 30 mg of amiloride free base added to another. Proton NMR spectra of amiloride HCl and amiloride free base were obtained under a wide range of temperatures. In a first step, the amiloride free base proton NMR spectra were recorded from -60°C to 20°C in increments of 5°C for the range of temperatures -60°C to -40°C, and in increments of 10° for the rest of the temperatures. Next, the amiloride HCl spectra were taken for the range 20°C to -60°C. Decrements of 10°C and 5°C were performed until -30°C and -60°C were reached, respectively. Carbon-13 NMR spectra of both compounds were recorded at room temperature. Proton and carbon-13 NMR spectra were recorded on the Varian VXR 400, at the Chemistry Department of Rutgers, the State University, Newark, NJ, using the 5 mm switchable probe.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Models Constructed by Laser Stereolithography

Among the models constructed with laser stereolithography were amino acids and analogs of amino acids (L-alanine, glycine, L-serine, β-chloro-L-alanine, and 1-amino-1 cyclopropane carboxylic acid), β-cyclodextrin and the transition state of the reaction of β-cyclodextrin with phenyl acetate, and the drug amiloride, as well as other two analogs of this compound, analog **18** and analog **19**. Models of water, methane, methyl chloride, cyclohexyl chloride, and phenyl acetate were also built.

The models were constructed on different scales. Those of smaller scales have the same characteristics as the larger ones, and they still can be used for understanding geometries of molecules. Some models were constructed solid and other models hollow. The hollow ones, while still being quite resistant to breakage, reduce by 60% the amount of resin used, and thus lead to a reduction in cost. The resin used in this technique is very expensive, so this aspect becomes particularly important when dealing with large models, and when building models in large numbers.

The time required to build the models in the stereolithography system depends on the complexity of the model. Table 8 shows the time required for the building of some of the models. In addition to the time of the building process, one or two more hours should be added for the last curing phase of the resin in an ultraviolet oven.

It should be noted that the models can be constructed with any desired radii. In our case, CPK radii were used for amiloride, analog **18**, analog **19**, water, phenyl acetate, methyl chloride, cyclohexyl chloride, methane models, and van der Waals radii were chosen for the rest of the models. It was found that decreasing the radii of the building spheres leads to models with less spherical overlap, and consequently, more distinct

atoms. As it was mentioned before, the number of facets is a characteristic that can be used to identify each atom type by touching or seeing. As the scale of the models become larger, it is better to have a larger number of facets, resulting in smoother spheres. Version 6 of I-DEAS has the capability of defining each sphere with a specific number of facets, however master series 1.3 of I-DEAS has not and the program assigns by default the number of facets for each atom type. For the purposes of this work, it is more convenient to have the capability of changing the number of facets, in order to identify each atom type. A possible solution for this could be to make the surface of the spheres with different textures adding distinguishable features, so that by touch each surface would feel different for each atom type.

Table 8. Building time for the construction of some of the models in the stereolithography system

Model	**Building Time** (hours)
amino acids	8
cyclodextrins	36
Amiloride	12

3.1.1 Amino Acids

Models of both amino acids and analogs of amino acids (shown in Figure 3 Appendix A) were constructed. These molecules include: L-alanine, glycine, L-serine, β-chloro-L-alanine (bottom row, Figure 3 Appendix A), and 1-amino-1-cyclopropane carboxylic acid (top row, Figure 3). All models are solid except those in top row. From an analysis of the constructed models, it can be clearly seen that the shapes obtained for these models are similar to each other. All of these molecules have a high affinity at the L-alanine receptor. These models illustrate and confirm the idea first suggested by Bryant et al.[12] about the

relationship between molecular volume and binding. Not only must volume requirements be met in order for a molecule to bind into the receptor, but also steric requirements must be met. The steric requirements are almost identical in these molecules.

3.1.2 Cyclodextrins

Two solid models of β-cyclodextrin were constructed. One of these β-cyclodextrin models was constructed using a smaller scale, reducing it by a factor of 0.07. Two models of the complex of the transition state of the reaction between β-cyclodextrin and phenyl acetate showing two different positions of acylation at 2' (Figure 4, Appendix A, solid model) and 3' (not shown, hollow model) were also built. The β-cyclodextrin models were constructed on a larger scale than the amino acid or amiloride models. The model of this molecule can be described as having a donut shape and presenting an internal cavity. From these models, it can be shown how the phenyl acetate contacts the macrocycle. In addition, it can be seen how the substrate in the complex of the transition state at the 2'-hydroxyl stays above the cavity[22].

3.1.3 Amiloride

Solid models of amiloride and analogs **18** and **19** were constructed. The amiloride model showed the planarity of the molecule, whereas models of analogs **18** and **19** showed the nonplanarity of these molecules. In addition, it was very clear in these models how the side chains of analogs **18** and **19** are twisted out of the plane. Figure 5 and 6, Appendix A show the models of analog **18** and **19**, respectively, displayed in a plastic block.

3.1.4 Other Models

The rest of the models (water, methane, methyl chloride, cyclohexyl chloride, and phenyl acetate) were made specifically for educational purposes. They have been sent to the

Washington State School for the blind to be used in part of their science courses. The teacher there will evaluate them and then suggest how we might improve them.

3.2 NMR Studies

Once the amiloride free base procedure was completed, some experiments were carried out in order to identify if indeed the free base was obtained. First, the melting points of the amiloride free base product and of the starting amiloride HCl were measured. There were considerable differences between these two melting points. For amiloride free base a melting point of 235°C was obtained, and for amiloride HCl a value of 288°C was obtained, in accordance with literature values of 240-242°C and 285-288°C, respectively[48,49]. Attempts were also made to identify the amiloride free base using mass spectrometry interfaced with gas chromatography. This technique failed to release the drug from the column, even when different solvents with different polarities were used. Probably the compound stuck to the column and another column with different polarity should be used. Since it was not possible to change the column, infrared spectrometry was performed in order to identify the compound. The spectrum for amiloride HCl compares qualitatively with similar frequencies as shown in the literature[48]. The amiloride free base spectrum presents almost the same vibrations as the amiloride HCl spectrum, except for the presence of a vibration at 770 cm^{-1} that is found in the spectrum of amiloride HCl and not in the free base. This vibration could be assigned to the NH rocking motion that could be present in the hydrochloride drug. All these experiments, measurements of melting points and infrared spectra, showed that the free base of the drug was obtained.

Rotations about single bonds occur so rapidly that NMR spectrometers see protons in their average environment. By lowering the temperature, the rates of rotation about single bonds can be slowed down, and a NMR spectrometer could then see different environments for protons in different conformations. This does not mean that the

molecule is frozen into a single conformation, but that the period between interconversions is long enough for the NMR spectrometer to see one conformation or the other or both. The mixture of solvents chosen to run the NMR spectra was DMSO-6d/methylene chloride deuterated (1/1.2). The cryosolvents could be brought to -50°C, and the solvent would remain liquid. Another requirement was that, once the drug was dissolved into the cryosolvent system and brought to -50°C, there should not be any precipitation. In Table 9 is shown some mixture of solvents used with the results observed.

