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SYNTHESIS AND ENZYME STUDIES USING CREATINE ANALOGS

by

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DISSERTATION

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To my parents

Mr. and Mrs. Nguyen Van Soan

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ABSTRACT

To probe the active site of creatine kinase (*E. C.* 2.7.3.2.), a number of new types of creatine analogs have been synthesized, fully characterized, and examined both as substrates and inhibitors: 3-amidinobuturic acid; R-, S-, and RS-C(α)-allylglycocyamine; N-amidino-N-cyanoethylglycine; N-amidinophenylglycine; N-amidinophenylalanine; guanidino-N,N-diacetic acid; 1-carboxymethyl-2-iminoimidazolidine-4-one; N,N'-diamidinoethylenediamine-N,N'-diacetic acid; 1,3-dicarboxymethyl-2-iminoimidazolidine; N-amidinoethylenediamine-N,N'-diacetic acid; tolazoline-N-acetic acid; 2-methyl-N-carboxymethylimidazole; and 2-methyl-N,N'-dicarboxymethylimidazole. These compounds have been found to be good competitive inhibitors for creatine kinase (K_i = 1-260 mM). 3-Amidinobutyric acid has been found to be an irreversible substrate (K_m = 7 mM) as well as a competitive inhibitor (K_i = 24 mM). Its K_m value is close to that of creatine (K_m = 9 mM) which indicates relatively tight binding to the enzyme. Its relative V_{max} is less than 1% as compared to creatine which shows the necessity of the nitrogen which has been replaced in the catalytic reaction. Those analogs that contain bulky groups are not well accepted by the enzyme. Compounds with additional electrostatic interactions and/or hydrogen

bonding bind well to the active site of the enzyme. Compounds which are analogs of cyclocreatine bind tightly to the enzyme (K_i = 1-36 mM). The cyclization of ethylenediamine derivatives with substituted cyanamide proved to be a useful method to synthesize cyclocreatine and N-substituted 2-iminoimidazolidine. A direct method for a high-yield synthesis of ethylenediaminemonoacetic acid, a precursor of cyclocreatine, is also presented.

The compound C(α)-(2,3-epoxypropyl)-glycocyamine (isoepoxycreatine) was also synthesized and preliminarily tested as an affinity label for creatine kinase. Incubation of creatine kinase with isoepoxycreatine completely inactivates the enzyme, and the inhibition follows pseudo-first-order kinetics. The enzyme shows a preference for the R-configuration at the α-carbon to the carboxylate group. All four isomers of isoepoxycreatine have been separated and spectrally analyzed by NMR, IR, GC-MS, and LSIMS. Selective heteronuclear decoupling and homonuclear decoupling allowed the determination of the NMR coupling constants and coupling patterns. Analysis of the coupling constants of vicinal methylene protons and ^{13}C NMR of the long-range three-bonded carbon-proton coupling of vicinal carbons established that the Karplus relations may be used to determine the absolute configurations of such chiral centers.

George L. Kenyon

TABLE OF CONTENTS

LISTS OF FIGURES

LISTS OF TABLES

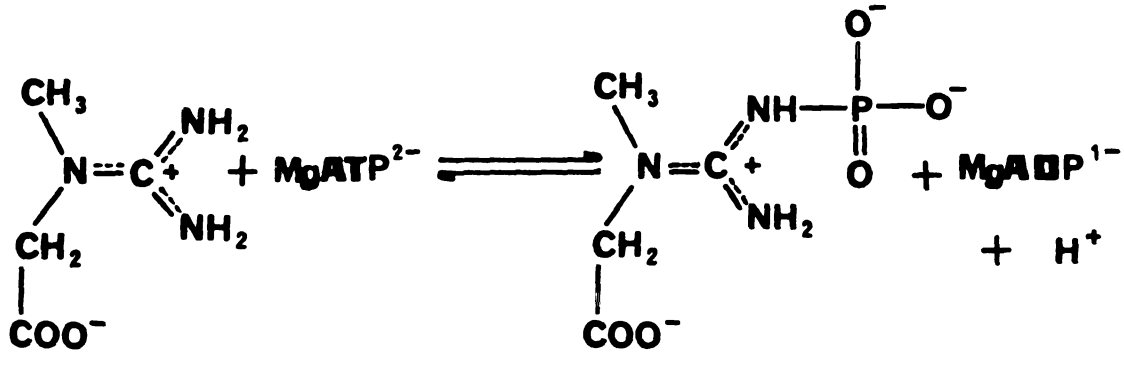
CHAPTER I

CREATINE KINASE REVIEW

INTRODUCTION

Creatine kinase (adenosine-5-triphosphate:creatine-N-phosphotransferase, E.C. 2.7.3.2.) is a very important enzyme in muscular metabolism (Bessman & Geiger, 1981; Lohmann, 1934). The enzyme has been found in high concentration in muscle, heart, and brain tissues of all vertebrates.

Creatine kinase catalyzes the reversible transfer of a phosphate residue between adenosinetriphosphate (ATP) and creatine as shown in the following reaction:



The reaction product, phosphocreatine, is a phosphoryl group donor of high group transfer potential and is an essential energy source for muscular contraction in vertebrates (Lipman, 1941; Fiske and Subbarow, 1929). The function of creatine kinase is, therefore, the rapid regeneration of ATP from phosphocreatine wherever high

levels of ATP are required for ATPase-dependent reactions. The enzyme otherwise maintains the concentration of ATP at a constant level in muscle tissue.

In an attempt to probe the active site of creatine kinase as part of a study of the mechanism of action of this enzyme, we have focused on the synthesis of creatine analogs as well as enzyme kinetic studies. With these specially designed synthetic substrate analogs, we will be able to see which functional groups of the substrate are most vital to the enzyme activity and also to see which functional groups can be permitted without loss of biological activity. The results of the substrate specificity studies are not only useful in probing the active site of enzyme but also in enabling us to control the activity of enzyme in the biological system by selective inhibition.

A large number of creatine analogs have been synthesized and tested as substrates or inhibitors by Kenyon and coworkers (Rowley et al.; Struve et al., 1977; Dietrich et al., 1980). In the present studies, new types of creatine analogs have been synthesized for such testing.

To date, a complete amino acid sequence of creatine kinase is not available. Even if it is available, amino acid sequencing does not give details about the three dimensional structure of the substrate-binding sites of

the enzyme. To fill in the lack of information concerning the active site of the enzyme, active-site-directed substrate analogs (affinity labels) have been designed. Affinity labels not only modify the amino acid residue of the active site, but also can give information about the geometry of the active site, where the catalytic reaction takes place.

CREATINE KINASE

The enzyme creatine kinase was reviewed thoroughly in 1973 by Watts (Watts, 1973). A brief review on the mechanism of action appeared in 1978 by Bickerstaff and Price (Bickerstaff & Price, 1978). A book about its clinical applications and pathology by Lang was published in 1981 (Lang, 1981). Recently, a chapter about structure-activity relationships was written by Kenyon and Reed, 1983 (Kenyon & Reed, 1983). The purpose of this chapter is to inform the reader who may be unfamiliar with creatine kinase.

Creatine kinase was first crystallized from rabbit skeletal muscle (Kuby et al., 1954). It was then isolated in pure form from human and many other vertebrates (Watts, 1973). While the levels of creatine kinase isoenzymes have been widely used in clinical settings for diagnosis of many diseases, most intensive investigations on the mechanism of the enzyme have used the enzyme isolated from rabbit skeletal muscle. This is also the source for the enzyme used in this thesis.

The catalytic reaction of creatine kinase is the transfer of a phosphoryl group from ATP to creatine. There is no evidence for any phosphorylated enzyme intermediate. It possesses two sites, one site for the nucleotide and another site for the guanidino substrate (Morrison &

James, 1965). Detailed kinetic studies also have shown that the catalytic mechanism is of the rapid equilibrium-random type with phosphoryl transfer in the rate-determining step for both directions, and that the presence of one substrate on the enzyme facilitates the binding of the second substrate (Morrison & James, 1965; Cleland, 1963).

It was further shown that two ternary complexes, namely enzyme-MgATP-creatine and enzyme-MgADP-phosphocreatine, have approximately the same free energy, that is, the equilibrium constant for the enzyme-bound substrates is nearly unity (Rao et al., 1979).

Creatine kinase requires a divalent metal cation for its catalytic reaction. While Mg^{2+} is the natural activator, other metal cations such as Mn^{2+}, Ca^{2+}, and Co^{2+} have also been found to be good activators. The V_{max} of the reaction with Mn^{2+} activator is approximately 80% of that of the natural activator, Mg^{2+}, in the forward direction. The first kinetic studies, performed by Kuby et al. in 1954 (Kuby et al., 1954) on rabbit muscle creatine kinase, showed a dependence of nucleotide upon the Mg^{2+} concentration. The authors found that MgATP and MgADP were active as substrates, whereas the free nucleotides were inactive. The true substrates of the enzyme should, therefore, be considered as the magnesium-nucleotide complex rather than just free nucleotides (ATP or ADP).

Creatine and phosphocreatine, on the other hand, failed to show any dependence of their activity on the Mg^{2+} concentration. Although the metal ion was bound to the enzyme as a complex with the nucleotide substrates (O'Sullivan & Cohn, 1966), it does not function as a bridging atom between substrate and the enzyme, as is the case with pyruvate kinase (Cohn, 1970). The role of the metal ion in the catalytic reaction has been investigated using spectroscopic studies of the transition state analog complexes (Reed et al., 1978; Reed & Leyh, 1980). Milner-White and Watts (1971) first reported certain small planar anions such as nitrate or formate stabilized a dead-end complex of creatine kinase which contained enzyme, creatine, and MgADP. The sizes and shapes of these anions suggested that they might mimic those of the planar phosphoryl group in the transition state of the catalytic reaction. Studies on the basis of this important finding revealed that the small anions occupied the same place of that normally occupied by the transfering γ-phosphoryl group of ATP, and that a complex is formed with a structure which resembles that of the transition state of the phosphoryl transfer reaction (Reed & Cohn, 1972; Reed & McLaughlin, 1973; James & Cohn, 1974; and McLaughlin et al., 1976). Since then, the complex of enzyme, creatine, nitrate or formate, and MgADP has been called the "transition state analog complex".

Magnetic resonance studies of the transition state analog complexes have revealed that the binding of anions to the dead-end complex with Mn^{2+} markedly affects the environment of the metal cation (Reed and Cohn, 1972; Reed and McLaughlin, 1973; and McLaughlin et al., 1972, 1976). Results of infrared spectral studies of the binding for NO_3^-, SCN^- and N_3^- with creatine kinase in the transition state analog complexes showed that these anions are directly bound to the metal ion in the quaternary complex (Reed et al., 1978). These authors also used ^{17}O superhyperfine coupling in EPR spectra for transition state analog complexes of creatine kinase to show that the activating metal ion is bound to all three phosphate groups in the transition state of the reaction (Reed and Leyh, 1980).

While the X-ray diffraction study of creatine kinase, which can give a clear picture of the active site, is still at a preliminary stage, some light has come to the active site of creatine kinase through the substrate specificity studies (Rowley et al., Dietrich et al., 1980). The structural information of the arrangement of the substrates and changes in conformation upon binding of substrates have relied on magnetic resonance studies. In order to use this method, it is necessary to introduce a paramagnetic metal ion such as Mn^{2+} or Co^{2+} in the enzyme's active site. These paramagnetic metal ions serve

as reference points to measure distances to substrate nuclei. The insertion of a second paramagnetic center or an organic spin label at the nearby -SH group in the active site allows the calculation of the distances of a three dimensional structure of creatine kinase-substrate complexes (Taylor et al., 1971; McLaughlin et al., 1976). Results have shown that the nucleotide and guanidino substrates are aligned at the active site of the enzyme such that the transferable phosphate group on one substrate is in apposition to the acceptor group of the second substrate (McLaughlin et al., 1976). Hansen and Knowles (1981) have also obtained stereochemical evidence to support such an "in-line" mechanism.

Because of the lack of structural information about creatine kinase, efforts have been extended to identify specific groups on the enzyme active site which are involved in the catalytic reaction. Most of the efforts involved the chemical modification of the amino acid side chains. The reactive -SH group at the active site has been the main subject for the chemical modification studies. Modification of this group can completely inhibit the enzyme (Reviewed by Kenyon and Reed, 1983). Other studies involved the chemical modification of a lysine residue (Clark and Cunningham, 1965; Lui and Cunningham, 1966; James and Cohn, 1974), an arginine residue (Borders and Riordan, 1975; Jame, 1976), and a histidine residue

(Pradel and Kassab, 1968).

The enzyme from rabbit skeletal muscle consists of two identical subunits with a total molecular weight of about 82,000 (Watts, 1973). The identity of two subunits was proposed from the functional behavior of the enzyme (Bickerstaff and Price, 1978). Each subunit with a molecular weight of about 41,000 is a single polypeptide chain containing 360 amino acids without a disulfide bridge. Each has one catalytic site with separate substrate binding sites and shows its own enzymatic activity. Creatine kinase can be dissociated into subunits by agents such as urea, guanidinium chloride, and sodium dodecylsulfate (Yue et al., 1967). Removal of the denaturing agents by dilution or dialysis leads to reassociation of the subunits and regaining the initial enzymatic activity (Dawson et al., 1967; Bickerstaff and Price, 1977).

Bickerstaff and Price (1977) used studies on matrix-bound derivatives of creatine kinase to propose that the dimeric structure of the enzyme was not required for catalytic activity. With the enzyme modification studies of the reactive thiol group on each subunit and the equilibrium dialysis technique for ADP binding, Price and Hunter (1976) showed that the subunits of the enzyme behave non-identically in the "transition state analog complex". Using the same method of study, Degani and

Degani (1979) and Denisov et al. (1982) have sugested that the functional non-identity of the subunits could arise from asymmetrical association of subunits or conformational changes induced by nucleotide binding. With a fluorescent analog of ATP: γ-(P-azidoanilide)-1-N^6-etheno-ATP, Denosov et al. (1982) found that one of the subunits is modified more rapidly than the other, but the difference in the rates of modification is only slight. Nevinsky et al. (1982) studied the differences of the subunits in affinity chromatography, electrophoretic mobility, stability, and pH-dependent activity and indicated that the subunits have non-identical active centers and that the functional non-identity of the subunits may reflect various catalytic rates of the transphosphorylation process.

In all vertebrates, especially in human tissues, there are two different kinds of subunits: muscle type subunit M, and brain type subunit B. Creatine kinase from human tissue contains 3 forms: muscle type isoenzyme CK-MM, brain type isoenzyme CK-BB, and myocardial type or hybrid type isoenzyme CK-MB. These forms were separated by their differences of their electrophoretic mobility toward the anode. Exchange of subunits among these isoenzyme occurs both in vitro (Keutel et al., 1972) and in vivo (Levy and Lum, 1975).

CK-MM + CK-BB ⟷ CK-MB + CK-BB + CK-MM

The method of hybridization used to prepare CK-MB is more practical than isolating the native CK-MB from human myocardium (Keutel et al., 1972).

In addition to the anodic migrating isoenzyme, a fourth band, which migrates toward the cathode in the electrophoretic separation of CK isoenzymes from human heart, was reported by Jacobs et al. in 1964. This band was a dimer of two identical subunits but did not show hybridization reaction with CK-MM and CK-BB. Its amino acid composition, aminoterminal amino acid, electrophoretic mobility, and certain immunological properties were all different from those of the other three CK isoenzymes (Lang and Wurzburg, 1982). This isoenzyme, designed as CK-Mt (mitochondrial CK isoenzyme), is, therefore, considered as a fourth creatine kinase isoenzyme.

In the last several years, with increasing numbers of electrophoretic CK isoenzyme separations, bands which exhibit their mobilities different from those of CK-MM, CK-MB, and CK-BB have been detected. These bands were found in "one per thousand" cases and designated as "variant", "atypical" or "macro-creatine kinase" isoenzymes. Their mobilities can be cathodic or anodic. The anodic band which was detected between CK-MM and CK-

BB, is called type 1 macro-CK. In most cases, macro-creatine kinase type 1 is a complex between CK-BB and immunoglobulin (IgG) (Urdal et al., 1980; Leroux et al., 1977; Sax et al., 1979; Bohner et al., 1982; and Meyer et al., 1980). The cathodic form is not an immunoglobulin complex but is closely related to mitochondrial creatine kinase, and for differentiation has been called type 2 macro-CK (Stein et al., 1982; James & Harrison, 1979). Macro-CK type 2 is not inhibited by anti-CK-MM or BB and also shows no cross reactivity toward immunoglobulins (Stein et al., 1982; Bayer et al., 1982).

The high levels of creatine kinase isoenzymes in serum have been widely used as indicators of muscular damage. Since the CK-MB isoenzyme in normal serum is negligible, its appearance in the circulation is a very specific indicator of myocardial infarction. The CK-MB isoenzyme was characterized as being cardiac specific and is only detected after acute myocardial infarction (Sjovall, 1964; and Van der Veen and Willebrand, 1966).

In many countries, screening of CK is performed for early diagnosis of Duchenne muscular dystrophy in newborns. Five to 6% of total CK activity was due to CK-MB in the case of Duchenne muscular dystrophy (Prellwitz, 1981).

Creatine kinase activity in the central nervous system is relatively high and is almost entirely due to the CK-BB isoenzyme. The CK-BB isoenzyme can be released

from tissues into the circulation. Due to its short half-life, CK-BB activity decreases very rapidly in the serum, and, therefore, in the serum of a normal person the CK-BB activity is very low and below the detection limit of most methods. As a result of disease or brain damage with concomitant breakdown of blood brain barrier, CK-BB can be released into the blood stream and its activity can be detected. The CK-BB activity has been measured in the sera of patients with cerebral diseases as well as diseases of the central nervous system, brain tumors, traumatic brain damage, and epilepsis (Prellwitz, 1981; Kaste et al., 1977; Phillips et al., 1980; Bell et al., 1978; Thompson et al., 1980).

ENZYME ASSAY

Creatine kinase catalyzes the reversible phosphorylation of creatine by ATP with the formation of phosphocreatine, ADP, and a proton:

$$\text{Creatine} + \text{MgATP}^{2-} \xrightleftharpoons{\text{CK}} \text{P-creatine}^{2-} + \text{MgADP}^{-} + \text{H}^{+}$$

Determination of creatine kinase activity in the forward reaction is through determination of the formation of either phosphocreatine or ADP or H^+. The reverse reaction, on the other hand, leads to formation of creatine and ATP as products, with the consumption of a proton, H^+.

a) FORWARD REACTION

i. pH-stat Method:

This is a very well-known and simple method for the assay of many enzymatic systems which generate a proton in their reactions (Cho et al., 1960; Mohowald et al., 1962). The initial rates of the enzymatic activity are measured by the continuous consumption of NaOH. This method has been used in our kinetic assays, and will be described in detail in the next chapter.

ii. Enzyme-coupled Method:

In this assay, the rate of ADP formation has been

measured by the coupling of the pyruvate kinase (PK) and the lactic dehydrogenase (LDH) reactions as follows:

$$\text{creatine} + \text{ATP} \xrightarrow{\text{CK}} \text{phosphocreatine} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

The decrease in the absorbance at 340 nm caused by the oxidation of NADH to NAD^+ can be followed by a UV spectophotometer. This method was first developed by Tanzer and Gilvarg (1959) and has been widely used. The procedure has been modified in our laboratory. Details will be discussed in the next chapter.

iii. Other Methods:

Several other methods have been reported for the estimation of creatine kinase activity based on the determination of inorganic phosphate after the hydrolysis of the phosphocreatine product (Askonas, 1951; Kuby et al., 1954; and Read & Rehorayan, 1959), or based on the determination of creatine consumption by its color reaction with diacetyl (Ennor and Stocken, 1953; Ennor and Rosenberg, 1954).

b) REVERSE REACTION:

i. pH-stat Method:

This method once again has been used for the reverse reaction by following the addition of perchloric acid (Milner-White and Rycroft, 1983).

ii. Enzyme coupled Method:

This sensitive method was first developed by Oliver (1955) and then refined by others (Rosalk, 1967; Nielson and Ludwigsen, 1963; and Swanson and Wilkinson, 1972). The method uses hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH) as coupled enzymes in the following reactions:

$$\text{phosphocreatine} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADPH}$$

The rate of the appearance of NADPH is indicated by the increase in the absorbance at 340 nm and followed by a spectophotometer.

iii. Other Methods:

Color reactions of creatine product with diacetyl and α-naphthol can also be used to determine the creatine

formed by the reverse reaction (Ennor and Stocken, 1953; Chappell and Perry, 1954; Dreyfus and Schapira, 1961; Ennor and Rosenberg, 1954; and Hughes, 1962).

Yashara et al. (1982) reported a new enzymatic method to determine creatine, especially in serum and urine, using creatine amidinohydrolase, sarcosine oxidase, and formaldehyde dehydrogenase. In this method, creatine is degraded to urea and sarcosine. The latter is measured with sarcosine oxidase and formaldehyde dehydrogenase in the presence of NAD^+. The absorption of NADH generated can be measured at 340 nm.

Recently, Tsuchida and Yoda (1983) proposed a multi-enzyme membrane electrode system for the determination of creatine in serum. The method involves two enzymes: creatine aminohydrolase and sarcosine oxidase. The hydrogen peroxide generated from the system is then detected with a polarographic electrode. This method is an alternative to the Jaffe alkaline picrate method. Another widely-used method for the determination of creatine in clinical laboratories is the Folin Method, which is also based on Jaffe reaction (Folin, 1904). However, this method is more time consuming and less sensitive than enzymatic methods.

Another sensitive method to determine both ATP and ADP is the PEI-cellulose thin layer chromatographic method which was proposed for creatine kinase assay by Rowley and

Kenyon (1974). This method serves as a rapid assay for screening creatine analogs in the creatine kinase reactions. Details of this method will be described in chapter II.

CHAPTER II

SYNTHESIS OF CREATINE ANALOGS

INTRODUCTION

The primary function of the well known enzyme creatine kinase is to supply ATP, which is involved in the mechanism of muscle contraction. Creatine kinase catalyzes the phosphoryl transfer reaction between creatine and ATP in the forward reaction and between phosphocreatine and ADP in the reverse reaction. The enzyme has an important role in the clinical diagnosis of many fatal diseases. The levels of creatine kinase isoenzymes in serum are strong indicators of myocardial infarction, Duchenne-type muscular dystrophy and many brain diseases including brain tumors (Lang, 1980).

Because of its importance in such biological systems, it is desirable to know about the mechanism of action of the enzyme and how to control its action. In an attempt to probe the active site of creatine kinase as part of the studies of mechanism of action for this enzyme, a large number of analogs of creatine have been synthesized and tested as substrates. One of those analogs, cyclocreatine (1-carboxymethyl-2-iminoimidazolidine), was shown to be a potential substrate for creatine kinase with a relative V_{max} of 90 percent of that of the natural substrate creatine (Rowley *et al.*, 1971; McLaughlin *et al.*, 1972).

Cyclocreatine has been introduced into biological systems and has been found to be a potential cardiac agent (Roberts and Walker, 1983; Walker, 1979; Woznicko and

Walker, 1980).

We have focused on the synthesis of new types of creatine analogs for similar studies. The present work includes the chemical synthesis of 15 new creatine analogs, including analogs which resemble that of cyclocreatine. Enzyme kinetic studies are described in Chapter III.

We report a simple method to synthesize ethylenediaminemonoacetic acid, a synthetic precursor of cyclocreatine, as a free base, with an excellent yield. We also report a new method to synthesize cyclocreatine and a general method to synthesize cyclic guanidines.

EXPERIMENTAL PROCEDURES

MATERIALS

The following starting compounds for the syntheses were purchased from Sigma Chemical Company and used as received: D-C(α)-phenylglycine, D-C(α)-phenylalanine, ethylenediamine-N,N'-diacetic acid, D,L-C(α)-allylglycine, D-C(α)-allylglycine, and L-C(α)-allylglycine.

The following starting compounds were purchased from Aldrich Chemical Company and used as received: tolazoline.HCL salt, iodoacetic acid, 2-methylimidazole, aminodiacetic acid, cyanoethylglycine, ethylenediamine, and ethyl crotonate.

Cyanamide was also obtained from Aldrich Chemical Company. The commercial compound contained a trace of stabilizer and was not suitable for reaction. It was freshly made from the commercial mixture by extraction by ether. The solvent was then removed using a rotary evaporator. The cyanamide was prepared just before reaction to avoid polymerization.

METHODS

Nuclear magnetic resonance spectra were determined on either Varian FT-80 spectrometer or a 240 MHz widebore

spectrometer equipped with a Nicolet 1180 Data System, Cryomagnet Systems for magnet and probes, and home-built electronics. For most creatine analogs, D_2O was used as solvent. Either DSS (sodium-2,2-dimethyl-2-silapentane-5-sulfonate) or TSP (3-trimethylsilyl-tetradeutero sodium propionate) were used as internal standards for proton NMR. With ^{13}C NMR, dioxane (67.4 ppm) was used either as internal or external standard in D_2O solvent.

GC-Mass spectra was performed on a Kratos MS-25 instrument equipped with a Varian Capilliary GC System and a OV-1 or a SE-52 column at the Mass Spectrometry Recource, School of Pharmacy, University of California, San Francisco.

All melting points are uncorrected.

Microanalysis were carried out by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

SYNTHESIS

CARBOCREATINE (3-AMIDINOBUTYRIC ACID) (1)

Synthesis of Ethyl-3-bromobutyrate

Ethyl crotonate (76 g) was dissolved in 50 mL of glacial acetic acid with stirring at 0° C using an ice-water bath. While stirring, HBr gas was introduced into

the reaction flask for 3 hr. The reaction mixture was then dissolved in 300 mL H_2O and extracted with ether. It was dried over anhydrous Na_2SO_4 before evaporation in vacuo. Product (103 g, 80% yield) was collected, bp 72° C, 12 mmHg.

^1H NMR in $CDCl_3$ with TMS as internal standard gave peaks at δ 1.27 (triplet, J = 7 Hz), 1.74 (doublet, J = 7 Hz), 2.87 (doublet, J = 7 Hz), 4.17 (quartet, J = 7 Hz), and 4.45 (sextet, J = 7 Hz).

Synthesis of Ethyl-3-cyanobutyrate

In a 1-L round-bottomed flask fitted with a magnetic stirrer and an addition funnel were placed 300 mL of 95% EtOH and 103 g (0.528 mole) of pure ethyl-3-bromobutyrate. The mixture was stirred at 75° C. In another flask, 69 g (1.056 mole) of KCN was dissolved in a minimum amount of hot water and stirred until the cyanide was completely dissolved. This solution was then added dropwise. The reaction mixture quickly turned to a dark red color and was stirred for 4 hr at 75° C. The flask was allowed to cool, and then the reaction mixture was poured into 1 L of ice water in a 2-L flask. The organic compounds were extracted with several 250 mL portions of ether. The extracted solution was washed with a minimum amount of ice-cold water and then dried over anhydrous Na_2SO_4. It

was filtered by gravity, and the filtrate was evaporated *in vacuo* before distilling. The yield of the product, which boiled at 102° C and 12 mmHg, was about 60% of theoretical amount based on the bromide compound used.

^1H NMR in $CDCl_3$ and 1% TMS showed peaks at δ 1.27 (3H, triplet, J = 7 Hz), 1.37 (3H, doublet, J = 7 Hz), 2.60 (2H, doublet-doublet, Js = 7 and 3.4 Hz), 3.05 (1H, septet, J = 7 Hz), 4.18 (2H, quartet, J = 7 Hz).

Synthesis of Carbocreatine (3-amidinobutyric acid) (1)

Method A

Ethyl-3-cyanobutyrate (4.794 g, 0.034 mole) was dissolved in 20 mL of ether and 3 mL of fresh distilled absolute EtOH in a 100 mL round-bottom flask. The reaction mixture was cooled to 0° C with an ice-water bath and stirred with a small magnetic stirring bar. An excess of HCl gas was slowly introduced into the solution for 4 hr while stirring at 0° C. The reaction mixture was allowed to stand for a few days. HCl gas was then blown off as much as posible by N_2 gas. MeOH was added several times to remove HCl. In a separate flask, MeOH (20 mL) was cooled in an ice-water bath before ammonia gas was added. This flask was weighed before and after the addition of ammonia gas. A total of about 2 g of NH_3 was used. This NH_3-MeOH mixture was then added to the reaction flask. It was stirred for 10 min. and then opened to the air for 20 min.

before closing again to stand for another day. The product appeared the next day, and more than 50% yield was collected by filtration. The compound was recrystallized twice to give a sharp melting point, 266-267° C (dec.).

Method B

Ethyl-3-cyanobutyrate (2.83 g, 0.02 mole) and pre-dried MeOH (1.9 g) were mixed together in a round-bottom flask which was cooled in an ice-water bath. The flask which contained the mixture was weighed carefully before the addition of HCl gas. After the addition of 1.56 g of HCl gas, the reaction flask was then closed and stored in a refrigerator for 3 days. White solid formed in about 12 hr. Ether was then added to the reaction flask, and the flask was placed for another day in a refrigerator. The flask was inserted into a dry-ice bucket for few hours before filtering under N_2 gas. Intermediate product (3.8 g) was collected and dissolved again in 10 mL of MeOH. In a separate round-bottom flask, MeOH (10 mL) was kept cold while introducing of 0.35 g NH_3 gas. This mixture of NH_3-MeOH was then added to the reaction flask which contained the intermediate product and MeOH. The white solid precipitated right after mixing them together and redissolved after few minutes of swirling. The mixture was left for 30 min. at room temperature. Both the solvent and excess NH_3 gas were removed by blowing N_2 gas through the

mixture with warming. NH_4Cl precipitated and was removed by filtering. Ether was added to the filtrate to precipitate the final product. A quantitative amount of product was collected by filtration and washed with MeOH. It was then recrystallized several times to a sharp melting point of 266-267° C (dec.). The pure product was barely soluble in water (1.3 g/ml) and was insoluble in organic solvents.

^1H NMR in D_2O showed peaks at δ 1.27 (3H, d, J = 7 Hz), 2.51 (2H, septet, J = 7 Hz), 2.96 (1H, sextet, J = 7 Hz)

GC-Mass spectrum gave peaks at m/e 112 $(M-18)^+$, 97 $(M-18-15)^+$ and a base peak at m/e 42 (HN=C=NH).

Anal. Calcd. for $C_5H_{10}N_2O_2$: C, 46.14; H, 7.74; N, 21.52. Found: C, 45.91; H, 7.58; N, 21.48.

DL-C-ALLYLGLYCOCYAMINE (4)

DL-C-Allylglycine (11.51 g) was added in small portions to a solution of cyanamide (7.98 g) dissolved in 10 mL of water. Each addition of the C-allylglycine was followed by the addition of several drops of 58% aqueous ammonia. Totals of 20 mL of water and 20 mL of aqueous ammonia were used. The reaction mixture was allowed to stand at room temperature for 12 hr. The crystals that had formed were filtered to give DL-C-allylglycocyamine (7.7

g, 49% yield). The crude product was recrystallized in water and washed with acetone. The pure compound, mp 234° C, is slightly soluble in water and insoluble in any organic solvents.

Anal. Calcd. for $C_6H_{11}N_3O_2$: C, 45.85; H, 7.05; N, 26.73. Found: C, 45.68; H, 6.98; N, 26.55.

D- and L-C-ALLYLGLYCOCYAMINE (2) and (3)

For each 2 g of D- and L-C-allylglycine, 2 g of cyanamide in 5 mL of water and 4 mL of aqueous ammonia were used as described above. The white crystals were filtered after 12 hr of reaction. The reaction using a large excess of cyamamide yielded the dimer of cyanamide as an impurity in the product. This dimer was separated from the product using the following HPLC method. With reverse phase M-9, C-18, ODS-3, 10μ column and water as solvent at a flow rate of 4 mL/min., the retention times of dimer of cyanamide and of the product are 4.4 min. and 7.4 min., respectively. Crude D-isomer (48% yield) and crude L-isomer (59% yield) were collected, respectively. The solids were then recrystallized by water to sharp melting points of 247-248° C (D-isomer) and 248-249° C (L-isomer).

Anal. Calcd. for $C_6H_{11}N_3O_2$: C, 45.85; H, 7.05; N, 26.73. Found: (L-isomer): C, 45.84; H, 7.08; N, 26.85.

(D-isomer): C, 45.72; H, 7.09; N, 26.88.

As expected, both the D-, L- isomers and the racemic mixture of C-allylglycocyamine all had the same spectral properties (proton NMR, ^{13}C NMR, GC-MS, and IR).

Proton NMR in D_2O showed peaks at δ 2.48-2.66 (2H, m), 3.99-4.04 (1H, d-d), 5.15-5.23 (2H, m), 5.69-5.83 (1H, m, Js = 17.15, 10.08, and 7.01 Hz).

Proton-decoupled ^{13}C NMR in D_2O showed peaks at δ 177.48, 157.26, 133.54, 119.71, 56.88, and 36.90.

GC-MS showed peaks at m/e 28, 43, 98 (base peak), 110, 111, 139 $(M-18)^+$, 140 $(M-17)^+$.

N-AMIDINO-N-(CYANOETHYL)GLYCINE (5)

Cyanamide (8.41 g) was dissolved in 30 mL of water with few drops of aqueous ammonia added. Cyanoethylglycine (25.63 g) was added in small portions with shaking. By the end of the addition, a total of 10 mL of 58% aqueous ammonia was used. The mixture was then heated with a heat gun. Just after the hot solution was filtered, product started to form. Product (19 g) was then collected by filtration, mp 230° C (dec.). It was slightly soluble in H_2O.

^1H NMR in D_2O-DCl showed peaks at δ 4.37 (2H, singlet), 3.81 (2H, triplet, J = 7 Hz), 2.89 (2H, triplet, J = 7 Hz).

Anal. Calcd. for $C_6H_{10}N_4O_2$: C, 42.35; H, 5.92; N,

32.92. Found: C, 42.27; H, 5.95; N, 32.72.

N-AMIDINO-D-PHENYLGLYCINE or C-D-PHENYLGLYCOCYAMINE (6)

Cyanamide (2.6 g, 0.06 mole) was dissolved in a minimum amount of water. Phenylglycine (7.71 g, 0.05 mole) was then added in small portions with shaking. Aqueous ammonia (58%) was needed to clear the mixture after each addition. The reaction flask was tightly closed, and, after a few days, crystalline product formed. Filtration gave approximately an 80% yield of N-amidino-D-phenylglycine. The solid was then recrystallized using a hot H_2O-NH_3 mixture to a melting point of 250° C (dec.).

Anal. Calcd. for $C_9H_{11}N_3O_2.H_2O$: C, 51.18; H, 6.20; N, 19.89. Found: C, 51.30; H, 5.88; N, 19.73.

^1H NMR in D_2O-DCl showed peaks at δ 7.45 (5H, m), 5.45 (1H, s).

HPLC with C-18, ODS-3, 10μ analytical column and water as mobile solvent (flow rate of 3 mL/min.) showed retention times for starting compound and for product at 5.1 min. and 15.9 min., respectively.

N-AMIDINO-D-PHENYLALANINE or C-D-BENZYLGLYCOCYAMINE (7)

D-Phenylalanine (8.26 g, 0.05 mole) was added in small portions to a reaction flask which contained 2.53 g of cyanamide and 2 mL of water. To the stirring solution

were added several drops of concentrated aqueous ammonia. After standing for few hours at room temperature, the crystals that formed were filtered to give approximately 85% yield of product. The solids were recrystallized in H_2O, mp 260-265° C.

HPLC using C-18, ODS-3, 10μ analytical column and water as mobile solvent (flow rate of 4 mL/min.) gave retention times for product and for starting compound at 13.1 min. and 3.4 min., respectively.

Anal. Calcd. for $C_{10}H_{13}N_3O_2$: C, 57.96; H, 6.32; N, 20.27. Found: C, 57.83; H, 6.26; N, 20.34.

Proton NMR in D_2O showed peaks at δ 7.33 (5H, s), 4.11-4.27 (1H, d-d, Js = 7.8 and 4.9 Hz); 2.31-3.38 (2H, m, Js = 14.0, 7.8, and 4.9 Hz).

GUANIDO-N,N-DIACETIC ACID (8)

Into a 25 ml Erlenmeyer flask was placed 5.2 g of cyanamide and 2.5 mL of water. Aminodiacetic acid (13.4 g) was added in small portions with shaking, followed by the addition of several drops of aqueous ammonia (58%). A total of 13 mL of concentrated aqueous ammonia was used. After standing at room temperature for several days, white crystalline product was formed. Filtration of the solid gave about 70% yield, mp 270° C (dec.). The compound was soluble in water. It was recrystallized from H_2O-EtOH. The solids were dissolved in a minimum amount of H_2O,

filtered, and then crystallized by the addition of EtOH.

^{1}H NMR in D_2O showed only one singlet peak at δ 3.91. To ascertain that the product was not starting compound, a small amount of starting compound was added to the NMR tube showing a second peak at δ 3.66.

^{13}C NMR in D_2O with dioxane as internal standard showed peaks at δ 53.81 (α- carbon), 158.19 (guanidino carbon), and 175.21 (carboxylate carbon).

Due to its ready cyclization, a microanalysis was not performed on this compound. Recrystallization of guanidinodiacetic acid with prolonged heating will convert the compound into a cyclic product, that is, 1-carboxymethyl-2-iminoimidazolidine-4-one (15), as described below.

1-CARBOXYMETHYL-2-IMINOIMIDAZOLIDINE-4-ONE (15)

This compound was prepared by prolonged heating of guanidino-N,N-diacetic acid (8) in water. Thus, a sample of guanidinodiacetic acid was dissolved in a mimimum amount of water and heated using a heat gun for 10 min. The crystals of 1-carboxymethyl-2-iminoimidazolidine-4-one (15) were formed after cooling. The compound was recrystalized from water. It was less soluble in water than its precursor.

^{1}H NMR in D_2O showed peaks at δ 4.31 (s) and 4.11 (s)

with equal intensities.

Anal. Calcd. for $C_5H_7N_3O_3$: C, 38.22; H, 4.49; N, 26.74. Found: C, 38.29; H, 4.47; N, 26.49.

N,N'-DIAMINOETYLENDIAMINE-N,N'-DIACETIC ACID or BISCREATINE (9)

Cyanamide (2.102 g, 0.05 mole) was dissolved in 20 mL of distilled water. Ethylenediamine-N,N'-diacetic acid (EDDA) (3.523 g, 0.02 mole) was added into reaction flask in small portions. After each addition, the reaction flask was then shaken and swirled until solution became clear. A few drops of aqueous ammonia were added after each addition to increase the solubility of the starting compound. A total of 1 mL of aqueous ammonia was used. After the addition of EDDA, the reaction flask was then sealed and allowed to stand for few days at room temperature to form crystals. The crystals, which were collected by filtration, contained a small amount of starting compound EDDA. They were separated by exploiting their differences in pKa values. Aqueous ammonia (58%) was added to the stirred mixture. The product was not soluble in aqueous ammonia and was collected by filtration, washed with water, and finally washed with acetone before drying in air. The product did not melt up to 360° C and was neither soluble in H_2O nor any organic solvents. It was

soluble in water as its hydrochloride salt, however.

^1H NMR in D_2O-DCl showed two singlets with equal intensities at δ 4.35 and 3.77.

^1H NMR in TFA-d showed two singlets at δ 4.54 and 4.03.

^{13}C NMR in D_2O-DCl with dioxane as an internal standard showed peaks at δ 171.99 (carboxyl carbon), 158.74 (guanidino carbon), 52.24 (α-carbon), and 48.81 (ethylene carbon).

Anal. Calcd. for $C_8H_{16}N_6O_4$. 0.444 H_2O: C, 35.82; H, 6.34; N, 31.32. Found: C, 36.22; H, 6.20; N, 30.89.

1,3-DICARBOXYMETHYL-2-IMINOIMIDAZOLIDINE (11)

This compound is a by-product of the synthesis of biscreatine (9). It is very soluble in water and was collected from the filtrate after biscreatine was filtered off. The filtrate was evaporated and the solid was recrystalized with water-methanol, mp 270-275° C (dec.).

Anal. Calcd. for $C_7H_{11}O_4N_3$. 1.15 H_2O: C, 37.89; H, 6.03; N, 18.94. Found: C, 37.78; H, 5.84; N, 19.02.

^1H NMR in D_2O showed two singlets with equal intensities at δ 4.04 and 3.71.

Proton decoupled ^{13}C NMR with dioxane as internal standard showed peaks at δ 174.58, 158.84, 49.18, and 47.73.

Proton coupled ^{13}C NMR with dioxane as an internal standard showed peaks at δ 174.58 (t, J = 3 Hz), 158.84 (s), 49.18 (t, J = 139 Hz), 47.73 (t-t, Js = 148.5 and 3 Hz).

N-AMIDINOETHYLENDIAMINE-N,N'-DIACETIC ACID (10)

EDDA (3.53 g, 0.02 mole) was dissolved in 5 mL of water and 2.5 mL of aqueous ammonia (58%). The mixture was heated to 95° C using a oil bath and stirred until a clear solution was obtained. Cyanamide (0.97 g, 15% molar excess) was added dropwise to the reaction flask and the temperature was raised up to 110° C. The reaction flask was then allowed to stand at room temperature overnight. Biscreatine (9) (0.059 g) was collected by filtration and identified by NMR. The filtrate was heated to 120° C with stirring for few more hours before standing again one more day at room temperature. Biscreatine (0.024 g) was collected the next day to bring the total to 0.083 g. The mixture was allowed to stand for another day, during which no additional crystals of biscreatine were formed. Unreacted cyanamide (if any) was extracted with CH_2Cl_2. The water layer was evaporated until dry. MeOH was added into the flask to wash the impure solid. Filtration of the white solid gave 1.3 g of N-amidinoethylendiamine-N,N'-diacetic acid. The product was recrystallized using a minimum amount of water and quickly washed with acetone.

The pure compound did not melt up to 320° C.

^1H NMR in D_2O showed peaks at δ 4.17 (2H, s), 4.00 (2H,s), 3.57-3.64 (2H, t), and 3.70-3.77 (2H, t).

Anal. Calcd. for $C_7H_{14}N_4O_4$: C, 38.50; H, 6.42; N, 25.67. Found: C, 38.52; H. 6.37; N, 25.40.

GC-MS gave a very strong peak at m/e $(M-17)^+$.

Proton-decoupled ^{13}C NMR in D_2O (dioxane) showed peaks at δ 175.74, 166.80, 156.48, 51.76, 48.66, 46.93, and 42.86.

TOLAZOLINE-N-ACETIC ACID (14)

Tolazoline-HCl (11.802 g, 0.06 mole) was dissolved in a cold mixture of NaOH and water with stirring. Free tolazoline was quickly extracted using ethyl acetate. The tolazoline was dried over anhydrous Na_2SO_4 and then gravity-filtered. The solvent was then evaporated to dryness using a rotary evaporator. Iodoacetic acid (3 g) was dissolved in 25 mL of acetone and the mixture was added dropwise into a reaction flask which contained tolazoline in 50 mL acetone and 50 mL EtOH. The reaction mixture was stirred overnight at room temperature. It turned yellow. It was then heated to complete the reaction. By this time it showed a dark color. The solvents were removed by a rotary evaporator to leave an oily product. A minimum amount of absolute EtOH was added

to dissolve the oil. The product was then crystallized with acetone and recrystallized with acetone-MeOH to give fine crystals, mp 230° C (dec.). Only one spot appeared on a silica gel-TLC plate with MeOH-acetone (5/5).

Anal. Calcd. for $C_{12}H_{14}N_2O_2 \cdot 1/3\ H_2O$: C, 64.27; H, 6.59; N, 12.49. Found: C, 64.09; H, 6.60; N, 12.49.