Table 9. Mixture of solvents carried out in order to find the most appropriate mixture to analyze the NMR spectra

DMSO	Methylene chloride	Observations
1	1	Frozen
4	3	Frozen
1	2	Not frozen
1	1.5	Not frozen
1	1.2	Not frozen

DMF	Methylene chloride	Observations
1	2	Not frozen
1	1	Not frozen

3.2.1 Spectra

Spectra are shown in Figures 7-15 in Appendix B. For the proton NMR spectrum of amiloride HCl at 20°C (Figure 7) the presence of three resonances can be seen, with chemical shifts of 7.3, 8.67, and 10.65 ppm. As the temperature is lowered, the resonance at 7.3 ppm starts to split into 4 resonances, and as the temperature is decreased, further the resonances get sharper, as is shown in the spectrum at -10°C in Figure 8, with chemical shifts of: 7.25, 7.34, 7.45, 7.93, 8.62, and 10.65 ppm. At -20°C (Figure 9) the resonances at 7.34 and 7.45 ppm start to converge into one resonance at 7.45 ppm, as it is shown in the spectrum at -30°C (Figure 10). This resonance starts to split again into two resonances at -40°C (shown in Figure 11), with chemical shifts of 7.45 and 7.5 ppm, which will merge again into one resonance at -45°C. All this behavior is still unexplained. More experiments should be performed in order to determine if the presence of two or more conformers of the molecule can be detected. Some examples in the literature can be found of similar behavior[50,51], where this is attributed to the rotations about C-N bonds and the presence of two conformations due to this rotation. Amiloride also has C-N bonds, and rotations about this bond can result in the presence of two or more conformers. In addition, another cause could be the presence of intramolecular hydrogen bonds. When the temperature is lowered the hydrogen bonds are more likely to occur, and the NMR spectrometer can see the hydrogen in different environments.

The ^{13}C NMR spectra carried out for amiloride HCl and its free base present a profile similar to that found in the work of Smith et al.[27]. In the proton NMR spectrum for amiloride free base at 19.6°C (room temperature), Figure 12, three resonances are present, with chemical shifts of 6.4, 7.3, and 8.65 ppm. When the temperature is lowered, four resonances appear which get sharper as the temperature goes down, with chemical shifts of 6.27, 6.73, 8.15, and 8.82 ppm, as can been seen in the spectrum at -20°C (Figure 13). At -30°C, spectrum shown in Figure 14, another resonance begins to be observed at 6.97 ppm. At -50°C (Figure 15) the first resonances start to split, but going lower than

this temperature causes the solvent to solidify and a considerable degree of noise appears. None of these resonances have yet been identified. Some additional experiments must be performed. Two-dimensional NMR techniques can be used first to assign most or all the carbon signals. Then with other two-dimensional techniques, it is possible to identify connectivities of proton and carbons.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

The unique feature of the models created by laser stereolithography compared to other molecular models is their ability to represent the molecular structure information obtained from accurate quantum mechanical calculations, representing a revolutionary new molecular modeling tool. The models created with stereolithography can be custom designed to suit the requirements of the user. These molecular models can serve as educational tools. They can be used in chemistry courses to assist in the learning about structures of molecules. Not only sighted students and researchers will benefit from these models, but also blind students and researchers can use them to understand geometries of molecules. These models offer accurate representations of molecules. This technique can be used to construct models of the transition state of a reaction, which would be impossible using standard molecular models because knowledge of the orientation and bonding of the substrate is required. In addition, this technique helps to enhance the communication between visually impaired and sighted researchers involving three-dimensional concepts. Another important advantage of this method is the possibility for blind or visually impaired scientists and students to acquire important information on three-dimensional images and apply this to their scientific research.

Future work should attempt to represent physical properties such as the electrostatic potential. This can be done by transforming images from the CAD program into mathematical functions and then translating them into a real model. In addition, larger molecular models such as proteins could be constructed.

Based on the NMR studies, a decision about the conformers of amiloride HCl and its free base cannot be made. The behavior presented by these compounds at low temperatures must first be explained. In order to accomplish this, more experiments are

necessary. Another solvent, perhaps dimethylformamide, could be utilized to run NMR spectra in the same range of temperatures. This would permit us to know if the solvent is responsible for this behavior. Amiloride HCl and its free base are very soluble in this solvent and could be cooled down to -50°C without precipitation. Another direction would be to do two-dimensional NMR spectroscopy. This data could give one the assignments for each proton and carbon in the molecule.

APPENDIX A

PICTURES OF SOME OF THE MODELS CREATED BY LASER STEREOLITHOGRAPHY

Figure 3. Plastic models of amino acids analogs

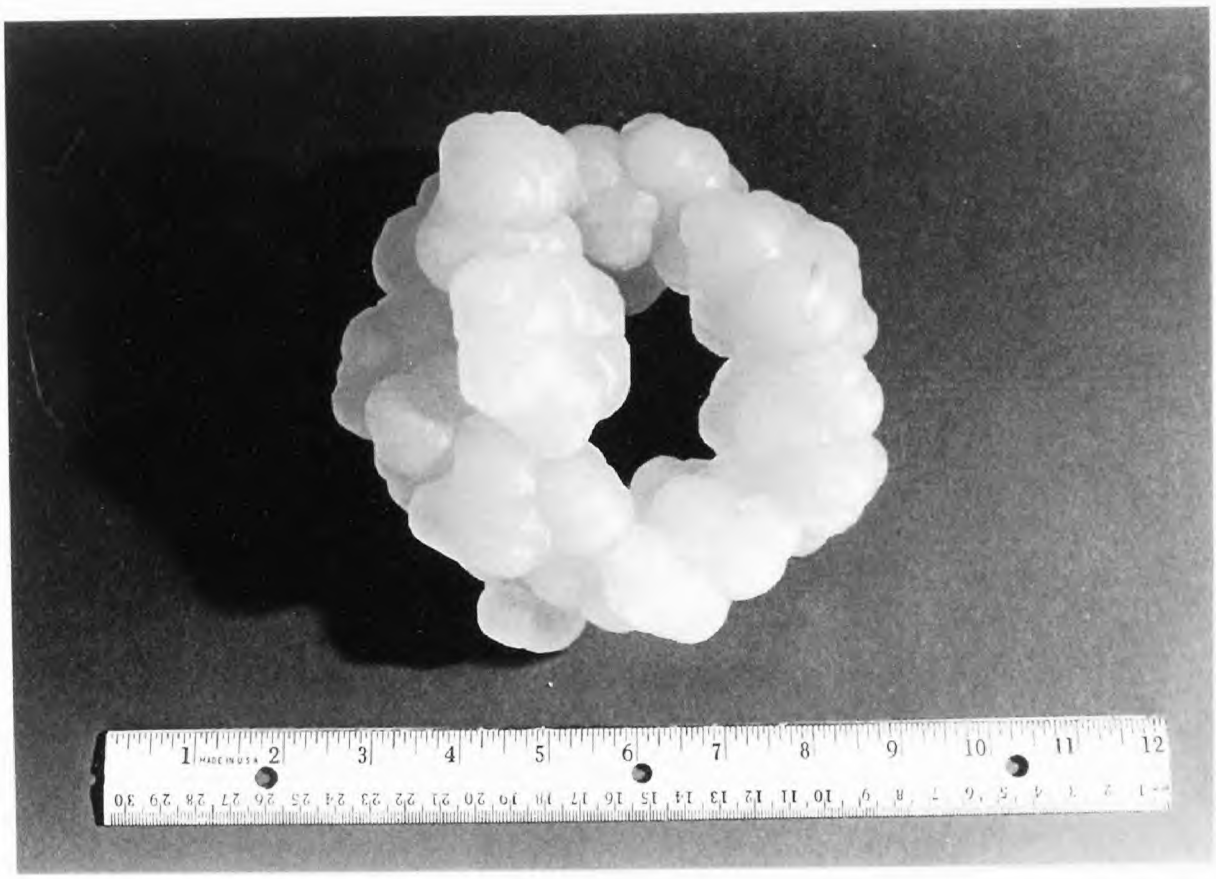


Figure 4. Model of the complex of the reaction of β-cyclodextrin with phenyl acetate