GC-MS showed a M^+ peak at m/e 219.

^1H NMR in D_2O showed peaks at δ 7.44-7.30 (5H, m), 4.82 (2H, s), 4.02 (2H, s), and 3.89 (4H, m).

Proton-decoupled ^{13}C NMR in D_2O (dioxane) showed peaks at δ 173.28 (carboxylate carbon), 131.94 (amidino carbon), 130.17 and 129.22 (aromatic carbons), 51.62 and 49.82 (ethylene carbons), 43.50 (α-carbon), and 32.21 (methylene carbon).

2-METHYL-N-CARBOXYMETHYLIMIDAZOLE (13) and 2-METHYL-N,N'-DICARBOXYMETHYLIMIDAZOLE (12)

2-Methylimidazole (16.42 g, 0.2 mole) was dissolved in 50 mL of absolute EtOH in a 250 mL reaction flask. Iodoacetic acid (18.59 g, 0.1 mole), which was dissolved in 50 mL acetone, was added dropwise to the reaction flask using an addition funnel. The reaction mixture was stirred for three days at room temperature to yield a large amount of a white precipitate. The product was then filtered and washed with acetone. An approximate yield of 70% was collected based on iodoacetic acid. The iodide salt and

unreacted starting compounds were separated from the products by washing with acetone. The products are a mixture of 2-methyl-N-carboxymethylimidazole (13) and a small amount of 2-methyl-N,N'-dicarboxymethylimidazole (12). These two products were separated from each other by their differences in solubility in water. The mixture was dissolved in a minimum amount of hot water and was then cooled to form crystals of 2-methyl-N,N'-dicarboxymethylimidazole (12).

2-Methyl-N-carboxymethylimidazole (13) was much more soluble in water and was collected after adding an equivalent amount of EtOH and again recrystallized several times with water-EtOH.

2-Methyl-N-carboxymethylimidazole (13), mp 245° C:

HPLC, with reverse phase, C-18, ODS-3, 10μ analytical column and water as mobile solvent at the flow rate 1 mL/min., gave a retention time of 4.4 min.

^1H NMR in D_2O showed peaks at δ 7.12 (s, 2H), 4.56 (s, 2H), and 2.36 (s, 3H).

^{13}C NMR in D_2O and dioxane as an internal standard showed peaks at δ 10.46, 51.14, 118.37, 123.53, and 172.67.

GC-MS showed a strong M^+ peak at m/e 140 and a base peak at m/e 95 $(M-45)^+$.

Anal. Calcd. for $C_6H_8O_2N_2$: C, 51.42; H, 5.75; N,

19.99. Found: C, 51.19; H, 5.69; N, 19.70.

2-Methyl-N,N'-dicarboxymethylimidazole (12), mp 280° C:

^1H NMR in D_2O showed peaks at δ 7.43 (2H, d), 4.95 (4H, s), 2.54 (3H, s).

Anal. Calcd. for $C_8H_{10}N_2O_4 \cdot 0.1\ H_2O$: C, 48.05; H, 5.14; N, 14.04. Found: C, 47.89; H, 5.26; N, 14.38.

ONE STEP SYNTHESIS OF ETHYLENDIAMINEMONOACETIC ACID.

Ethylenediamine (40.16 mL, 0.6 mole) and 50 mL water were mixed together with stirring in an ice-water bath. A mixture of 11.56 g (0.06 mole) of iodoacetic acid and 40 mL water (endothermic mixture) was added in small portions with stirring. The ice-water bath was removed after 5 min. Mixing was continued with stirring at room temperature for 3 hr. It was then kept cold in a refrigerator for 2 days. The mixture was filtered and solvent and excess ethylenediamine were removed *in vacuo* to leave product as yellow oil. The oil was dissolved several times, each with 50 mL 95% EtOH and the solvent was removed again to remove any trace of unreacted ethylenediamine. About 500 mL 95% EtOH was added into reaction flask and the mixture was heated to dissolve the oil again. The flask was placed in a refrigerator overnight. An oily solid formed the next day. The flask was shaken by hand again and then placed in

dry-ice for few minutes. After slowly shaking by hand, the clear solution became cloudy and a precipitate started to form. The remaining oil in the flask turned solid at the same time. The solid was collected by filtration and washed with ether, (8.13 g, 100% yield). The crude product gave a proton NMR spectrum with no trace of impurity. The first recrystallization with 95% EtOH required cooling in dry ice to obtain crystals. The second recrystallization, however, gave good crystals in only a few minutes with cooling at room temperature.

Anal. Calcd. for $C_4H_{10}O_2N_2$. 1.8 H_2O: C, 31.91; H, 9.10; N, 18.60; I, 0.00. Found: C, 32.25; H, 8.89; N, 18.24; I, 0.00.

^1H NMR in D_2O showed peaks at δ 3.30 (2H, s); 2.93-3.05 (4H, d-d).

GC-MS showed a small peak at m/e 118 $(M)^+$, a strong peak at m/e 100 $(M-18)^+$, a base peak at m/e 88 ($HOOC-CH_2-NH_3^+-CH_2$), and a peak at m/e 73 $(M-COOH)^+$.

A NEW WAY TO SYNTHESIZE CYCLOCREATINE

Ethylenediaminemonoacetic acid (2 g, 0.017 mole) was added in small portions into a mixture of 2 g of cyanamide, 1 mL of water and 1 mL of aqueous ammonia (58%). The mixture was filtered with a sintered-glass filter, 2 mL of aqueous ammonia was used to clean the

flask and added into the mixture. The mixture was then warmed with a heat gun. Polymer of cyanamide which formed during reaction was removed by filtration. The filtrate was then allowed to stand at room temperature for 3 days, and no crystals were formed. The solvent was then removed. MeOH and ether were added and the solid was collected by filtration with an approximate yield of 60%. The solid was recrystallized twice with water.

^{1}H NMR in D_2O and 1% DSS showed peaks at δ 3.88 (2H, s), 3.69 (4H, t).

GC-MS showed peaks at m/e 125 $(M-18)^+$ (base peak), 124, 98 $(M-45)^+$, 84 $(M-CH_2-COOH)^+$, 75, 69, and 55.

Anal. Calcd. for $C_5H_9N_3O_2$. 0.75 H_2O: C, 38.34; H, 6.74; N, 26.82. Found: C, 38.48; H, 6.62; N, 27.01.

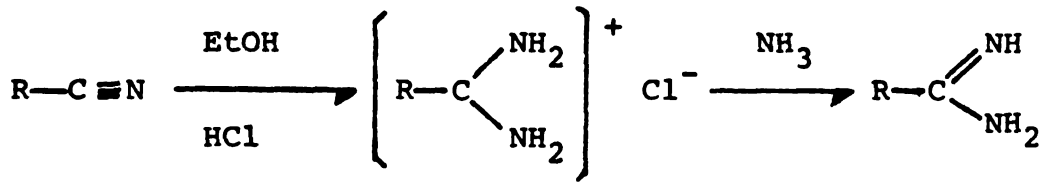
RESULTS AND DISCUSSION.

CARBOCREATINE.

3-Amidinobutyric acid (1), an analog of creatine, in which a nitrogen atom of guanidino group is replaced by a carbon, has been synthesized. Since this compound is a carbon analog of creatine, for convenience it will be called carbocreatine. The synthesis of carbocreatine involved several steps, starting with ethyl crotonate, as shown in scheme II-1.

In this scheme, ethyl 3-bromobutyrate, a known compound, was made by bromination of ethyl crotonate by treatment with HBr gas in glacial acetic acid. The bromine atom of this compound was replaced by a cyano group and was then converted to an amidino group by the Pinner method. This method, which is widely used for the synthesis of amidines, was introduced by Pinner in 1892. It involves the formation of an imidate salt by the reaction of a nitrile with an anhydrous alcohol, usually EtOH or MeOH, in the presence of an acid catalyst. This imidate salt is then converted into an amidine by treatment with ammonia in absolute alcohol. MeOH was chosen for this second step, due to its low boiling point and ready evaporation.

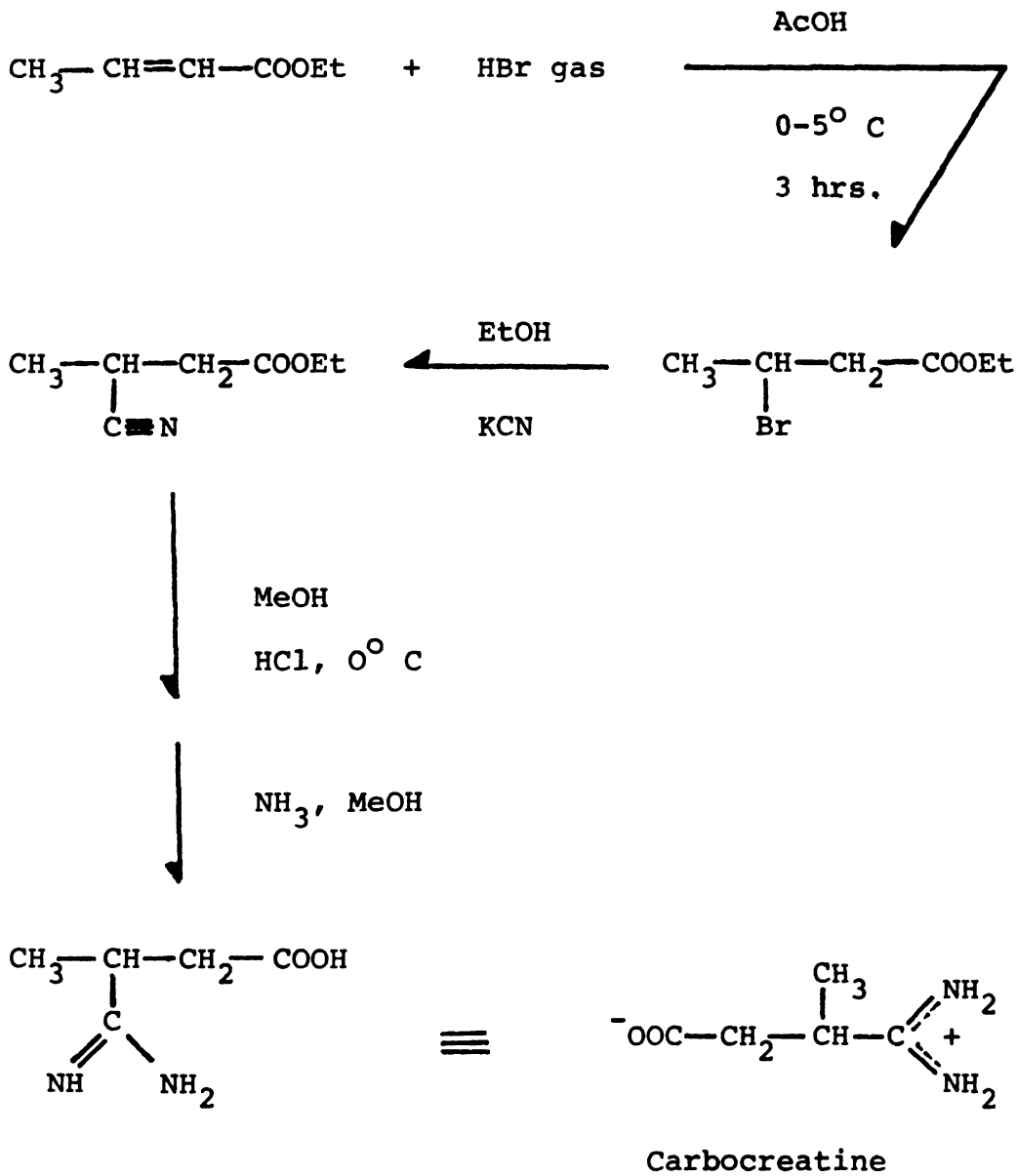
PINNER METHOD



Imidate salt.

Figure II-1 shows the ^{1}H NMR spectrum (240 MHz) of carbocreatine. The α-protons of carbocreatine are non-equivalent because of the chirality of their adjacent carbon. Under the high field NMR, these two protons show separate peaks, each with a doublet-doublet pattern.

Scheme II-1: SYNTHESIS OF CARBOCREATINE



Carbocreatine

SYNTHESIS OF OTHER CREATINE ANALOGS.

Other analogs which were thought to be good inhibitors for creatine kinase have also been synthesized. The synthesis of these compounds follows several methods as described below.

Acyclic creatine analogs.

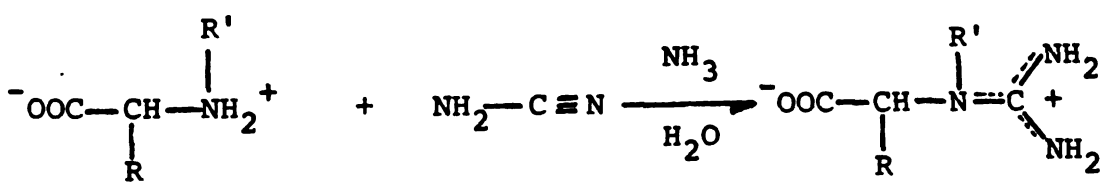
One of the general methods for the synthesis of creatine analogs is the reaction of cyanamide with the nitrogen atom of substituted glycine to form guanidine. This method is probably the most reliable method for the synthesis of glycocyamine derivatives. This general method has been described and utilized by Kenyon and coworkers previously (Rowley *et al.*, 1971). The reaction is very slow, usually requiring several days at room temperature. The reaction seems to work very well with water-soluble starting compounds. With slightly soluble compounds we found that an excess amount of cyanamide in liquid ammonia could help to dissolve the compound, increasing the yield and speeding up the reaction. This is also the case with D- and L-C-allylglycine, both freely water-soluble compounds. While the racemic mixture DL-C-allylglycine formed the product after 12 hours, the more water soluble isomers D- and L-C-allyglycine did not form any crystals of product after 3 days of reaction under the same conditions. On treatment with more concentrated ammonia, the crystals started to form in one hr. Table II-1

outlines the results of increasing the yield by increasing the amount of cyanamide and aqueous ammonia with C-allylglycine.

While a large excess amount of cyanamide can cause formation of its trimer or dimer, a large excess amount of aqueous ammonia did not change the course of reaction. We found that by recrystallization of the crude product with H_2O-EtOH and washing it with acetone we could free the product from the polymers of cyanamide. Although the polymer of cyanamide can not be detected by ^1H NMR in D_2O solvent, owing to its structure containing only exchangeable protons, its presence in the final product can be easily detected using HPLC. Using a reverse phase C-18, ODS-3 analytical column with water as mobile phase at the flow rate of 1 ml/min, the presence of the polymer can be detected by a refractive index detector with a retention time of 4.2 min. Creatine analogs usually have longer retention times. As an example, C-allylglycocyamine has a retention time at 7.6 min under the same conditions.

The general reaction for this method is shown below:

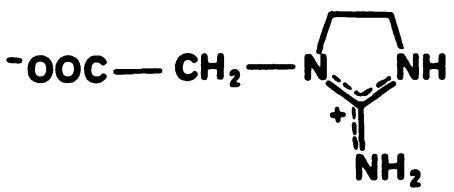
Method A:



We noticed a tendency toward cyclization of the creatine-type analog to give the corresponding creatinine-type analog when synthesizing guanidino-N,N-diacetic acid (8), using this method. Upon recrystallization of guanidino-N,N-diacetic acid (8), we found its corresponding cyclization product, namely 1-carboxymethyl-2-iminoimidazolidine-4-one (15). Rowley et al. (1971) noted the same problem with DL-N-methyl-N-amidinoalanine, which had been prepared by the same method. The problem, however, was overcome when gentler conditions were used for the isolation of guanidino-N,N-diacetic acid.

Cyclic creatine analogs.

Other analogs are conformationally-restricted analogs with structures that resemble cyclocreatine, a synthetic substrate for creatine kinase (Rowley et al., 1971).



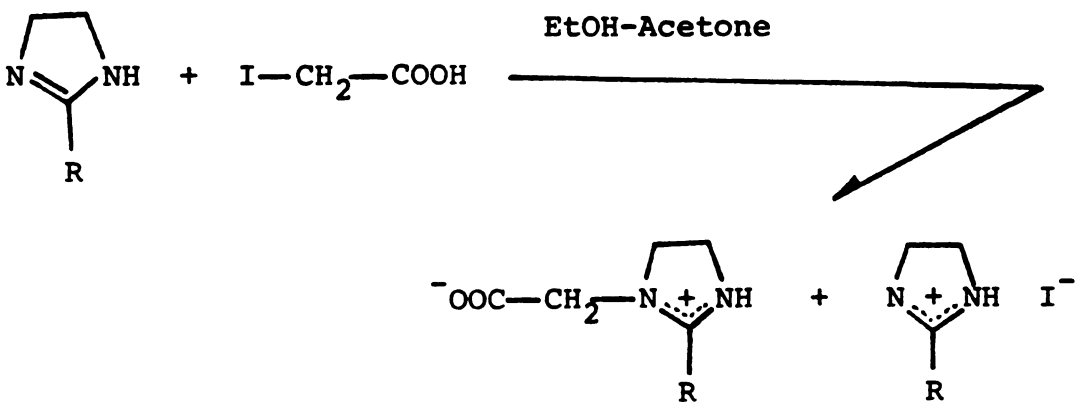
cyclocreatine

These types of analogs have been synthesized either by the formation of the C-N bond between the nitrogen atom of the five-membered ring and the carbon atom of carboxymethyl group, or by the cyclization of the five-

membered ring. Choosing one of the two methods depends on the final structures of the analogs, or on the availability of the precursors.

The first method used to synthesize this type of analog is shown below:

Method B:

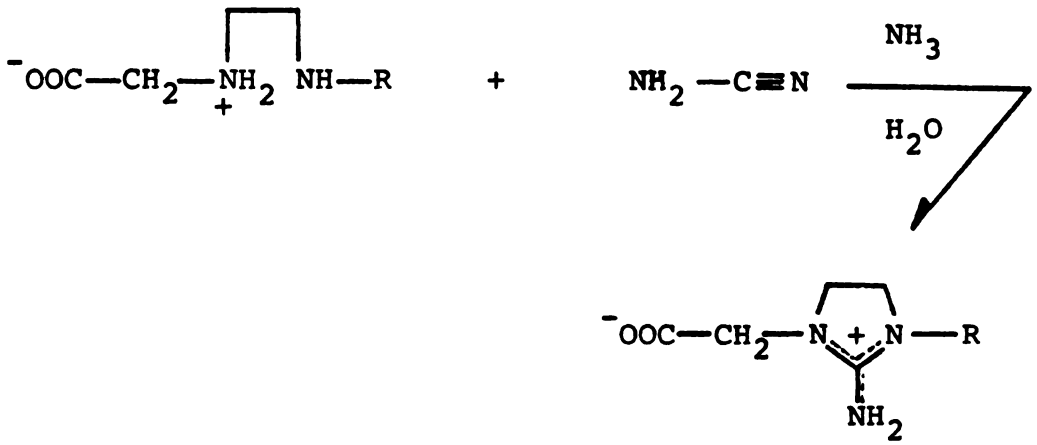


This method has been used in the past for the making of N-C bonds. Rowley et al. (1971) reported the synthesis of N-ethylglycine and N-propylglycine using this method. Creatine analogs are insoluble in acetone, whereas the iodide salts are well-known acetone-soluble compounds. We have used the different solubilities in acetone as a convenient way to separate acetone-insoluble creatine analogs and acetone-soluble compounds, such as iodide salt and starting compounds. We have used this method to

synthesize 2-methyl-N,N'-dicarboxymethylimidazole (12), tolazoline-N-acetic acid (14), and 2-methyl-N-carboxymethylimidazole (13) in high yields.

The second method for making this type of analog is presented as follows:

Method C:



We have synthesized 1,3-dicarboxymethyl-2-iminoimidazolidine (11) using Method C. This method may serve as a general method for the synthesis of 2-iminoimidazolidine derivatives by the cyclization of ehylenediamine derivatives with cyanamide. Cyclocreatine, which was previously prepared by Rowley et al. (1971) using the method of cyanogen bromide, has also been synthesized by this method with a high yield.

While the method with cyanogen bromide failed in the synthesis of N-substituted 2-iminoimidazolidine, Method C

may overcome the problem by starting with N-substituted cyanamide and serving, then, as a general method for making this type of analog.

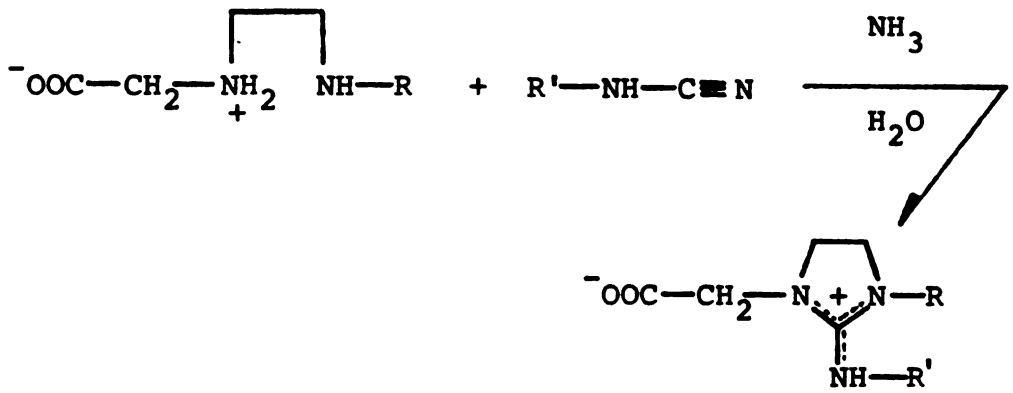
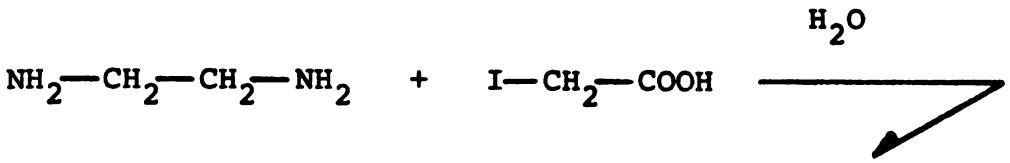


Table II-2 shows all of new creatine analogs along with their proposed names, ^1H NMR spectra, and methods of synthesis. ^1H NMR spectra of new creatine analogs were taken in D_2O using a 240 MHz instrument, IR, and mass spectra are presented in Figures II-2 to II-11.

The precursor of cyclocreatine, 2-(2-aminothylamino)-ethanoic acid or ethylenediamine-N-monoacetic acid, was previously synthesized as the dihydrochloride salt by other workers using different methods (Rowley et al., 1971; Dietrich et al., 1980). These methods were complicated and involved many steps. The free base of this compound, that was not previously obtained, has been synthesized as follows:



$NH_2—CH_2—CH_2—NH—CH_2—COOH$

Ethylenediamine-N-monoacetic acid

This is the most simple synthesis of ethlenediamine-N-monoacetic acid. The product obtained was pure and in quantitative yield based on iodoacetic acid. To avoid a di-N-substituted or tri-N-substituted product, we have used a stoichiometry of 10 to 1, that is, 10 moles of ethylenediamine to 1 mole of iodoacetic acid. Since ethylendiamine is low-boiling, it is readily separated from the product. The structure of this new base was proven by the microanalysis (which shows no trace of iodide salt), NMR spectrum, and GC-MS analysis.

Table II-1

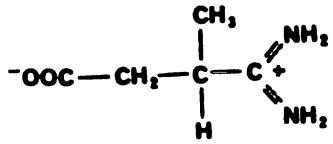
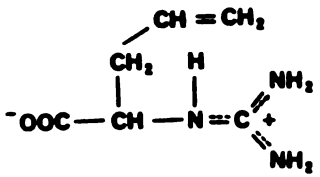
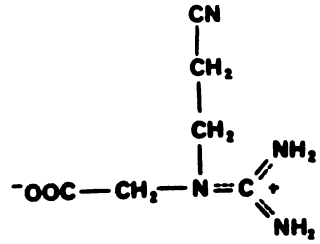
IMPROVED SYNTHESIS OF C(α)-ALLYLGLYCOCYAMINE

Allylglycine (Moles)	Cyanamide (Moles)	NH_4OH 58% ml/g *	Time	Yield
DL 1	1.10	0.69	2 days	20%
DL 1	1.15	1.00	1 day	29%
D or L 1	2	1	3 days	00%
DL 1	1.9	2	12 hours	49%
DL 1	2.7	2	4 hours	70%
D or L 1	2.7	2	12 hours	50-60%

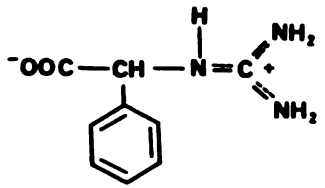
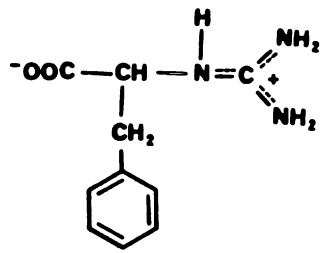
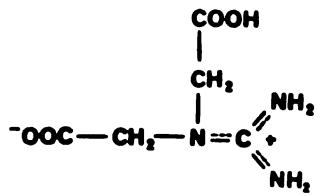
* ml/g of allylglycine used

TABLE II-2

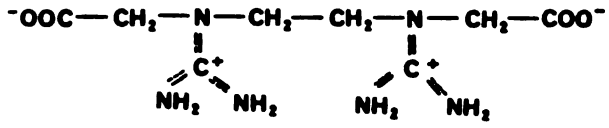
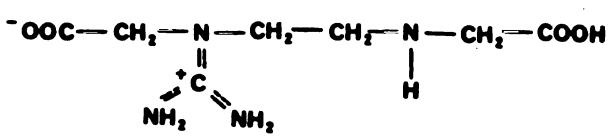
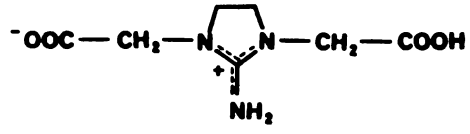
NEW SYNTHETIC CREATINE ANALOGS

Name	Structure	NMR in D_2O (ppm)	Synthetic method.
3-Amidinobutyric acid (Carbocreatine). (1)		1.27 (3H, d, J = 7 Hz); 2.51 (2H, m, J = 7 Hz); 2.96 (1H, m, J = 7 Hz).	
D-, L-, and DL-C-allylglycocyamine (2), (3), and (4)		2.48-2.66 (2H, m); 3.99-4.04 (1H, d-d); 5.15-5.23 (2H, m); 5.69-5.83 (1H, m).	A
N-amidino-N-(cyanoethyl)-glycine. (5)		4.37 (2H, s); 3.81 (2H, t, J = 7 Hz); 2.89 (2H, t, J = 7 Hz).	A

(Table II-2, cont.)

Name	Structure	NMR in D_2O	Synthetic method
C-phenylglycocyamine or N-amidino-D-phenyl-glycine. (6)		7.45 (5H, m); 5.45 (1H, s).	A
C-benzylglycocyamine or N-amidino-D-phenylalanine. (7)		7.33 (5H, s); 4.11-4.27 (1H, d-d, Js = 7.8 and 4.9 Hz); 2.31-3.38 (2H, m, Js = 14.0, 7.8, and 4.9 Hz).	A
Guanidino-N,N-diacetic acid. (8)		3.91 (s).	A

(Table II-2, cont.)

Name	Structure	NMR in D_2O	Synthetic method
N,N'-diamidinoethylen-diamine-N,N'-diacetic acid (Biscreatine). (9)		in D_2O-DCl: 4.35 (4H, s); 3.77 (4H, s).	A
N-amidinoethylen-diamine-N,N'-diacetic acid. (10)		4.17 (2H, s); 4.00 (2H, s); 3.57-3.64 (2H, t); 3.70-3.77 (2H, t).	A
1,3-Dicarboxymethyl-2-iminoimidazolidine. (11)		4.04 (4H, s); 3.71 (4H, s).	C

(Table II-2, cont.)

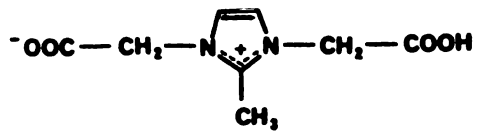
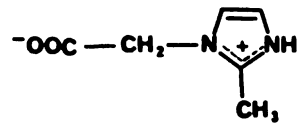
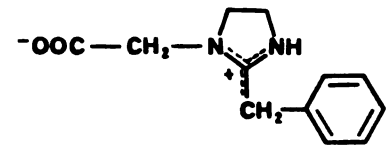
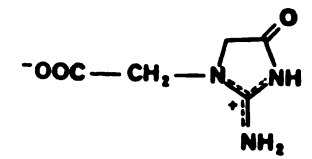
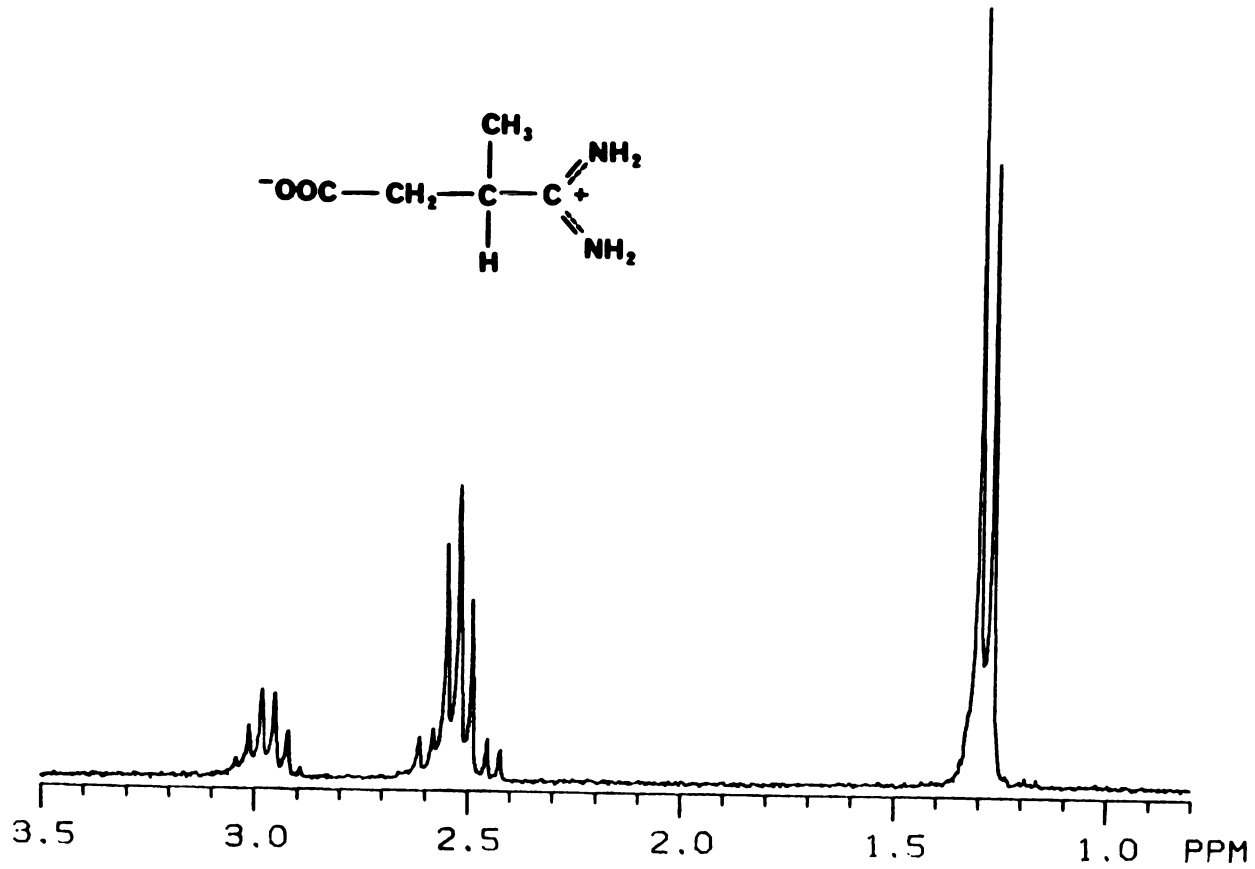
Name	Structure	NMR in D_2O (ppm)	Synthetic method
2-Methyl-N,N'-dicarboxy-methylimidazole. (12)		7.43 (2H, d); 4.95 (4H, s); 2.54 (3H, s).	B
2-Methyl-N-carboxy-methylimidazole. (13)		7.12 (2H, s); 4.56 (2H, s); 2.36 (3H, s).	B
Tolazoline-N-acetic acid. (14)		7.44-7.30 (5H, m); 4.82 (2H, s); 4.02 (2H, s); 3.89 (4H, m).	B
1-Carbozymethyl-2-imino-imidazolidine-4-one. (15)		4.31 (2H, s); 4.11 (2H, s).	

Figure II-1 to II-11:

The ^{1}H NMR spectra shown in Figures II-1 to II-11 were analyzed in D_2O with either DSS (2,2-dimethyl-2-silapentane-5-sulfonate) or TSP (3-trimethylsilyl-tetradeutero sodium propionate) as internal reference. All chemical shifts are given in the Experimental Section. Except for N-amidino-N-(cyanoethyl)-glycine, which was performed on a FT-80 Varian NMR Spectrometry, all others were obtained on a 240 MHz instrument as described in the Method Section. The spectra were obtained using the program One-Pulse Sequence (1PULS) at the spectral frequency of 240 MHz, spectral width of ±2500 Hz, and the size of 32K or 16K data points.

FIGURE II-1: ^{1}H NMR OF CARBOCREATINE



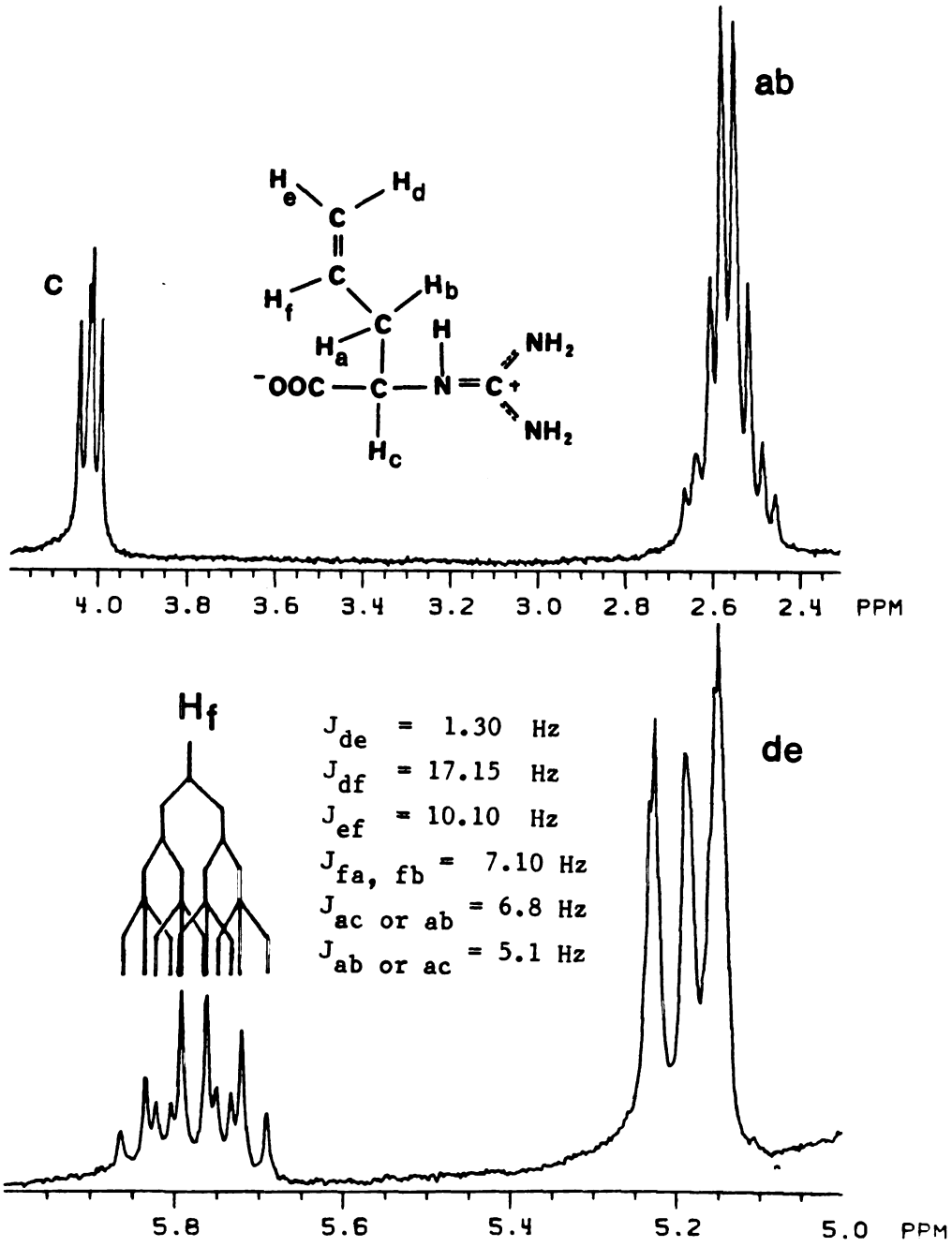


FIGURE II-2:

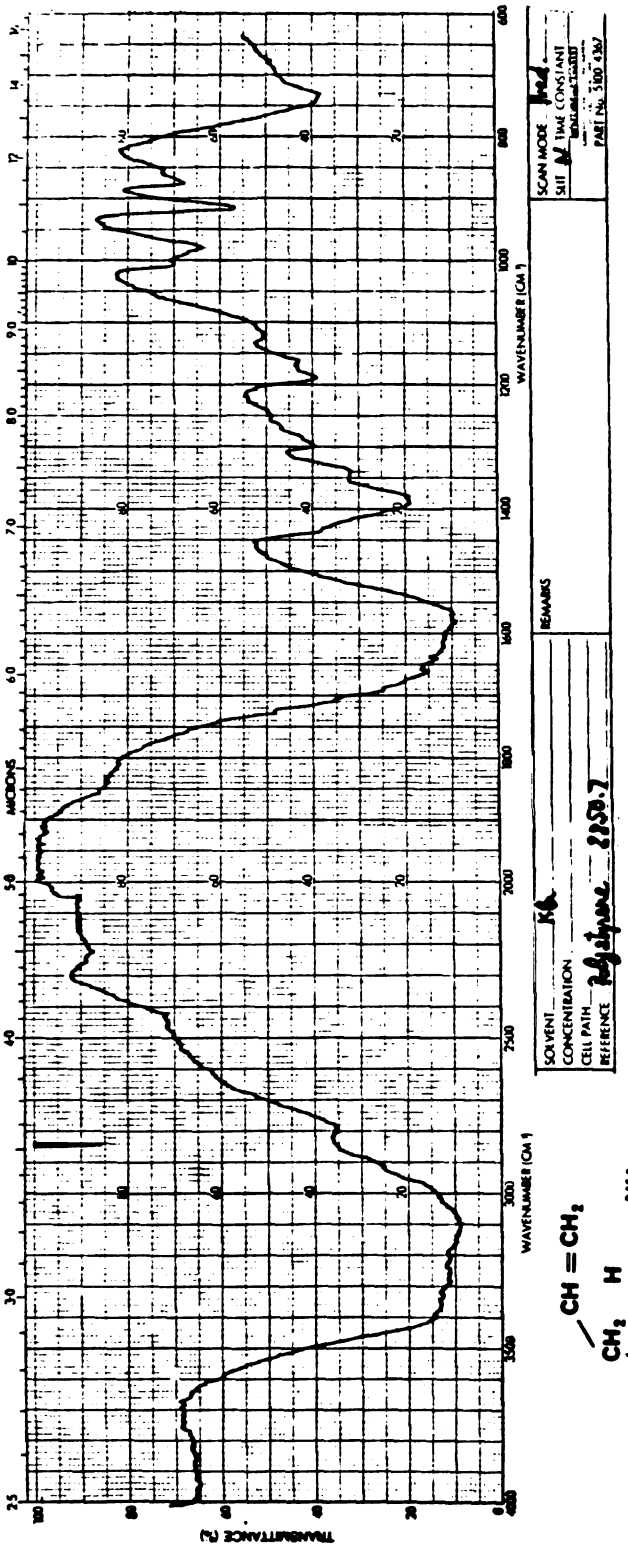
^{1}H NMR OF C(α)-ALLYLGLYCOCYAMINE

Figure II-3:

IR spectrum of C(α)-allylglycocyamine was taken as KBr pellet on a Perkin-Varian IR Spectrometer with medium scan mode. Polystyrene at the wavenumber 2850.7 cm^{-1} was used as an external reference.

FIGURE II-3:

IR OF c(α)-ALLYLGLYCOCYAMINE



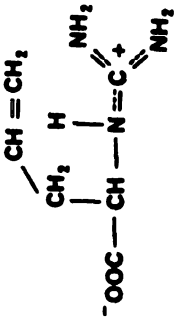


FIGURE II-4: ^1H NMR OF N-AMIDINO-N-(CYANOETHYL)-GLYCINE

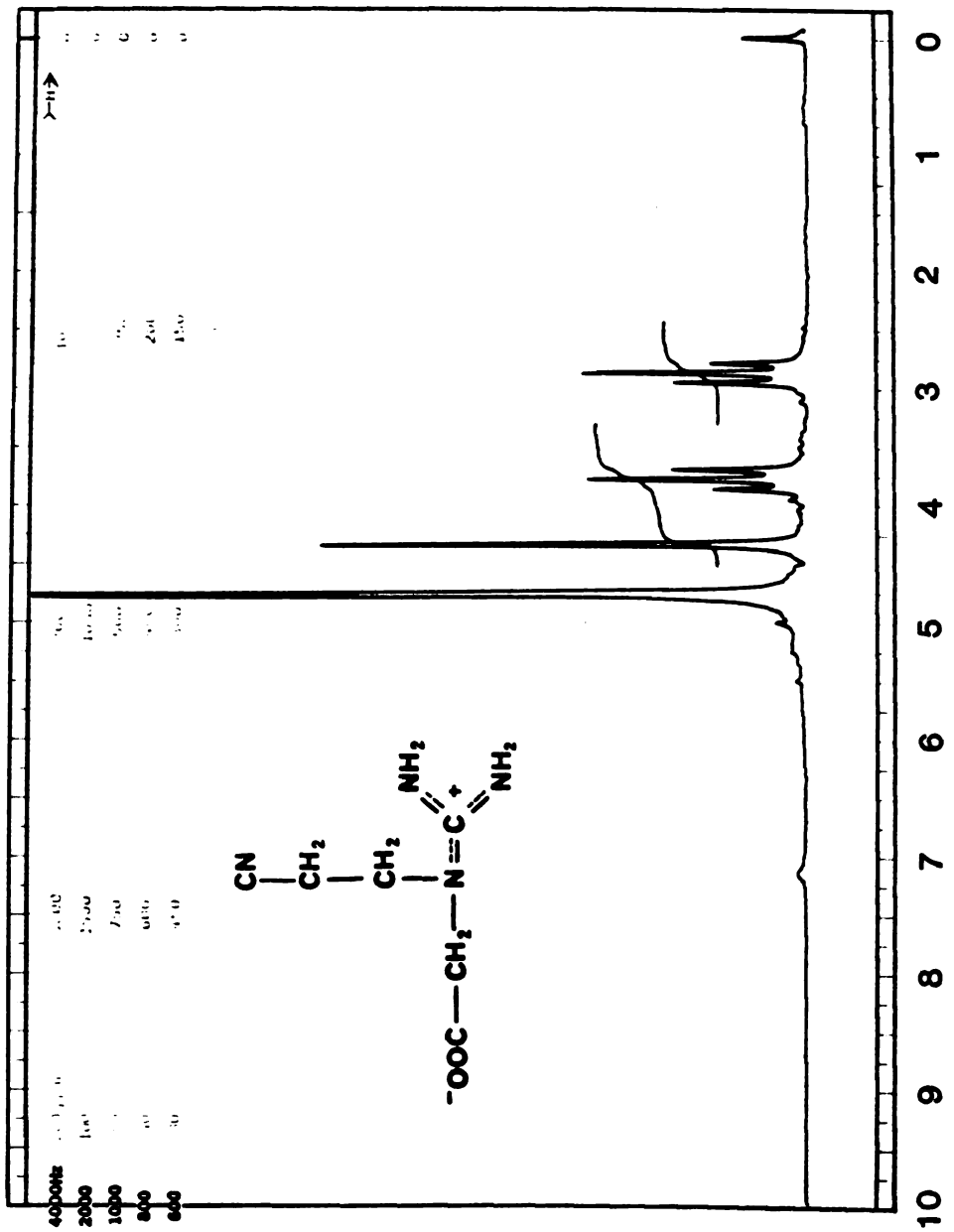


FIGURE II-5: ^{1}H NMR OF N-AMIDINOPHENYLALANYL

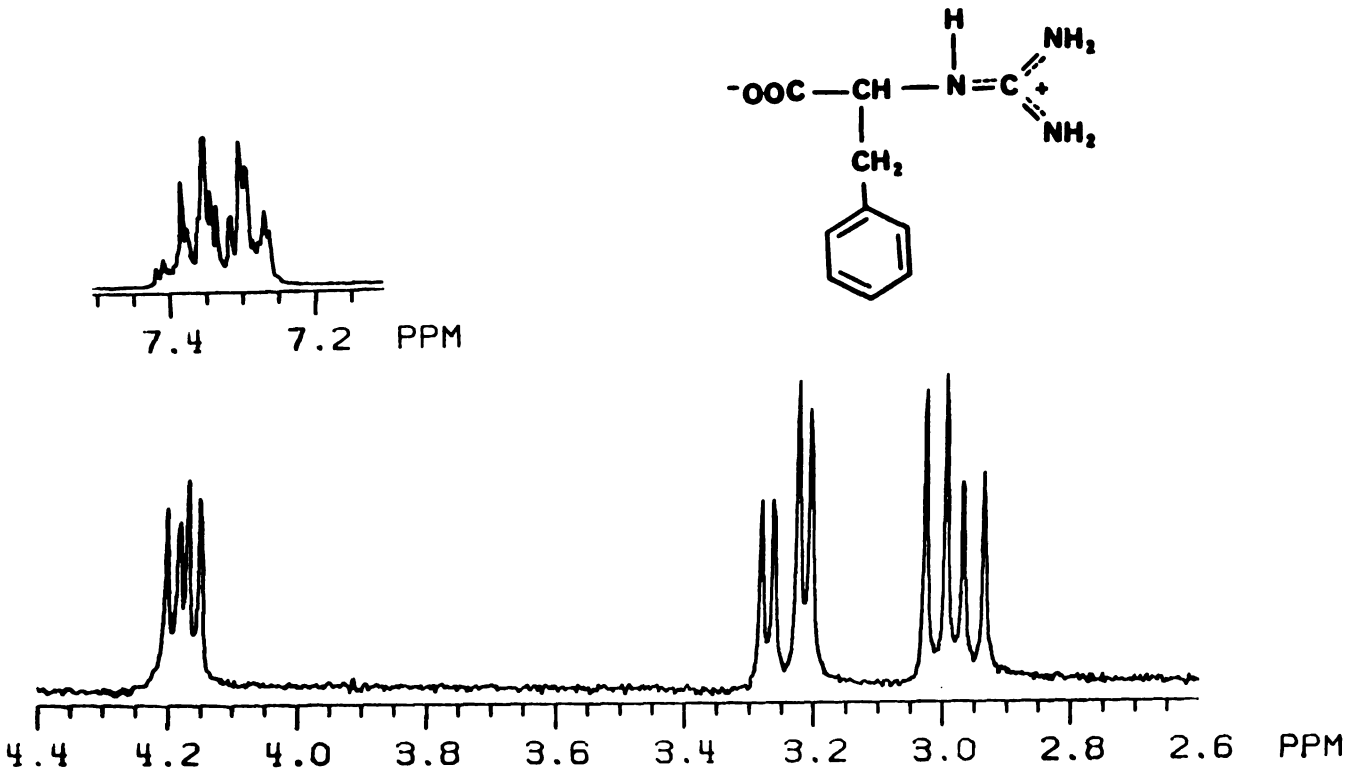


FIGURE II-6: ^{1}H NMR OF GUANIDINO-N,N'-DIACETIC ACID

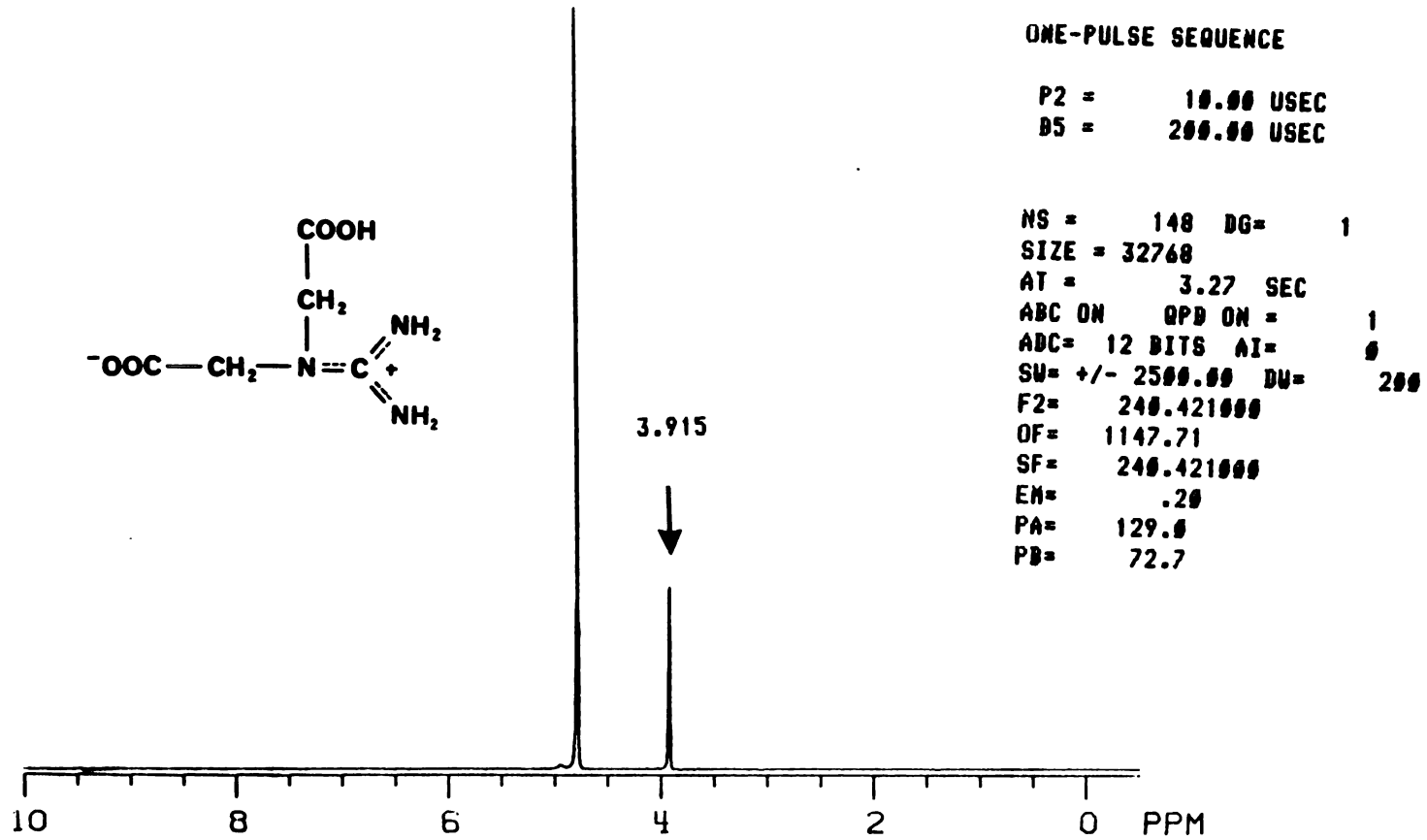
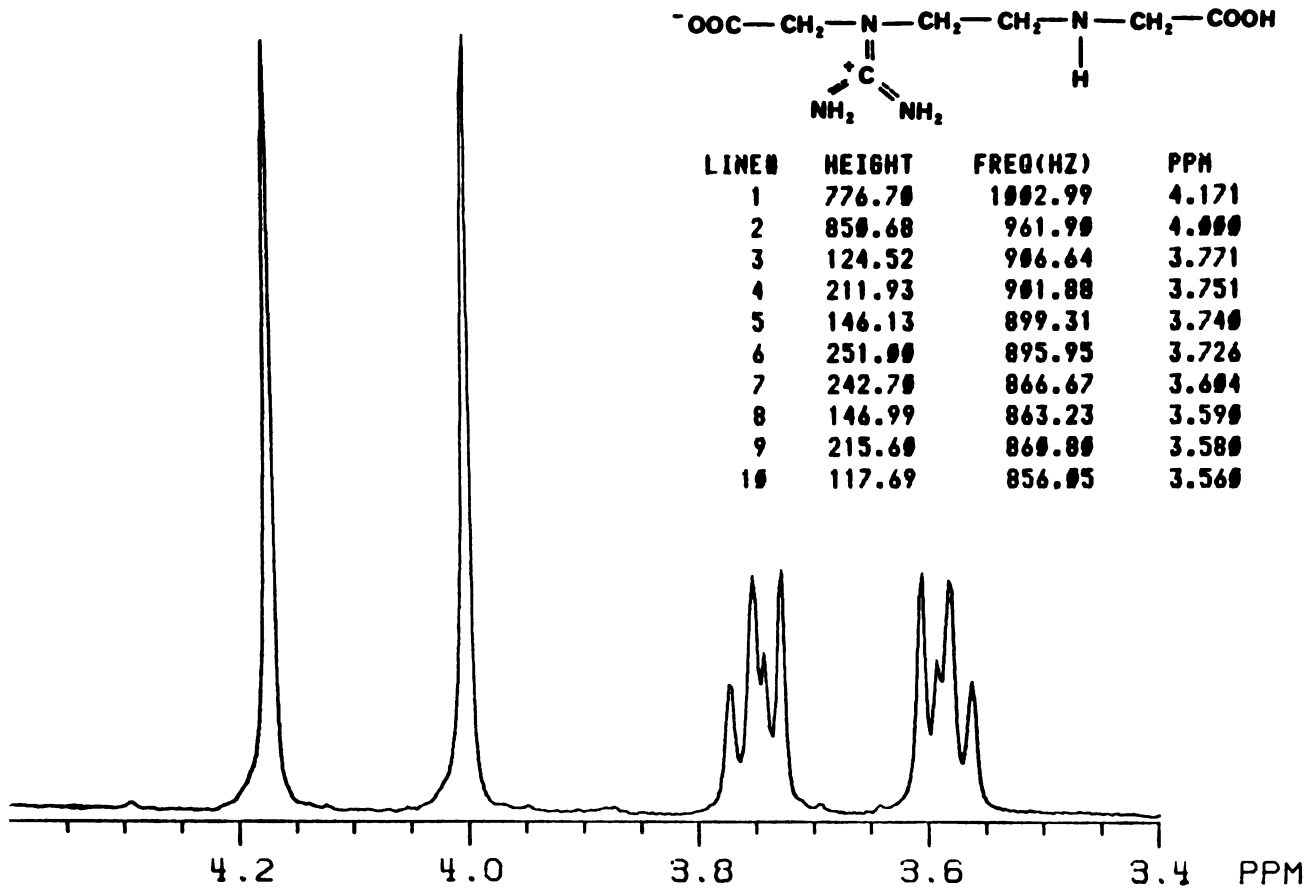


FIGURE II-7: ^{1}H NMR OF N-AMIDINOETHYLENDIAMINE-N,N'-DIACETIC ACID



LINE#	HEIGHT	FREQ(HZ)	PPM
1	776.78	1002.99	4.171
2	850.68	961.90	4.000
3	124.52	906.64	3.771
4	211.93	901.88	3.751
5	146.13	899.31	3.740
6	251.00	895.95	3.726
7	242.78	866.67	3.604
8	146.99	863.23	3.590
9	215.60	860.80	3.580
10	117.69	856.05	3.560

FIGURE II-8: ^{1}H NMR OF 1,3-DICARBOXYMETHYL-2-IMINOIMIDAZOLIDINE

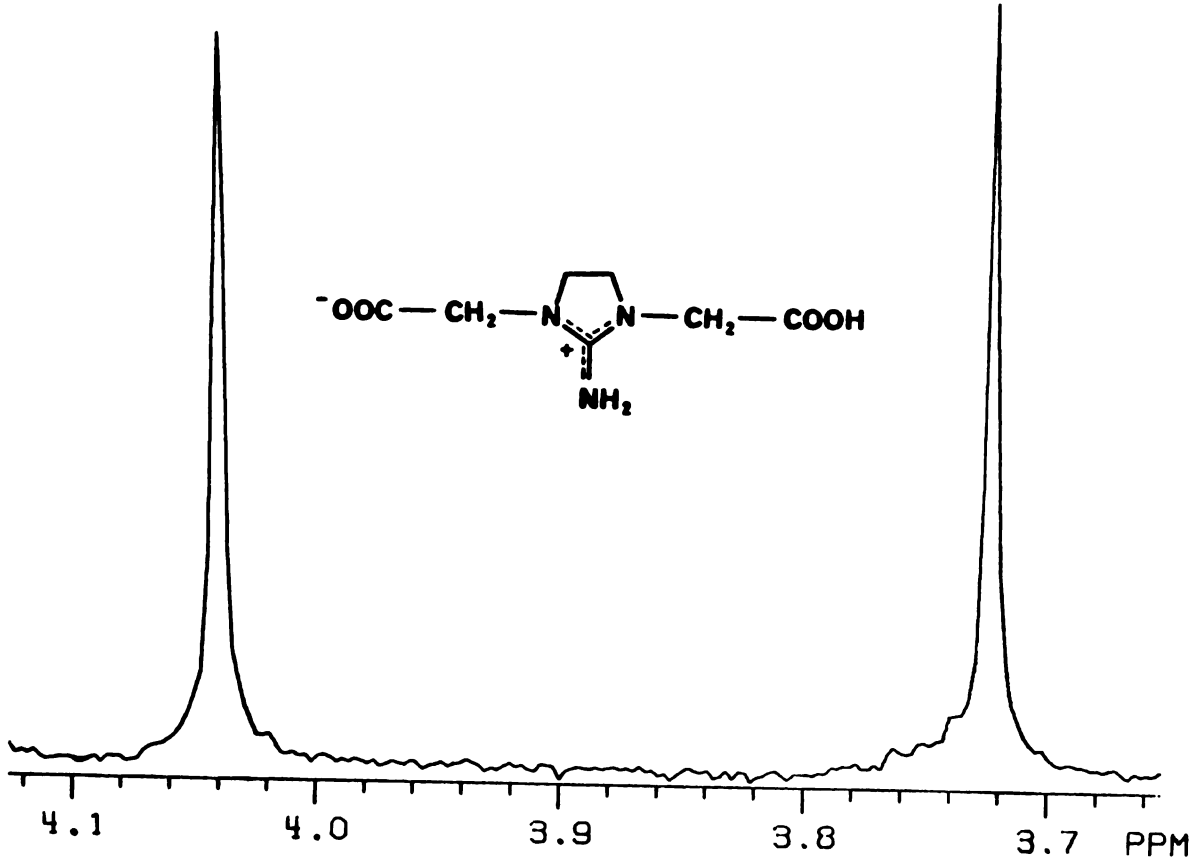


FIGURE II-9: ^{1}H NMR OF 2-METHYL-N-CARBOXYMETHYLIMIDAZOLE

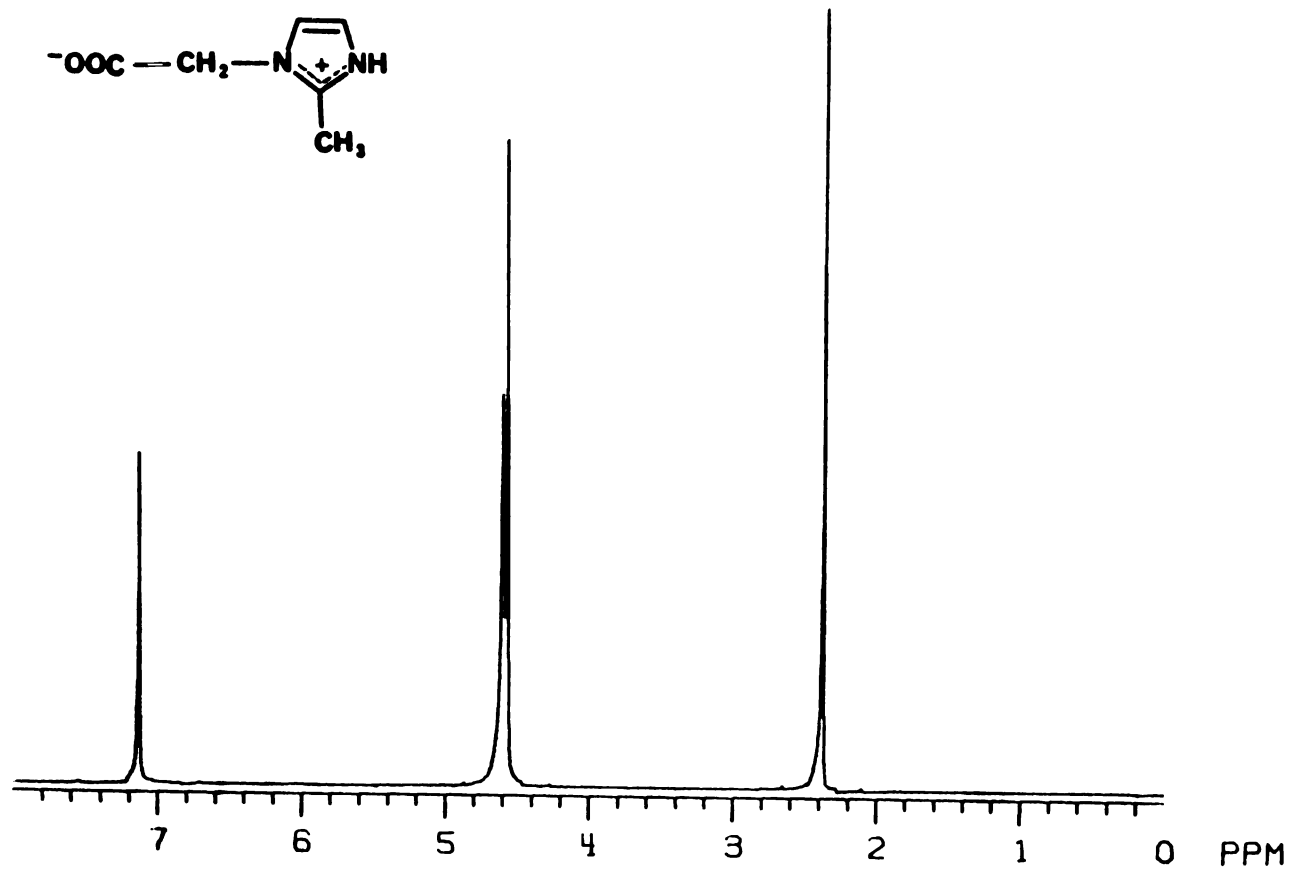


Figure II-10:

GC-MS spectrum of 2-methyl-N-carboxymethylimidazole (13) was obtained by the chemical ionization at the source of 240° C on a Kratos MS-25 instrument equipped with a Varian Capilliary GC System at the Mass Spectrometry Resource, School of Pharmacy, UCSF.

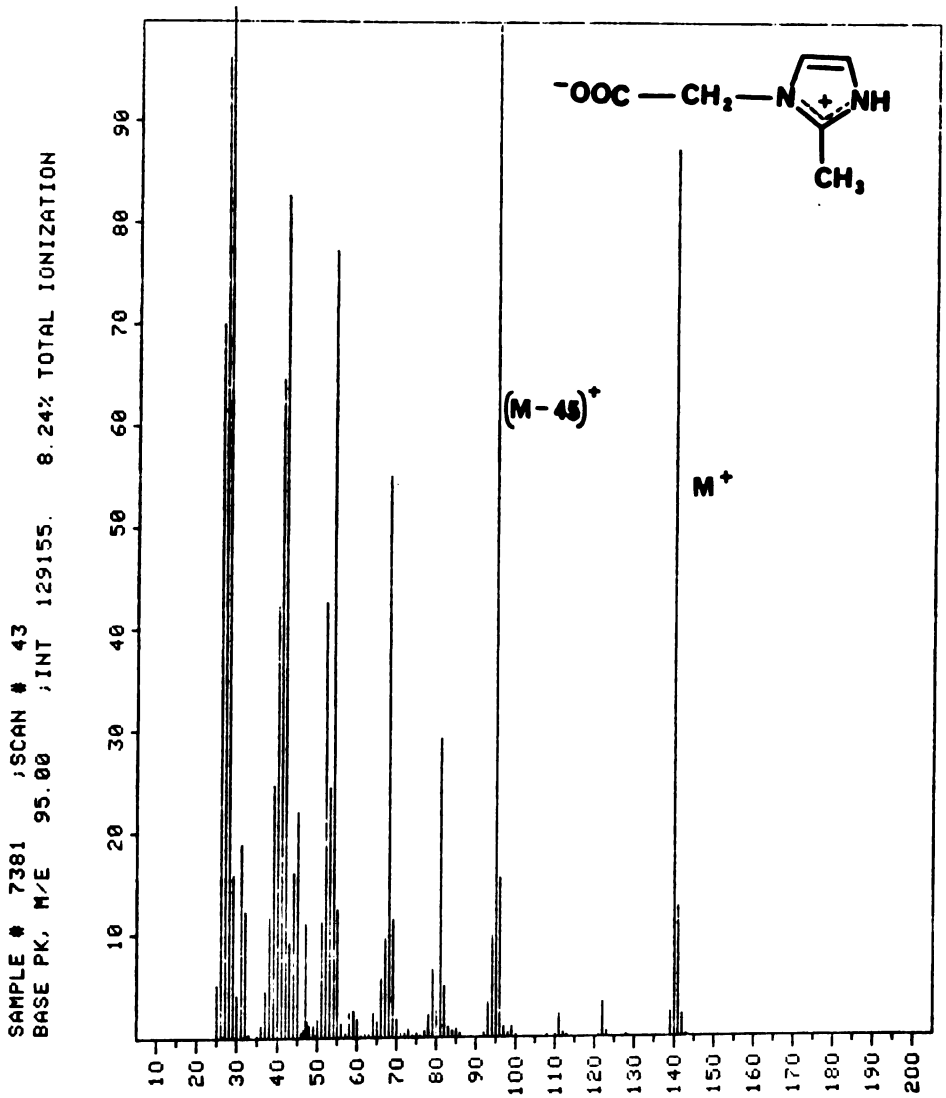
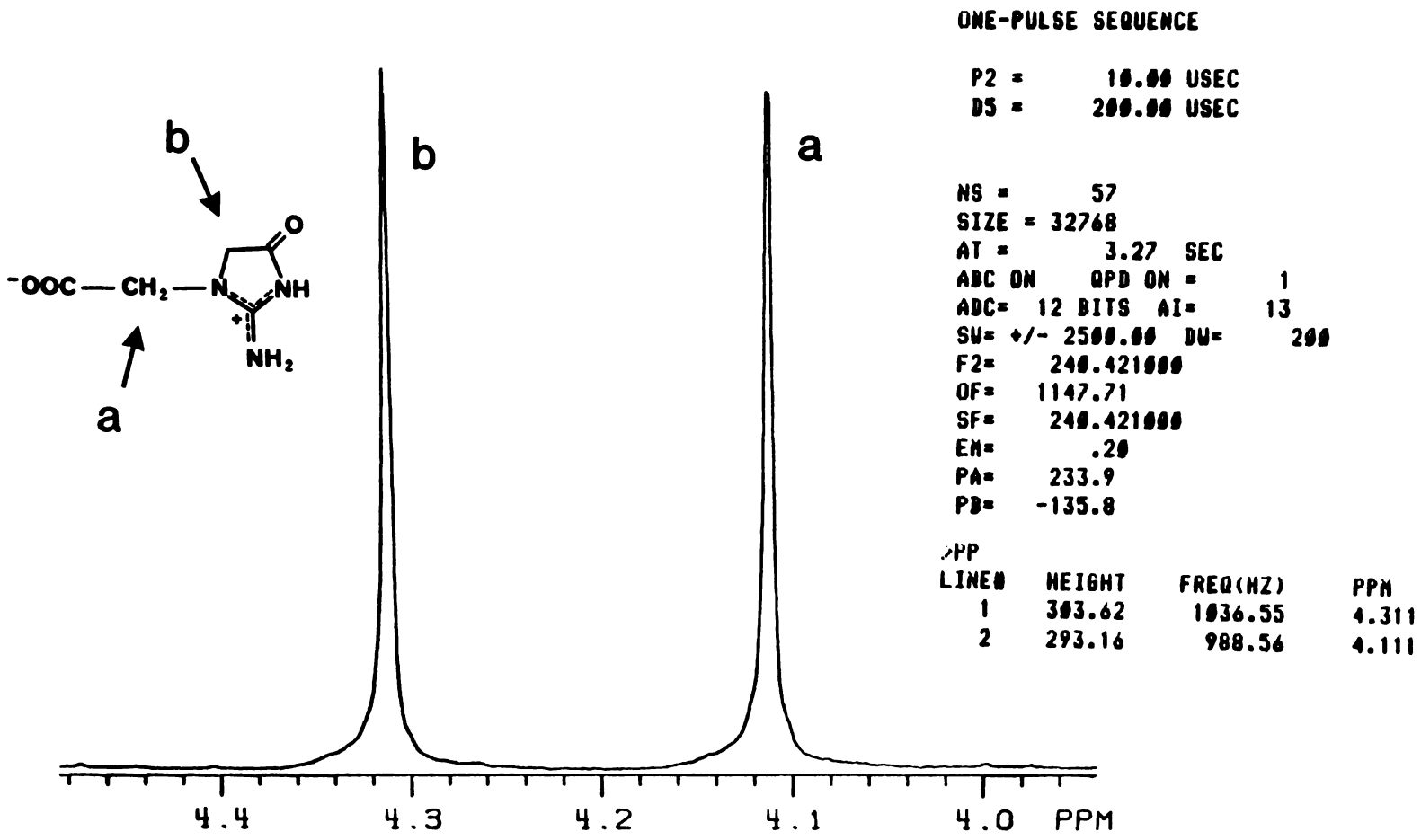


FIGURE II-10:

GC-MS OF 2-METHYL-N-CARBOXYMETHYLIMIDAZOLE

FIGURE II-11: ^{1}H NMR OF 1-CARBOXYMETHYL-2-IMINOIMIDAZOLIDINE-4-ONE



ONE-PULSE SEQUENCE

P2 = 10.00 USEC
D5 = 200.00 USEC

NS = 57
SIZE = 32768
AT = 3.27 SEC
ABC ON QPD ON = 1
ADC= 12 BITS AI= 13
SW= +/- 2500.00 DW= 200
F2= 240.421000
OF= 1147.71
SF= 240.421000
EM= .20
PA= 233.9
PB= -135.8

PP

LINE#	HEIGHT	FREQ(HZ)	PPM
1	303.62	1036.55	4.311
2	293.16	988.56	4.111

CHAPTER III

ENZYME STUDIES USING CREATINE ANALOGS

EXPERIMENTAL PROCEDURE

MATERIAL

Creatine monohydrate was purchased from Aldrich Chemical Company. All other materials needed for enzyme assays were obtained from Sigma Chemical Company. They are listed below along with the catalog number: creatine kinase (# C-3755); ATP (# A-5394); phospho(enol)pyruvate monopotassiun salt (PEP), (# P-7127); L-lactic dehydrogenase (# L-1254); pyruvate kinase (# P-9136); NADH (# N-8129); and bovine serum albumine (# A-4503).

Other materials used were obtained from the following sources: $Mg(OAc)_2$ (J. T. Baker Chemical Co.); N-2-hydroxymethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) (Calbiochem); glycine (Aldrich); NaOH (J. T. Baker Chemical Co.); NaOH solution N/10 (Fisher Scientific Co.); and KOAc (Merck & Co., Inc.).

3-Carboxymethyloxazolidine was made using the method of Marletta and Kenyon (Marletta's Ph.D. Thesis, 1978).

METHOD

PEI Method.

All creatine analogs were preliminarily tested as substrates for the enzyme creatine kinase using the

polyethylenimine (PEI) cellulose thin layer chromatography method of Rowley and Kenyon (1974). The PEI plates were either prepared as described in the above reference or purchased from Macherey-Nagel Co.. Each enzymatic reaction was a mixture of following solutions:

Solution		Mixture
Na_2ATP	0.04 M	0.050 mL
$Mg(OAc)_2$	0.04 M, pH 8.5	0.050 mL
glycine	0.10 M, pH 9	0.350 mL
creatine kinase	20 mg/mL of 0.01 M HEPES, pH 7.5	0.050 mL
creatine or analogs		5 mg

Solutions were kept at 0° C. All components of the mixture, except for creatine kinase, were combined in a 1 mL reaction cell. Two control reactions were also performed without substrate analogs: one with 5 mg creatine and another without it. At time 0, creatine kinase was added each reaction cell to initiate the reaction. The reaction cells were again placed in an ice bath to maintain the temperature at 0° C. At time t, 5 μL of each sample was spotted on a PEI thin layer chromatography plate. The plates took about 40 to 45 minutes to develop, using 1.2 M LiCl as eluent. If PEI plates prepared by the procedure of Rowley and Kenyon (1974) were used, it is necessary to use their

visualization technique to reveal all the phosphorylated compounds. If fluorescent indicator PEI plates were used, a simple UV lamp was needed to see all UV absorbent components, such as ATP and ADP. The appearance of ADP and disappearance of ATP would indicate an essentially irreversible substrate. A control with creatine would have approximately 50/50 of ATP and ADP, and a control without creatine would have 100% of ATP with no trace of ADP.

pH-stat method.

Preliminary testing of all inhibitors was performed on a thermostatted Radiometer pH-stat microtitration apparatus. A Radiometer TTT-2 titrator, an ABU-12 autoburet unit (0.25 mL volume), and a REC-51T recorder were used to control and record the addition of sodium hydroxide to the reaction mixture. The initial rates were measured by the consumption of sodium hydroxide at 30° C under nitrogen flow.

Each kinetic run was organized as follows:

Solution	Volume
$Mg(OAc)_2$ 0.04 M	0.3 mL
bovine serum albumin 1%	0.3 mL
ATP 0.04 M	0.3 mL
creatine .08 M	varied
creatine kinase 1 mg/mL of 0.01 M HEPES pH 7.5	10 µL

water	added
Total volume	3 mL

The total volume of each reaction mixture was 3 mL. A solution of all components, and, where present, inhibitor, was stirred magnetically in the reaction vessel for a few minutes. Before the addition of the enzyme, the pH of the reaction mixture was brought to the desired value (usually pH 8 for pH-stat method) by the addition of concentrated base, using a syringe initially, and then the instrument. The enzymatic reaction was initiated by the addition of 10 μL of creatine kinase. The uptake of base was recorded as a function of time, and the initial velocity was calculated by measuring the base uptake starting immediately after the addition of the enzyme to the preincubated reaction solution. It was found to be linear with time for the first few minutes of reaction and started bending as ADP accumulated as product. ADP itself is an inhibitor of the forward reaction (Maggio et al., 1977). The concentration of creatine in each reaction mixture was in the range of 60 to 2.4 mM.

Enzyme coupled assay.

In the forward direction, that is creatine phosphorylated by ATP, creatine kinase was coupled to pyruvate kinase and lactic dehydrogenase. The initial velocity was measured by the slope of the change in the absorbance at 340 nm. The procedure used for this thesis work was modified from that of McLaughlin et al. (1973). The procedures are as follows:

Buffer solution:

glycine	0.2 M pH 9
$Mg(OAc)_2$	0.12 M
KOAc	0.2 M

Glycine was used as a buffer at pH 9. Glycine (3.8301 g) was dissolved in about 200 mL of distilled and deionized water, and the solution was adjusted to pH 9 using NaOH. $Mg(OAc)_2$ (0.6435 g) and KOAc (4.907 g) were added to the above buffer. The solution was then adjusted again to pH 9 before filling to 250 mL using a volumetric flask.

Assay solution:

Assay solution consisted of the following components:

ATP	178.8 mg
PEP	20 mg

NADH	12 mg
PK	8 mg
LD	14 mg

ATP (0.1788 g) was introduced into a 50 mL volumetric flask. Two-thirds of the total volume was added with buffer solution before adding all other components. The flask was then filled up to 50 mL with buffer solution.

The stock enzyme solution of creatine kinase had a concentration of 1 mg/mL of 0.01 M HEPES (pH 7.5). This stock enzyme was diluted to 0.1 mg/mL just before use to avoid a pipetting problem. The stock enzyme is stable for several months when stored in a refrigerator at concentration of 1 mg/mL. The less concentrated enzyme is not sufficiently stable for storing.

For the determination of V_{max}, K_m, and K_i, the concentration of components in the auxiliary system of the coupled reactions was raised until the addition of each component did not change the rate of the overall reaction. The components in the auxiliary system were then used in excess to make certain that the auxiliary system did not limit the rate of the overall reaction. The assay solution is stable for at least a week. It was kept cold in a refrigerator.

Each final kinetic run contained the following solutions in its 1 mL UV cell, and was prepared just before the assay:

Cell solution:

assay solution	0.5 mL
creatine 0.05 M	0.4 mL to 0.01 mL
creatine kinase 0.1 mg/mL	0.012 mL
water added	0.088 mL to .478 mL
Total volume	1 mL

All components, except for creatine kinase, were added to the UV cell and warmed to 30° C for at least one minute before assay. Creatine kinase was finally added to the reaction cell to initiate catalysis. All kinetic assays were done at 30° C and pH 9. A constant temperature was maintained by circulation of thermostatically controlled water through an outer jacket system.

The final concentrations are outlined in Table III-1. Under these conditions, the initial rate was linear until the reaction terminated. The conversion of NADH to NAD was then monitored with a spectrophotometer at 340 nm. The initial velocity was calculated from the slope of this progress curve which is measured as OD/min.

$$\text{Initial velocity } Vi = \frac{\text{Slope OD/min.}}{6.22} \text{ mmoles/min.}$$

Our K_m value of 9.42 ± 0.58 mM determined for creatine kinase using creatine as a varied substrate is

within experimental error the same as that reported by Maggio *et al.* (1977) (K_m = 8.61 ± 0.57 *mM*). A set of control runs without inhibitor were performed every day before inhibition studies, and each was found to be virtually identical. For testing purposes, the concentrations of pyruvate kinase and lactic dehydrogenase were only one-third of those in Table III-1.

TABLE III-1

ENZYME COUPLED ASSAY

Component	Final concentration
Creatine	Varied from 20 mM to 0.5 mM
ATP	3 mM
Glycine buffer, pH 9	100 mM
$Mg(OAc)_2$	6 mM
KOAc	100 mM
PEP	0.20 mg/mL
NADH	0.12 mg/mL
Pyruvate kinase	0.08 mg/mL
Lactic dehydrogenase	0.14 mg/mL
Creatine kinase	1.2 g/mL

RESULTS

CREATINE ANALOGS AS INHIBITORS

A PEI-TLC method was used for screening of creatine analogs as substrates for the enzyme creatine kinase. Figure III-1 shows the PEI plates with 13 substrate analogs. Plate A was taken after 1 hr reaction, whereas plate B was taken after 1 day. Controls were performed at the same time and under the same conditions. Spot number 0 shows the control without creatine, whereas spot number 1 shows the control with 5 mg of creatine. Other spots from numbers 2 to 15 show the enzymatic reactions of creatine kinase using creatine analogs as substrates, each with 5 mg of analog. The enzyme catalytic reactions are determined from the appearance of ADP (Rf = 3.2 cm) and the disappearance of ATP (Rf = 2.4 cm). Results from this PEI method indicate that with carbocreatine (spot # 5), an essentially irreversible substrate, all ATP had disappeared after one day's reaction. Cyclocreatine (spot # 13) which has been synthesized using new method presented in this thesis was also spotted here as an additional proof for its structural assigment. After 1 hour's reaction, no trace of ATP was left for this compound, indicating a powerfully irreversible substrate.

McLaughlin *et al.* (1972) reported a relative V_{max} for cyclocreatine about 90% of that of creatine. Two other compounds DL-C-allylglycocyamine (4) (spot # 3) and N-amidino-N-cyanoethylglycine (5) (spot # 4) are poor substrates, as the appearance of ADP can be seen in the plate. Since they are poor substrates and poorly soluble as well, there were no further attempts to measure their K_m values. Isoepoxycreatine (spot # 2), 2-phenylimidazolidine iodoacetic acid salt (spot # 6), tolazoline-N-acetic acid (14) (spot # 7), biscreatine (9) (spot # 8), N-amidinoethylendiamine-N,N'-diacetic acid (10) (spot # 9), 2-methyl-N-carboxymethylimidazole (13) (spot # 10), N-amidino-D-phenylalanine (7) (spot # 11), N-amidino-D-phenylglycine (6) (spot # 12), guanido-N,N-diacetic acid (8) (spot # 14), and 1-carboxymethyl-2-iminoimidazolidine-4-one (15) (spot # 15) all showed no detectable substrate activities.

A kinetic study of carbocreatine (1) as a substrate for creatine kinase was also performed. Figure III-2 shows the typical Lineweaver-Burke plots for the catalytic reaction of creatine kinase using carbocreatine as a substrate. The concentration of creatine kinase was kept constant at 0.05 mg/mL. The carbocreatine concentration was varied from 0.5 mM to 2.0 mM.

An enzyme coupled assay method was used to measure the K_m for carbocreatine. Other substrates and auxiliary

system were used as: ATP (1 mM); $Mg(OAc)_2$ (6 mM); KOAc (0.1 M); PEP (0.2 mg/mL); NADH (0.1 mg/mL); pyruvate kinase (0.032 mg/mL); and lactic dehydrogenase (0.032 mg/mL) in glycine buffer (0.1 M) at pH 9 and 30° C.

Results from kinetic studies of carbocreatine (1) as a substrate is shown below as determined by a computer program developed by Cleland (1963):

$K_m = 7.64 \pm 1.45$ mM

$V_{max} = 0.096 \pm 0.010$ mmoles/min.mg protein

$1/V_{max} = 10.39 \pm 1.64$ (mmoles/min.mg protein)$^{-1}$

$V_{max}/K = 0.0125 \pm 0.0004$

Thus, the K_m value of carbocreatine determined from above condition is nearly equal to that of natural substrate creatine (9.42 ± 0.58 mM). Maggio et al. (1977) reported a K_m (creatine) for the native creatine kinase of 8.61 ± 0.57 mM. The relative V_{max} of carbocreatine is, however, less than 1% of that of creatine.

CREATINE ANALOGS AS INHIBITORS

The inhibition of creatine analogs were performed by measuring the progress curves for the enzymatic reactions at different concentrations of the substrate creatine. The enzyme concentration was kept constant for all inhibition studies (1.2 g/mL). The progress curves which recorded the disappearance of NADH at 340 nm with time were linear. The initial rates were calculated from the slopes of these

progress curves as described in the Methods section. Typical Lineweaver-Burke plots for the phosphorylation and the inhibition of creatine by its synthetic analogs are shown in Figures III-3 to III-12.

The fact that the reciprocal plots of the initial velocity versus substrate concentration in the presence and absence of inhibitor are linear and intersect at the same point on the vertical axis indicates that the inhibitors are strictly competitive. Thus, the inhibitors apparently bind to the same site of the enzyme as the variable substrates.

The values of K_i, inhibition constant, of the competitive inhibition were calculated from the slopes of the reciprocal plots of the initial rates versus the substrate concentrations in the presence of the inhibitor and from the values of K_m/V_{max} which were obtained from the slopes of the reciprocal plots in the absence of inhibitor.

$$\text{Slope}_I = \frac{K_m}{V_{max}} \left[1 + \frac{I}{K_i} \right]$$

or

$$\text{Slope}_I = \text{Slope}_{NI} \left[1 + \frac{I}{K_i} \right]$$

Where:

I : concentration of inhibitor

$Slope_I$ = slope of L.-B. plots in the presence of inhibitor.

$Slope_{NI}$ = slope of L.-B. plots in the absence of inhibitor.

K_m = Michaelis constant.

V_{max} = maximum velocity.

The values of K_i for all creatine analogs are outlined in the Tables III-2 and III-3. Creatine and cyclocreatine structures were added as references. K_i values of N-amidino-D-phenylalanine (7) (IV), N-amidino-D-phenylglycine (6) (V), N-amidino-N-(cyanoethyl)glycine (5) (VI), and biscreatine (9) (VIII) are not available, due to the low solubility of these compounds. Preliminary tests for these compounds showed that, except for N-amidino-N-(cyanoethyl)glycine (5) (VI), all others are inhibitors.

DISCUSSION

Carbocreatine, an analog in which a nitrogen atom of guanidino group of creatine is replaced by a carbon atom, has been found to be a substrate for creatine kinase in the forward reaction with a V_{max} relative to creatine of less than 1%. The fact that carbocreatine is a poor substrate for creatine kinase clearly indicates the importance of the nitrogen atom that has been replaced.

In order to have transphosphorylation, it is necessary to have the attacking nitrogen atom be a good nucleophile. For this to happen, the positive charge of the guanidinium group must be localized mainly on the two other nitrogens. The nitrogen atom that has been replaced in carbocreatine may therefore play an important role in the catalytic reaction, even though it is not directly involved in the transphosphorylation process. This tertiary nitrogen atom may interact ionically with the carboxyl group at the active site of creatine kinase. Just such a carboxyl group must be ionized for binding of either creatine or phosphocreatine under all conditions as found by Cook *et al.* (1981). This carboxyl group may also be the one that forms a covalent bond to the epoxide group of epoxycreatine as reported by Marletta and Kenyon

(1979). Via such an ionic interaction, this carboxyl group could assist in localizing the positive charge away from the nitrogen being phosphorylated. Our results with carbocreatine support a SN_2 mechanism for the phosphoryl transfer between substrates, in which one of the two primary guanidino nitrogens of creatine would attack the γ-phosphoryl group of ATP and, simultaniously, weaken the P-O bond.

Kinetic studies reveal that carbocreatine binds to the active site of the enzyme as well as creatine does. Thus, the K_m value for carbocreatine (7.65 ± 1.45 mM) in within experimental error the same as that of creatine (8.61 ± 0.57mM). The loss of activity as a substrate may also be explained by the rotation of the amidino group of carbocreatine at the active site of the enzyme. The rotation of the single C-C bond between the nitrogen-replaced carbon and the carbon atom of the amidino group may greatly reduce the activity of the compound as a substrate. The result of the low K_m value also indicates the unimportance of the binding role of the tertiary nitrogen in the binding to the active site of the enzyme.

Inhibition studies using carbocreatine also demonstrate that it is a competitive inhibitor in the forward reaction with a K_i value of 24 mM. The fact that inhibition is strictly competitive reinforces the idea that carbocreatine binds to the enzyme at the active site

(Santi and Kenyon, 1979).