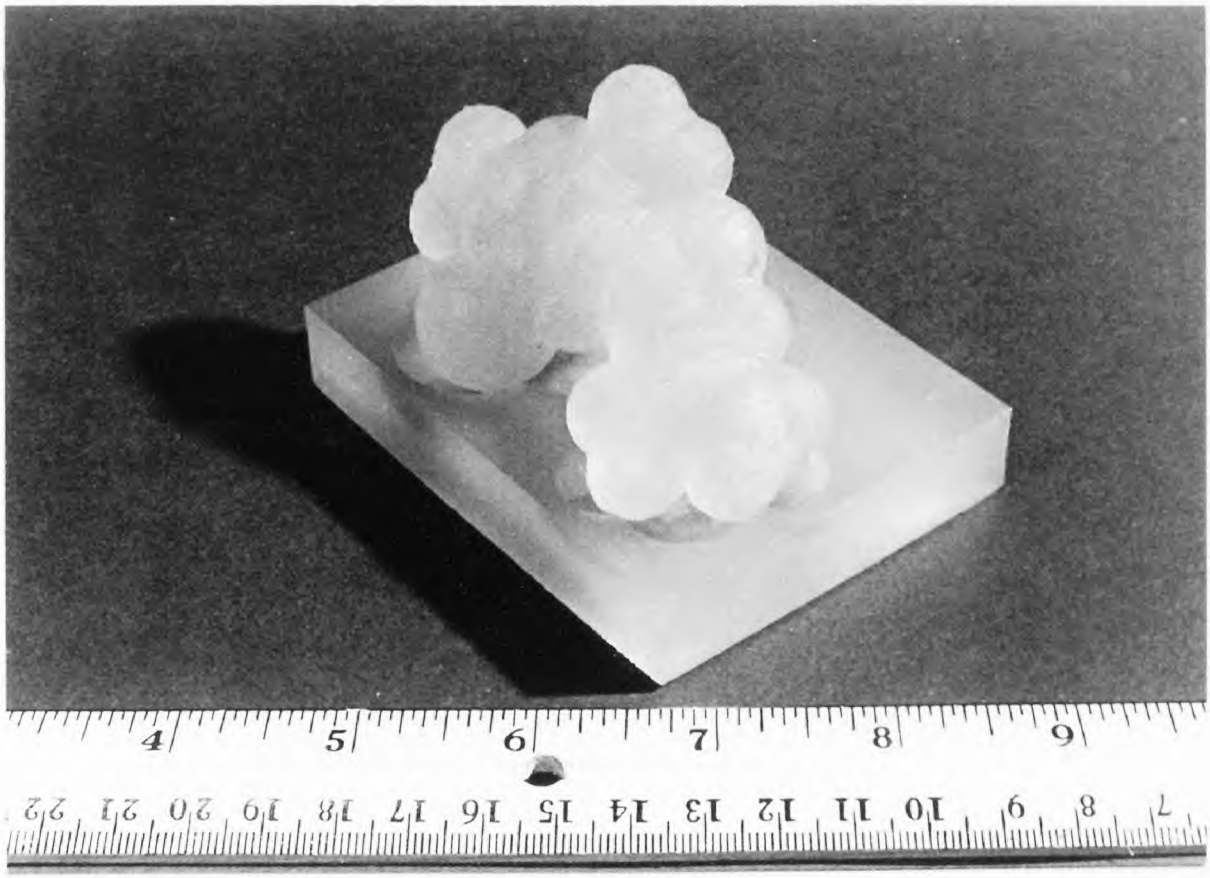


Figure 5. View of model of analog **18** displayed on a plastic block

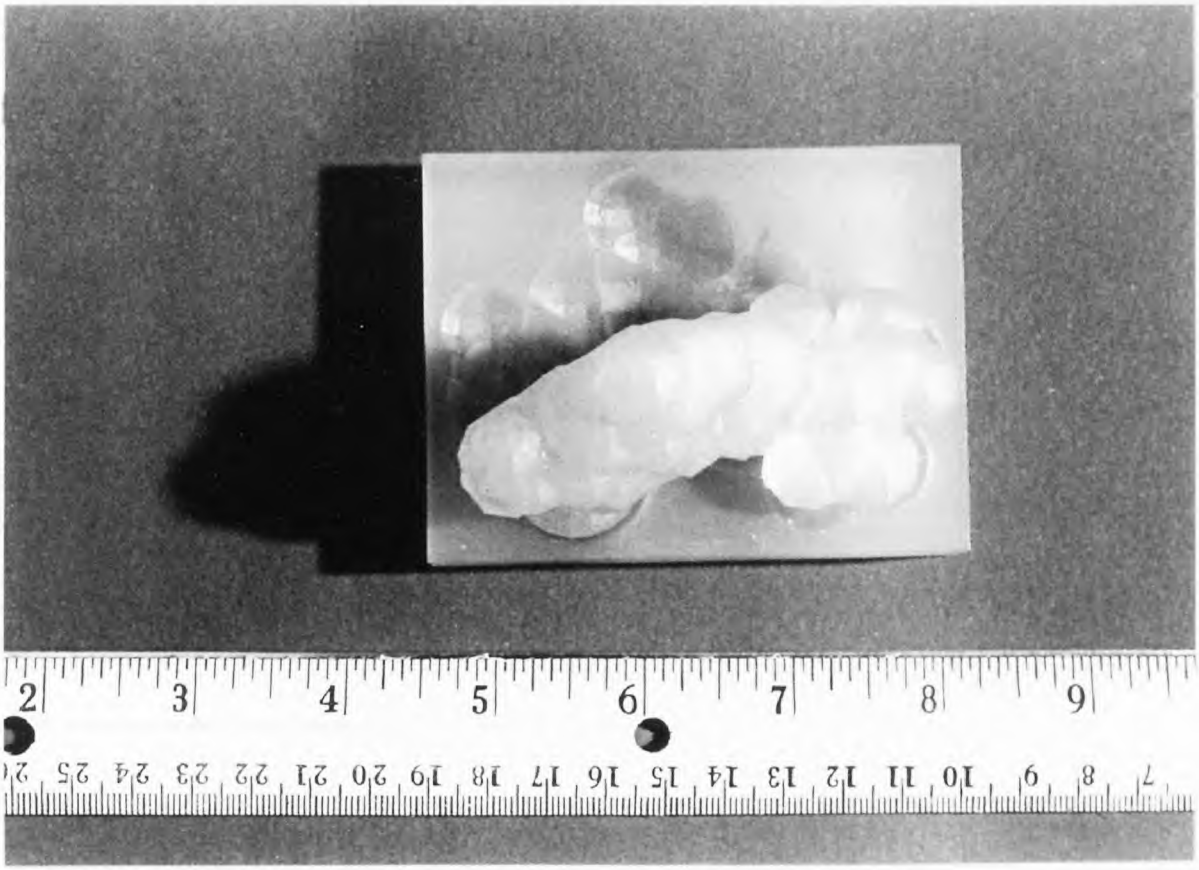


Figure 6. View of solid model of analog **19** displayed on a plastic block

APPENDIX B

NMR SPECTRA FOR AMILORIDE HYDROCHLORIDE AND ITS FREE BASE

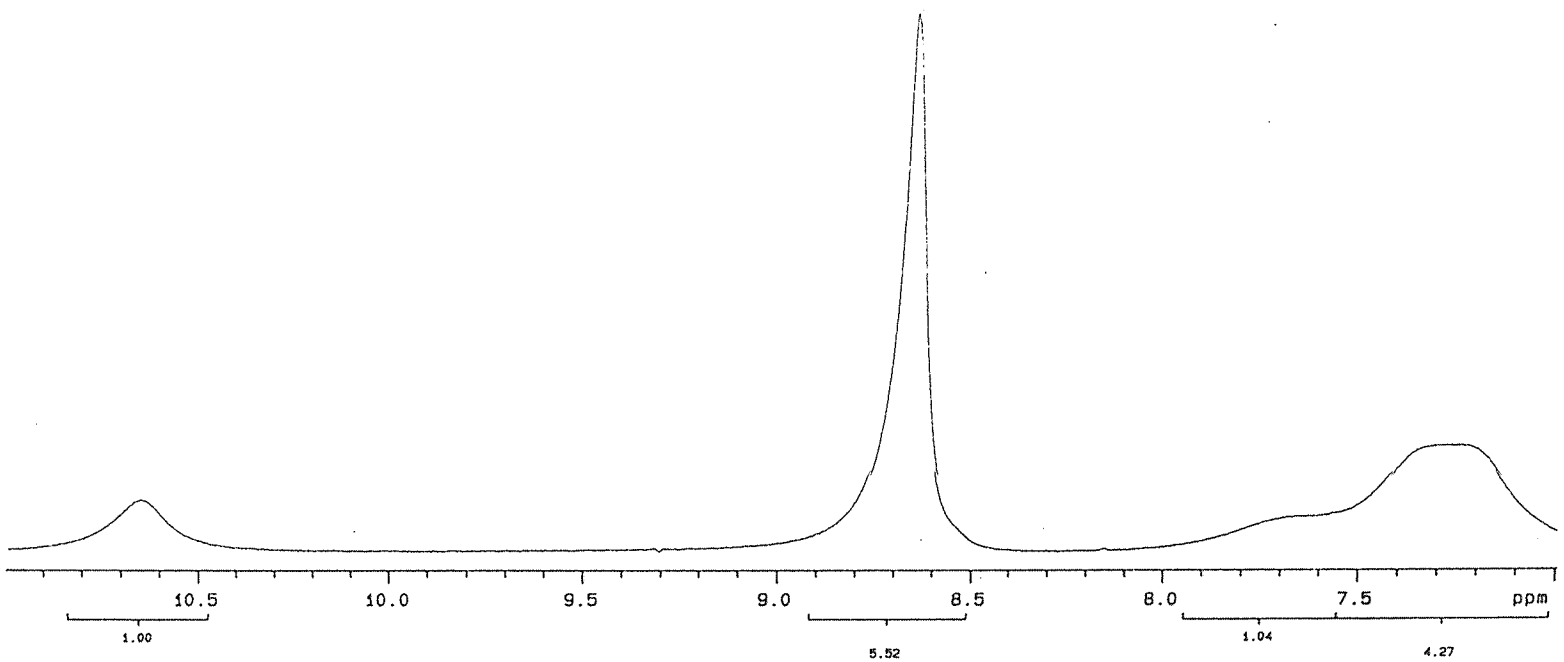


Figure 7. ^{1}H spectrum of amiloride HCl at 20°C

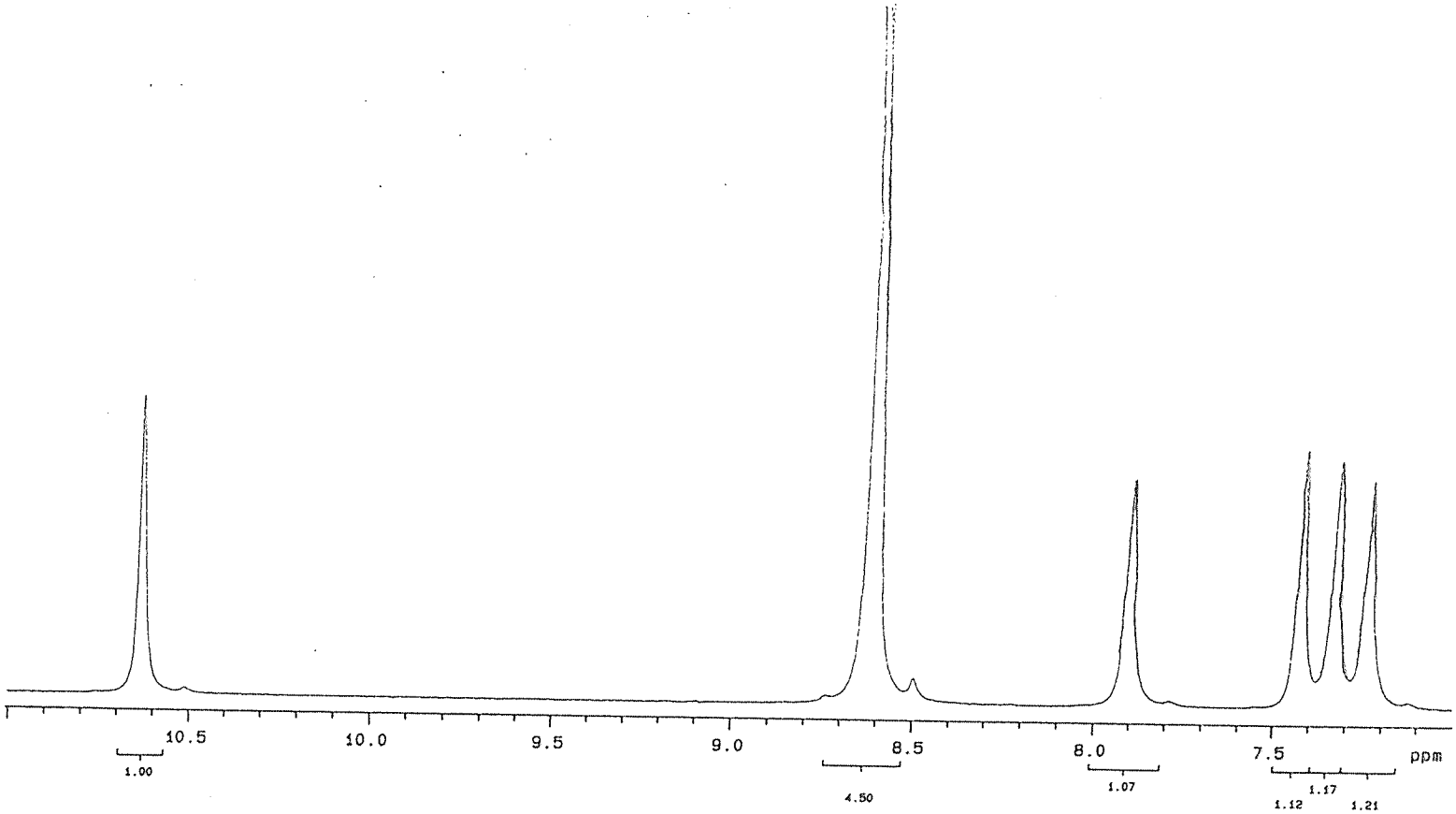


Figure 8. ^{1}H spectrum of amiloride HCl at -10°C

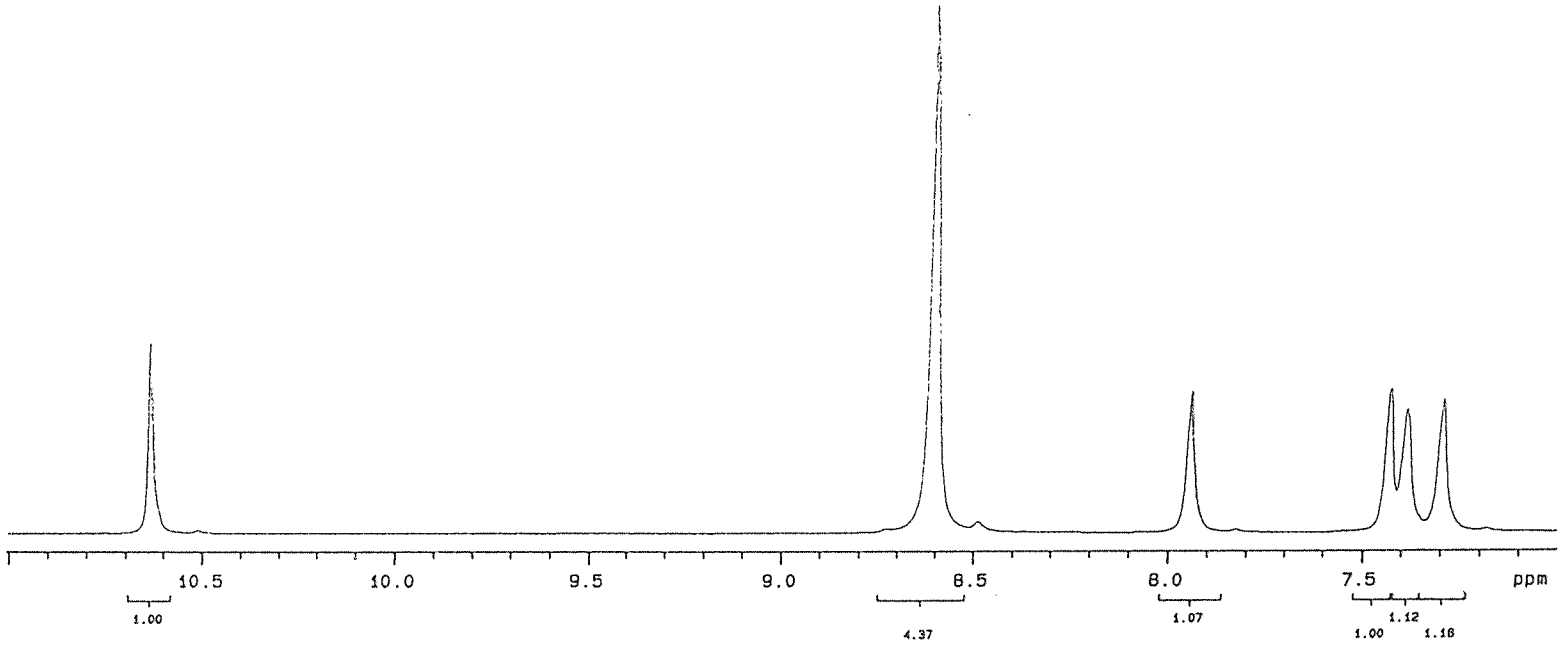


Figure 9. ^{1}H spectrum of amiloride HCl at -20°C

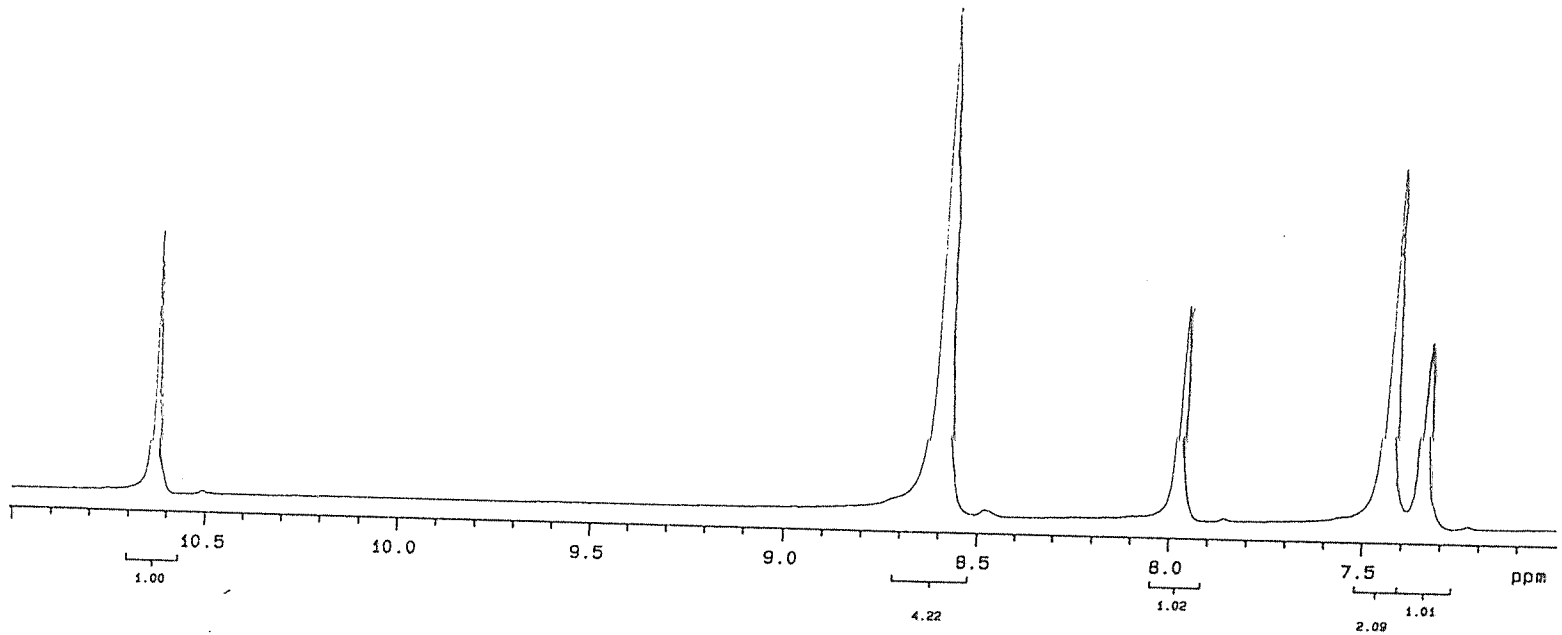


Figure 10. ^{1}H spectrum of amiloride HCl at -30°C