Both N-amidino-D-phenylalanine and N-amidino-D-phenylglycine are not substrates indicating that bulky groups such as benzyl or phenyl group are not tolerated. Similar results had been found by Rowley and Kenyon (Rowley's Ph.D. Thesis, 1971). Thus, enzyme apparently does not have room for an aromatic residue in its active site, especially on the carbon α- to the carboxylate group. On the other hand, a methyl group is rather well tolerated on that carbon. McLaughlin et al. (1972), for example, reported a V_{max} of DL-N-methyl-N-amidinoalanine of about 24% as compared to that of creatine. Groups bulkier than methyl group in that region are much less desirable. Thus, D,L-C-allylglycocyamine shows a very little activity as a substrate. Results from kinetic studies do show, on the other hand, that D,L-C-allylglycocyamine is a competitive inhibitor for creatine kinase, with a K_i value of 143 mM. The D-isomer has a K_i value of 100 mM wherease its L-isomer has a K_i value of 50 mM.

The N-methyl group of the natural structure creatine is obviously desirable since creatine is more reactive as a substrate than glycocyamine (McLaughlin et al., 1972). Bulkier groups on that nitrogen are less desirable. Thus, a cyanomethyl group in place of that methyl group completely destroys the activity of the compound either as

a substrate or an inhibitor.

Compounds with bulkier groups (VII, VIII, and IX) than cyanoethyl group in place of the N-methyl have also been found to be competitive inhibitors for creatine kinase. These compounds have additional electrostatic groups and therefore may bind tightly to the enzyme by specific hydrogen bonds with H-bond receptors at the active site of the enzyme and/or electrostatic interactions between the charged groups and those of the amino acid residues at the active site.

As can be seen from table III-3, the low K_i values for a number of cyclocreatine analogs show the strong affinity of the enzyme toward the planar cyclic guanidines. As compared to other types of creatine analogs, these analogs have relatively low K_i values in the range from 1 to 36 mM, except for tolazoline-N-acetic acid (XV, K_i = 115 mM) which has a benzyl group in place of the primary nitrogen atom. Once again, the enzyme does not seem to have any tolerance of aromatic residues at its active site.

1,3-Dicarboxymethyl-2-iminoimidazolidine (XII) and 1-carboxymethyl-2-iminoimidazolidine-4-one (XV) have structures close to cyclocreatine which results in low K_i values. Except for a small amount of added bulk at the C-4 position, the structure of 1-carboxymethyl-2-iminoimidazolidine-4-one (XV) is very much the same as

that of cyclocreatine. The loss of activity as a substrate can be explained by the presence of the carbonyl carbon at the 4-position of cyclic guanidinium group which deactivates the nearby secondary nitrogen as a nucleophile toward the γ-phosphoryl group of ATP.

1,3-Dicarboxymethyl-2-iminoimidazolidine (XII) has a hydrogen of cyclocreatine replaced by a carboxymethyl group. This is the hydrogen that normally would be replaced by a phosphoryl group in the enzymatic reaction. This carboxymethyl group, then, resembles that phosphoryl group. The strong binding of the compound to the active site of the enzyme may come from the electrostatic interaction of this additional carboxymethyl group with the ϵ-ammonium group of the lysyl residue postulated by James and Cohn (1974) to be in the active-site region. This should also be the case with 2-methyl-N,N'-dicarboxymethylimidazole (XIV).

The imidazole type analogs (XIII and XIV), which have absolutely flat 5 membered rings, also inhibit the enzyme competitively.

Comparison between the K_i values of 2-methyl-N,N'-dicarboxymethylimidazole (XIV) (K_i = 1.4 mM) and 1,3-dicarboxymethyl-2-iminoimidazolidine (XII) (K_i = 2.6 mM) where the primary nitrogen being replaced by a methyl group indicate the unimportance of the primary nitrogen atom in the binding.

3-Carboxymethyloxazolidine (XVI), where the secondary nitrogen of cyclocreatine is replaced by an oxygen, is also an inhibitor with a K_i value (260 mM) lower than other cyclocreatine analogs. The lower affinity toward the active site compared to other cyclocreatine analogs can be explained be the property of oxygen atom which may distort the planar structure of the 5-membered ring cyclic guanidines.

Conclusions

The fact that carbocreatine binds to the enzyme as well as creatine does and with a relative V_{max} of less than 1% indicates the necessity of the nitrogen being replaced in the catalytic reaction and its relative unimportance in binding.

The enzyme does not tolerate aromatic residues at its active site.

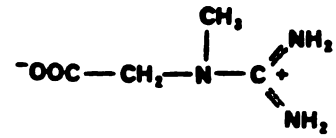
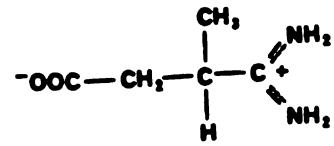
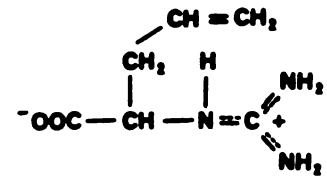
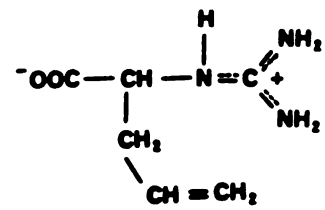
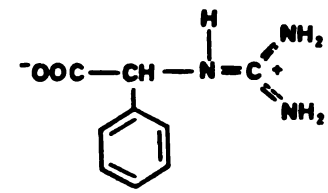
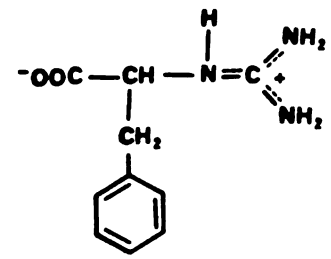
Compounds related to cyclocreatine but with additional electrostatic attraction and/or hydrogen bonding abilities bind rather well to the active site of the enzyme.

The K_i values of cyclocreatine analogs, which fall in a relatively low range between 1 to 36 mM, indicate the preference of the enzyme for relatively flat, 5-membered ring cyclic guanidines.

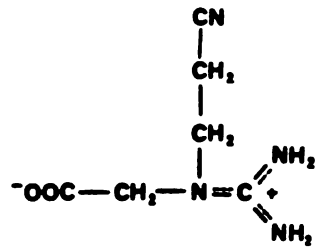
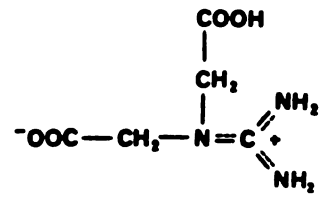
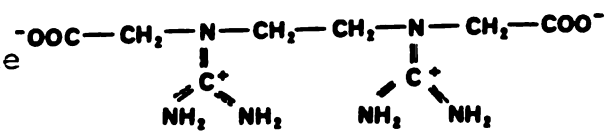
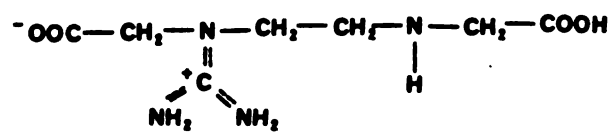
The additional carboxymethyl groups of 1,3-dicarboxymethyl-2-imidazolidine (XII) and 2-methyl-N,N'-dicarboxymethylimidazole (XIV) may take the place of the phosphoryl group being transferred during catalysis.

TABLE III-2

K_i CONSTANTS OF CREATINE ANALOGS

No.	Name	Structure	K_m	K_i mM
I	Creatine		9	
II	Carbocreatine		8	24
III	D-C-allyl-glycocyamine			100
	DL-C-allyl-glycocyamine			143
	L-C-allyl-glycocyamine			55
IV	C-Benzyl-glycocyamine			-
V	C-Phenyl-glycocyamine			-

(Table III-2 cont.)

No.	Name	Structure	Ki
VI	N-amidino-N-(cyanoethyl)-glycine		-
VII	Guanidino-N,N-diacetic acid		80
VIII	Biscreatine		-
IX	N-amidino-ethylen-diamine-N,N'-diacetic acid		166

(Table III-2 cont.)

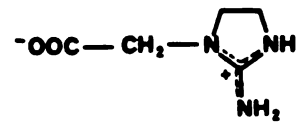
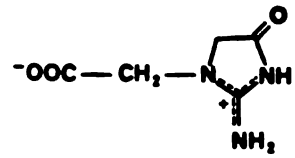
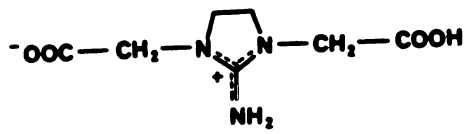
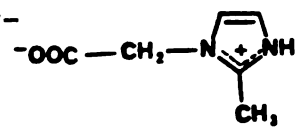
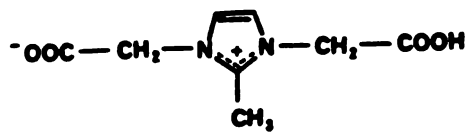
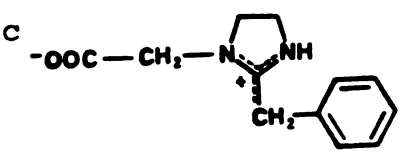
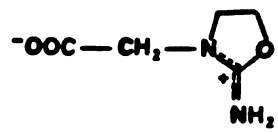
No.	Name	Structure	Ki
X	Cyclocreatine		-
XI	1-carboxymethyl-2-iminoimidazolidine-4-one		2
XII	1,3-Dicarboxymethyl-2-iminoimidazolidine		3
XIII	2-Methyl-N-carboxymethylimidazole		36
XIV	2-Methyl-N,N'-dicarboxymethylimidazole.		1
XV	Tolazoline-N-acetic acid.		115
XVI	3-carboxymethyloxazolidine		260

Figure III-1:

Polyethylenimine (PEI) cellulose thin layer chromatography of creatine analogs. The plates were prepared as described in the Method Section. Plate A was taken after 1 hour's reaction, whereas plate B was taken after 1 day's reaction.

Spot # 0: control without creatine

1: control with creatine

2: isoepoxycreatine

3: C(α)-allylglycocyamine (4)

4: N-amidino-N-(cyanoethyl)-glycine (5)

5: carbocreatine (1)

6: 2-phenylimidazdolidineiodoacetic acid salt

7: tolazoline-N-acetic acid (14)

8: biscreatine (9)

9: N-amidinoethylendiamine-N,N'-diacetic acid (10)

10: 2-methyl-N-carboxymethylimidazole (13)

11: N-amidino-D-phenylalanine (7)

12: N-amidino-D-phenylglycine (6)

13: cyclocreatine

14: guanidino-N,N-diacetic acid (8)

15: 1-carboxylmethyl-2-iminoimidazdolidine-4-one (15)

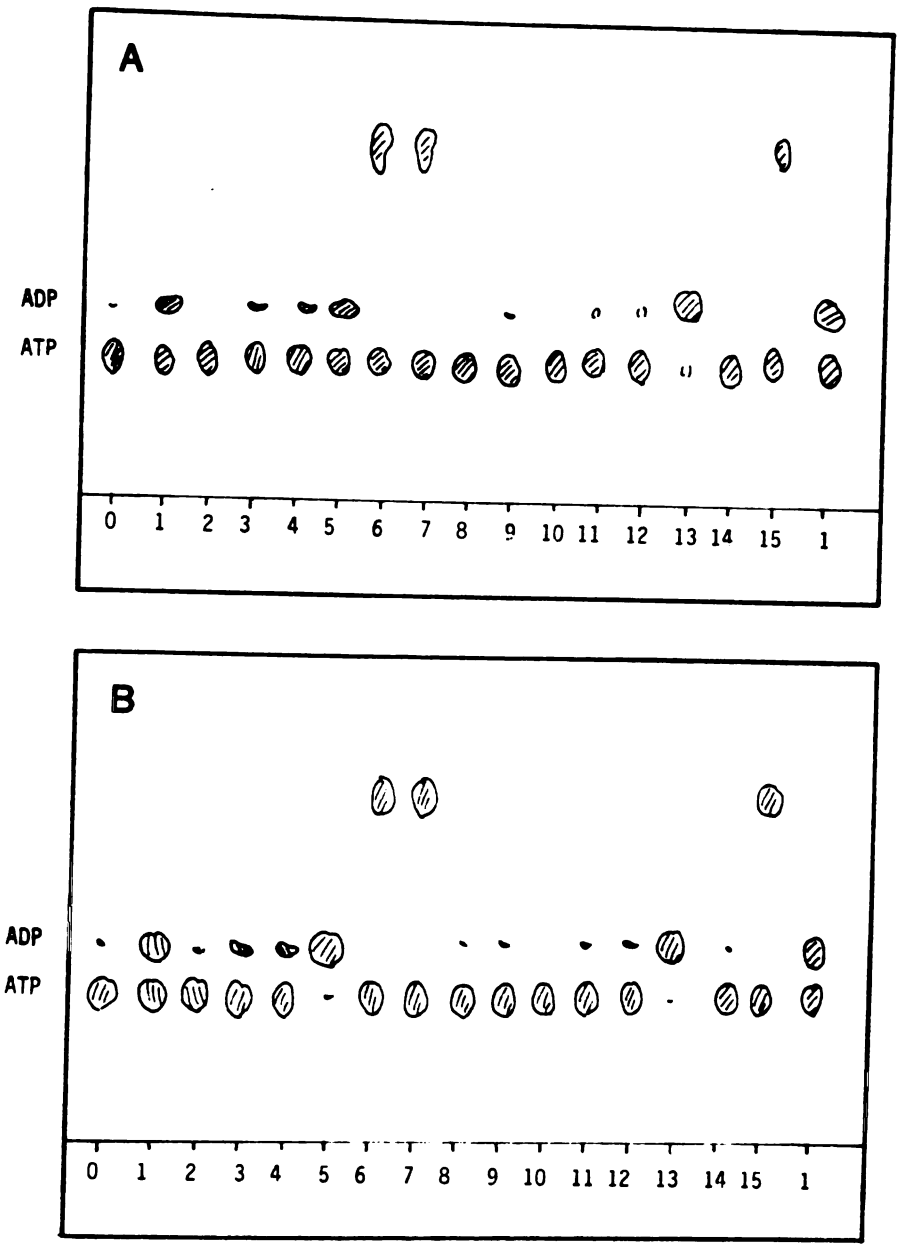


FIGURE III-1:

PEI PLATES: CREATINE ANALOGS AS SUBSTRATES

Figure III-2:

Reciprocal plots of the initial velocity 1/V (μmole/min.mg of protein)$^{-1}$ vs the carbocreatine concentration 1/S (mM^{-1}) at pH 9 (glycine buffer 0.1 M, pH 9) and at 30° C. The concentration of creatine kinase was 50 μg/mL. Concentrations of carbocreatine were in the range 0.5 to 20 mM. Concentrations of other substrates and of auxiliary system were ATP (1 mM), $Mg(OAc)_2$ (6 mM), KOAc (0.1 M), PEP (0.2 mg/mL), NADH (0.1 mg/mL), pyruvate kinase (0.032 mg/mL), and lactic dehydrogenase (0.032 mg/mL).

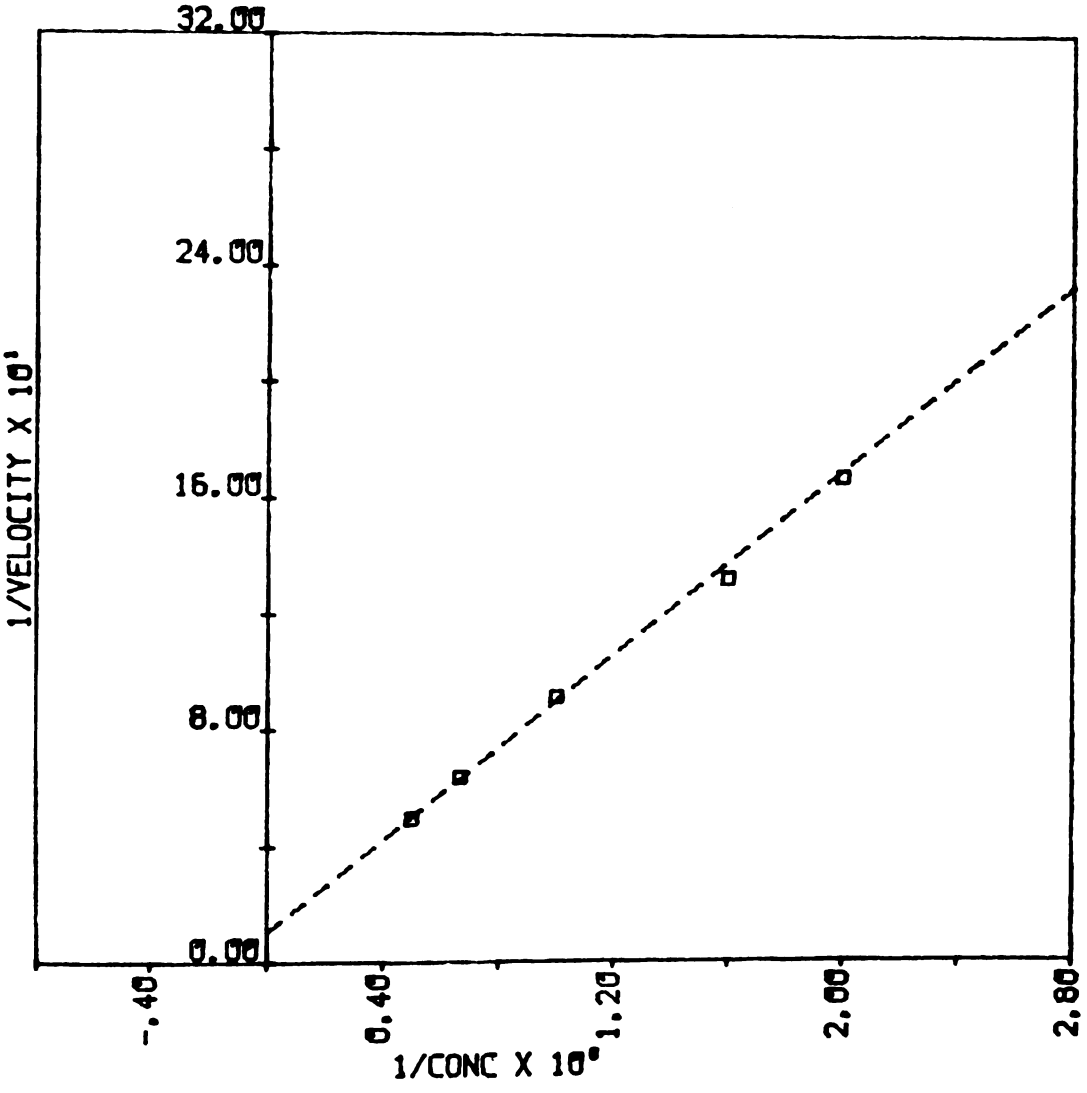


FIGURE III-2:

CARBOCREATINE AS A SUBSTRATE

Figure III-3 to III-12:

Lineweaver-Burke plots for the inhibitions of creatine kinase by creatine analogs (as stated on the figure) at pH 9 (buffer glycine 0.1 M, pH 9) and at 30° C. Initial velocities were determined from the slopes of progress curves as described in the Methods Section. The concentrations of inhibitors were stated on the figures. The reaction mixtures contained 0.5-20 mM of substrate creatine and an enzyme creatine kinase of 1.2 μg/mL ($1.49 \cdot 10^{-8}$ M). The concentration of the other substrates and the auxiliary system were those in Table III-1.

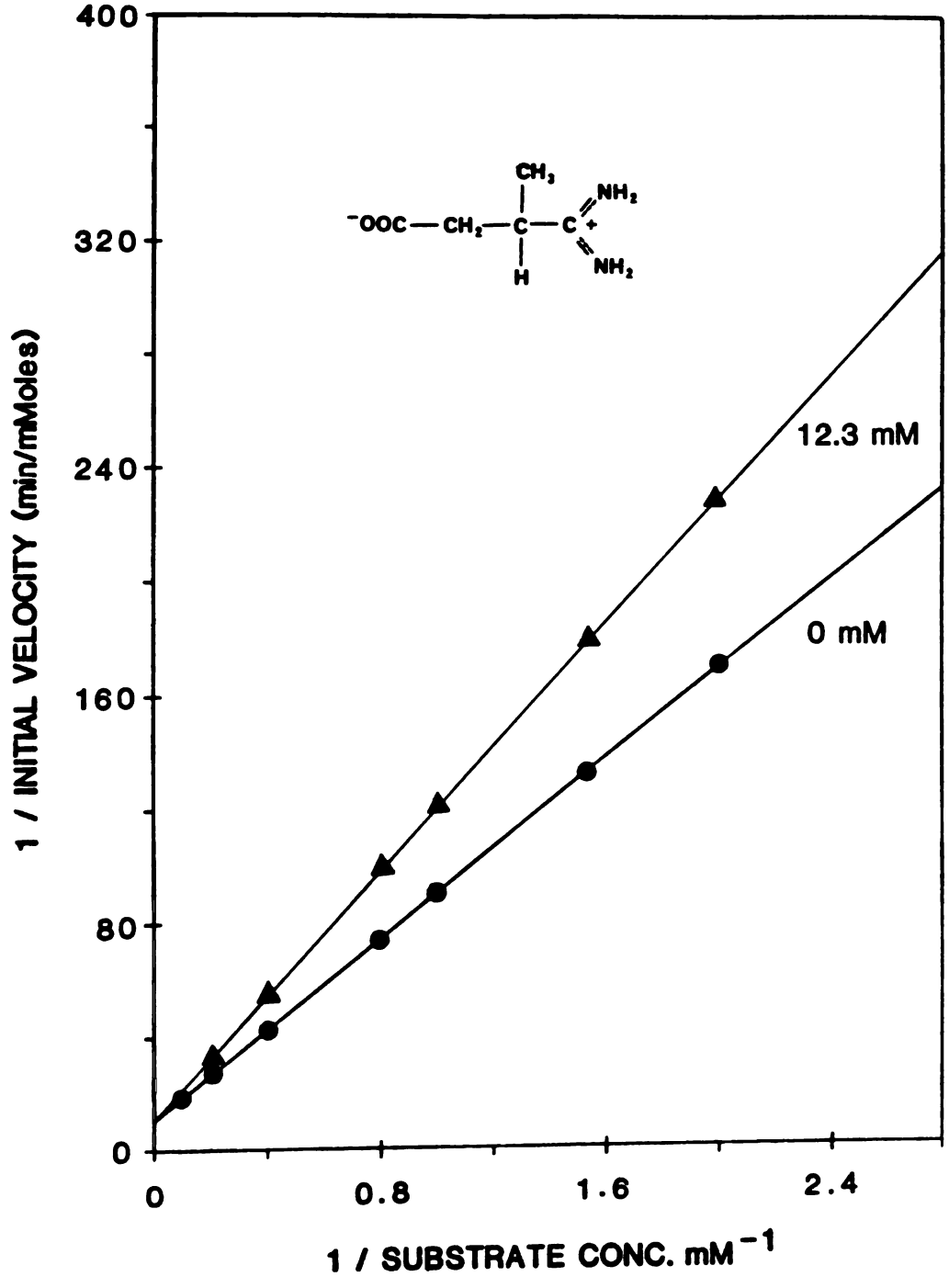


FIGURE III-3:

LINEWEAVER-BURKE PLOTS OF CARBOCREATINE

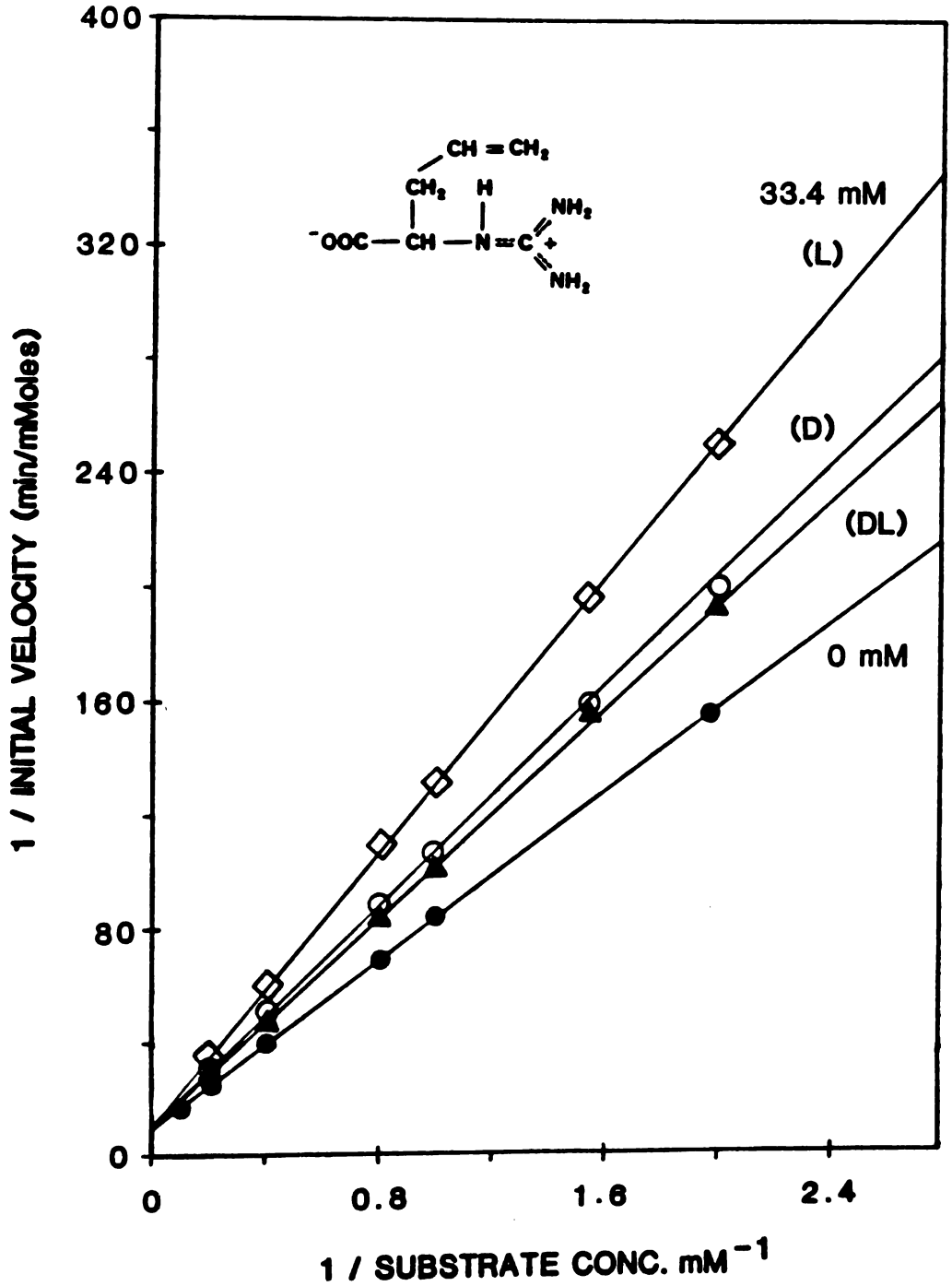


FIGURE III-4:

LINEWEAVER-BURKE PLOTS OF C(α)-ALLYLGLYCOCYAMINE

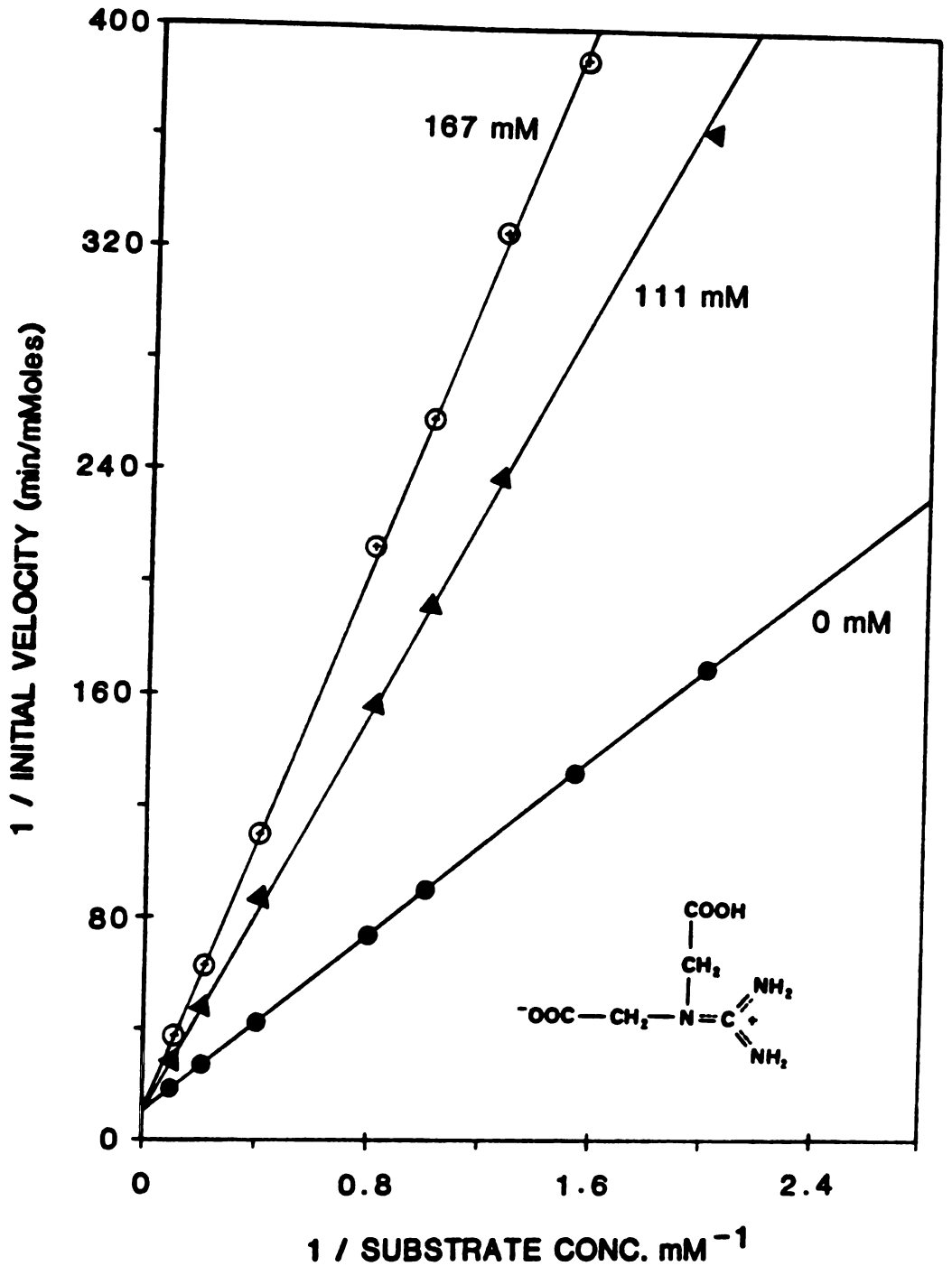


FIGURE III-5:

LINEWEAVER-BURKE PLOTS OF GUANIDO-N,N-DIACETIC ACID

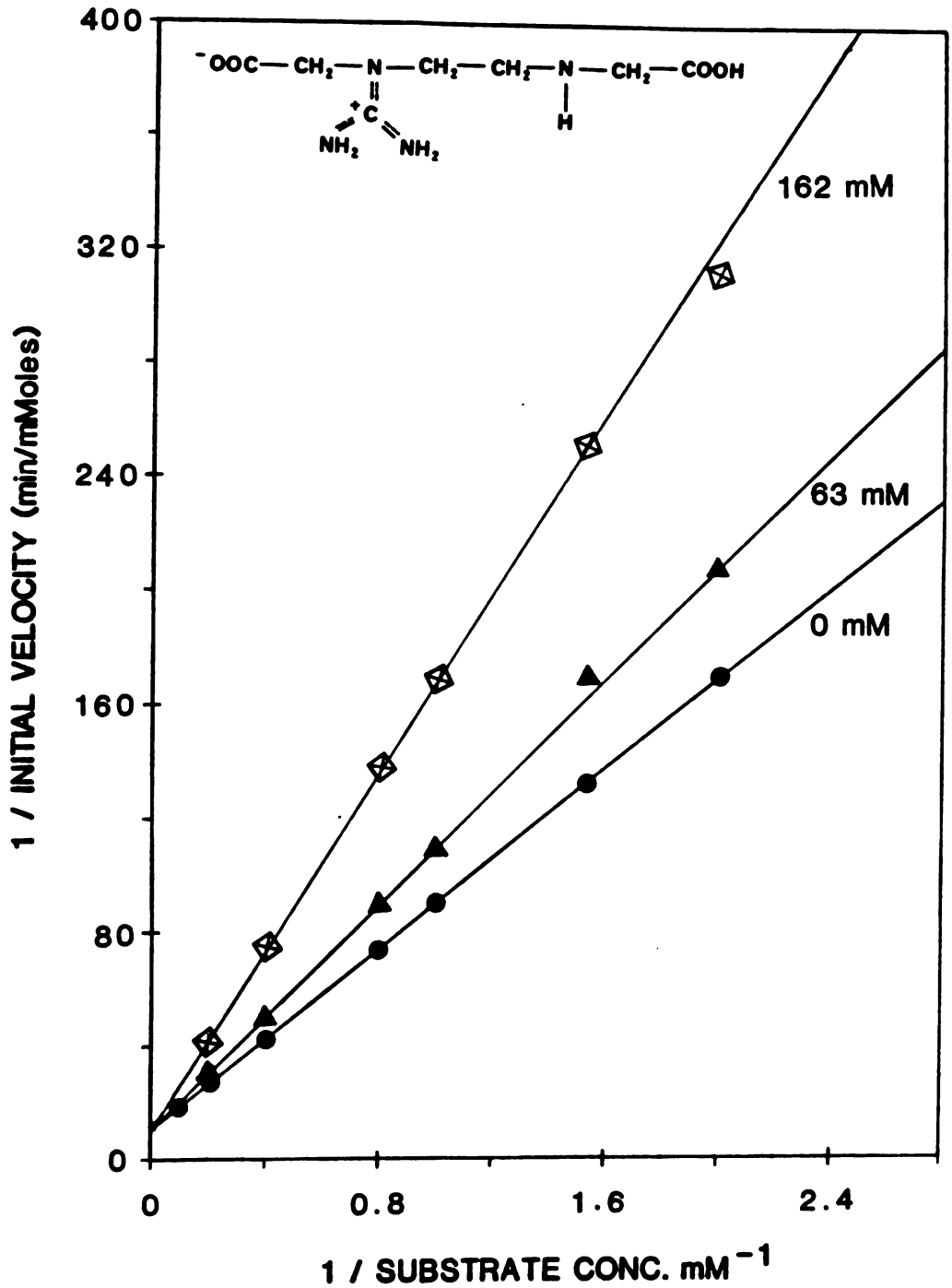


FIGURE III-6: LINEWEAVER-BURKE PLOTS OF N-AMIDINOETHYLENDIAMINE-N,N'-DIACETIC ACID

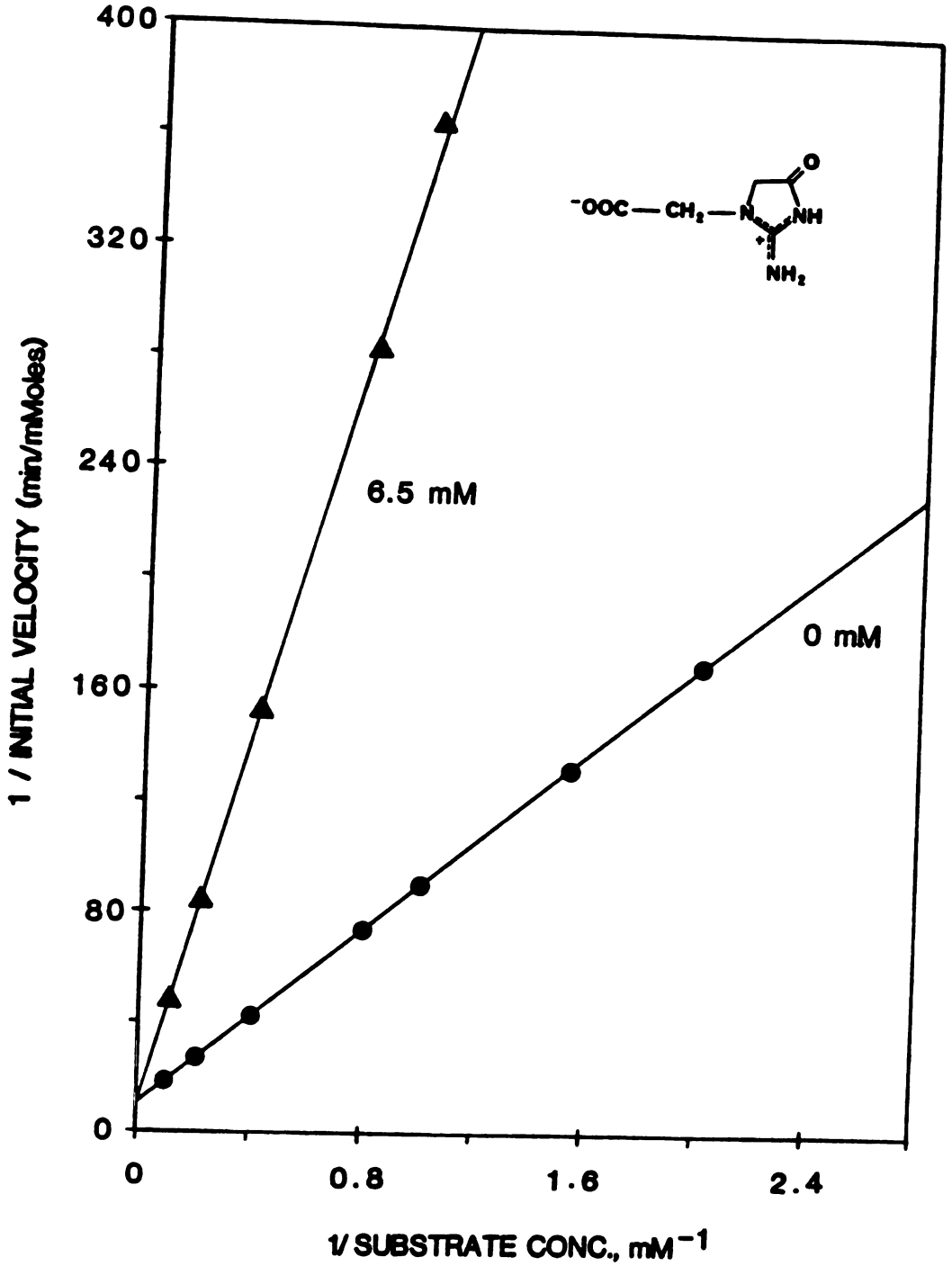


FIGURE III-7: LINEWEAVER-BURKE PLOTS OF

1-CARBOXYMETHYL-2-IMINOIMIDAZOLIDINE-4-ONE

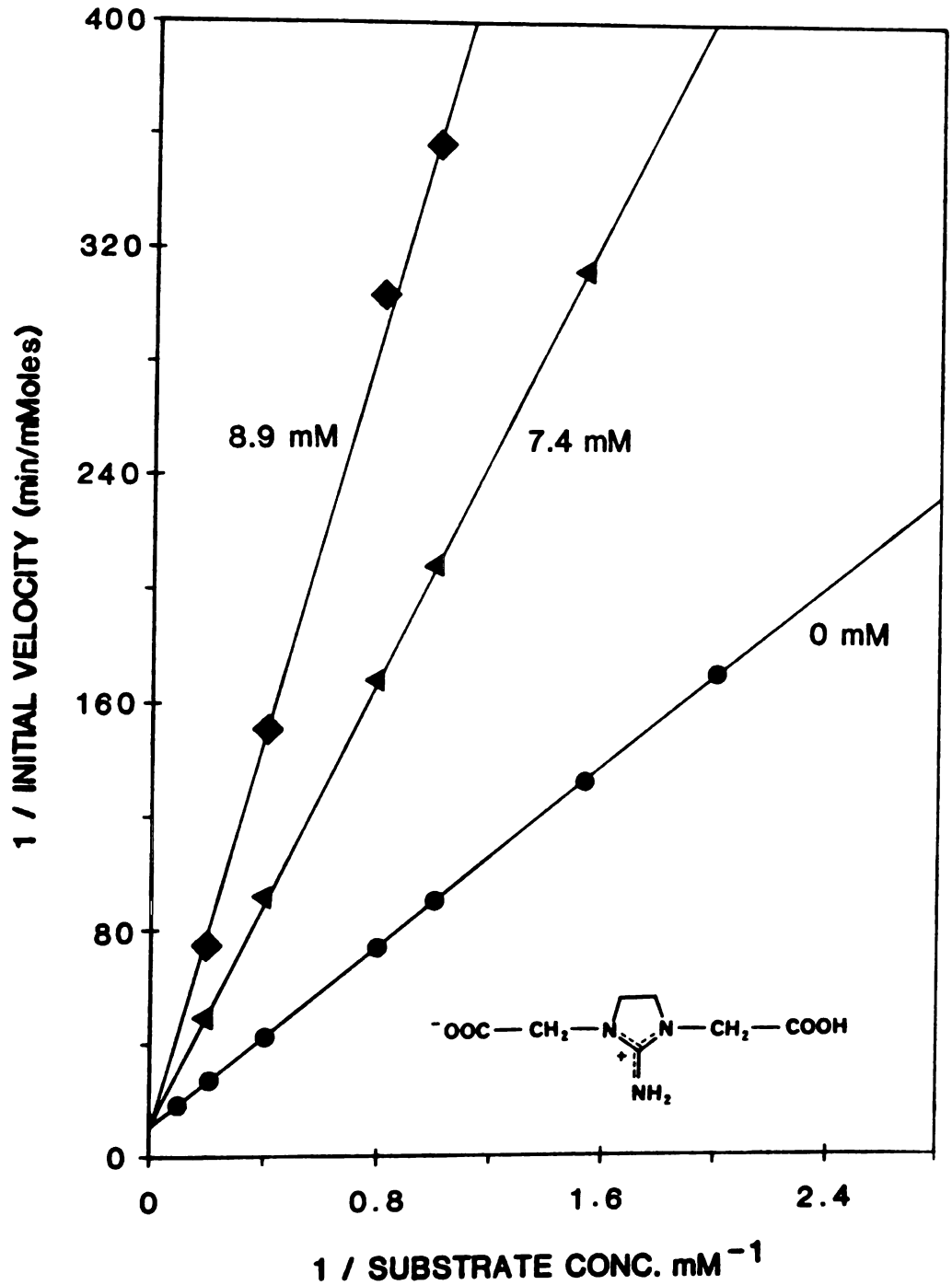


FIGURE III-8: LINEWEAVER-BURKE PLOTS OF

1,3-DICARBOXYMETHYL-2-IMINOIMIDAZOLIDINE

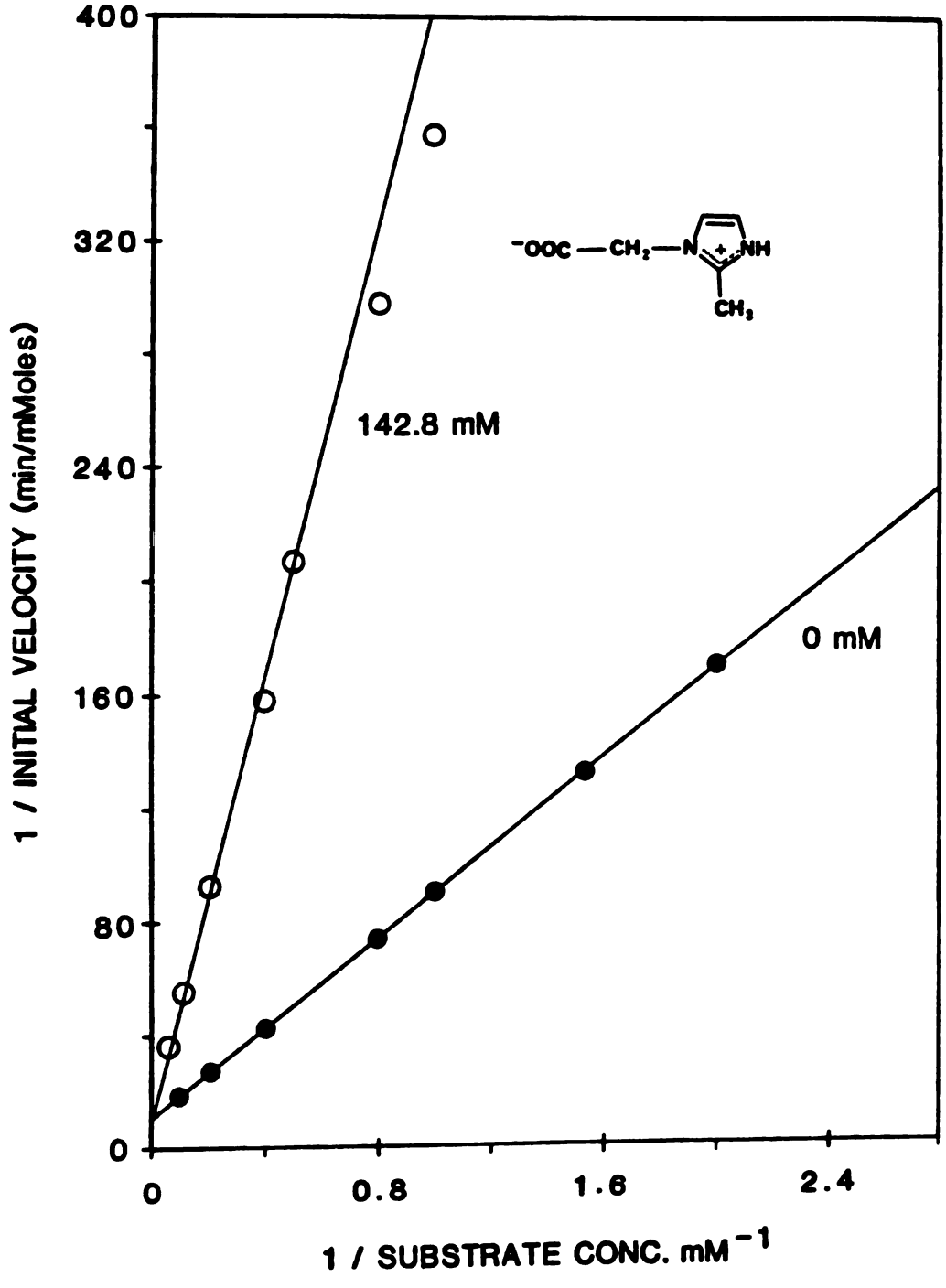


FIGURE III-9: LINEWEAVER-BURKE PLOTS OF 2-METHYL-N-CARBOXYMETHYLIMIDAZOLE

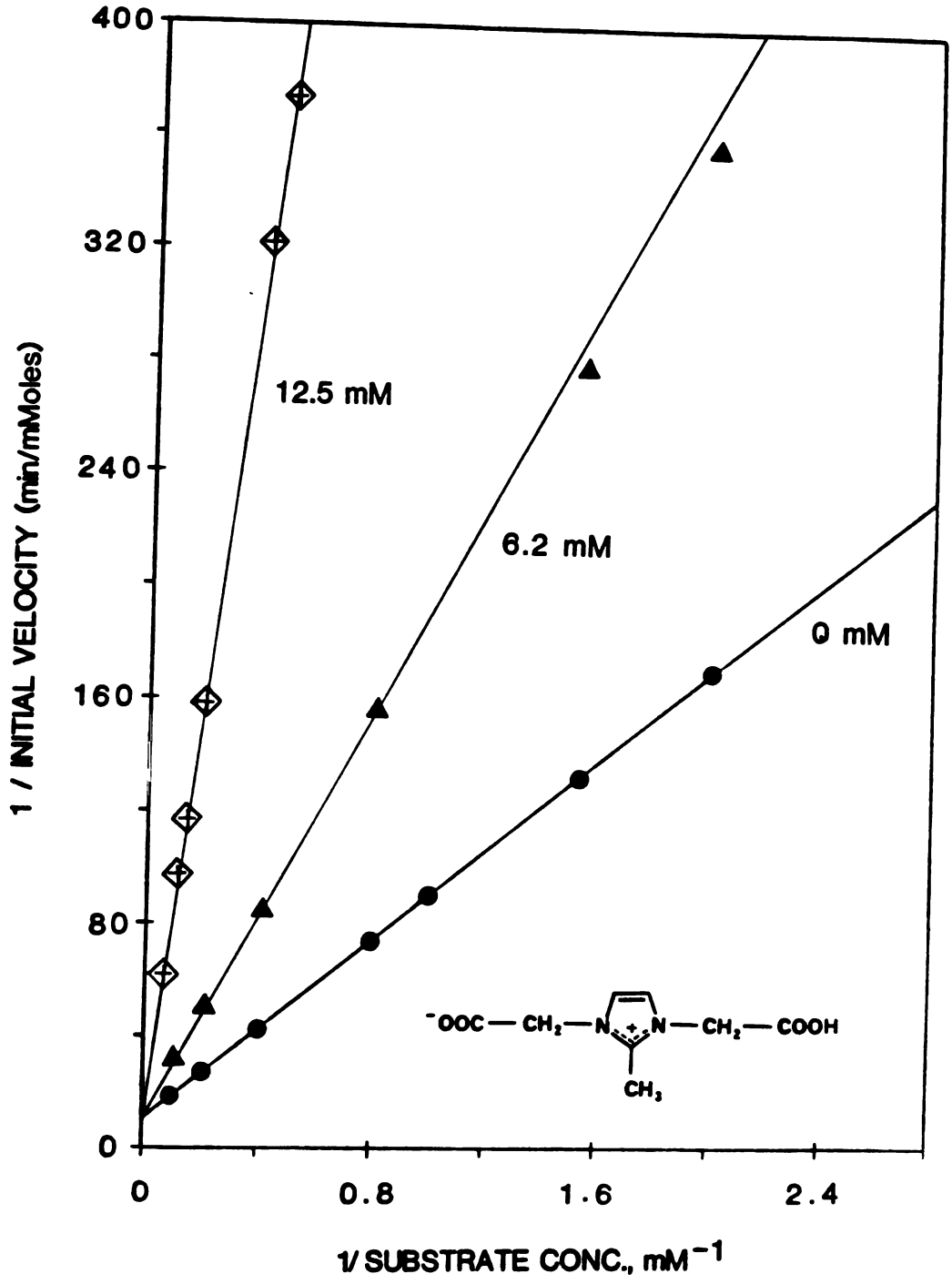


FIGURE III-10: LINEWEAVER-BURKE PLOTS OF 2-METHYL-N,N'-DICARBOXYMETHYLIMIDAZOLE

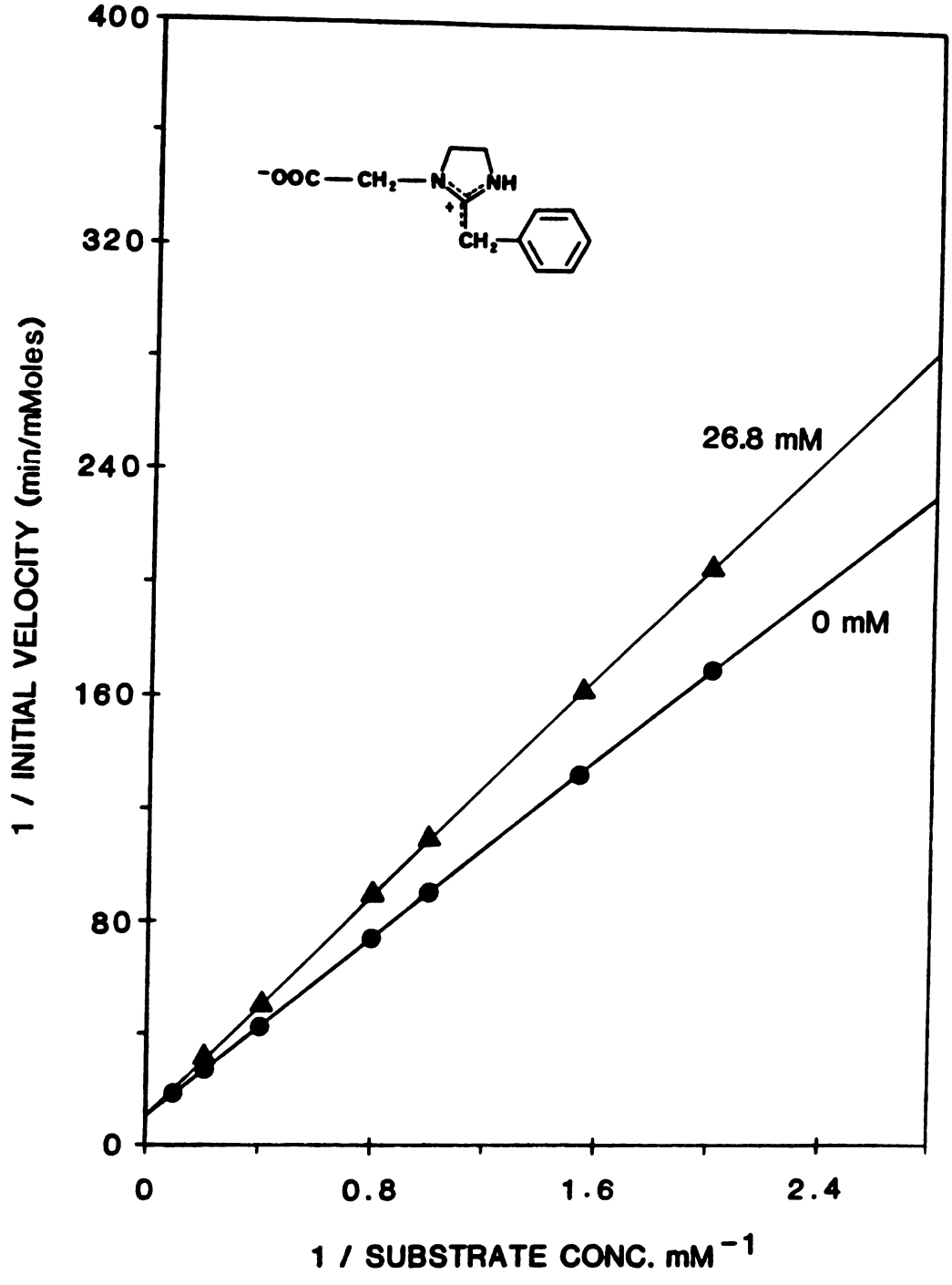


FIGURE III-11:

LINEWEAVER-BURKE PLOTS OF TOLAZOLINE-N-ACETIC ACID

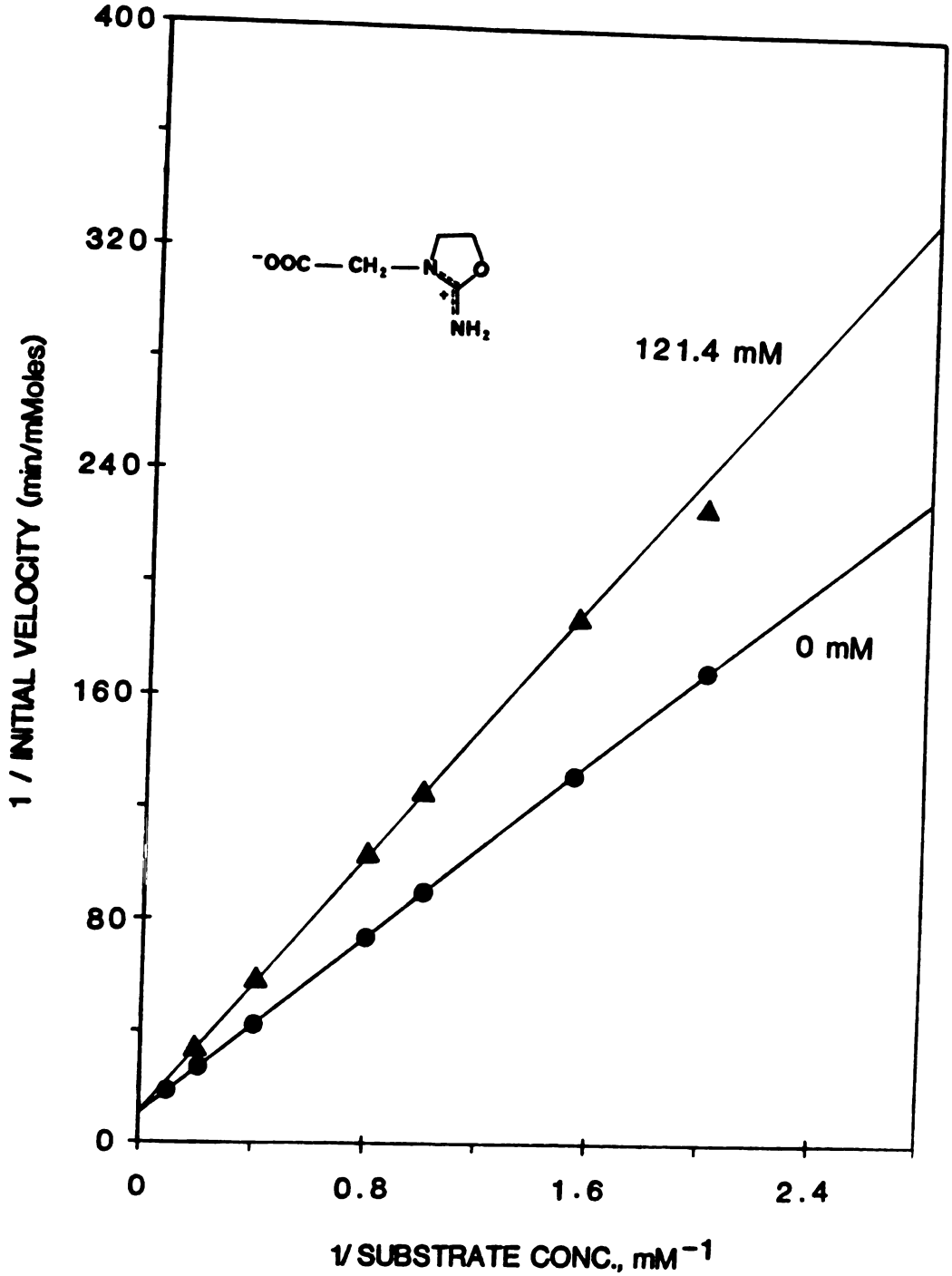


FIGURE III-12:

LINEWEAVER-BURKE PLOTS OF 3-CARBOXYMETHYLOXAZOLIDINE

CHAPTER IV

ISOEPOXYCREATINE

INTRODUCTION

Creatine kinase is an important enzyme in the mechanism of muscle contraction which catalyzes the reversible transfer of a phosphoryl residue from ATP to creatine. To study the active site of creatine kinase, Kenyon and coworkers have been involved in the design and synthesis of active-site directed reagents (Marletta and Kenyon, 1979). Their results suggested a carboxylate group of either an aspartic or a glutamic acid residue at the active site of the enzyme which was the nucleophile involved (Kenyon and Reed, 1983; Marletta and Kenyon, 1979).

Effort has been extended to study the geometry of the active site, while designing an affinity label structurally related to creatine. We report here the synthesis and structural analysis of C(∝)-(2,3-epoxypropyl)-glycocyamine (isoepoxycreatine) as well as the preliminary enzyme kinetic studies. This compound has a structure related to epoxycreatine (Marletta and Kenyon, 1979), but with additional chiral centers that can allow an investigation of the geometry of the active site of the enzyme.

Methods commonly used to determine the absolute configurations of chiral centers of a compound rely either on chemical correlations with optically active precursors

(Matzinger et al., 1972; Tramontini et al., 1972; and Mori, 1975) or on chemical degradations to optically-known substances (Larsen, 1967; and Wielan et al., 1968). The absolute configurations are then determined by comparisons of spectral properties with those of model compounds in which the absolute configurations are known. Here we propose a direct method to determine the absolute configuration of a chiral carbon by interpretation of the coupling constants of long-range three-bonded carbon-proton couplings (^{13}C NMR) as well as the couplings of vicinal methylene protons (^{1}H NMR). The selected hetero-decoupling and homo-decoupling experiments of ^{13}C NMR and ^{1}H NMR which confirmed the couplings are also presented.

EXPERIMENTAL

MATERIALS

Rabbit muscle creatine kinase, creatine, ATP, coupling enzymes, and all chemical used for the auxiliary system were purchased from Sigma Chemical Company as described in Chapter III.

D-, L-, and DL-C(α)-allylglycine were also obtained from Sigma Chemical Company and used as received. Other materials were obtained from the following sources: H_2O_2 50% (Dupont), H_2O_2 90% (FMC Chemical Company), sodium tungstate (Powers-Weightman Rosengarten Co.). Water for HPLC and for buffer solutions was distilled and deionized.

Detectors used for HPLC were the Waters Associates Differential Refractometer R-401 and the Kratos-797 (used at 210 nm). Columns used for analytical testing was the reverse phase 10μ, C-18, ODS-3 column manufactured by the Whatman Company. The preparative column was also a Whatman reverse phase, Partisil M-9, 10/50, 10μ, C-18, ODS-3.

Spectrophotometric assays were performed on the Hitachi 100-80 spectrophotometer with a built-in recorder.

SYNTHESIS OF ISOEPOXYCREATINE

C(α)-Allylglycocyamine (1.5 g)was dissolved in 10 mL of H_2O_2 50% with stirring. Sodium tungstate was added as a

catalyst to initiate the reaction. The reaction became pale-yellow immediately after the addition of sodium tungstate as the intermediate sodium pertungstate, WO_8^{2-}, appeared. The mixture was stirred for 2 hr and then poured into 200 mL of acetone to stop the reaction. White solid, which precipitated, was filtered through a fine-scintered glass filter and washed with acetone. Crude solid (1.6 g) was collected. An equal amount of this solid (80 mg) was dissolved in a minimum amount of cold water, millipore-filtered, and immediately injected into the HPLC system. The mobile solvent H_2O and the HPLC system which includes the loop, column, injector, and collector were also immersed in ice baths. Using preparative M-9 column and at a flow rate of 2 mL/min, the retention times for the product and its precursor were 20 min and 30 min, respectively. The by-product diol and a trace of impurity of sodium tungstate were found in the solvent front with a retention time of 13 min. The compound was collected in a round-bottomed flask, immediately frozen by dry-ice before lyophylization.

Anal. Calcd. for $C_6H_{11}O_3N_3$: C, 41.61; H, 6.40; N, 24.26; Found: For RS-C(α) isomer: C, 41.66; H, 6.33; N, 24.14. For R-C(α) isomer: C, 41.38; H, 6.40; N, 23.99.

^1H NMR (FT 80) in D_2O (TSP) gave peaks at δ 1.80 (2H, m), 3.53 (2H, m), 3.82 (1H, m), 4.22 (1H, m).

^1H NMR (240 MHz) in D_2O (TSP) of the diastereoisomers

$S_\alpha S_\gamma$ and $R_\alpha R_\gamma$ gave peaks at δ 1.69 (1H, m), 1.91 (1H, m), 3.53 (2H, m), 3.78 (1H, m), and 4.28 (1H, d-d). Diastereoisomers $S_\alpha R_\gamma$ and $R_\alpha S_\gamma$ gave peaks at δ 1.74 (1H,m), 2.03 (1H, m), 3.53 (2H, m), 3.89 (1H, m), and 4.21 (1H, d-d).

IR in KBr pellet gave peaks at wavenumbers (cm^{-1}) 3400 (broad), 1690, 1640, 1490, 1420, 1260, 1190, 930, and 880.

GC-MS gave peaks at m/e 173, 155, 142, 129, 125, 124, 112, 111, 99, 98, 86, 85, 84, 72, 70, 69, 57, 56, 55, 45, 44, 43, 32, 31, 29, 28, and 27.

RESULTS AND DISCUSSION

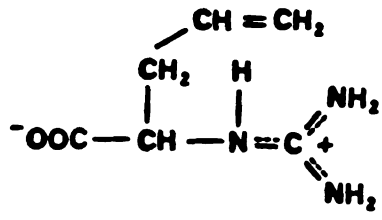
SYNTHESIS OF ISOEPOXYCREATINE

C(α)-(2,3-Epoxypropyl)-N-amidinoglycine or C(α)-(2,3-epoxypropyl)-glycocyamine was synthesized as shown in Scheme III-1. This epoxy compound, for simplicity, will be called isoepoxycreatine. Isoepoxycreatine is actually so named because its structure resembles that of epoxycreatine, N-(2,3-epoxypropyl)-N-amidinoglycine (Marletta and Kenyon, 1979). The synthesis includes two steps. The first step is the synthesis of the creatine analog, C(α)-allylglycocyamine or C(α)-allyl-N-amidinoglycine. The second step is to make isoepoxycreatine from this material.

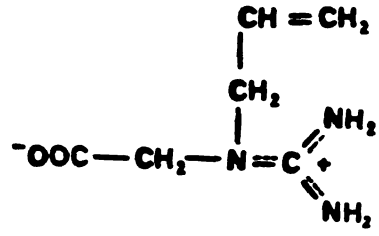
C(α)-Allylglycocyamines were prepared by the reactions of D-, L-, and DL-C(α)-allylglycine with cyanamide using aqueous ammonia as a catalyst. This follows a general method for the synthesis of glycocyamines using cyanamide as a precursor which was widely utilized by Kenyon and coworkers (Rowley et al., 1971; Dietrich et al., 1980). C(α)-Allylglycocyamine has one chiral center at its α-carbon and, therefore, has two stereoisomers. The synthesis of D-, L-, and DL-C(α)-allylglycocyamine have been described in Chapter II along with many other new analogs of creatine. It should

be stated that the names D-, L-, and DL- were after their precursor D-, L-, and DL-C(α)-allylglycine, natural amino acids. More correct names would be R-, S- and RS-C(α)-allylglycocyamine, respectively. As expected, the R-isomer, S-isomer and RS- racemic mixture all have the same spectral properties (IR, NMR, and mass spectra). These spectra are shown both in chapter II and also in this chapter in order to compare them with those of isoepoxycreatine.

Hydrogen peroxide (H_2O_2) was used for epoxidation of C(α)-allylglycocyamine with sodium tungstate (Na_2WO_4) as a catalyst. The choice of H_2O_2 was made because allyglycocyamine was soluble only in water and H_2O_2 works best in an aqueous solvent. The insolubility of these creatine analogs in organic solvents made it impractical to use other epoxidizing agents such as metachloro-peroxybenzoic acid or peroxyacetic acid, which require organic solvents. The pH 4 of 50% hydrogen peroxide solution was also considered to be neutral enough not to cause formation of diol from epoxide over a short period of time. The synthesis of epoxycreatine by Marletta and Kenyon (1979) also utilized H_2O_2 and Na_2WO_4. They reported difficuties in their epoxidation reaction, with a low yield of product tentatively explained by the inductive effect of the guanidinium group allylic to the double bond.

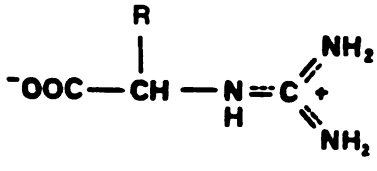


C(α)-allylglycocyamine

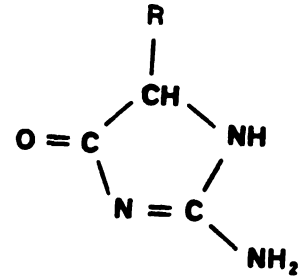


N-allylglycocyamine

C(∝)-Allylglycocyamine, on the other hand, has an allylic group bonded directly to the ∝-carbon of glycocyamine (one carbon away from electron withdrawing guanidinium moiety) and does not have the same electronic effect as that of N-allylglycocyamine. As a result, the yield of isoepoxycreatine was higher, and the compound was considerably more stable toward hydrolysis of the epoxide ring. The main difficulty in the isolation of this isoepoxycreatine is, however, the facile cyclization of the compound to form the corresponding glycocyamidine, which has proven a common problem for many glycocyamine analogs (Rowley et al., 1971).



glycocyamine
(R = H)



glycocyamidine
(R = H)

The presence of this cyclic compound can be detected both by its slight solubility in H_2O as compared to isoepoxycreatine and by the loss of activity as a time-dependent inhibitor, which will be described later in this chapter.

Trying to monitor directly the epoxidation reaction by observing the appearance of peaks using 240 MHz proton NMR did not yield much success. In a 5 mm NMR tube, H_2O_2 (90%) and D_2O (1/3 and 2/3 by volumes, respectively) were used as solvent, and all chemical shifts of allylglycocyamine (5 mg) were shifted upfield using TMS as external standard. A trace of sodium tungstate catalyst was used to initiate the reaction, small and broad peaks at approximately expected chemical shifts were detected above the noise about 45 min. after the reaction started. The probe temperature was 20° C. There were not any intermediates detected after 2 hr of reaction. The difficulty of this method is, however, that in order to see the appearance of peaks in a short time, a higher percentage of hydrogen peroxide is required which is, in turn, not allowed by the NMR method.

High pressure liquid chromatography proved to be a better method to monitor the epoxidation reaction. The columns used for analytical testing and for preparative separation were C-18, ODS-3, 10μ reverse phase columns. At certain times, approximately equal amounts of reaction

mixture were withdrawn from the reaction flask, diluted into water and immediately injected into the HPLC system. Using a 25 cm long analytical column with water as mobile solvent, the retention times for the diol, isoepoxycreatine, and the starting compound C(α)-allylglycine were 3.4 min., 5.4 min., and 7.4 min., respectively. Marletta and Kenyon (1979) also reported the retention time for their epoxycreatine between those of diol and N-allylglycine.

The epoxidation reaction was stopped at 2 hr as diol peak started to form. Proton NMR and ^{13}C NMR of crude product after 2 hr reaction are shown in Figures IV-1 and IV-2, respectively. As references, ^{1}H NMR and ^{13}C NMR of C(α)-allylglycocyamine, the precursor, are also shown here in Figures IV-3 and IV-4, respectively.

SCHEME IV-1

SYNTHESIS OF ISOEPOXYCREATINE

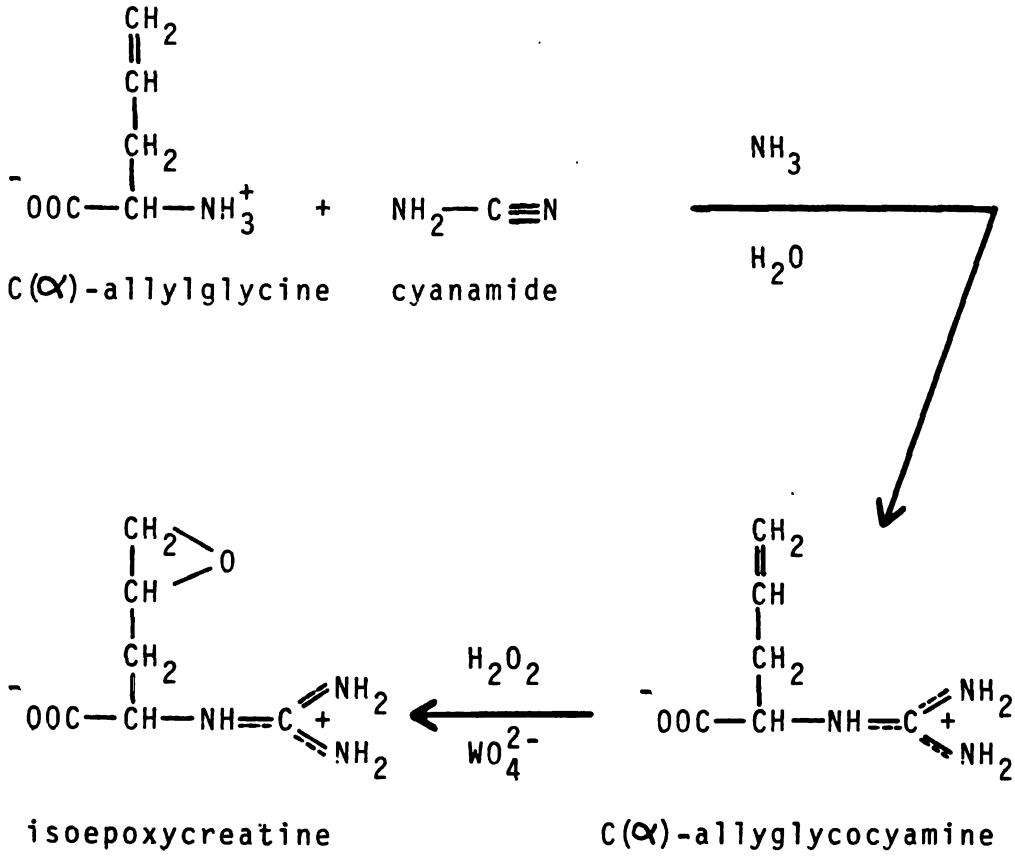


Figure IV-1 to IV-28:

^{1}H NMR spectra were performed of a 240 MHz instrument at the School of Pharmacy, UCSF. The program one-pulse sequence (1PULS) was used at the spectral frequency of 240 MHz, spectral width of ±2000 Hz, and the size of 16K data points. With selected proton-decoupled ^{1}H NMR, the program 1PULS was also use with decoupler turned on and the irradiation at the frequency of transition of selected proton.

^{13}C NMR spectra were also performed on a 240 MHz instrument. The program 1PULS with the decoupler turned on was used for the proton-decoupled ^{13}C NMR. For the fully-proton-coupled ^{13}C NMR, the program 1PDFA (one-pulse decoupler off during acquisition) was used with the decoupler off to get the same NOE effect as that of the proton-decoupled ^{13}C NMR. The size of 32K data points, spectrum frequency of 60 MHz, and spectrum width of ±6300 Hz were used for all ^{13}C NMR spectra.

For partially proton-decoupled ^{13}C NMR, the proton NMR was obtained while in ^{13}C NMR probe, and in ^{13}C NMR setting of the instrument. The program "JR" (jump and run) was used to eliminate the solvent water peak in order to get a ^{1}H NMR spectrum. The right irradiation frequency was found for the selected proton, the decoupler power was then reduced to 40 milisecond at 180 pulse before changing the program back to 1PULS with decoupler on to obtain the partially proton-decoupled ^{13}C NMR.

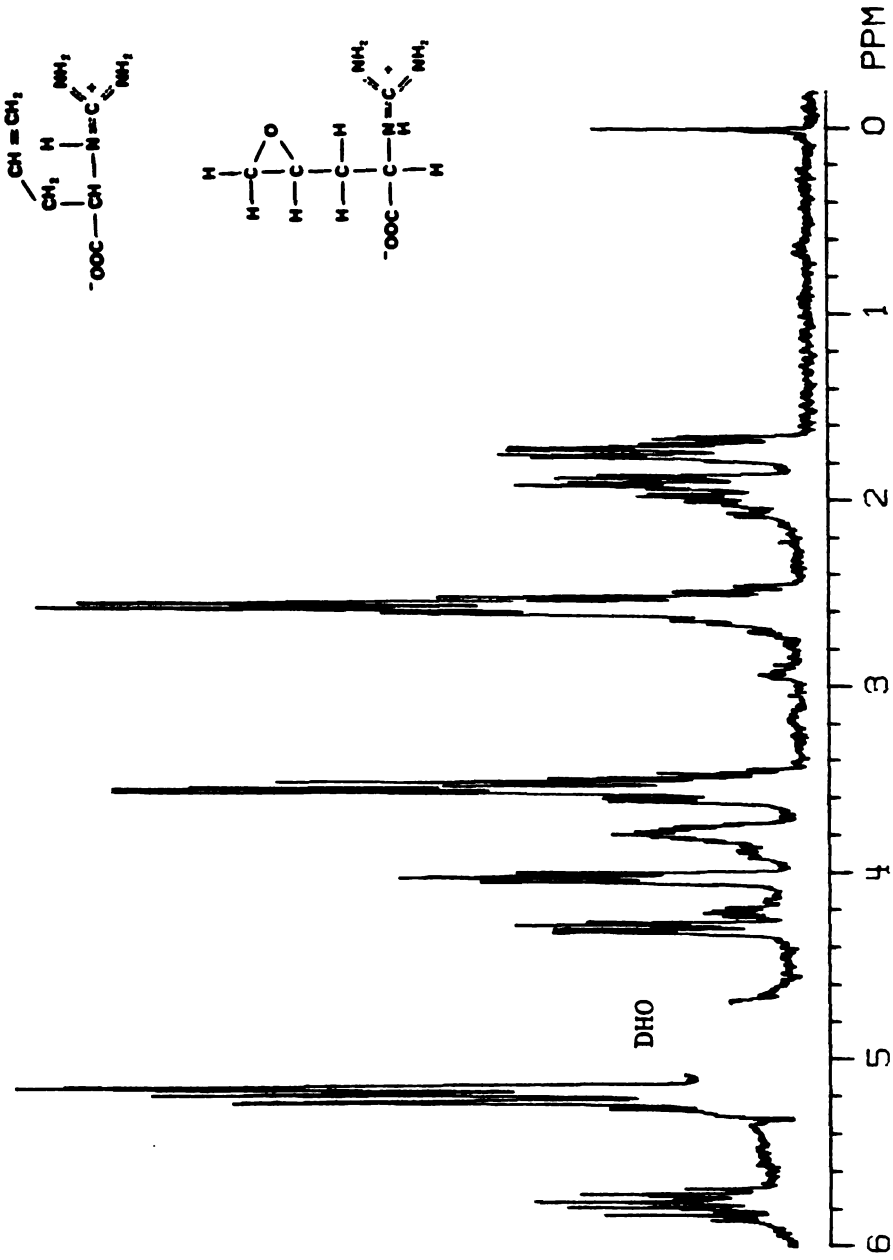
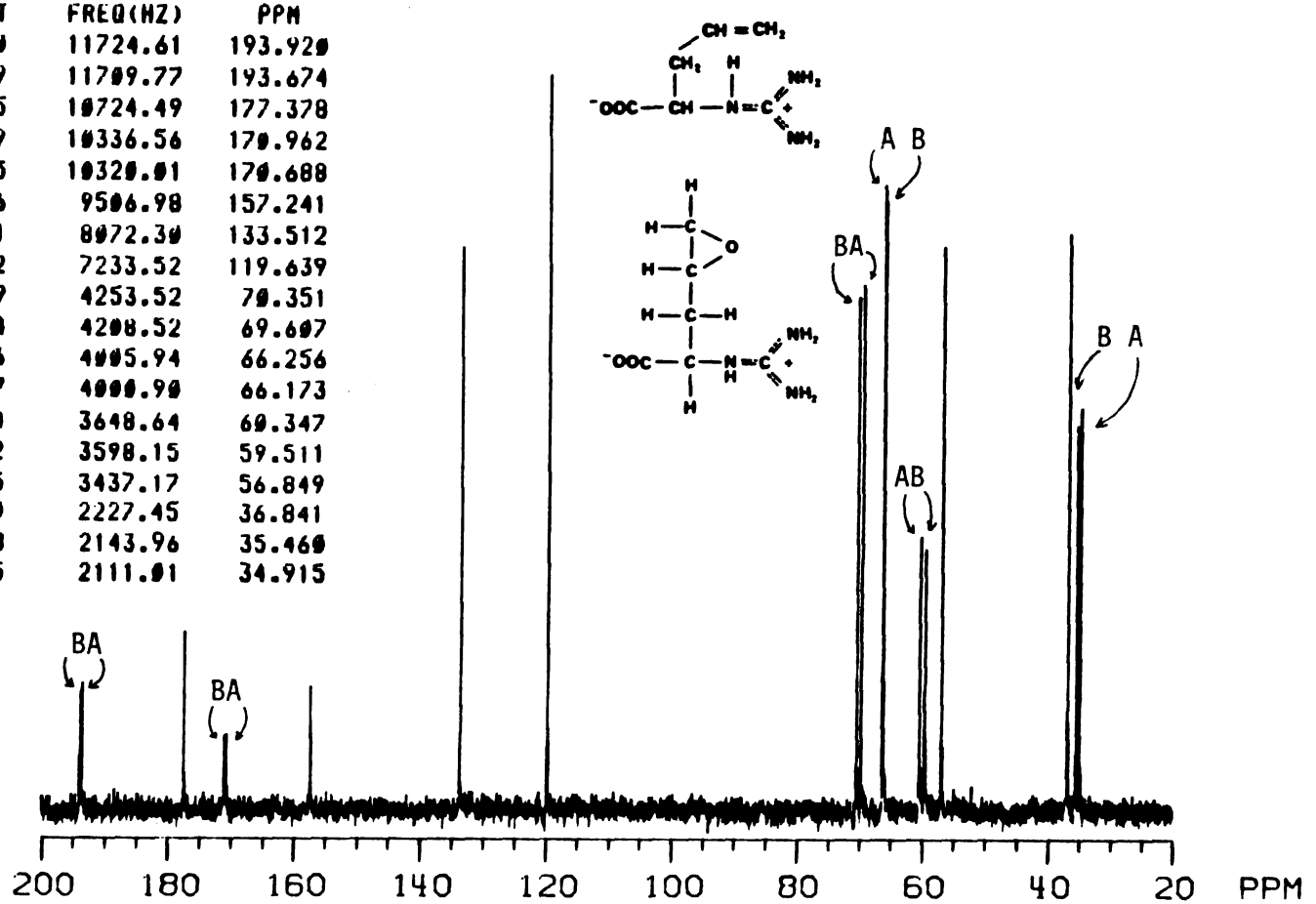


FIGURE IV-1: ^{1}H NMR OF THE CRUDE PRODUCT OF ISOEPOXYCREATINE

FIGURE IV-2: ^{13}C NMR OF THE CRUDE PRODUCT OF ISOEPOXYCREATINE

LINE#	HEIGHT	FREQ(HZ)	PPM
1	213.60	11724.61	193.920
2	230.59	11709.77	193.674
3	324.25	10724.49	177.378
4	136.39	10336.56	170.962
5	139.45	10320.01	170.688
6	224.16	9506.98	157.241
7	1010.71	8072.30	133.512
8	1328.92	7233.52	119.639
9	932.09	4253.52	70.351
10	953.54	4208.52	69.607
11	1134.36	4005.94	66.256
12	1060.27	4000.90	66.173
13	497.24	3648.64	60.347
14	475.72	3598.15	59.511
15	1023.35	3437.17	56.849
16	1040.00	2227.45	36.841
17	699.48	2143.96	35.460
18	731.75	2111.01	34.915



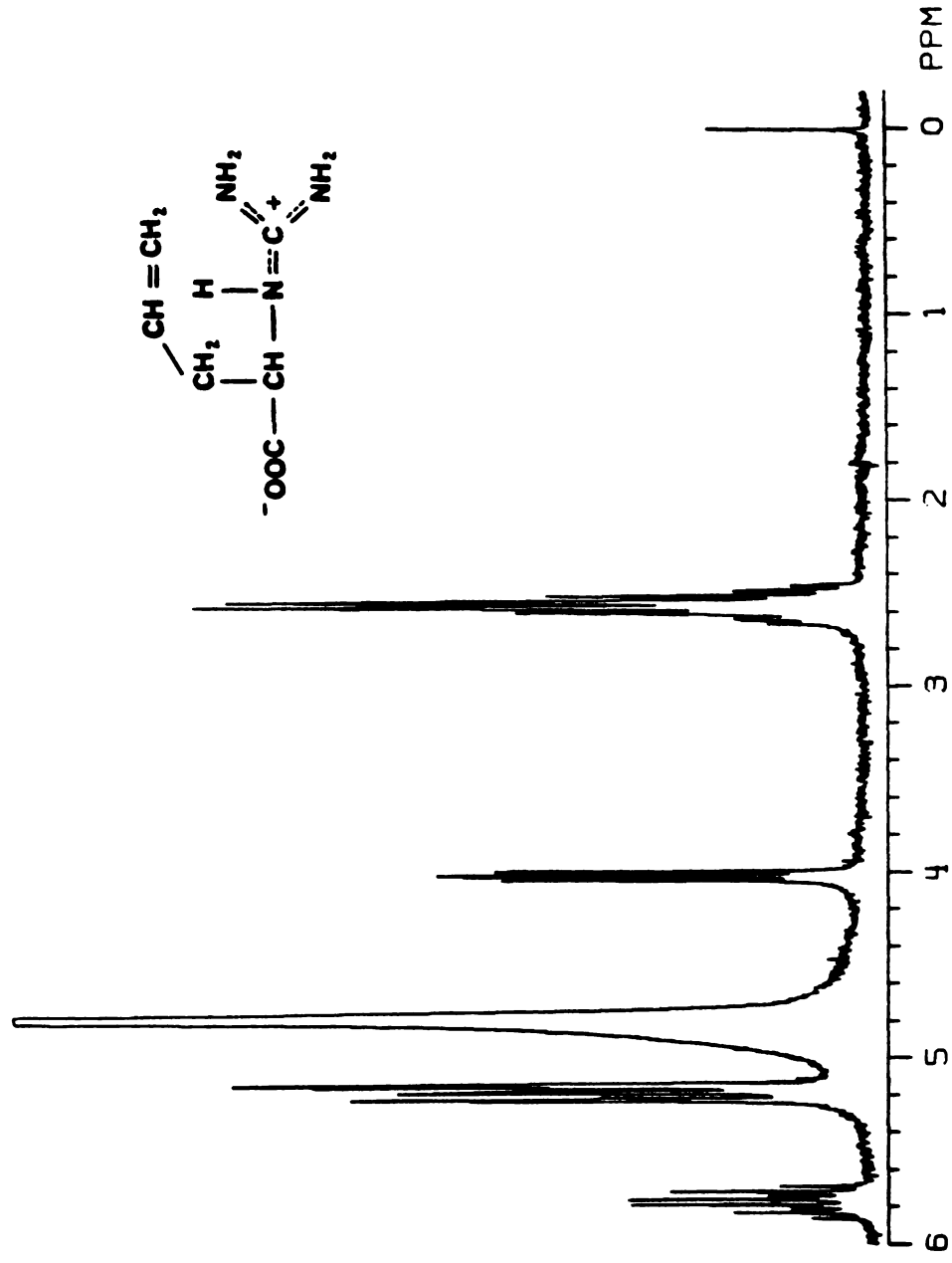
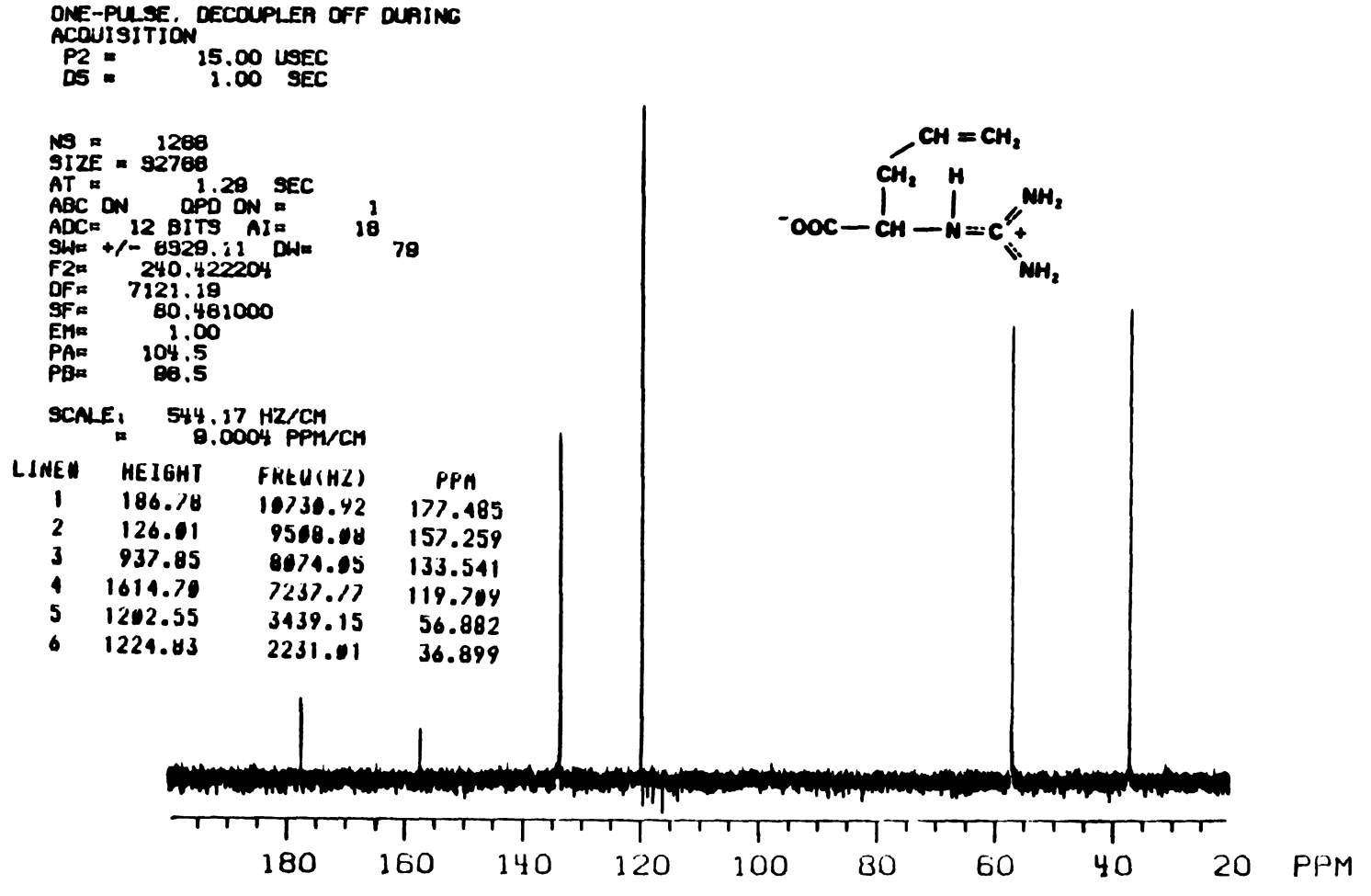


FIGURE IV-3: ^{1}H NMR OF C-ALLYLGLYCOCYAMINE

FIGURE IV-4: PROTON-DECOUPLED ^{13}C NMR OF C(α)-ALLYLGLYCOCYAMINE

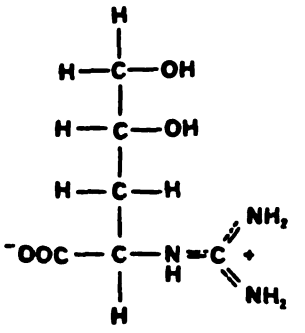


ISOLATION OF ISOEPOXYCREATINE

High pressure liquid chromatography once again served as the only method to separate isoepoxycreatine from its starting compound and its by-product diol. We found that it was difficult to separate the compound without having concomitant cyclization. Prolonged storage of compound in water caused loss of its activity as an inhibitor for creatine kinase. Attempts to crystallize pure epoxycreatine also resulted in a cyclic compound which was poorly soluble in water. After trying different types of columns with different solvents, including solutions of inorganic salts, we found that C-18 reverse phase columns and water as mobile solvent could be used to purify isoepoxycreatine. A crude product of isoepoxycreatine in the mixture of diol by-product and starting compound (including a trace amount of sodium tungstate and sodium pertungstate) was dissolved quickly in a minimum amount of ice-water, millipore-filtered, and then injected into the precooled HPLC system (injector, loop, precolumn, and column). The mobile solvent water was also precooled in an ice-water bath. The compound eluted from the column was also collected in a ice-cold round-bottomed flask, immediately frozen with dry-ice, and then subjected to lyophylization. The compound was detected using a

refractometer. The C-18, ODS-3, 10μ reverse phase column was manufactured by the Whatman Company.