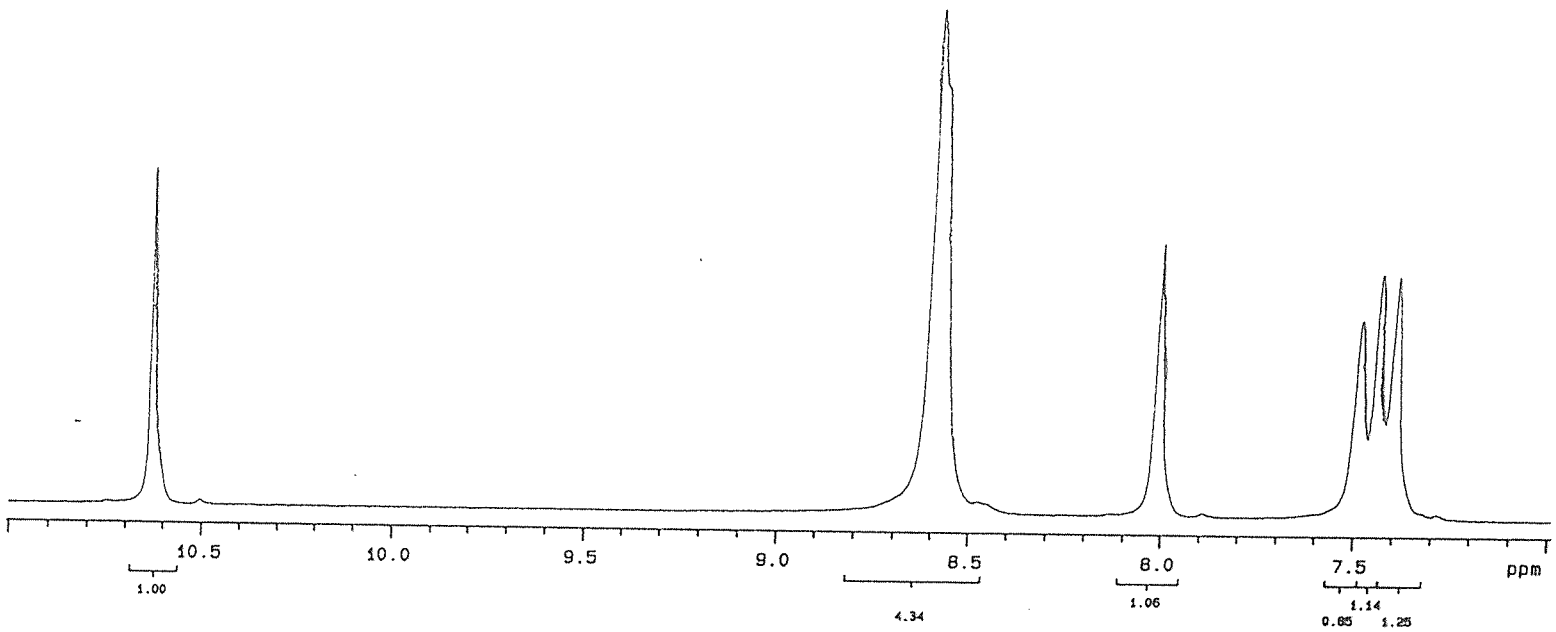


Figure 11. ^{1}H spectrum of amiloride HCl at -40°C

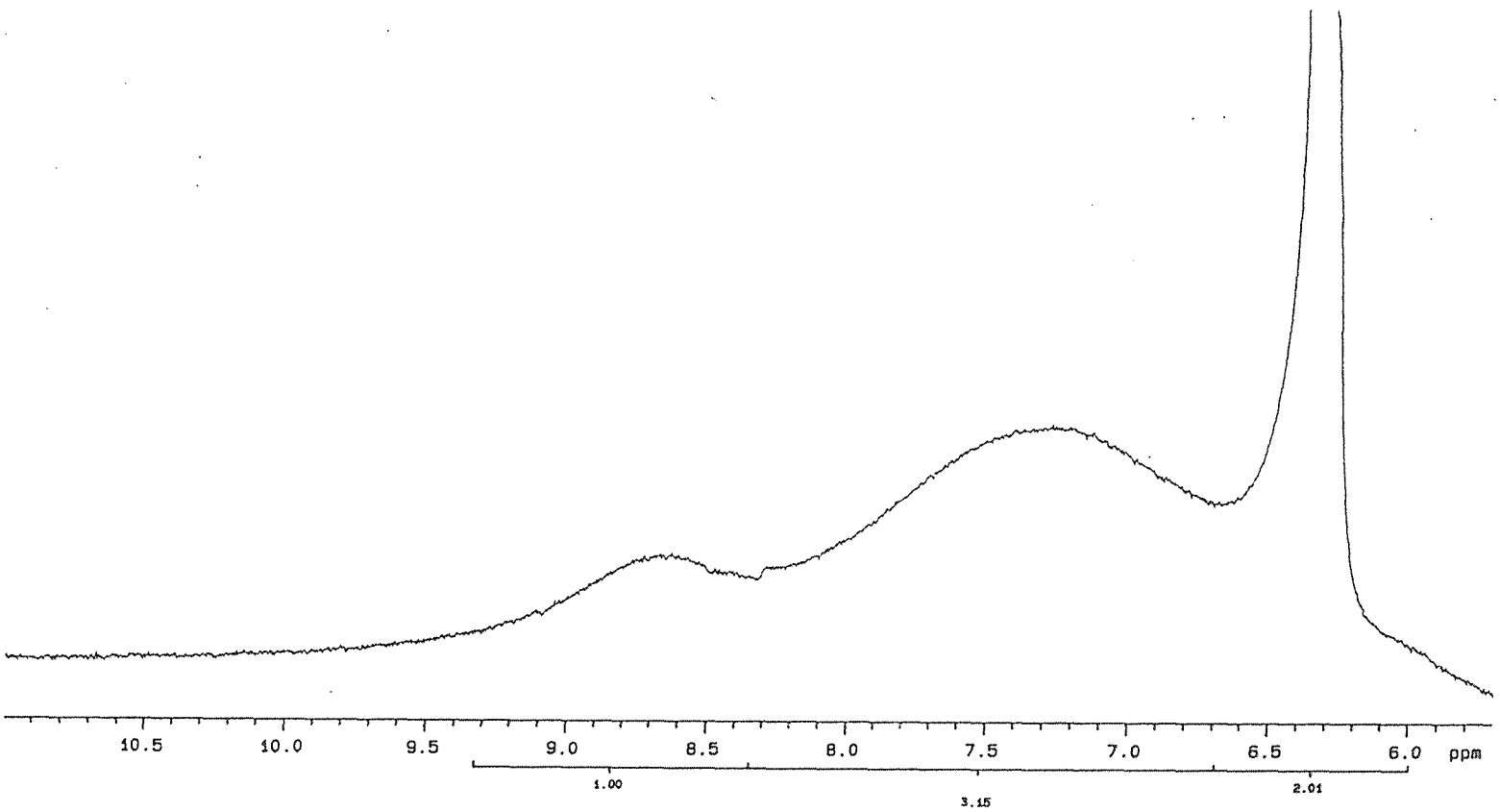


Figure 12. ^{1}H spectrum of amiloride free base at 19.6°C

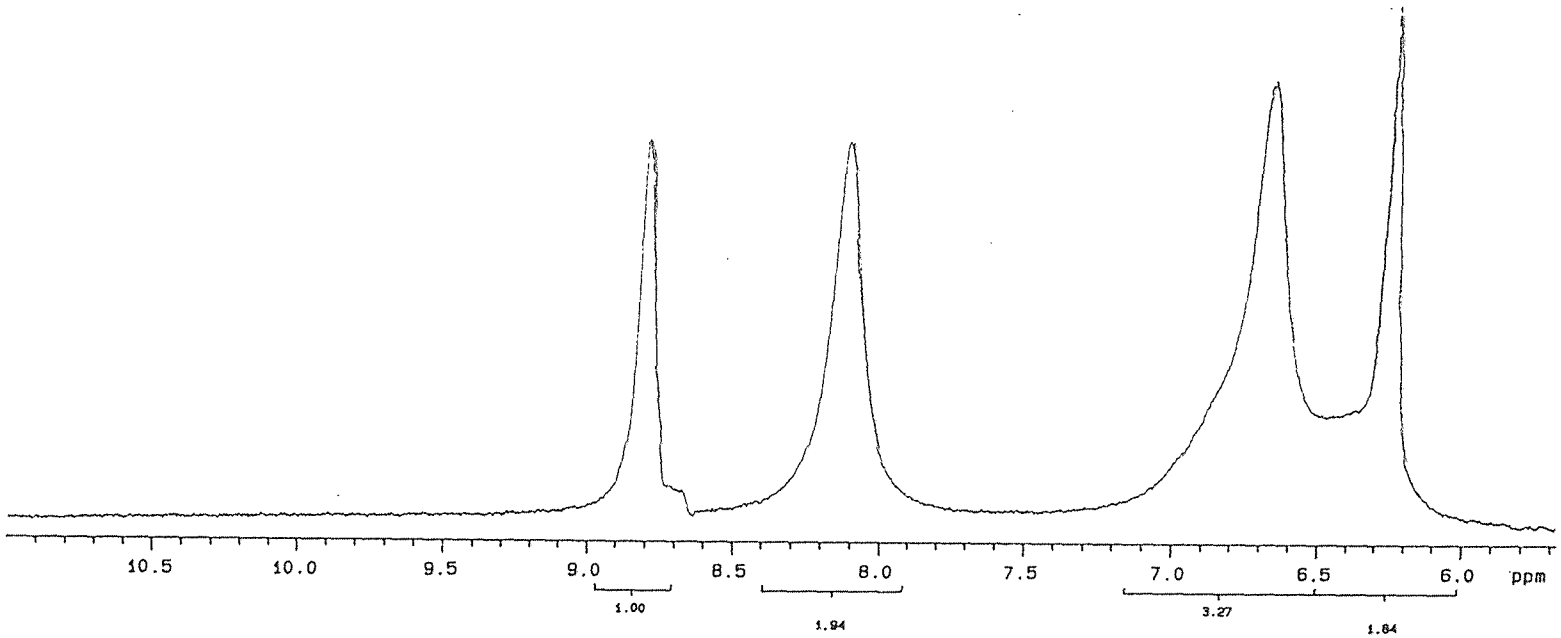


Figure 13. ^{1}H spectrum of amiloride free base at -20°C