The product after lyophylization was considered pure enough for enzyme studies. The ^{1}H NMR showed no trace of impurity as seen in Figure IV-5. Its microanalysis corresponded to that expected for isoepoxycreatine. A sample of this compound after lyophilization showed the same retention time without impurity. To test the presence of epoxide group in the final product, a small amount of this isoepoxycreatine was heated at reflux for 1 hr. The mixture was then cooled and injected into the same HPLC system. A new peak appeared at the place which was proposed for diol by-product. Cyclic compound isoepocycreatinine and its precursor, isoepoxycreatine, had the same retention times. ^{1}H NMR of diol, which was collected from HPLC after refluxing, is shown in Figure IV-6. The HPLC of isoepoxycreatine after refluxing is shown below with C(α)-allylglycocyamine added as reference.



C(α)-(2,3-dihydroxypropyl)-glycocyamine

(the diol by-product)

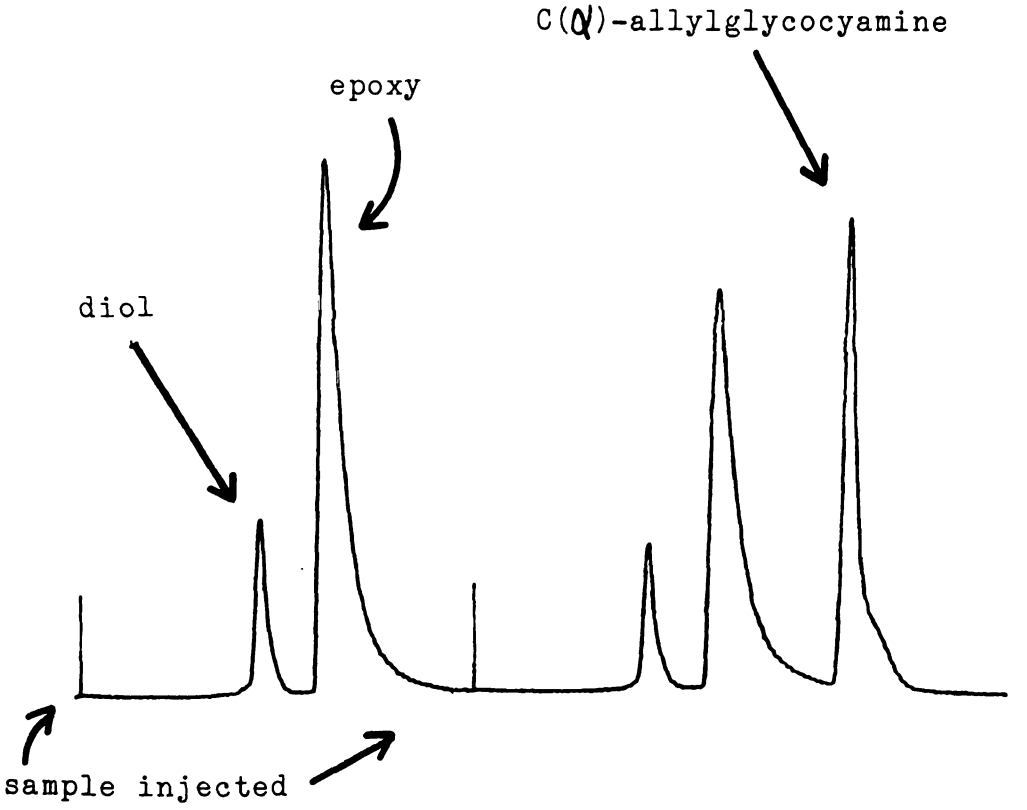
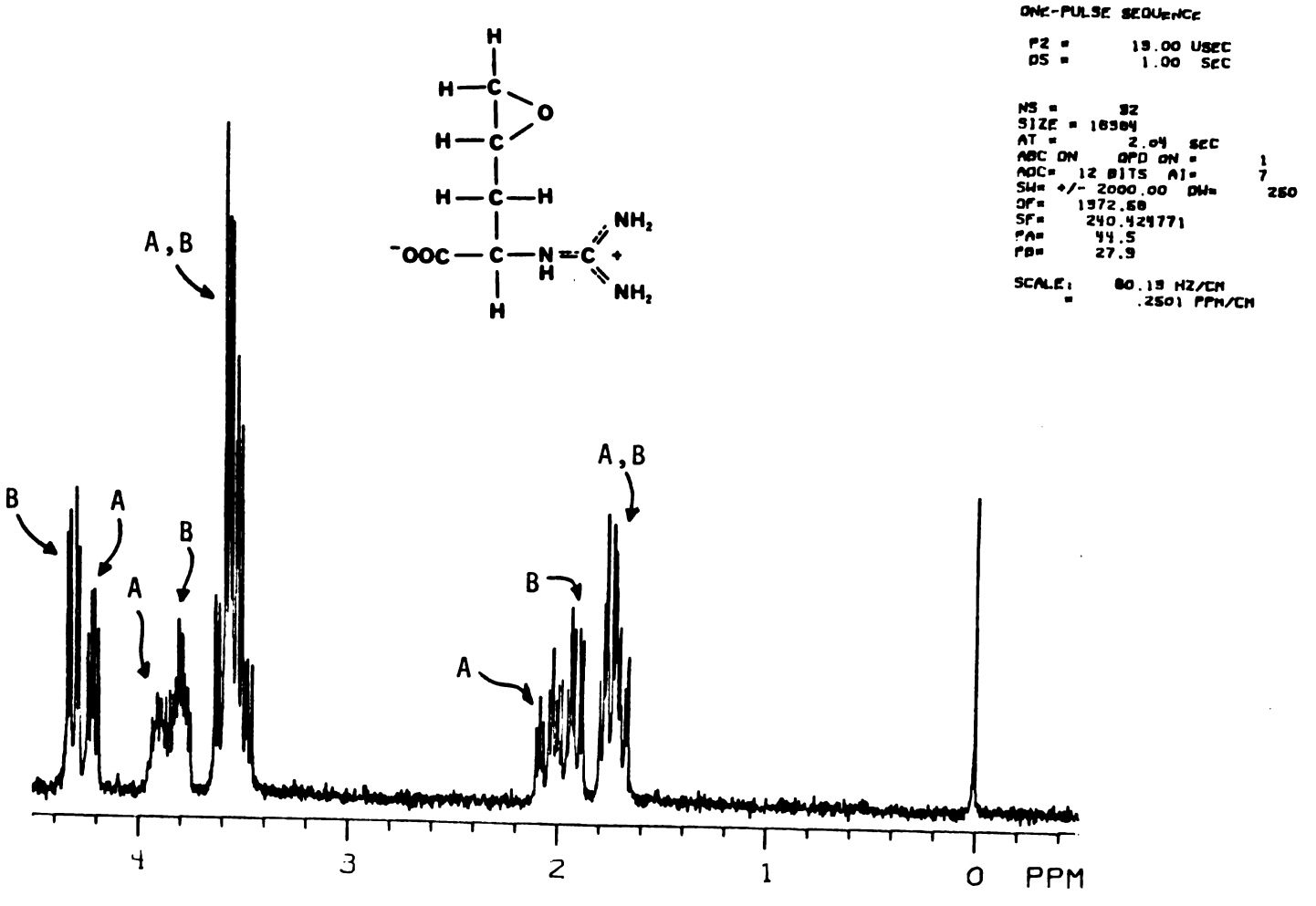


FIGURE IV-5: ^{1}H NMR OF ISOEPOXYCREATINE



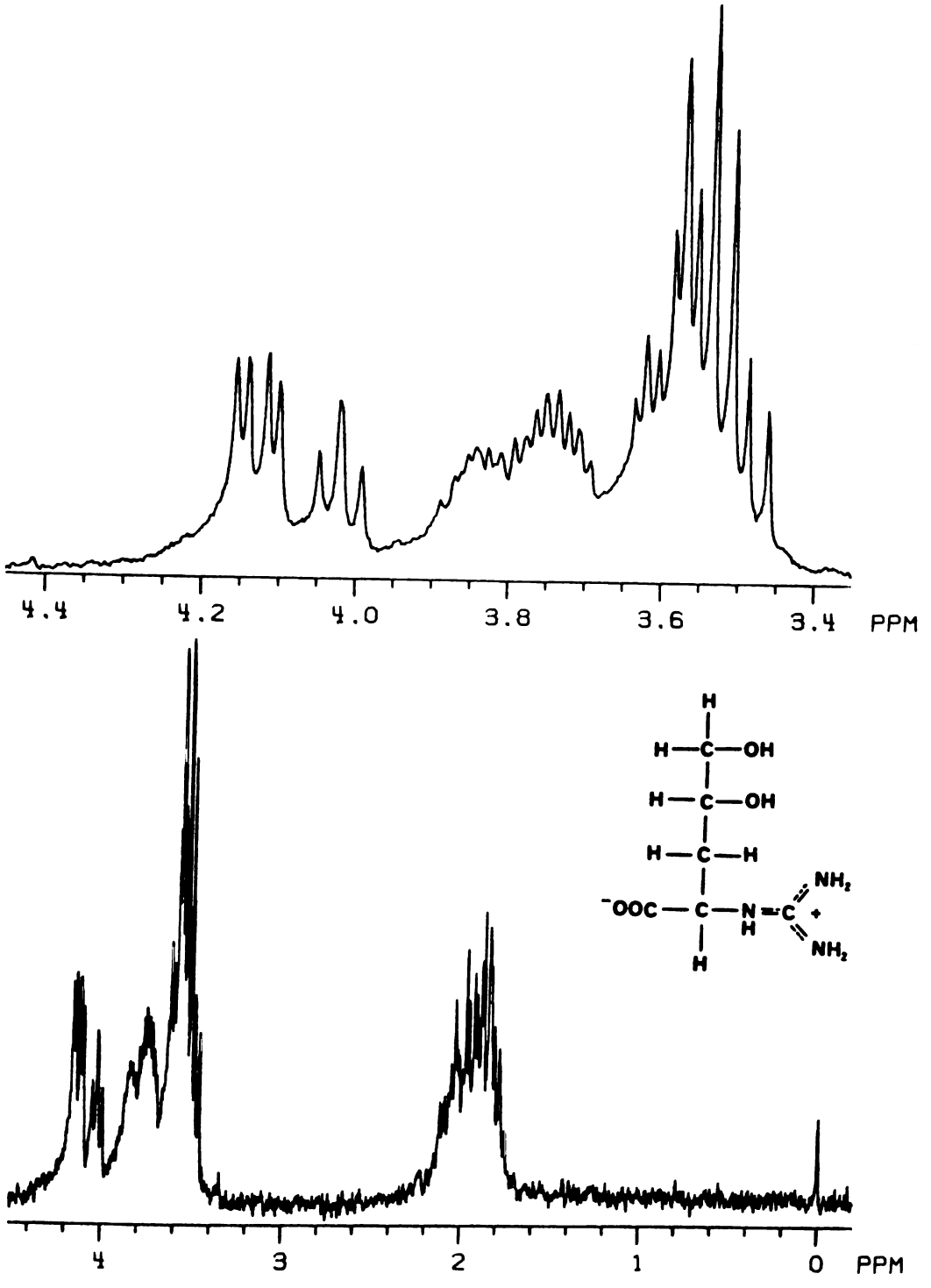
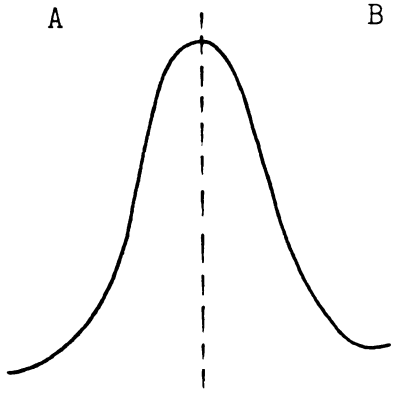


FIGURE IV-6:

^{1}H NMR OF C(α)-(2,3-DIHYDROXYPROPYL)-GLYCOCYAMINE

As expected for a two-chiral-center molecule, isoepoxycreatine had 4 different isomers RR, RS, SR, and SS as shown in scheme IV-2. Each pair of diastereoisomers (RR, SS or RS, SR) had the same spectral properties which were, in turn, different from those of another pair. For simplicity, one pair will be called A and the other pair will be called B. All four isomers had the same retention times on HPLC and, therefore, it was difficult to separate one pure isomer from another without cyclization.

The HPLC peak of isoepoxycreatine which contained two diastereoisomers A and B is as below:

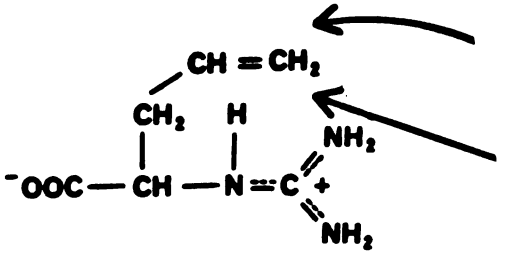


The A diastereoisomer, which was the first isomer to elute from HPLC column, presented the second quartet at δ 4.20 ppm (downfield from TSP) in Figure IV-5. The second isomer to emerge from the HPLC column (B diastereoisomer) gave the first quartet at δ 4.28 ppm shown in the same figure (Figure IV-5). The structural assignments of A and B will be discussed latter in this

chapter.

If the compounds were made from the natural amino acid L-C(∝)-allylglycine, two diastereoisomers, SR and SS, were obtained. If the starting compound for the synthesis was D-C(∝)-allylglycine, then RS and RR were the final products.

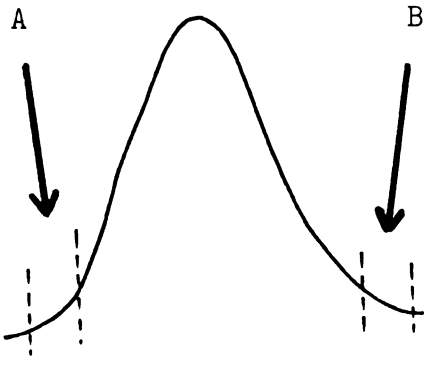
We found that at different concentrations of H_2O_2, there was stereoselectivity for the epoxidation of C(∝)-allylglycocyamine. If a low concentration of H_2O_2 (less than 50% by weight) was used, an approximate ratio of 50:50 of A and B were collected as products. At higher concentration of H_2O_2 (50% of H_2O_2 or more) and at shorter reaction times, B is preferred over A. The stereospecific epoxidation can be explained by the availability of the H_2O_2 molecules around the allylic bond during reaction. The greater the concentration of H_2O_2, the more is available at the less hindered face. When the allylic bond is more heavily solvated with water in case of less concentrated H_2O_2, the H_2O_2 molecules will have more of an equal chance to interact with both faces.



less hindrance

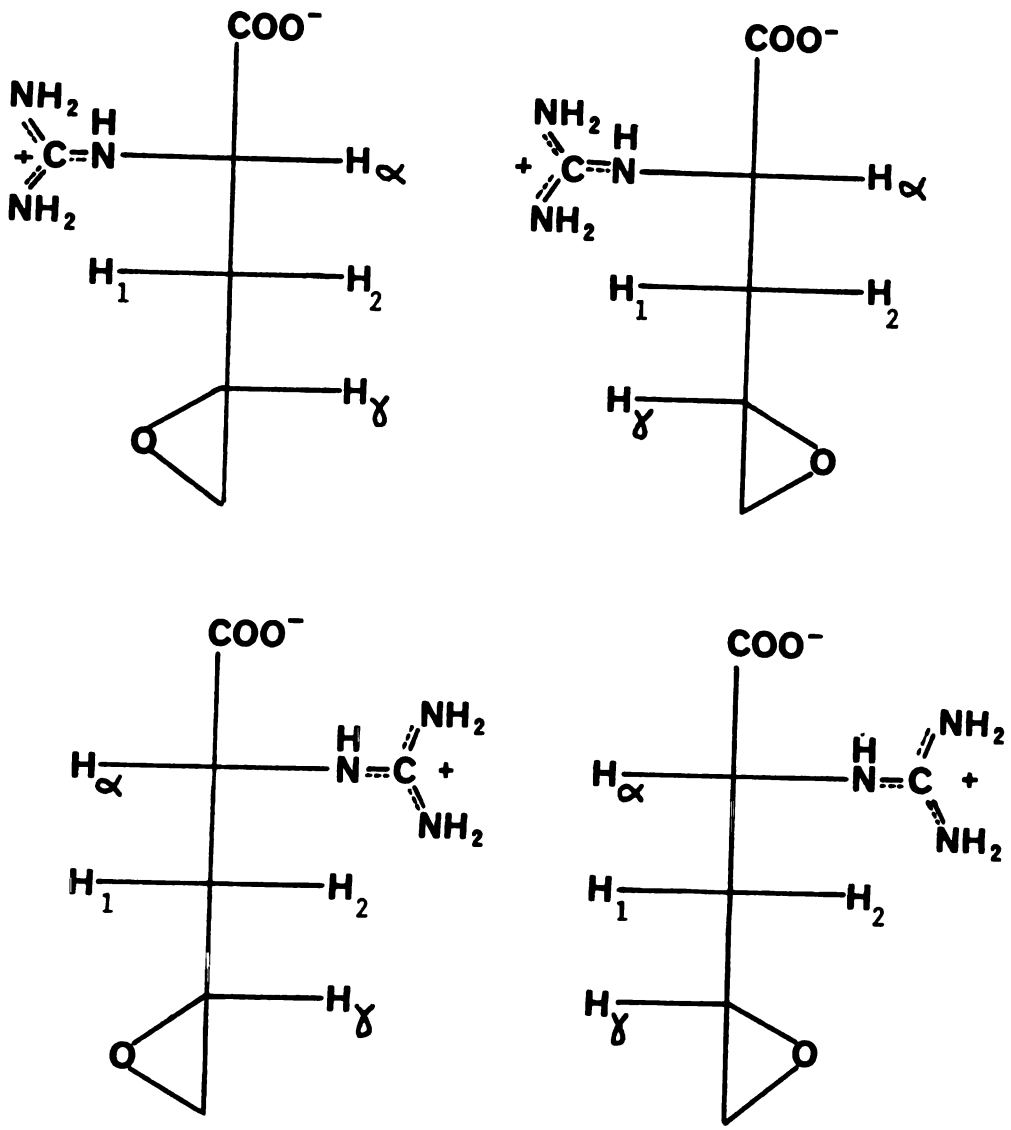
more hindrance

Separation of A and B was a difficult problem. Different types of columns, including chiral columns (such as a Pirkle chiral column), were used unsuccessfully. Recycle-HPLC with reverse phase columns did a better job, but the cyclic compounds were obtained as final products. Small amounts of approximately pure A and B, which were obtained by collecting the product as shown below, were subjected to spectral analysis.



SCHEME IV-2

FOUR ISOMERS OF ISOEPOXYCREATINE



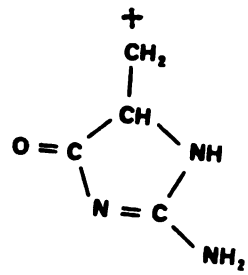
IDENTIFICATION OF ISOEPOXYCREATINE

The identification of isoepoxycreatine mainly relied on its microanalysis, spectral analysis, and the HPLC retention times. The epoxide was eluted between the diol and the olefin as reported for epoxycreatine by Marletta and Kenyon, 1979. The fact that hydrolysis of the epoxide gave an additional peak at the place which was proposed for the diol is also an added proof for its structure.

Mass Spectroscopy

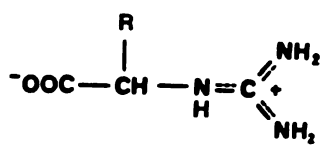
All four isomers of isoepoxycreatine had the same mass spectral properties. Mass spectra of A, B, and the mixture are shown in Figures IV-7 to IV-9, respectively. Structural assignments for peaks are shown in Table IV-1. The mass spectrum of the precursor C(∝)-allylglycocyamine is also shown here in Figure IV-10 along with the fragment assignments in Table IV-2.

The molecular ion peak ($M^{+\cdot}$) of isoepoxycreatine (m/e 173) is relatively small using GC-MS spectrometry. The base peak at m/e 112 of isoepoxycreatine spectrum is assigned as the following fragment ion:

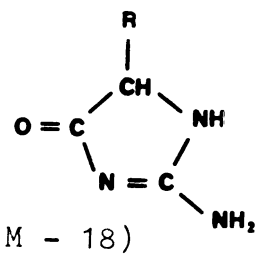


m/e 112

$(M - H_2O)^+$ peaks obtained in both spectra of isoepoxycreatine and its precursor could be due to the formation of cyclic glycocyamidine fragment:



M

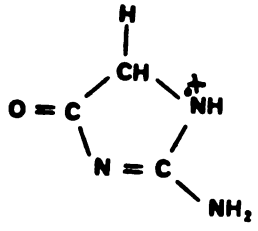


(M - 18)

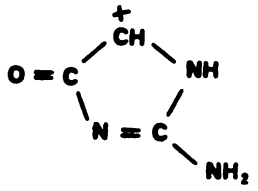
The base peak (m/e 43) of allylglycocyamine is also a strong peak in the mass spectrum of isoepoxycreatine:

m/e 43

Peaks at m/e 99 and 98 are strong in both compounds and could be assigned as the following stable fragment ions:



m/e 99



m/e 98

A new technique has been developed to detect the molecular ion of a molecule which is a nonvolatile, labile, polar organic substance (Aberth et al., 1982). This technique uses a cesium ion "gun" to produce the primary beam for secondary ion mass spectrometry (S. I. M. S.). This soft-ionization technique imparts less energy to the molecule, and as a result, causes less fragmentation and produces a higher molecular ion peak.

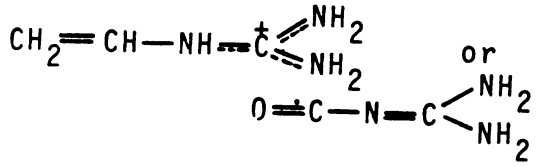
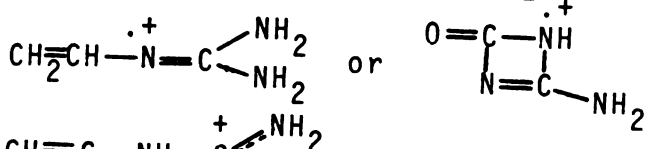
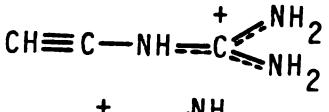
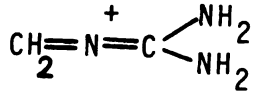
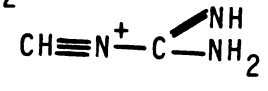
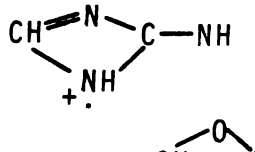
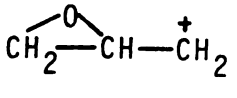
Using a liquid SIMS source on a Kratos MS-50 with a cesium gun as the primary beam and the liquid target matrix glycerol-MeOH, molecular ion peaks of isoepoxycreatine and the diol are the base peaks. Figure IV-11 shows the mass spectrum of isoepoxycreatine using this technique. The pure glycerol-MeOH matrix is also shown here in Figure IV-13 to compare the spectrum with and without isoepoxycreatine. Under the same system, the mass spectrum of the diol was also observed as well as shown in Figure IV-12. Molecular ions of both diol and isoepoxycreatine appeared as negative ions (M^-) at m/e 190

and 172. In Figure IV-11, peak at m/e 264 is a complex of isoepoxycreatine and glycerol. Peak at m/e 282 in Figure IV-12 is also a complex of glycerol and the diol.

The molecular ion peaks of the epoxy and the diol as base peaks and peaks of the complex between epoxy and glycerol and between diol and glycerol obtained from this technique, provide strong evidence for the structural assignment of these compounds.

TABLE IV-1

MS FRAGMENTS OF ISOEPOXYCREATINE

m/e	Fragments
173	M^+
155	$(M - H_2O)^+$
142	$(M - 31)^+$ or $(M - CH_2OH)^+$
129	$(M - 44)^+$ or $(M - CO_2)^+$
125	$(M - CH_2OH - OH)^+$
124	$(M - CH_2OH - H_2O)^+$
112 (base)	$(M - \text{epoxy} - H_2O)^+$
111	$(M - 44 - H_2O)^+$
99	$(M - 44 - CH_2O)^+$
98	$(M - 44 - CH_2O - H^+)\cdot$
86	
85	
84	
72	
70	
69	
57	

(Table IV-1 cont.)

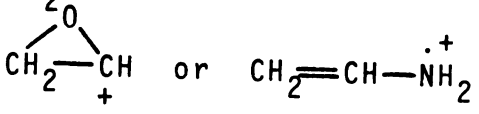
m/e	Fragments
56	$CH_2{=}CH{-}CHO$
55	$\cdot O{\equiv}C{-}CH{=}CH_2$
45	$^{+}CH_2{-}CH_2{-}OH$ or $\cdot COOH$
44	CO_2
43	
32	$\cdot CH_2{-}\overset{+}{O}H_2$
31	$^{+}CH_2{-}OH$
29	$H{-}C{\equiv}O\cdot$
28	$^{+}C{\equiv}O\cdot$
27	$CH_2{=}\overset{+}{C}H$

TABLE IV-2

MS FRAGMENTS OF C(∝)-ALLYGLYCOCYAMINE

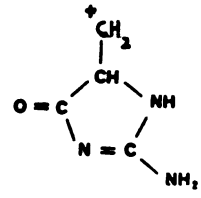
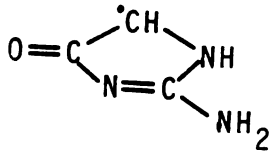
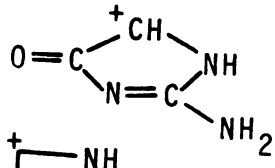
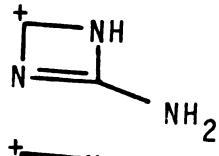
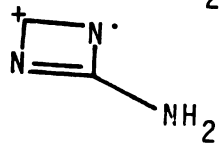
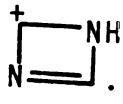
Fragments	m/e
M - 18	139
M - CO_2	113
M - COOH or 	112
M - COOH - H^+	111
 + H^+	99
	98
	70
	69
	54
CO_2 or $^{+}NH_2{=}\dot{C}{-}NH_2$	44
$NH{=}C^{+}{-}NH_2$ or $CH_2{=}CH{-}\overset{+}{\dot{N}}H_2$	43
$\overset{+}{N}H{\equiv}C{-}NH_2$	42
$CH_2{=}CH{-}CH_2^{+}$	41
$CH_2{=}CH{-}\dot{C}H^{+}$	39
$\dot{C}{\equiv}O^{+}$ or $\dot{C}H_2{-}\overset{+}{C}H_2$	28

Figure IV-7:

GC-MS spectrum of $S_{\alpha}R_{\gamma}$-C(α)-(2,3-epoxypropyl)-glycocyamine ("A" isoepoxycreatine). This mass spectrum was performed using chemical ionization at the source temperature of 240° C on a Kratos MS-25 equipped with Varian Capillary GC System at the School of Pharmacy, UCSF.

Figure IV-8:

GC-MS spectrum of $S_{\alpha}S_{\gamma}$-C(α)-(2,3-epoxypropyl)-glycocyamine ("B" isoepoxycreatine). The legends are the same as those of the Figure IV-7.

Figure IV-9:

GC-MS spectrum of the mixture of $S_{\alpha}S_{\gamma}$- and $R_{\alpha}R_{\gamma}$-isoepoxycreatine. The legends are the same as those of Figure IV-7.

Figure IV-10:

GC-MS spectrum of C(α)-allylglycocyamine, the precursor of isoepoxycreatine. The legends are the same as those of the Figure IV-7.

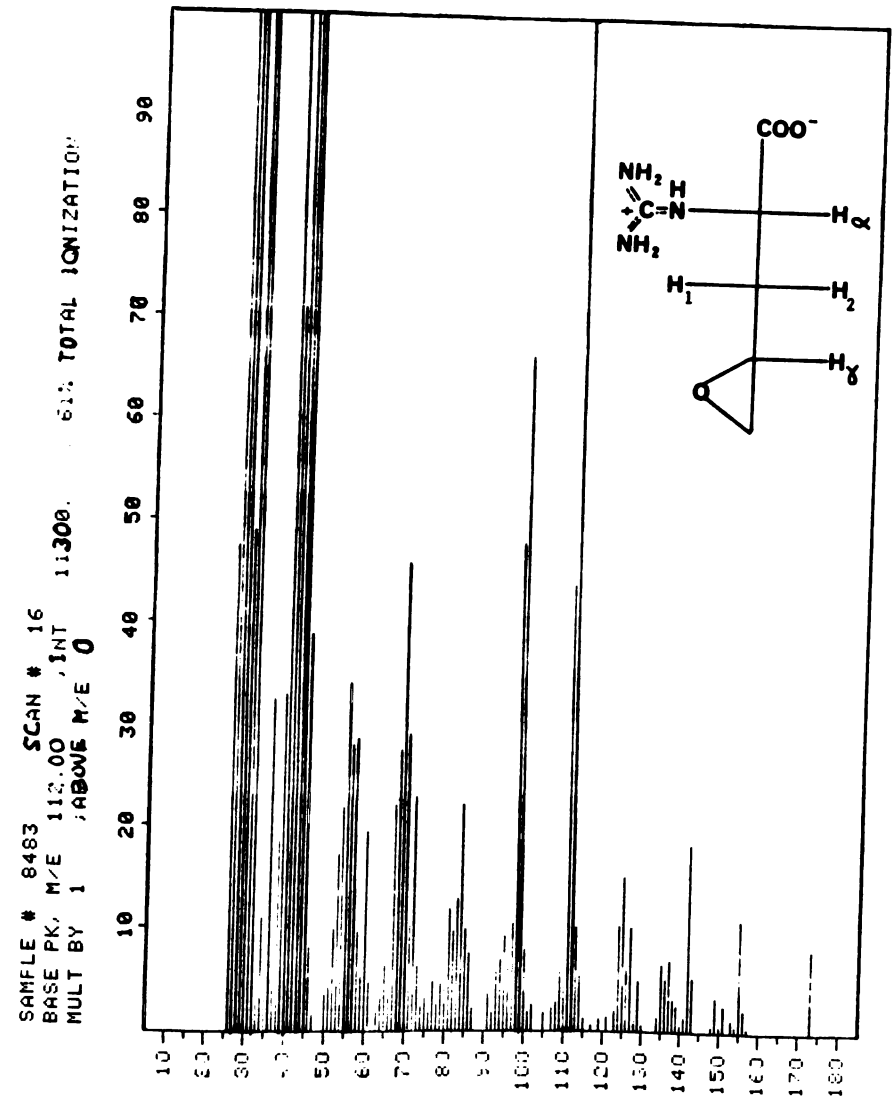


FIGURE IV-7:

GC-MS OF "A" ISOEPOXYCREATINE

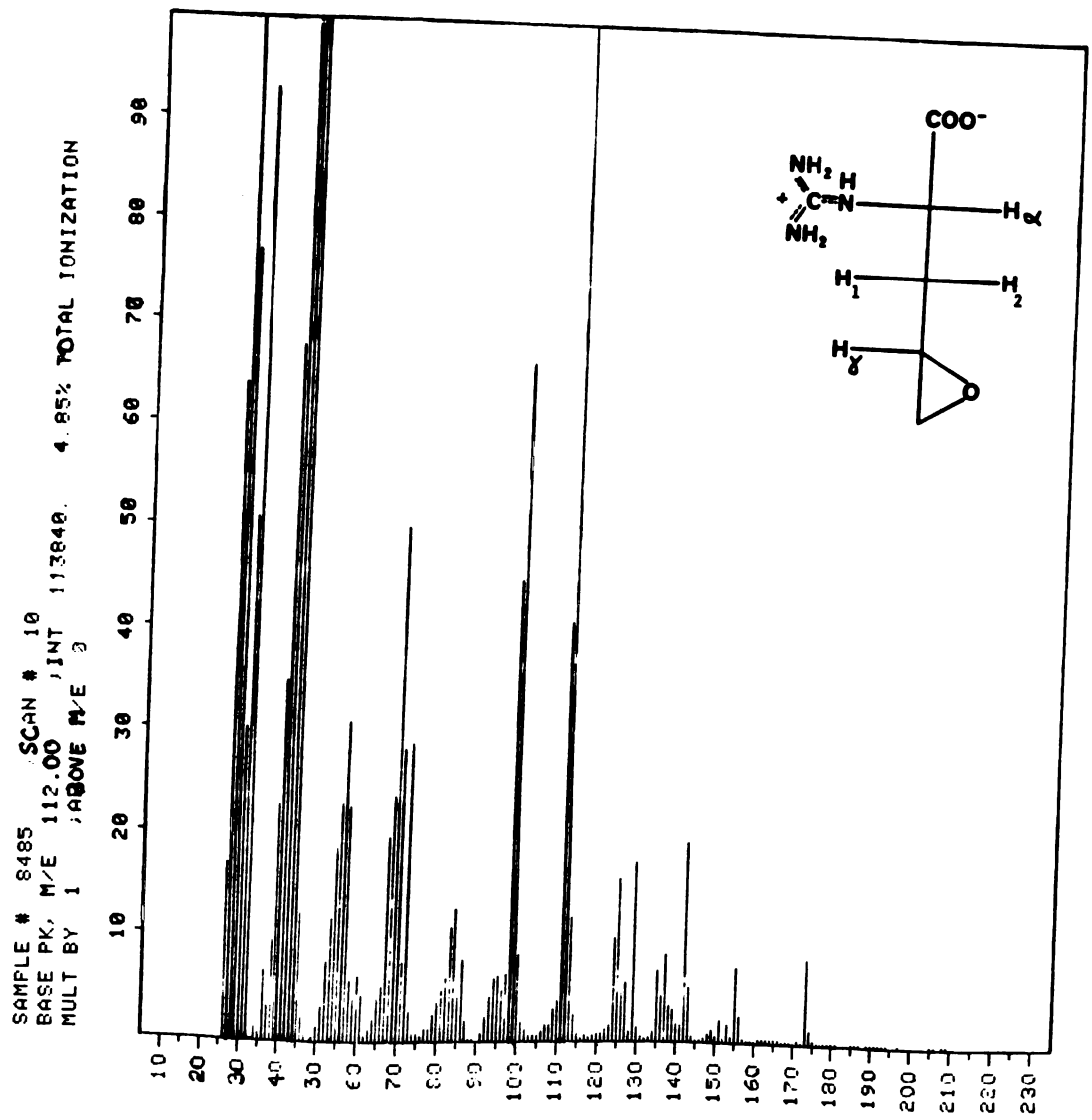


FIGURE IV-8:

GC-MS OF "B" ISOEPOXYCREATINE

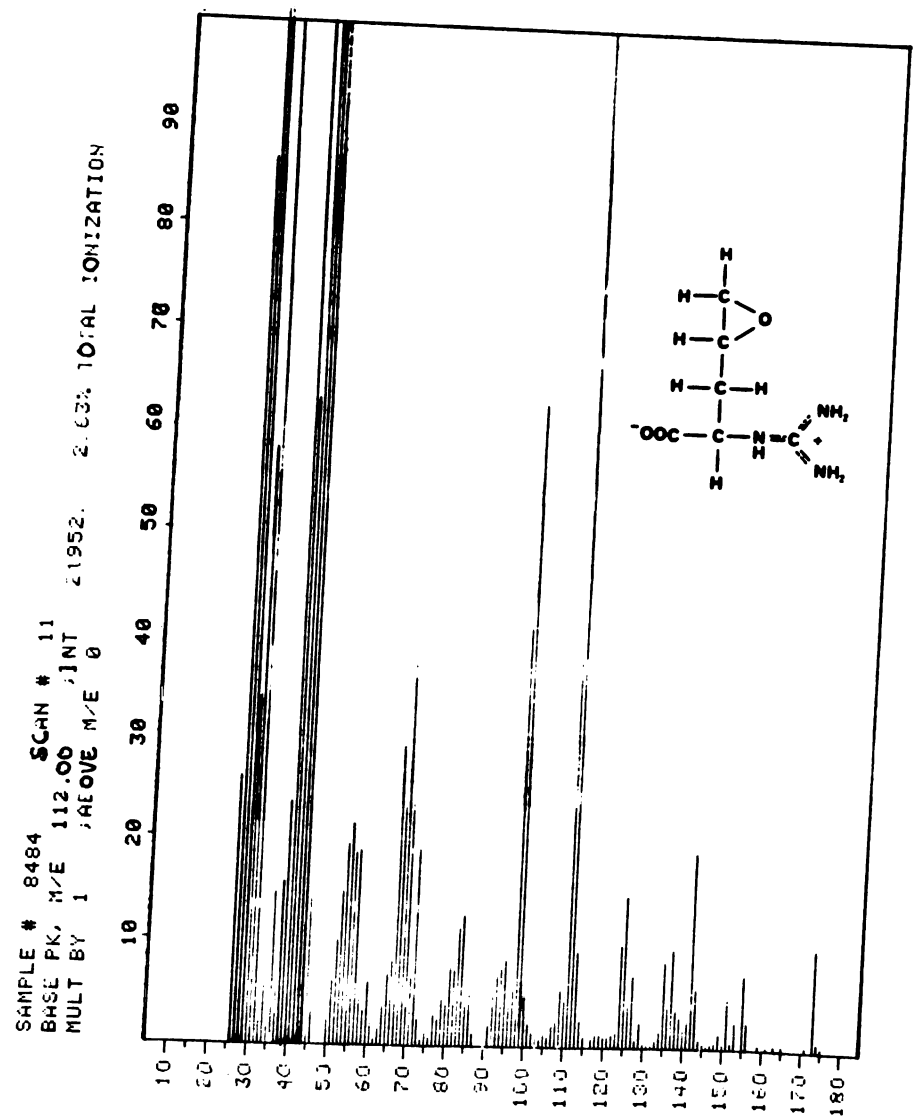


FIGURE IV-9:

GC-MS OF ISOEPOXYCREATINE

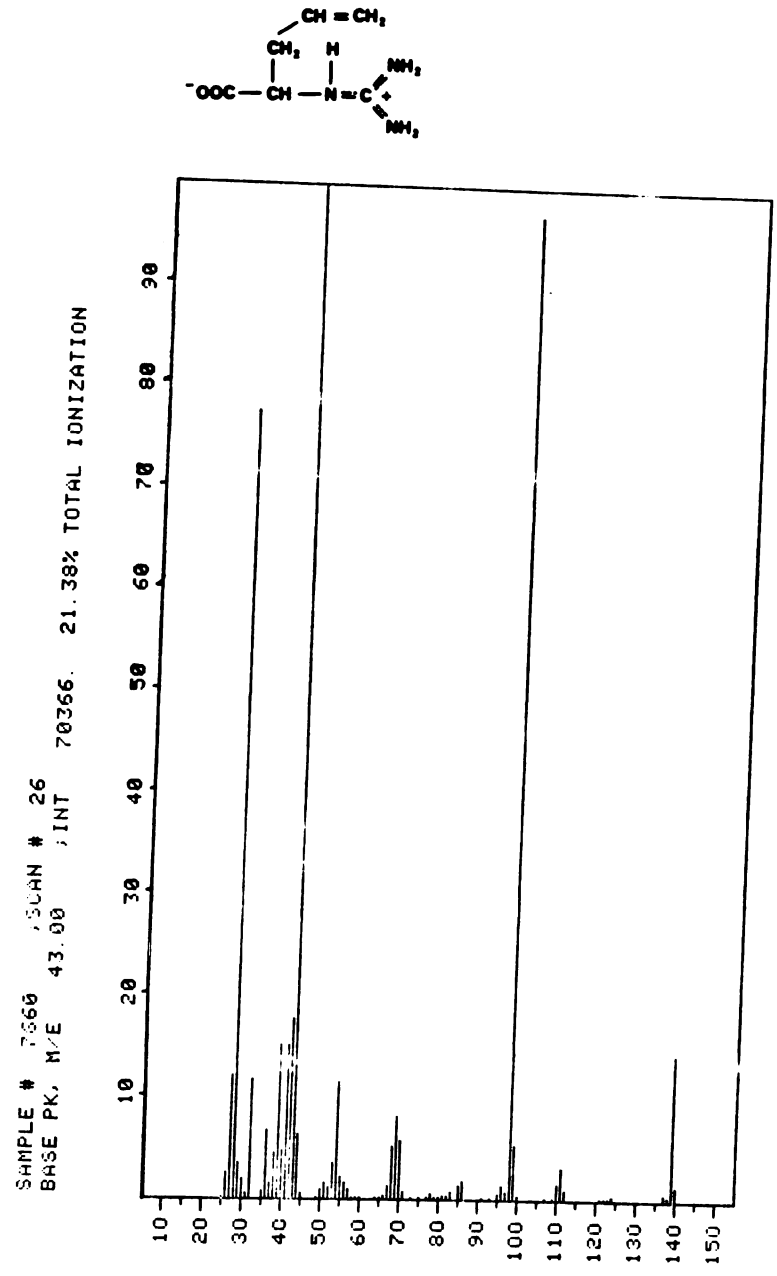


FIGURE IV-10:

GC-MS OF C(α)-ALLYLGLYCOCYAMINE

Figure IV-11:

Liquid Secondary Ion Mass Spectrum of isoepoxycreatine was obtained at the Department of Chemistry, U. C. Berkeley. It was performed on a Kratos MS-50 Mass Spectrometer equipped with a 23-KG Magnet and negative ion swithching. The molecular ion was obtained as negative ion (M^-). The cesium ion gun (C_s^+) produced a primary beam which focused on the liquid target matrix of glycerol-MeOH which contained the sample. This "soft" ionization technique has the ability to produce gas-phase molecular ions without prior evaporation of the sample (Aberth et al., 1982).