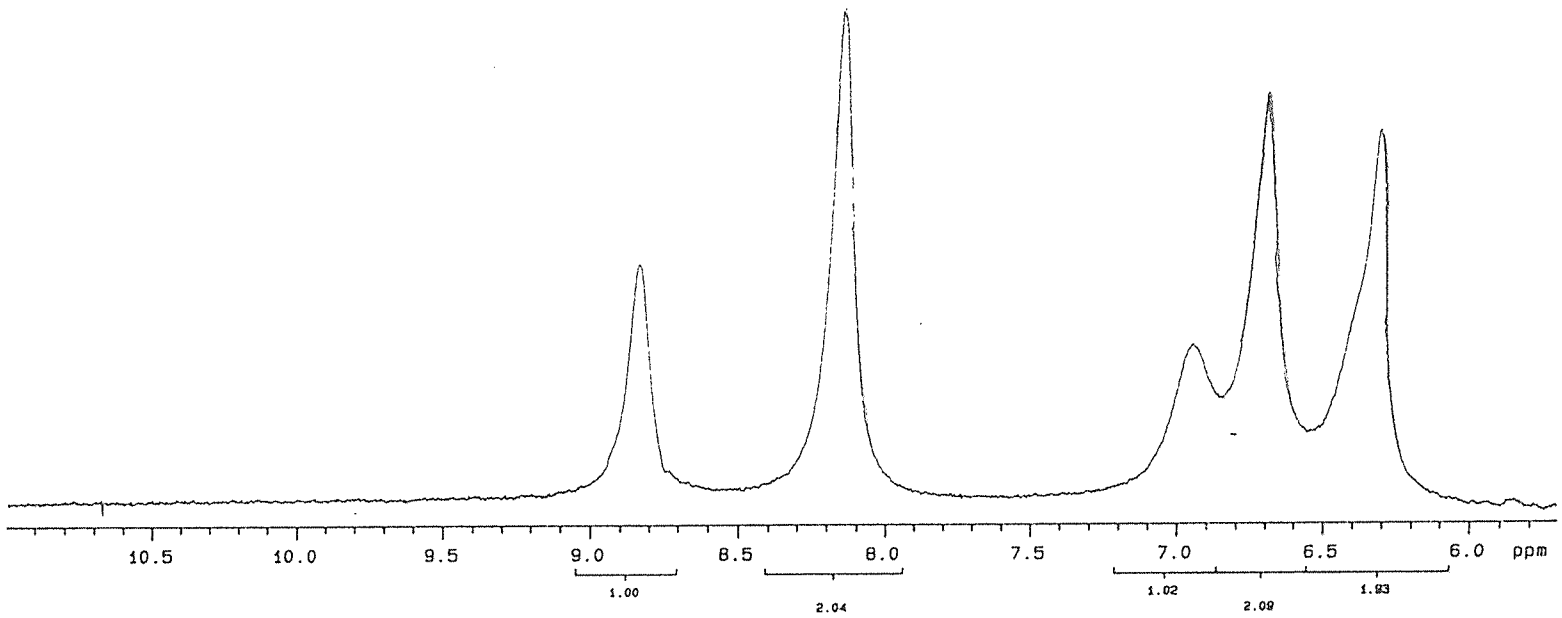


Figure 14. ^{1}H spectrum of amiloride free base at -30°C

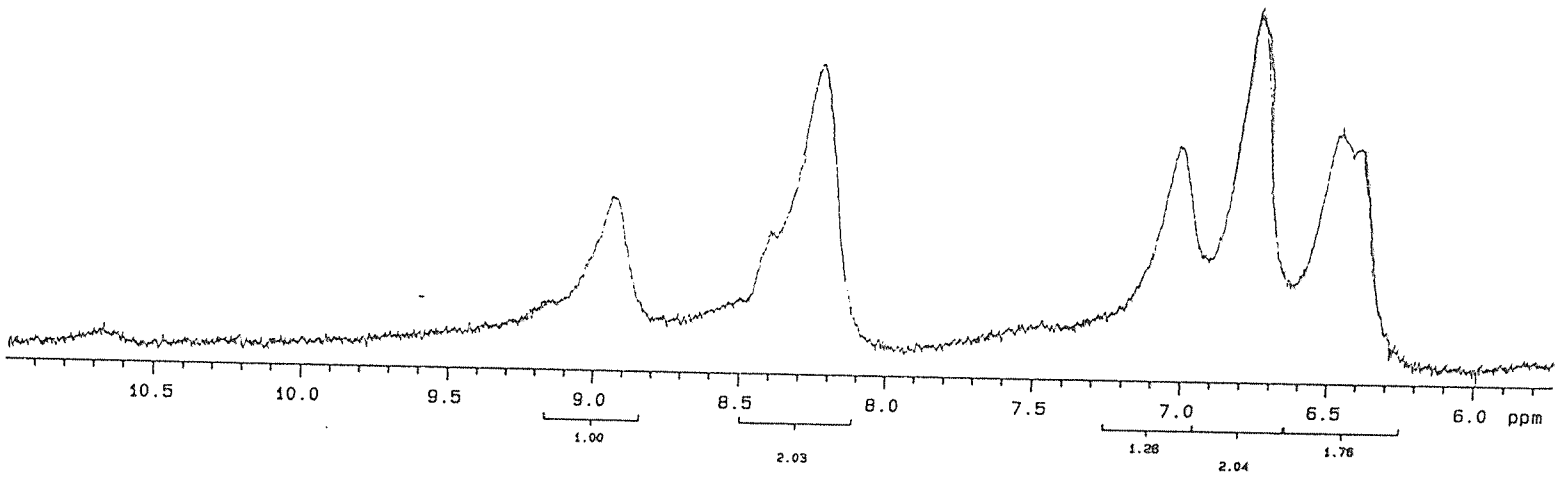


Figure 15. ^{1}H spectrum of amiloride free base at -50°C

REFERENCES

1. Brodlie, K. *Nuclear Instruments and Methods in Physics Research,* **1995**, *A 354*, 104.

2. Skawinski, W. J., Busanic, T. J.; Ofsievich, A. D., Luzhkov, V. B., Venanzi, T. J., Venanzi, C. A. *Information Technologies and Disabilities,* **1994**, (No 4, Article 6)

3. Kempfer, L. *Medical Device and Diagnostic Industry,* **1994.**

4. Jacobs, P. *Medical Device & Diagnostic Industry,* **November 1994**.

5. *Machine Design,* **July 1987**, *9*, 62.