Figure IV-12:

Liquid Secondary Ion Mass Spectra of C(∝)-(2,3-dihydroxypropyl)-glycocyamine (the diol). The legends are the same as those of Figure IV-11.

Figure IV-13:

LSIMS of the pure matrix glycerol-MeOH. The legends are the same as those of Figure IV-11.

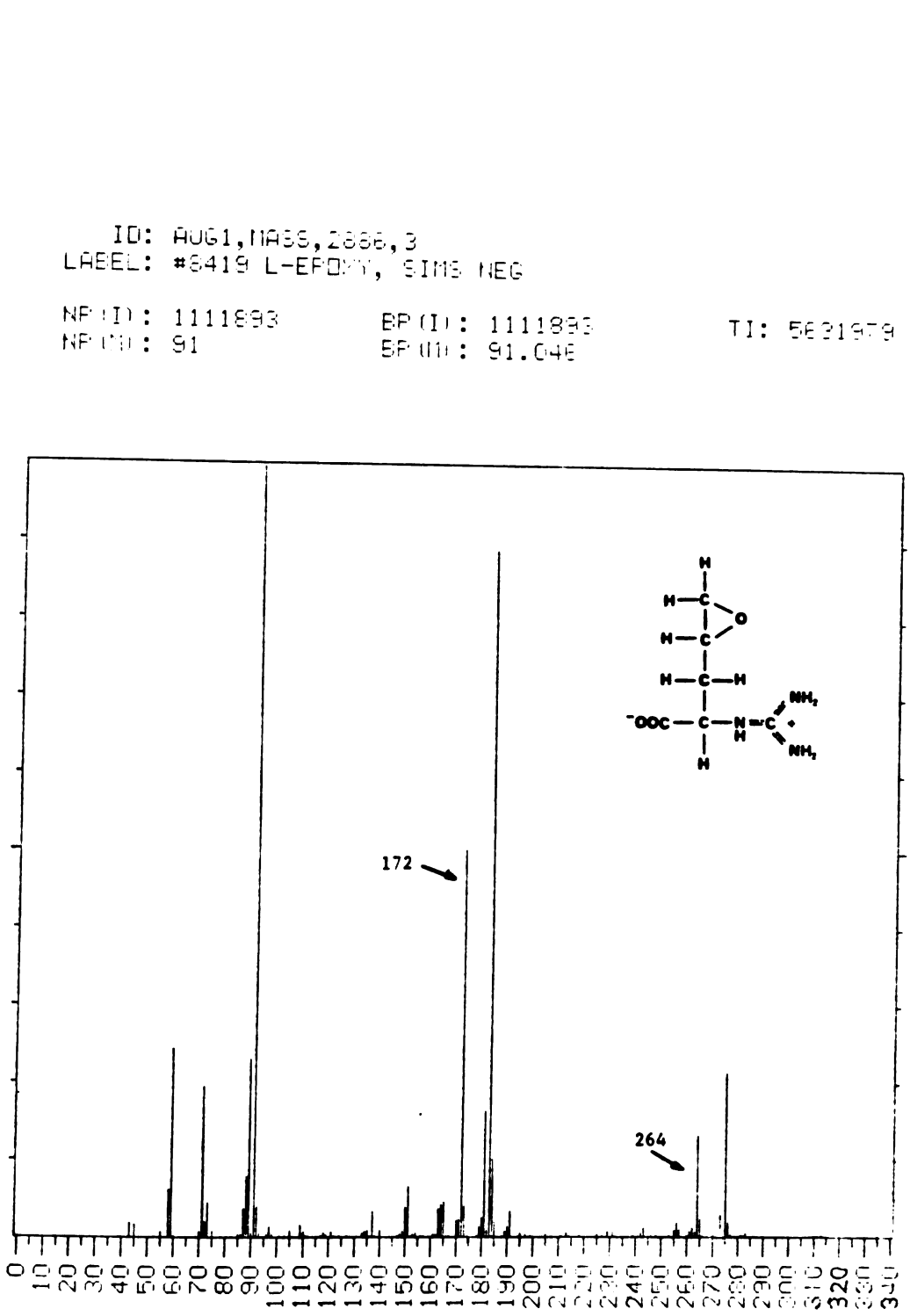


FIGURE IV-11:

LSIMS OF ISOEPOXYCREATINE

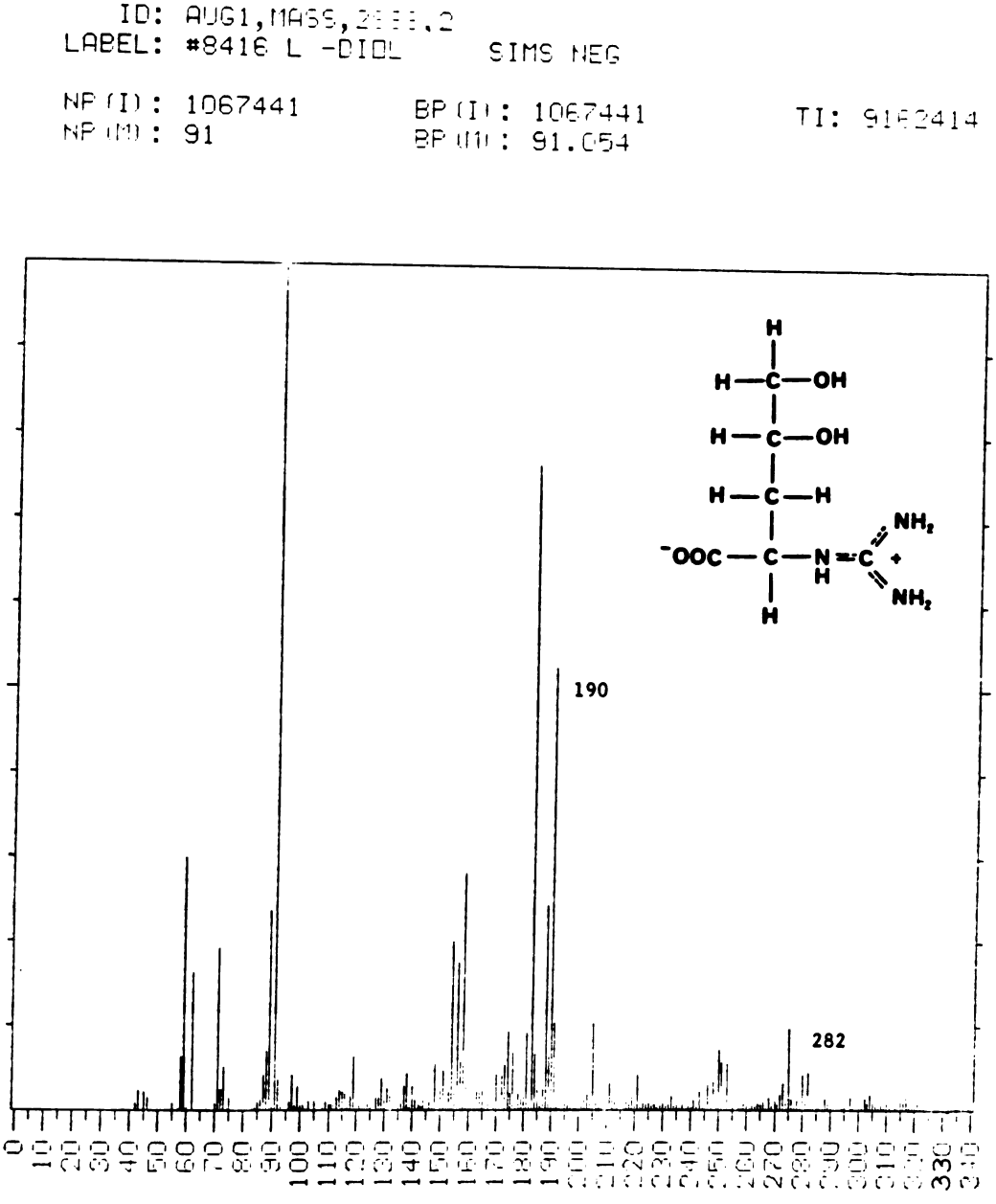


FIGURE IV-12:

LSIMS OF C(α)-(2,3-DIHYDROXYPROPYL)-GLYCOCYAMINE

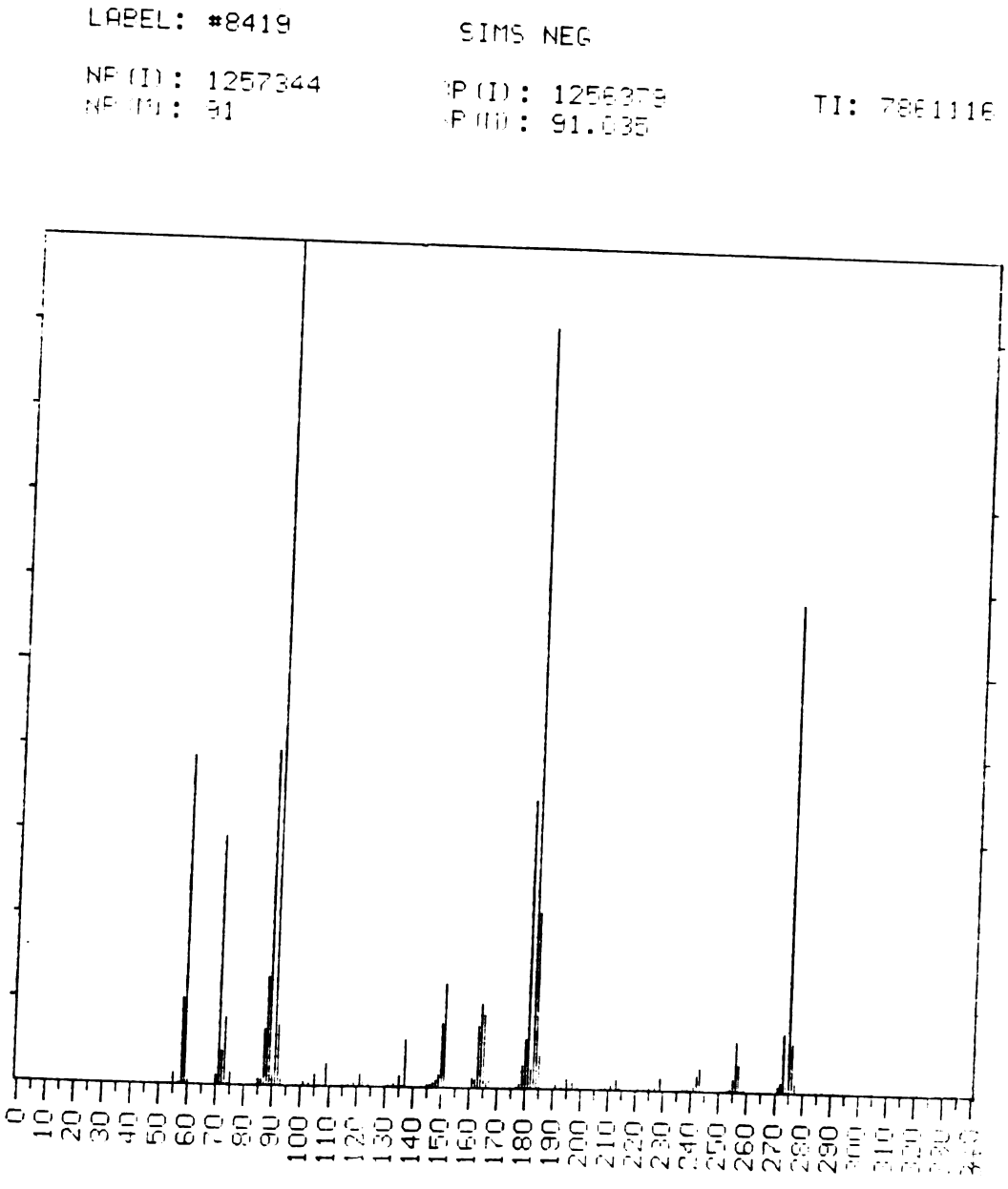


FIGURE IV-13:

LSIMS OF THE PURE MATRIX GLYCEROL-MEOH

IR Spectroscopy

The IR spectra of A, B, and the mixture of isoepoxycreatine in KBr pellets are shown in Figures IV-14a, b, and c, respectively. These spectra are similar to that of epoxycreatine, which was reported by Marletta and Kenyon, 1979. The IR spectrum of isoepoxycreatine showed absorption at 1260 cm^{-1}, which is characterized as a bond stretching and contracting in the plane of the epoxide ring. The bands appearing at 930 cm^{-1} and 880 cm^{-1} are attributed to the ring stretching of the epoxide group. The C=N stretching vibration of the guanidine group shows absorption at 1690 cm^{-1}. A strong band at 1640 cm^{-1} is attributed to asymmetrical stretching of the carboxylate group, along with a weak symmetrical stretching at 1420 cm^{-1}. There is no evidence for the formation of γ-lactone which would require a strong stretching band at 1780-1760 cm^{-1}.

Figure IV-14:

IR spectra of isoepoxycreatine in KBr pellets. The spectra were obtained at medium scan modes and with polystyrene as an external standard (wavenumber 2850.7 cm^{-1}). The first spectrum is $S_{\alpha}R_{\gamma}$-C(α)-(2,3-epoxypropyl)-glycocyamine ("A" isoepoxycreatine), the second spectrum is $S_{\alpha}S_{\gamma}$-C(α)-(2,3-epoxypropyl)-glycocyamine ("B" isoepoxycreatine), and the third spectrum is the mixture of $S_{\alpha}S_{\gamma}$- and $R_{\alpha}R_{\gamma}$-C(α)-(2,3-epoxypropyl)-glycocyamine.

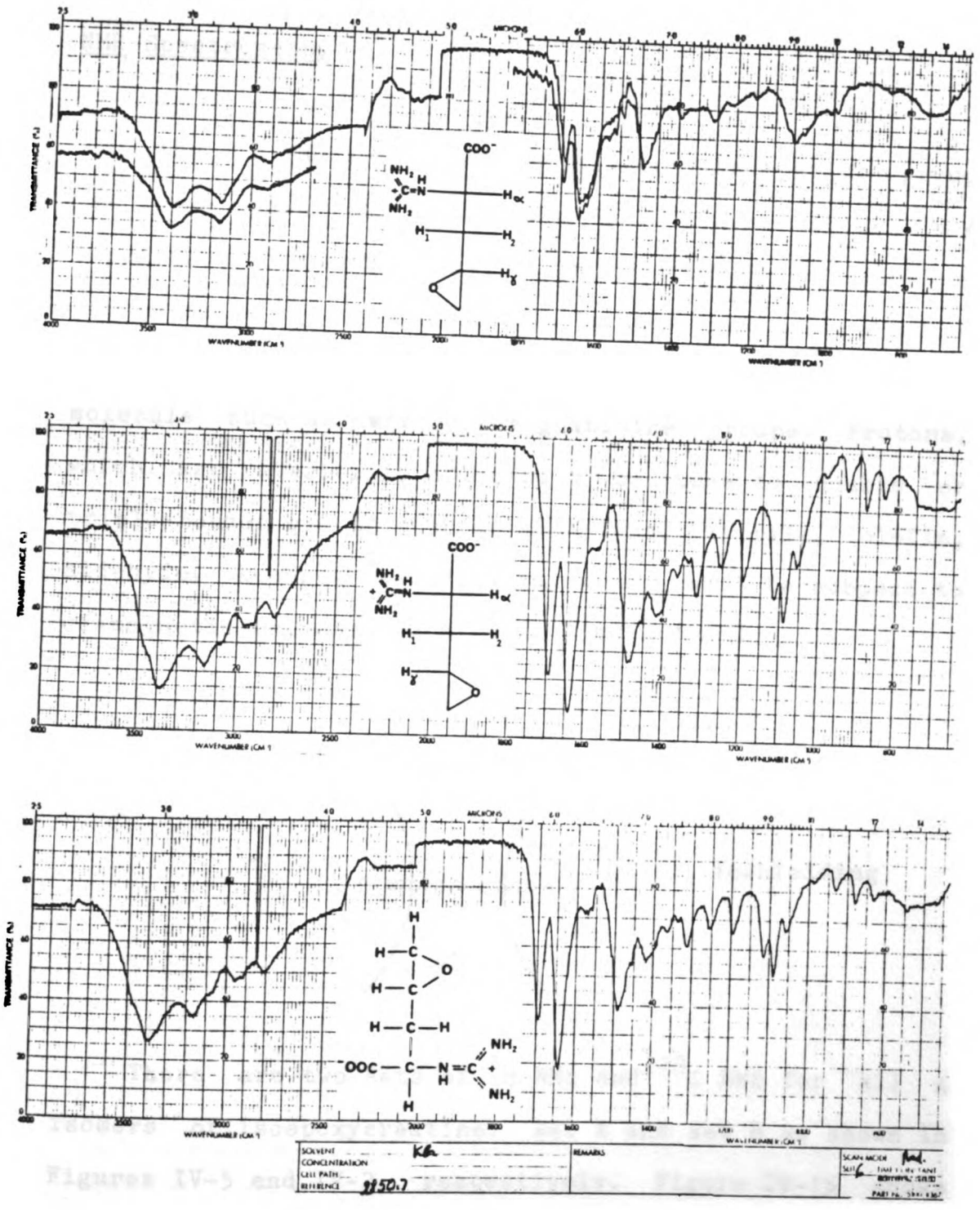
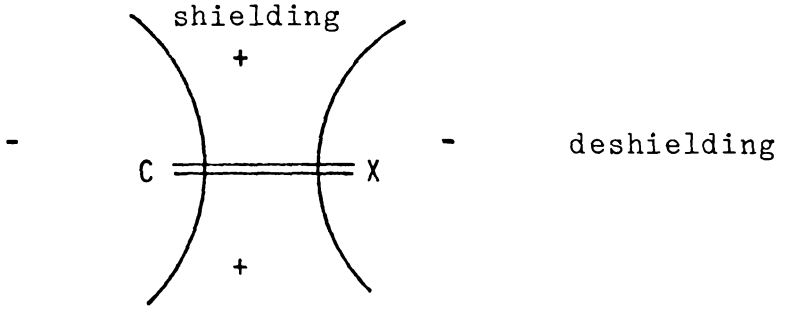


FIGURE IV-14:

IR OF "A", "B", AND THE MIXTURE OF ISOEPOXYCREATINE

NMR Spectroscopy

The ^{1}H NMR and ^{13}C NMR signals of the epoxide group are more downfield than expected. These atoms may experience deshielding effects caused either by paramagnetic electronic circulations or diamagnetic anisotropic effects that originate in other parts of the molecule such as carbonyl or guanidine groups. Protons, which are sterically oriented to be close to double or triple-bonded groups such as acetylene, nitrile, olefin, aldehyde, ketone, ester, acid, etc., will be subject to diamagnetic anisotropic effects (Dyer, 1965).



There are two sets of ^{1}H NMR and ^{13}C NMR for all 4 isomers of isoepoxycreatine: set A and set B as shown in Figures IV-5 and IV-2, respectively. Figure IV-15 shows the expanded ^{1}H NMR of isoepoxycreatine along with the assignments which were determined from the selected decoupling experiments.

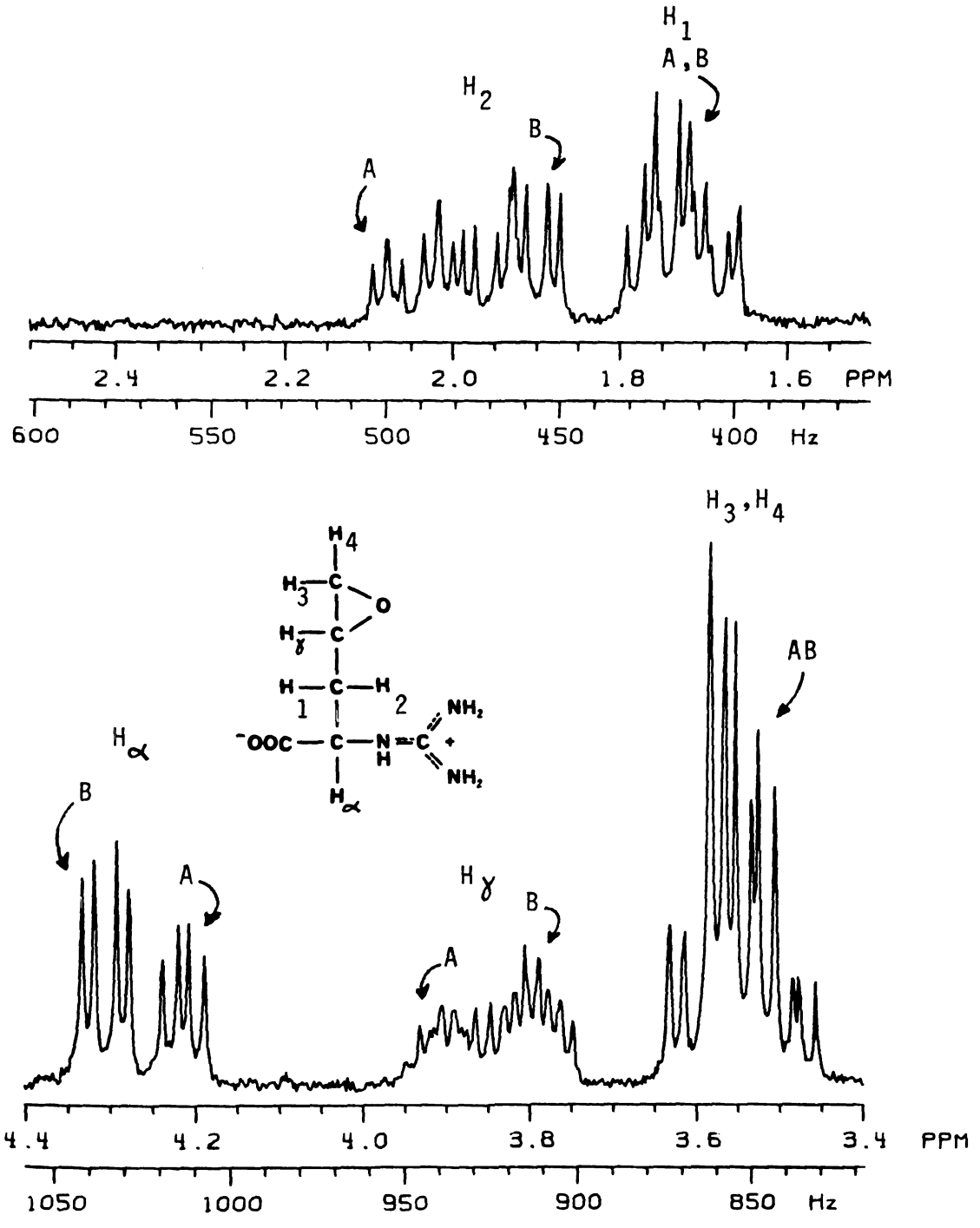


FIGURE IV-15:

EXPANDED ^{1}H NMR OF ISOEPOXYCREATINE

SELECTED DECOUPLING EXPERIMENTS

Selected decoupling experiments were performed on samples of pure A and pure B which were made from either L-C(α)-allylglycocyamine or DL-C(α)-allylglycocyamine and which were separated using HPLC. The ^1H NMR of pure A and pure B are shown in Figures IV-16 and IV-17, respectively. The chemical shift assignment of each proton of isoepoxycreatine was confirmed by irradiation at the frequency of transition of the vicinal protons.

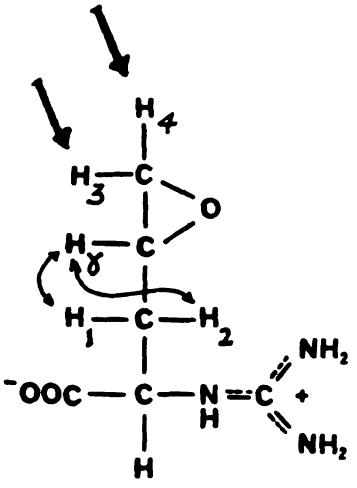
The protons (H_1 and H_2) of the methylene group near asymmetric centers are not chemical shift equivalent. They are coupled to one another, and each, in turn, has different couplings to vicinal protons. They are diastereotopic and are not interchangeable even with fast rotation of the C-C bond. The nonequivalent protons of the methylene group between two chiral centers absorb at δ 1.74 and δ 2.91 in Figure IV-16 and at δ 1.71 and δ 1.91 in Figure IV-17.

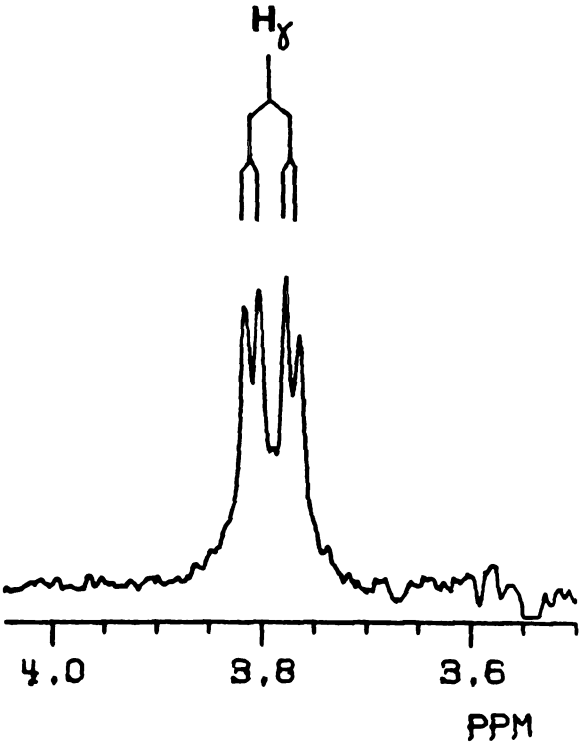
The well-known Karplus relation has been applied to vicinal couplings (Karplus, 1963). The coupling between two vicinal protons depends on the dihedral angle between these protons. The trans coupling constants are about 11 Hz, whereas the gauche coupling constants are about 3 Hz (Whitesides *et al.*, 1967). The coupling constants of protons of the methylene carbon between two chiral centers

have been determined by selected homo-decoupling experiments.

Irradiation at H_3 and H_4

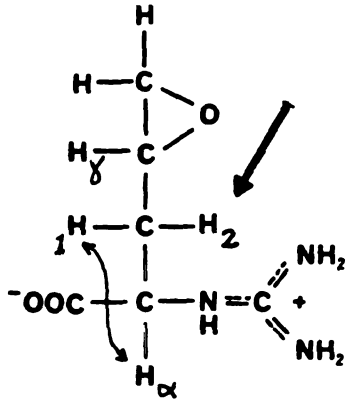
The irradiation of the methylene protons (δ -C) of the epoxide group is shown below and in Figure IV-18 as well. By this irradiation, γ-proton of vicinal epoxide carbon (γ-C), which was a multiplet, collapsed to a pair of doublets. This proton (H_γ) is unequally split by the neighboring methylene protons (H_1 and H_2), that is, gauche to one proton and trans to the other with the coupling constants of 3.1 Hz and 9.4 Hz, respectively. As expected, other protons were not affected by this irradiation.

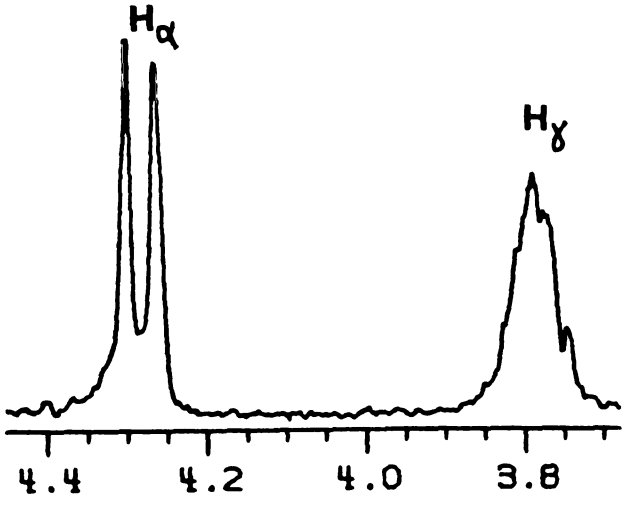




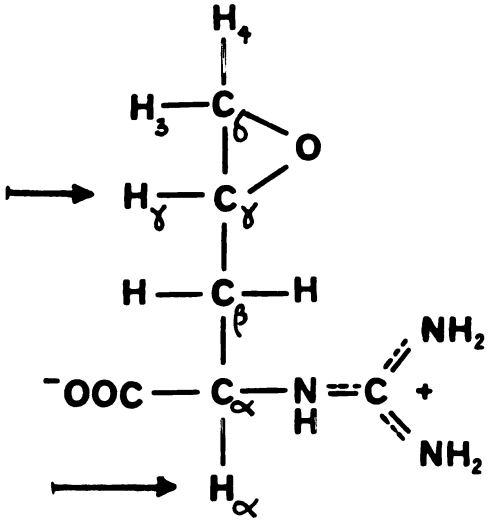
Irradiation at H_1 or H_2

Under irradiation at one of two protons of methylene carbon (H_1 or H_2) as shown below and also in Figure IV-19, proton H_α of α-carbon collapsed from a pair of doublets to a simple doublet (J = 8.5 Hz) (T). Proton H_γ of epoxide ring was also affected by this irradiation as expected.



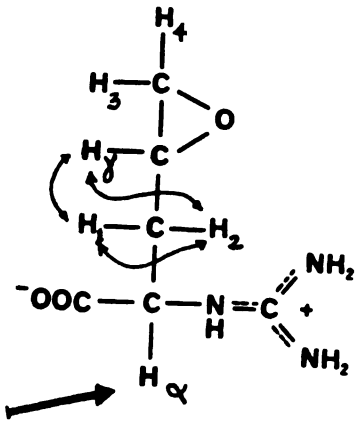


While the homonuclear decouplings at H_3, H_4, and H_1 or H_2 confirmed the chemical shift assignments for all protons of isoepoxycreatine, the selected irradiation of the α-carbon proton H_α and the epoxide proton H_γ are necessary for the determination of the absolute configuration of the chiral centers (C_α and C_γ) of the molecule.

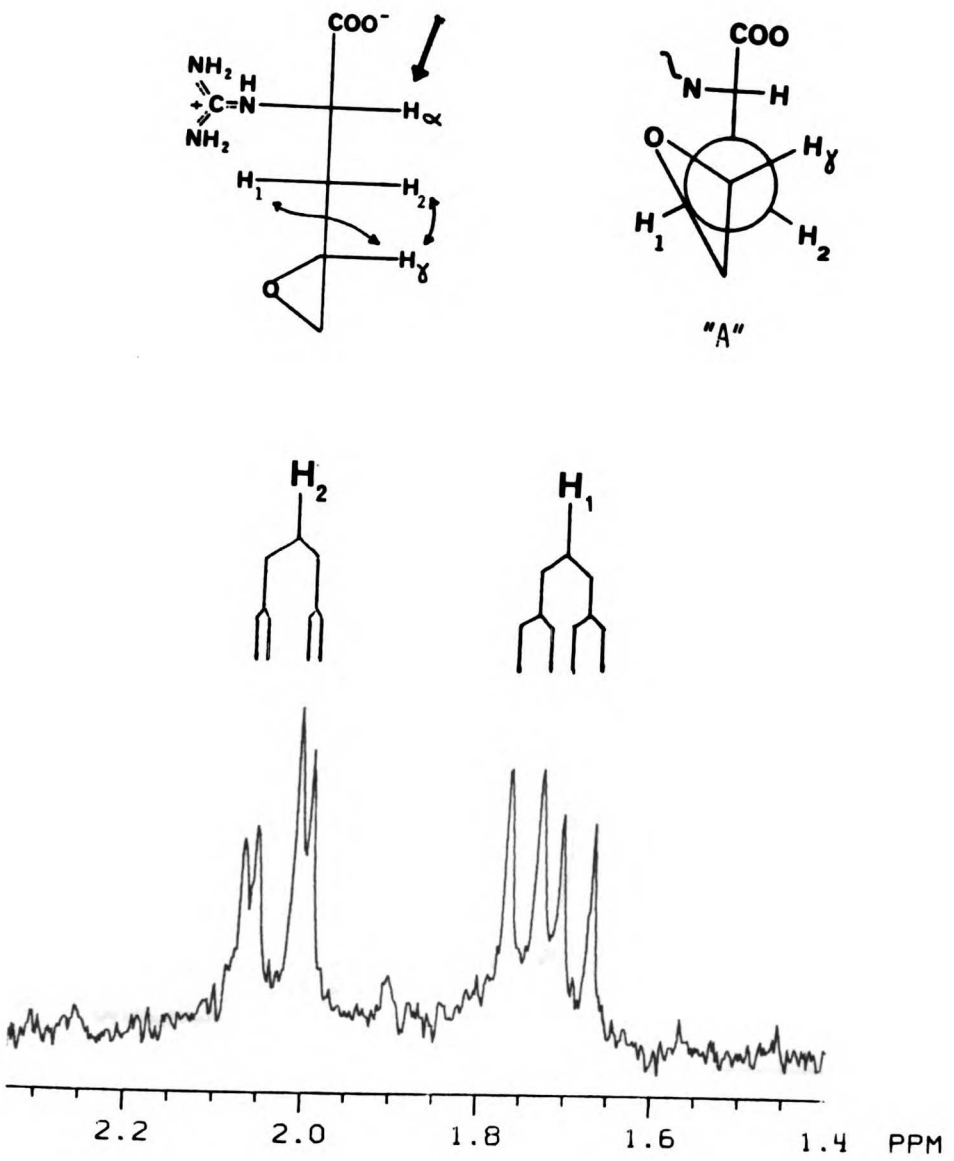


Irradiation at proton H_α

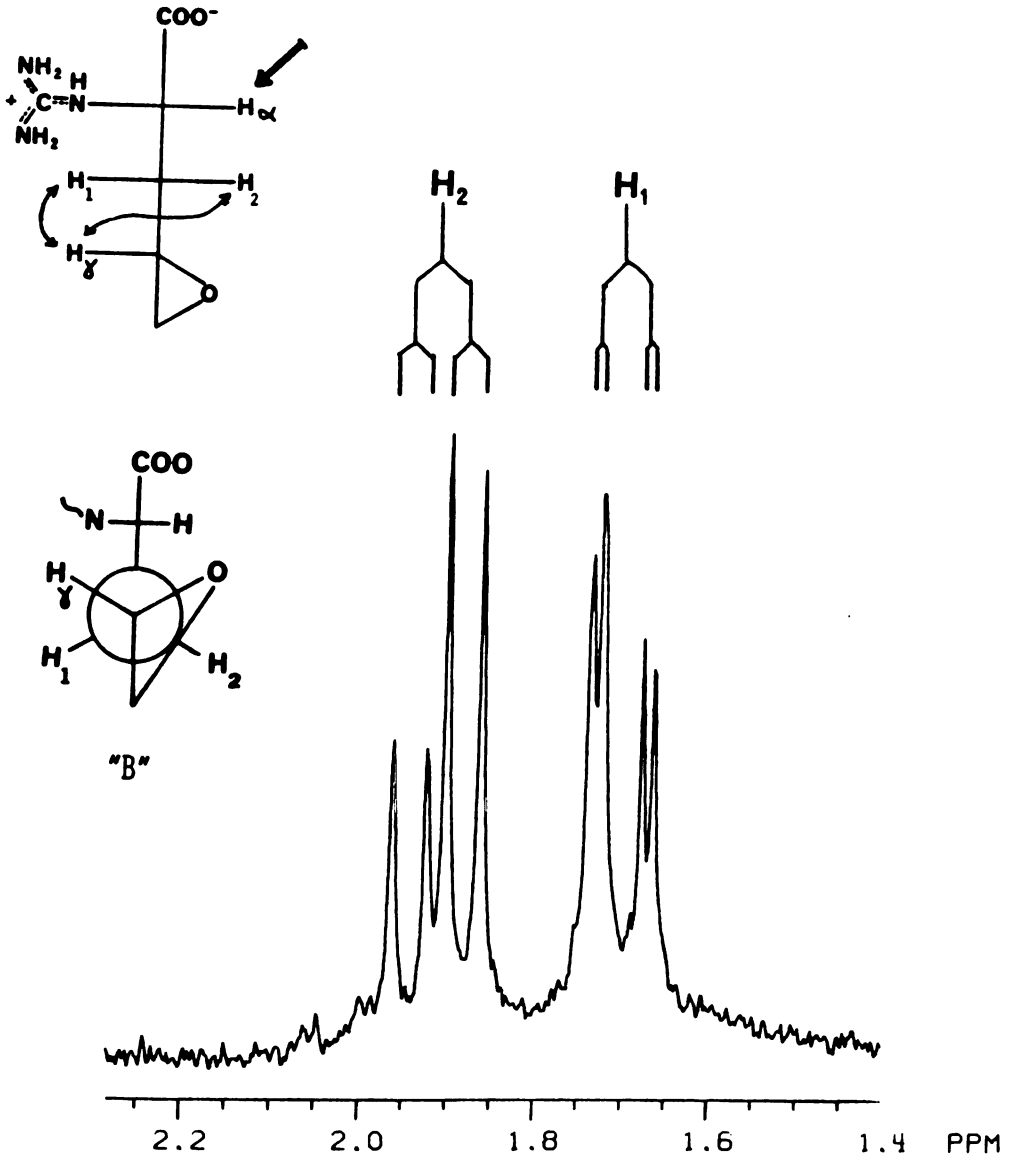
Under irradiation of frequency at α-proton, the multiplet patterns of H_1 and H_2 collapsed to a pair of doublets with different coupling constants.



^1H NMR of selected decoupling at H_α of A-isomers is shown below and as well in Figure IV-20. From this experiment, H_1 and H_2 are no longer coupled to H_α. Each proton is split by the other, and unequally by the neighboring proton H_γ of epoxide carbon. The new coupling constants for H_1 are -14.6 Hz and 9.1 Hz (T), whereas the new coupling constant for H_2 are -14.6 Hz and 3.4 Hz (G). The geminal coupling constants are negative numbers and are in the range of 12-15 Hz (Silverstein et al., 1974).



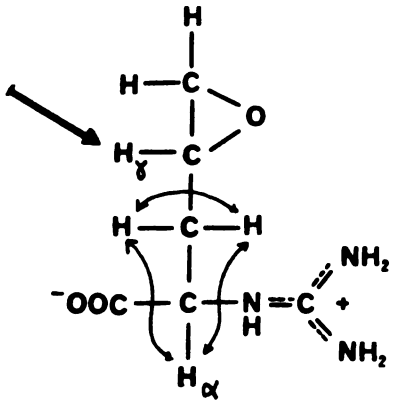
Selected decoupling at proton H_α of B isomers is shown in Figure IV-21 and below. The H_1 is now split by H_2 and H_γ with the coupling constants of -14.6 Hz and 2.7 Hz (G). The coupling constants of H_2 to H_1 and H_γ are -14.6 Hz and 9.4 Hz (T).



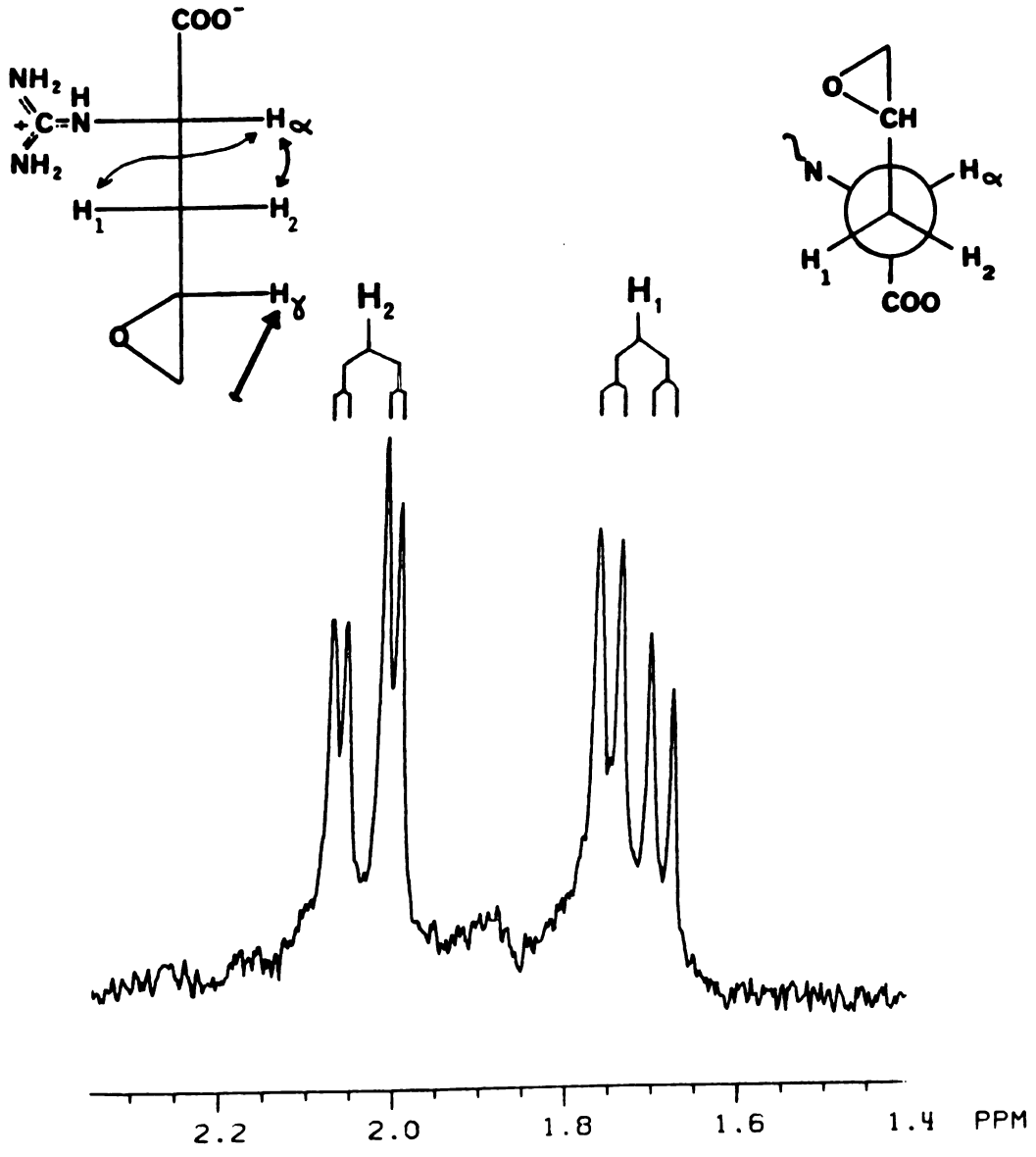
The result from the irradiation at H_α gives better coupling information for each isomer than the previous irradiations. Thus, in A isomers, proton H_γ is trans to H_1 and gauche to H_2, whereas in B isomers, proton H_γ is gauche to H_1 and trans to H_2.