6. Neckers, D. C. *Chemtech,* **October 1990**, *20*, 615.

7. Neckers, D. C. *Polymer Engineering and Science,* **October 1992**, *32*, 1481.

8. Maddox, R., Knesek, J. *Aerospace America,* **June 1993**, 28.

9. Kochan, D. *Computers in Industry,* **1992**, *20*, 133.

10. Gabriele, M. C. *Plastics Technology,* **June 1991**, 48.

11. Deitz, D. *Mechanical Engineering,* **February 1990**, 34.

12. Bryant, B. P., Leftheris, K., Quinn, J. V., Brand, J. G. *Amino Acids,* **1993**, *4*, 73.

13. Venanzi, T. J., Bryant, B. P., Venanzi, C. A. *Journal of Computer-Aided Molecular Design,* **in press**.

14. Breslow, R., Trainor, G., Ueno, A. *Journal of the American Chemical Society,* **1983**, *105*, 2739.

15. D'Souza, V. T., Hanabusa, K., O'Leary, T., Gadwood, R.C., Bender, M.L. *Biochemical Biophysical Research Communications,* **1985**, *129*, 727.

16. van Etten, R. L., Clowes, G.A., Sebastian, J.F., Bender, M.L. *Journal of the American Chemical Society,* **1967**, *89*, 3242.

17. Mallick, I. M., D'Souza, V.T., Yamaguchi, M., Lee, J., Chalabi, P., Gadwood, R.C., Bender, M.L. *Journal of the American Chemical Society,* **1984**, *106*, 7252.

18. Breslow, R. *Advances in Chemisty Series,* **1980**, *191*, 1.

19. Trainor, G. L., Breslow, R. *Journal American of the Chemical Society,* **1981**, *13*, 158.

20. Ueno, A., Breslow, R. *Tetrahedron Letters,* **1982**, *23*, 281.

21. Fujita, K., Tahara, T., Imoto, T., Koga, T. *Journal of the American Chemical Society* **1986**, *108*, 2030.

22. Luzhkov, V. B., Venanzi, C. A. *Journal of Physical Chemistry* **1995**, *99*, 2312.

23. Goodman, L. S., Gilman, A., Gilman, A.G. *Pharmacological Basis of Therapeutics,* eigth edition, Pergamon Press.: New York, **1990**, pp 1811.

24. Cragoe, E. J., Jr., Kleyman, T. R., Simchowitz, L. *Amiloride and Its Analogs: Unique Cation Transport Inhibitors*, VCH: New York, **1992**.

25. Li, J. H.-Y., Cragoe, E. J., Jr., Lindemann, B. *Journal of Membrane Biology,* **1985**, *83*, 45.

26. Li, J. H.-Y., Cragoe, E. J., Jr., Lindemann, B. *Journal of Membrane Biology,* **1987**, *95*, 171.

27. Smith, R. L., Cochran, D. W., Gund, P., Cragoe, E. J., Jr. *Journal of the American Chemical Society,* **1979**, *101*, 191.

28. Venanzi, C. A., Plant, C., Venanzi, T. J. *Journal of Computational Chemistry,* **1991**, *12*, 850.

29. Buono, V. T. J., Zauhar R.J., Luzhkov V.B., Venanzi C.A. *Journal of the American Chemical Society,* **1994**, *116*, 1502.

30 Skawinski, W. J., Venanzi, C. A. **in preparation**.

31. Venanzi, C. A., Plant, C., Venanzi, T. J. *Journal of Medicinal Chemistry,* **1992**, *35*, 1643.

32. Venanzi, C. A., Venanzi, T. J. *In Mechanisms of Taste Transduction*, S. A. Simon and S. D. Roper, Ed., CRC: Boca Raton, **1993**, pp 428.

33. Venanzi, C. A., Buono, R. A., Luzhkov, V. B., Zauhar, R. J., Venanzi, T. J. *In Structure and Reactivity in Aqueous Solution: Characterization of Chemical and Biological Systems*; C. J. Cramer and D. G. Truhlar, Ed.; American Chemical Society: Washington, D.C., **1994**, ACS Symposium Series 568, pp 260.

34. Ault, A., Dudek, G.O. *NMR: An Introduction to Proton Nuclear Magnetic Resonance Spectroscopy.*, Holden-Day: San Francisco, **1976**, pp 141.

35. Abraham R.J., Fisher. J., Loftus, P. *Introduction to NMR Spectroscopy*; John Wiley & Sons: New York, **1988**, pp 271.

36. Williams, D. A. R. *Nuclear Magnetic Resonance Spectroscopy*; John Wiley & Sons: London, **1986**, pp 272.

37. Wiberg, K. B., and Wong, M. W. *Journal of the American Chemical Society,* **1993**,*115*, 1078.

38. Wong, M. W., and Wiberg, K. B. *Journal of Chemical Physics,* **1991**, *95*, 8991.

39. Wong, M. W., Frich, M. J., and Wiberg, K. B. *Journal of the American Chemical Society,* **1991**, *113*, 4776.

40. Wong, M. W., Wiberg, K. B., and Frisch, M. J. *Journal of the American Chemical Society,* **1992**, *114*, 523.

41. Wong, M. W., Wiberg, K. B., and Frisch, M. J. *Journal of the American Chemical Society,* **1992**, *114*, 1645.

42. Betzel C., S. W., Hingerty B.E., Brown G.M. *Journal of the American Chemical Society,* **1984**, *106*, 7545.

43. Allen, F. H., Kennard, O., Taylor, R. *Accounts of Chemical Research,* **1983**, *16*, 146.

44. Stewart, J. J. P. *MOPAC-93 manual*, Fujitsu, Ltd.: Tokyo, Japan., **1993**.

45. Available from Molecular Simulations: Boston, MA.

46. *User reference manual. Software release 3.60.*, 3D Systems Inc., **June 1990**.

47. Skawinski, W. J., Busanic, T. J., Ofsievich, A. D., Venanzi, T. J., Luzhkov, V. B., Venanzi, C. A. *Journal of Molecular Graphics,* **1995**, *13*, 126.

48. Florey, K. *Analytical Profiles of Drug Substances.*, Academic Press.: New York, **1986**.

49. Clarke, E. G. C. *Clarke's Isolation and Identification of Drugs.;* Second edition; The Pharmaceutical Press.: London, **1986**, pp 1223.

50. Cholli, A. L., Lau, M.L. *Applied Spectroscopy,* **1995**, *49*.

51. Zhao, Y., Raymond, M.K., Tsai, H., Roberts, J.D. *The Journal of Physical Chemistry,* **1993**, *97*, 2910.