Irradiation at H_γ

Selected decoupling at proton H_γ of the epoxide carbon resulted in a changes at protons H_3, H_4, H_1, and H_2. Protons H_3 and H_4 each collapsed to a doublet (J = 12 Hz) by coupling to each other. Protons H_1 and H_2 collapsed from multiplets to a pair of doublets by coupling to each other (J = -14.6 Hz), and by unequal couplings to H_α.



Proton NMR of the selected decoupling at proton H_γ of A isomers is shown in Figure IV-22 and below. The large coupling constant of H_1 to H_α corresponds to a trans relationship between these two protons (J = 7.4 Hz), whereas the small coupling between H_2 and H_α (J = 3.9 Hz) indicates that they are gauche to each other.



The selected decoupling at H_γ of B isomers is shown below and also in Figure IV-23. The result from selected irradiation at H_γ indicates the gauche relationship between H_α and H_2 (J = 9.1 Hz) and the trans relationship between H_α and H_1 (J = 3.3 Hz).

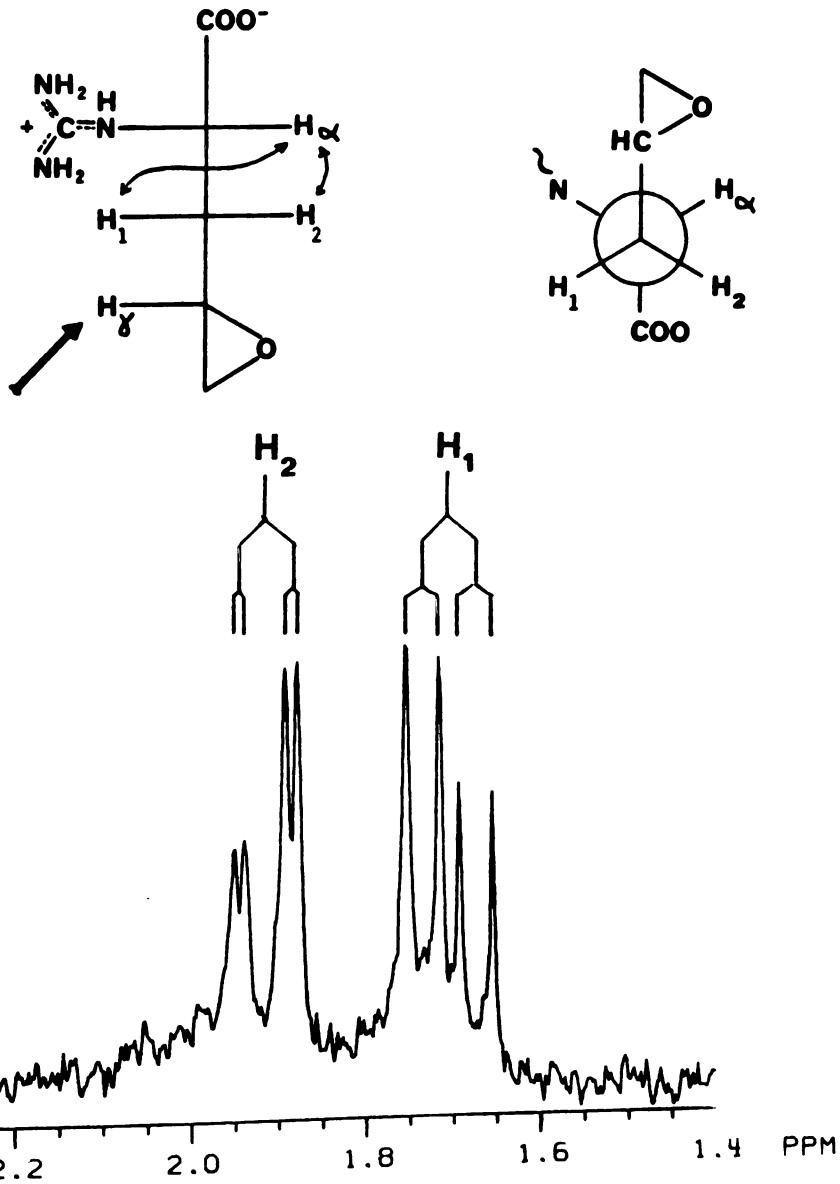


Figure IV-16:

Fully-coupled ^{1}H NMR spectra of $S_{\alpha}R_{\gamma}$- and/or $R_{\alpha}S_{\gamma}$-isoepoxycreatine ("A" isomer). The legends are shown in the figure and in page 124. The peaks are shown below:

PP

LINE#	HEIGHT(L)	FREQ(HZ)	PPM
1	268.80	1018.39	4.235
2	283.79	1013.76	4.216
3	271.31	1011.14	4.205
4	197.04	1006.54	4.186
5	144.28	941.40	3.915
6	151.85	938.25	3.902
7	188.12	935.01	3.889
8	184.32	931.55	3.874
9	141.08	929.34	3.865
10	129.92	925.61	3.849
11	227.61	869.37	3.616
12	210.24	865.35	3.599
13	132.15	863.67	3.592
14	488.98	857.67	3.567
15	398.94	853.58	3.550
16	154.58	850.22	3.536
17	416.64	845.47	3.516
18	341.98	838.92	3.489
19	148.18	833.75	3.467
20	119.42	827.17	3.440
21	143.62	501.30	2.085
22	191.28	497.13	2.067
23	136.98	492.86	2.050
24	180.77	486.58	2.023
25	234.97	482.56	2.007
26	132.73	478.26	1.989
27	172.60	430.03	1.788
28	203.23	422.69	1.758
29	179.47	420.74	1.750
30	163.18	415.29	1.727
31	159.73	413.29	1.719
32	111.29	408.03	1.697
33	106.58	406.00	1.688

FIGURE IV-16:

^{1}H NMR OF "A" ISOEPOXYCREATINE

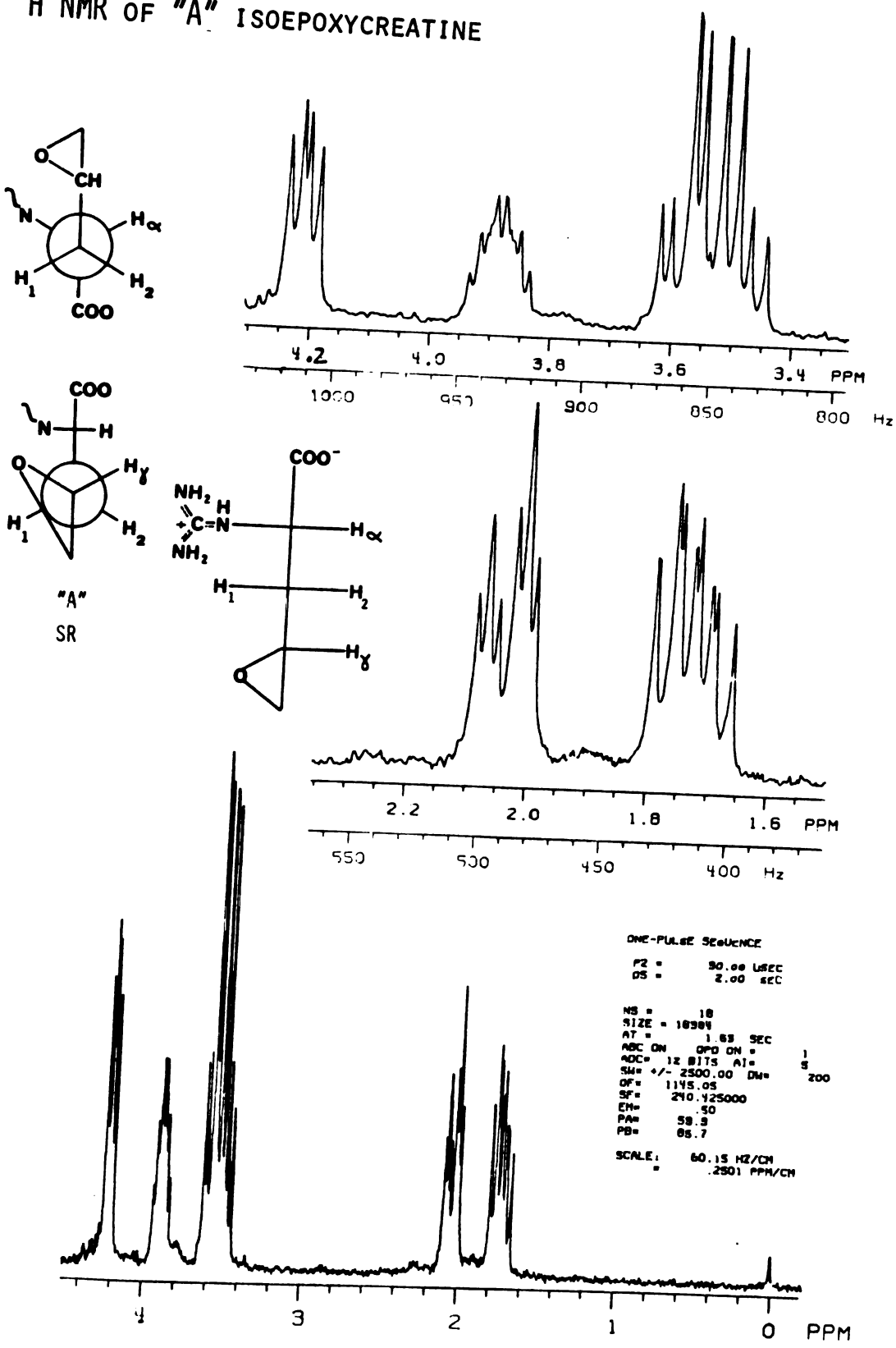


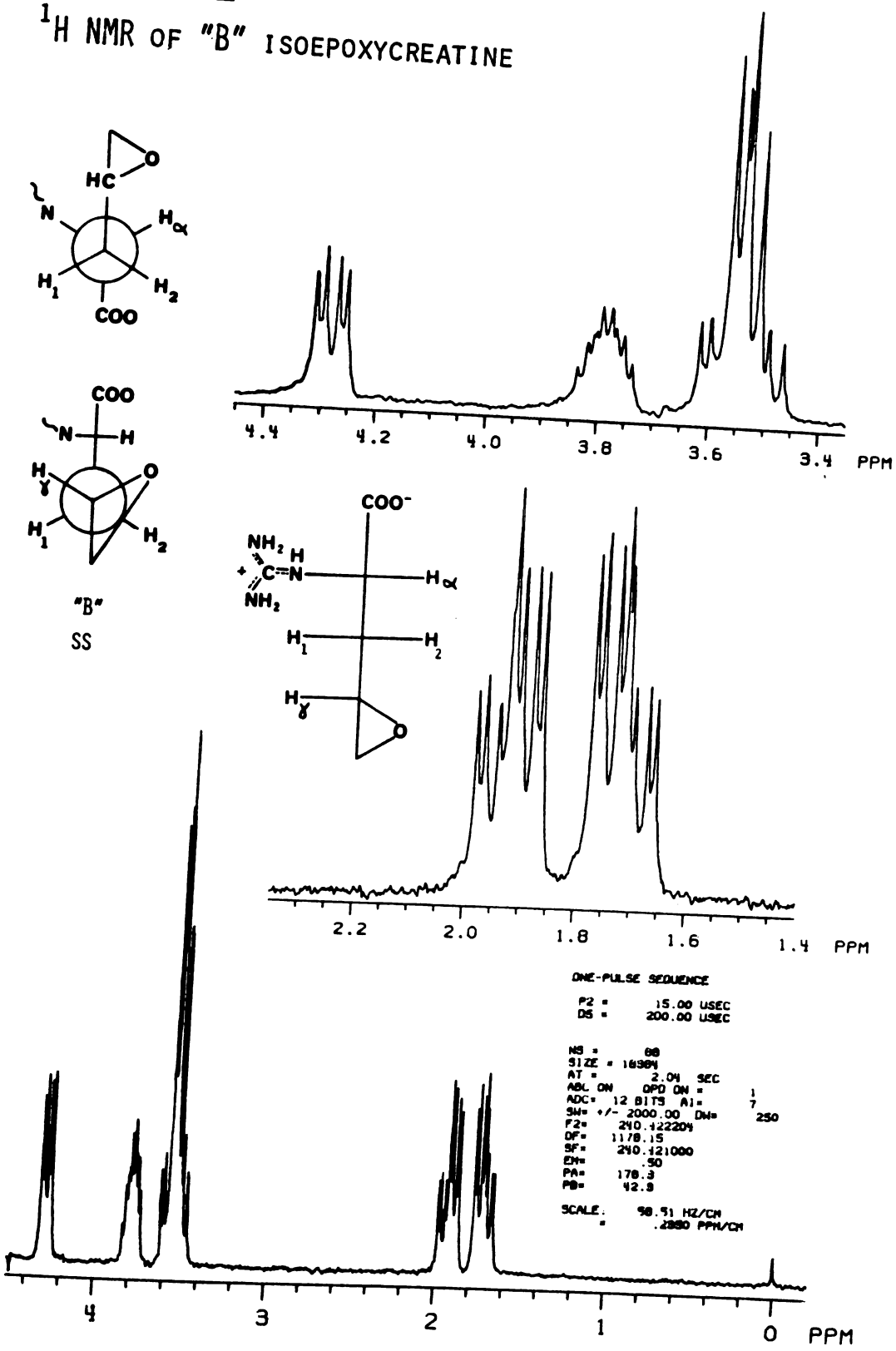
Figure IV-17:

Fully-coupled ^{1}H NMR spectra of $S_{\alpha}S_{\gamma}$- and/or $R_{\alpha}R_{\gamma}$-isoepoxycreatine ("B" isomers). The legends are shown in the figure and in page 124. The peaks are shown below:

PP LINE#	HEIGHT	FREQ(HZ)	PPM
1	293.85	1037.50	4.315
2	287.02	1033.88	4.300
3	308.87	1027.76	4.274
4	245.63	1024.18	4.259
5	93.02	920.41	3.828
6	147.23	916.86	3.813
7	168.23	913.46	3.799
8	230.13	910.69	3.787
9	236.35	906.77	3.771
10	183.49	904.14	3.760
11	170.67	900.80	3.746
12	122.32	897.00	3.730
13	241.24	868.55	3.612
14	266.63	864.33	3.595
15	138.68	861.65	3.583
16	813.20	856.84	3.563
17	742.40	852.61	3.546
18	769.50	849.95	3.535
19	606.51	843.40	3.508
20	263.09	838.33	3.486
21	189.68	831.70	3.459
22	170.30	474.36	1.973
23	202.90	470.75	1.958
24	172.38	464.70	1.932
25	337.56	459.96	1.913
26	293.49	456.27	1.897
27	290.56	450.21	1.872
28	254.18	446.58	1.857
29	321.45	423.30	1.760
30	313.02	420.05	1.747
31	125.01	417.46	1.736
32	323.64	413.59	1.720
33	326.94	410.27	1.706
34	178.00	405.65	1.687
35	174.45	399.07	1.659
36	144.67	395.88	1.646
37	405.56	.00	.000

FIGURE IV-17:

^{1}H NMR OF "B" ISOEPOXYCREATINE



Figures IV-18 and IV-19:

Selective homonuclear decoupled ^1H NMR spectra of $S_\alpha S_\gamma$-isoepoxycreatine ("B" isomer). The legends are shown in the figures and in page 124.

Figures IV-20 to IV-23:

Selective homoneclear decoupled ^1H NMR spectra of $S_\alpha R_\gamma$- (Figures IV-20 and IV-23) and $S_\alpha S_\gamma$-isoepoxycreatine (Figures IV-21 and IV-23). Decoupling frequencies are shown in the figures. Scales of the spectra are 64% reduced from the scales stated in the figure. Other legends are shown in page 124.

FIGURE IV-18: ^1H NMR OF SELECTED DECOUPLING AT H_3 AND H_4

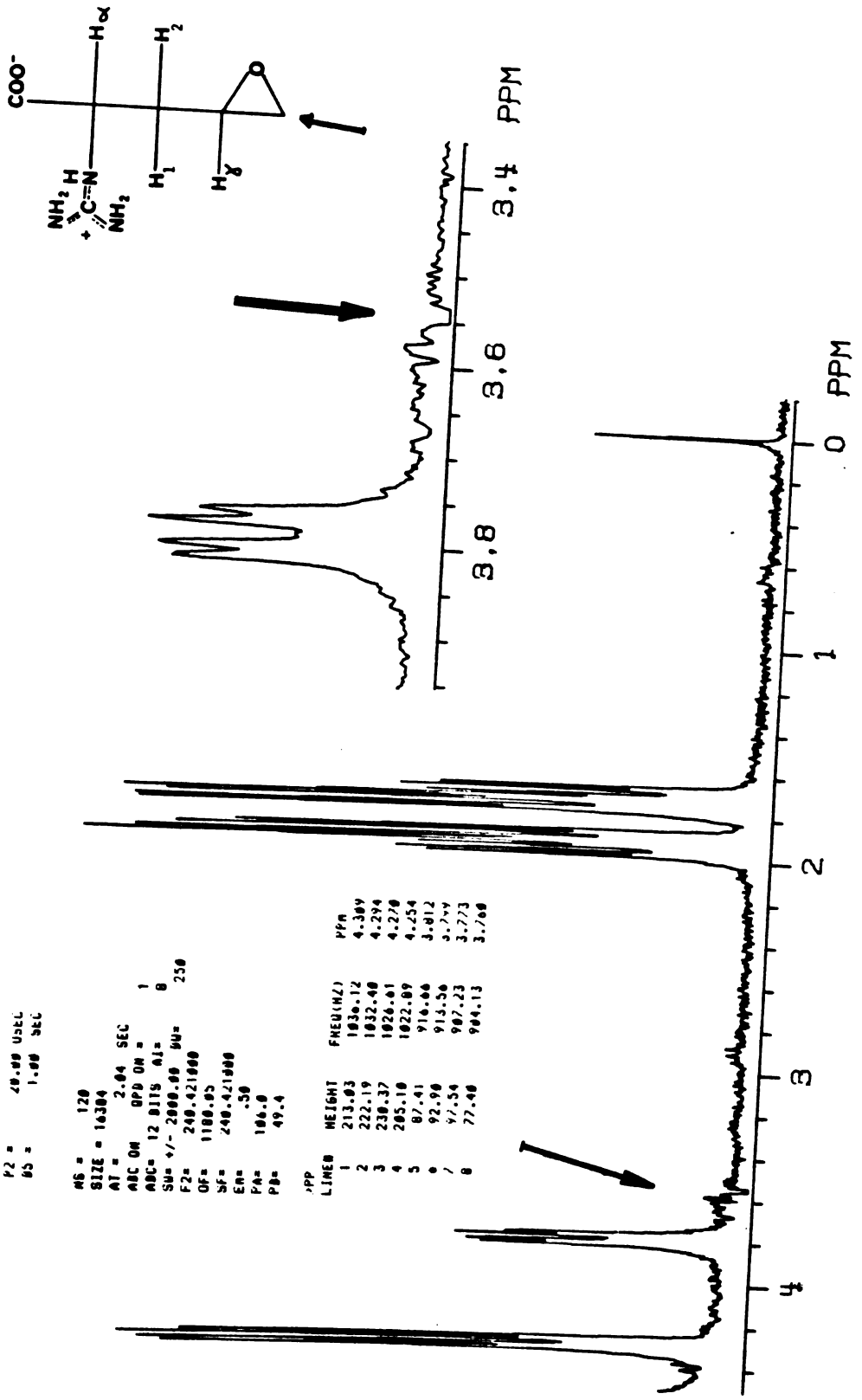
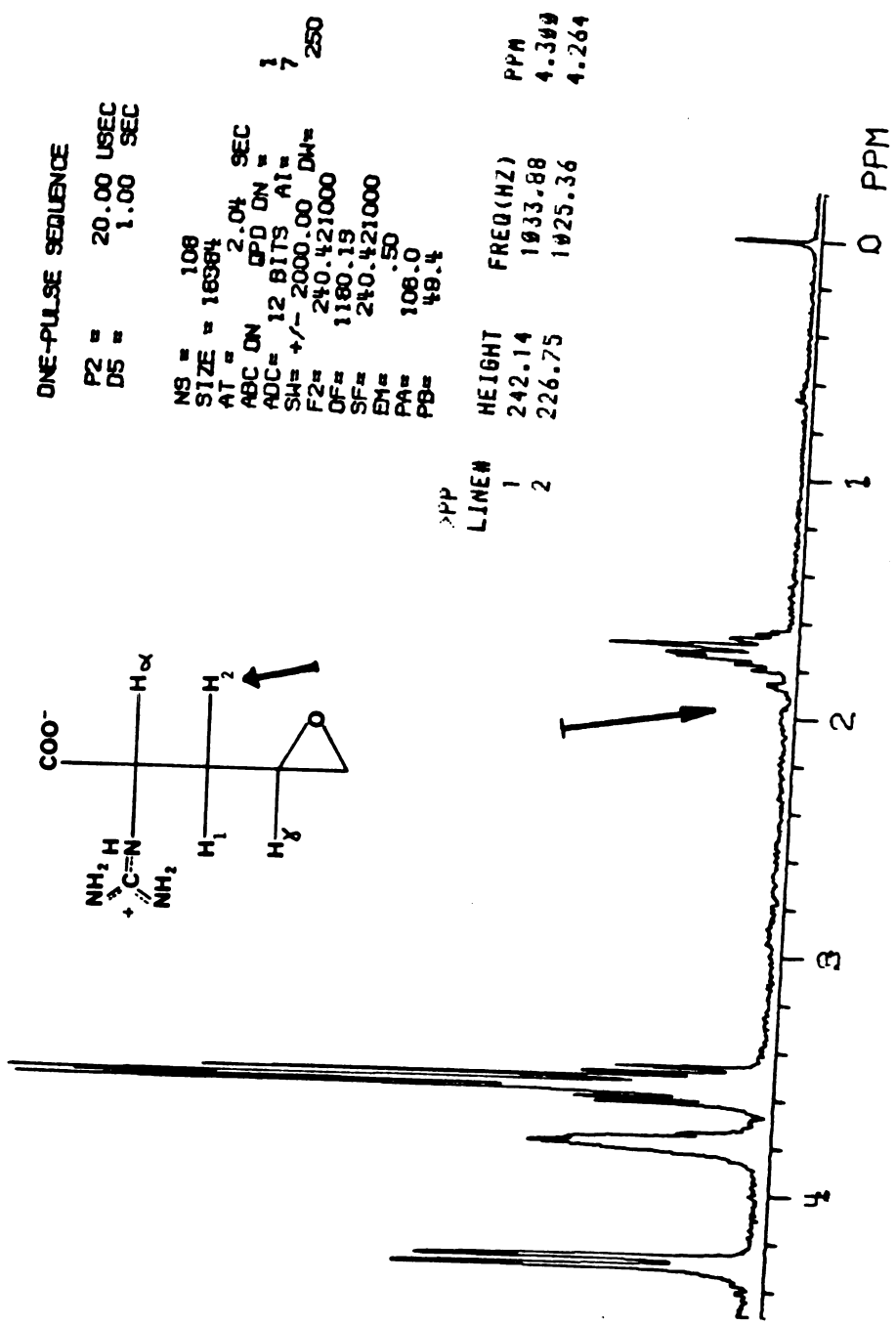
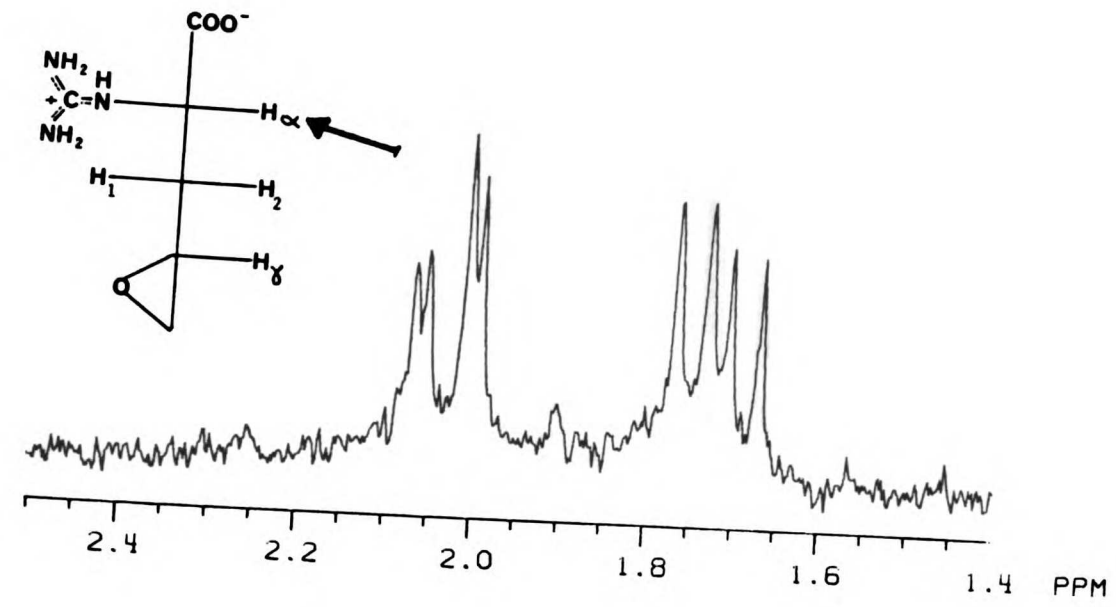


FIGURE IV-19: ^1H NMR OF SELECTED DECOUPLING AT H_2





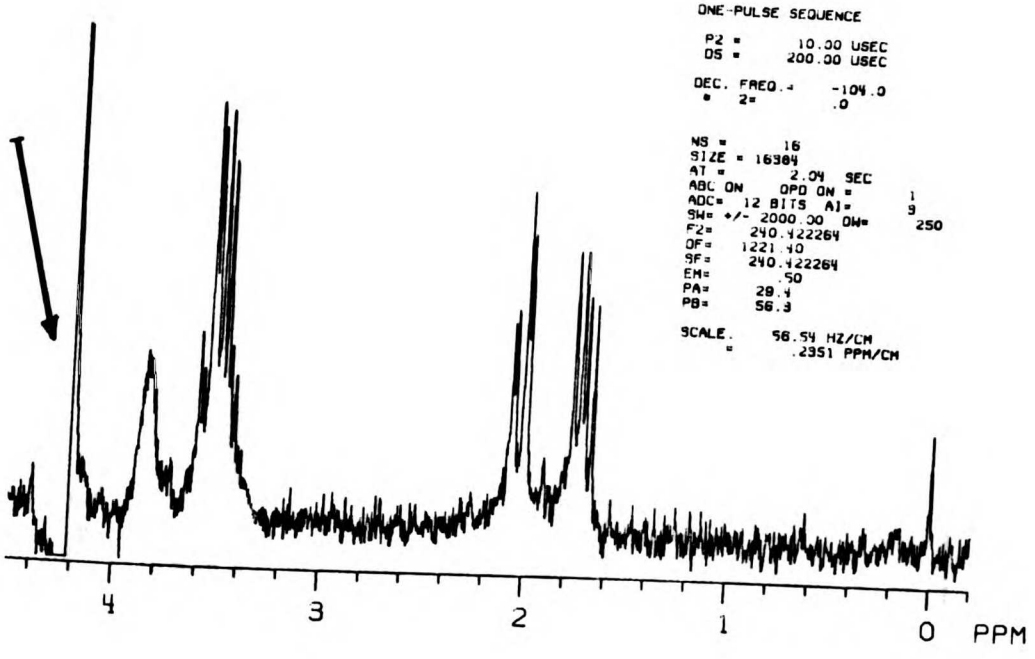


FIGURE IV-20:

^{1}H NMR OF SELECTED DECOUPLING AT H OF "A" ISOMER

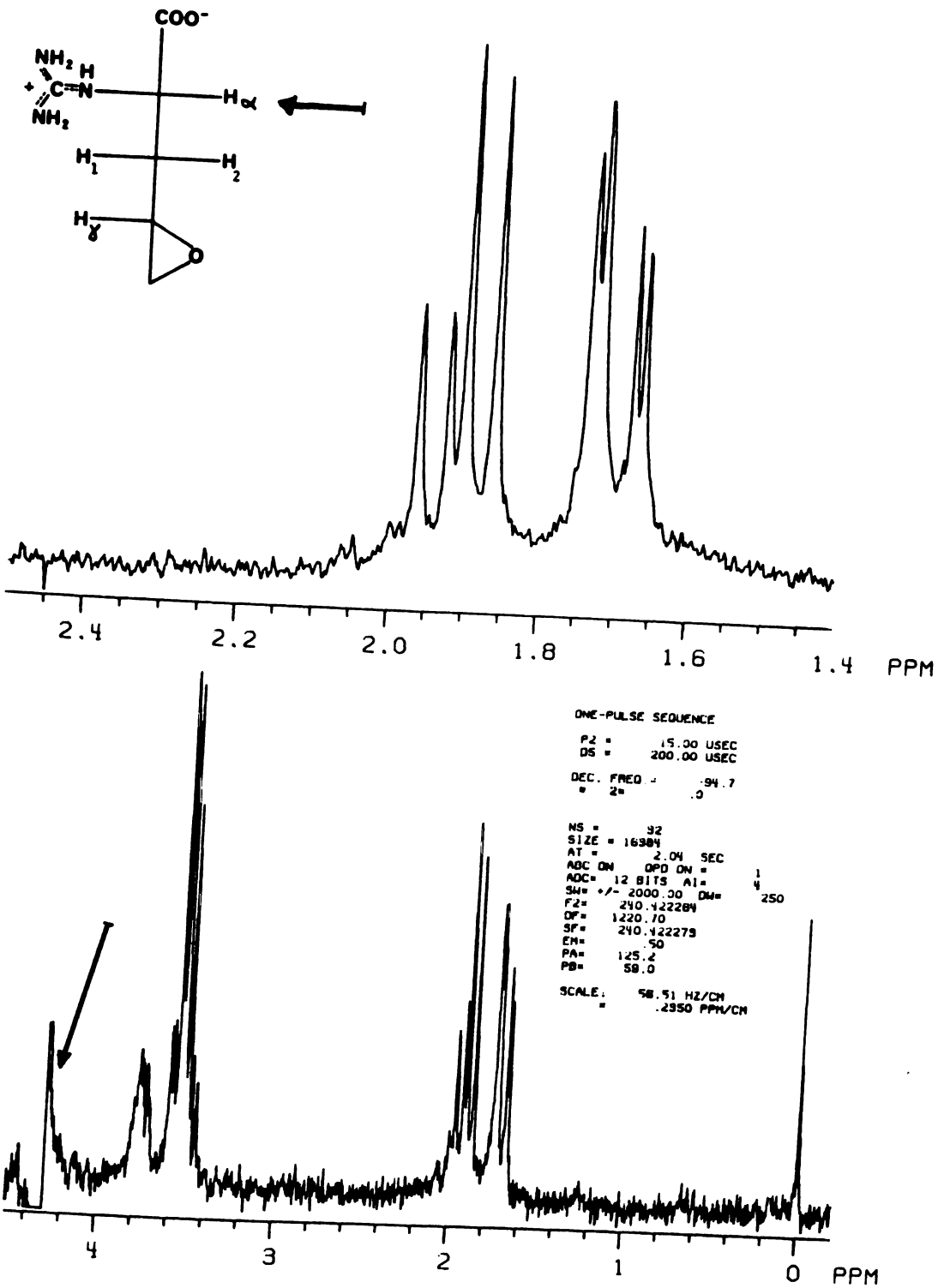


FIGURE IV-21:

^{1}H NMR OF DECOUPLING AT H_α OF "B" ISOMER

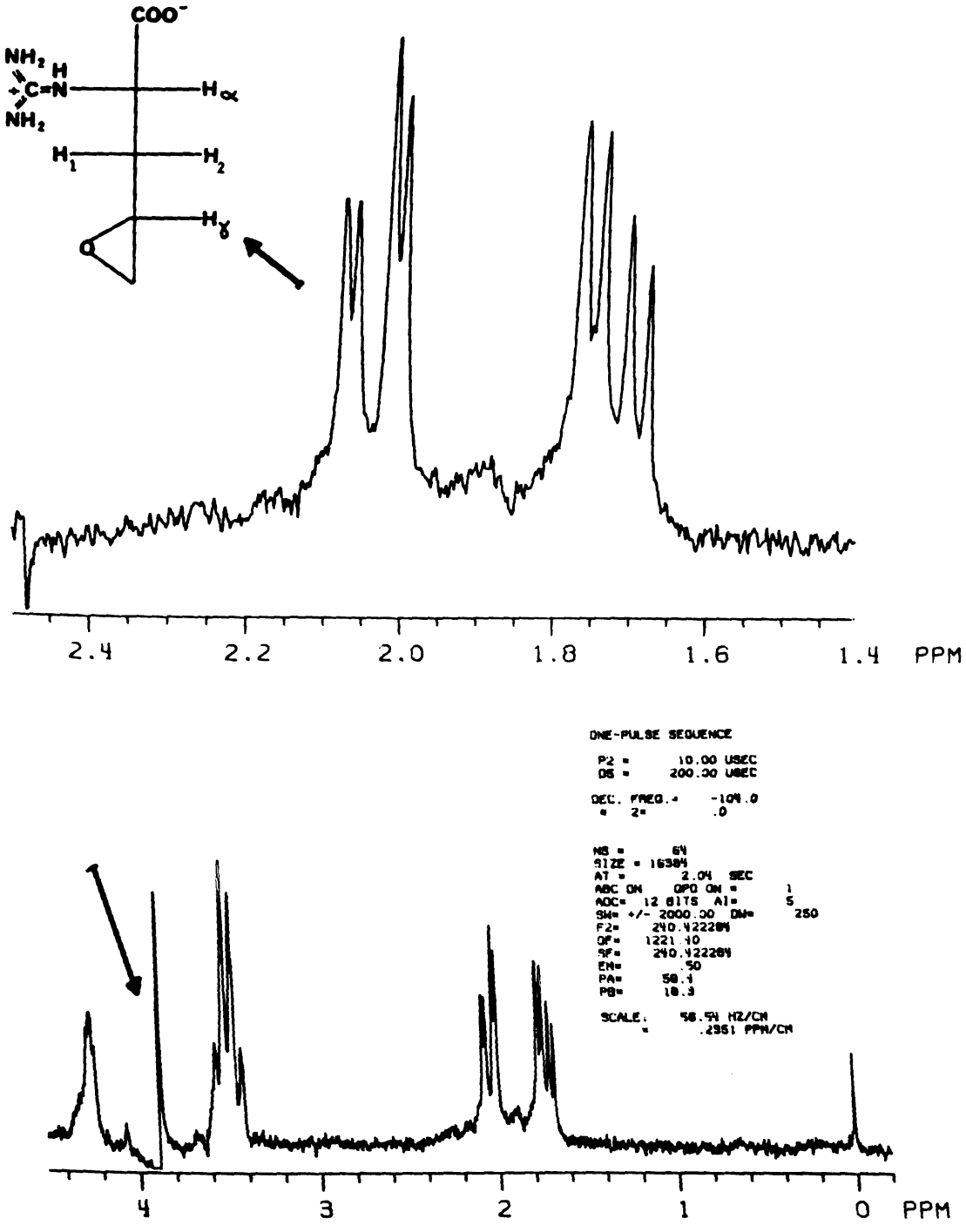


FIGURE IV-22:

^{1}H NMR OF DECOUPLING AT H_γ OF "A" ISOMER

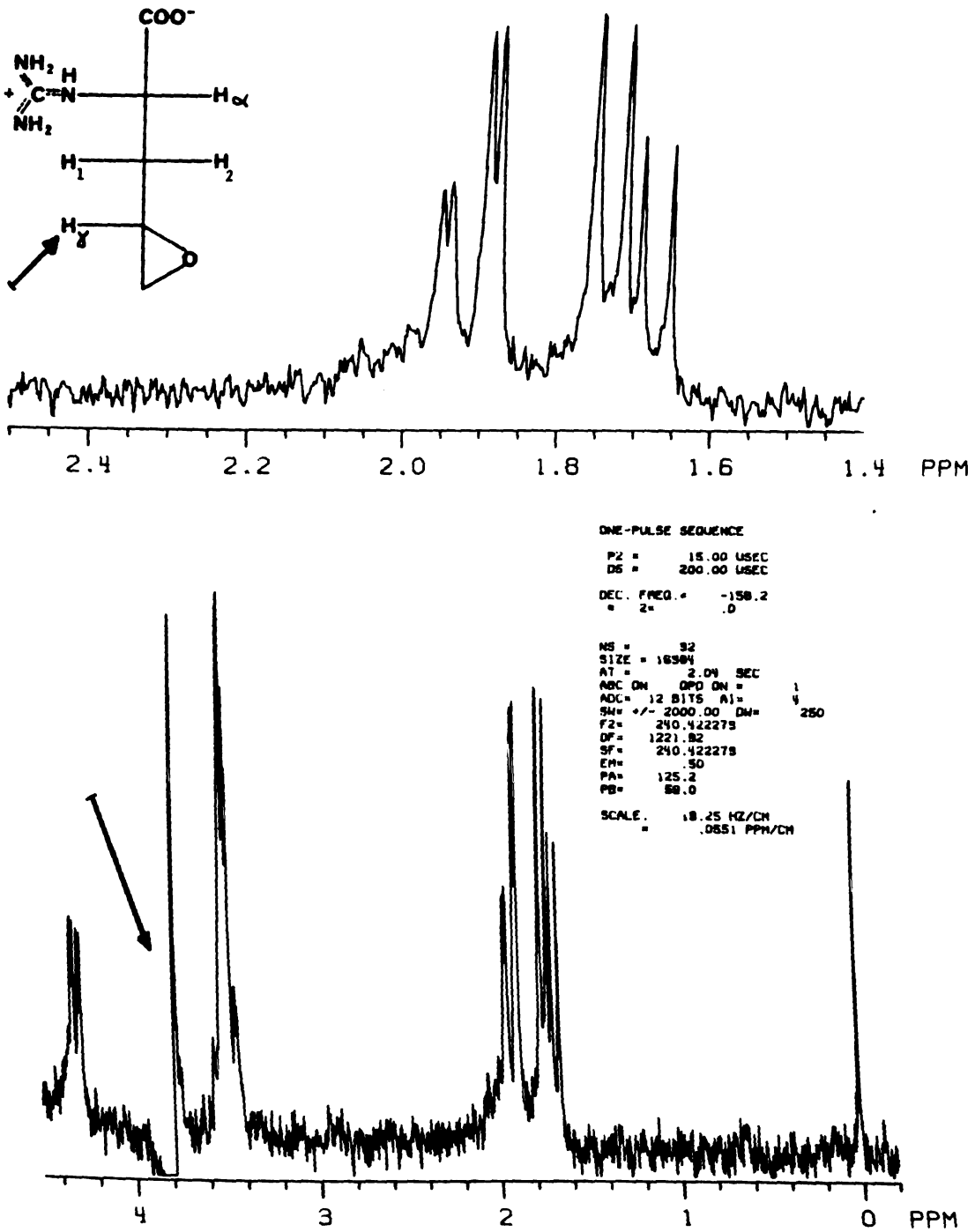


FIGURE IV-23:

^{1}H NMR OF SELECTED DECOUPLING AT H_γ OF "B" ISOMER

DETERMINATION OF ABSOLUTE CONFIGURATION OF DIASTEREOMERS

The absolute configurations of the asymmetric centers of isoepoxycreatine have been determined by ^{1}H NMR with selected homo-decoupling experiments and by consideration of the angular dependence of the three-bonded carbon-proton couplings ($^{3}J_{C-H}$) using ^{13}C NMR.

Proton NMR spectra of the expanded regions of methylene protons H_1 and H_2 are shown in Figures IV-24 (A isomer) and IV-25 (B isomer) along with coupling patterns as determined from the selected decoupling experiments. The coupling constants are summarized in Table IV-3.

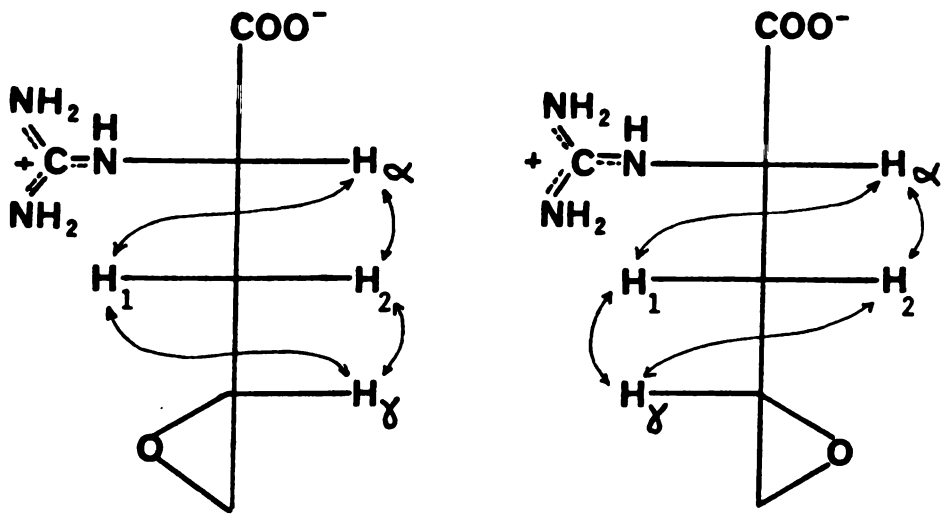
Considering only proton couplings from Table IV-3 and from the utility of the well-known Karplus relationship between vicinal proton coupling constants and dihedral angles (Karplus, 1963), structures of A isomer and B isomer can be roughly drawn as shown in the following scheme (scheme IV-3). The finding from proton couplings indicates a gauche relationship between H_2H_α and H_2H_γ and a trans relationship between H_1H_α and H_1H_γ in A isomers. The methylene protons H_1 and H_2 in B isomer are more equivalent. In B isomers, H_1 is gauche to H_γ but trans to H_α and H_2 is gauche to H_α but trans to H_γ.

TABLE IV-3

COUPLING CONSTANTS OF H_1 AND H_2

Pure isomers	A (Hz)	B (Hz)
$J_{H_1H\alpha}$	7.4	9.1
$J_{H_1H\gamma}$	9.1	2.7
$J_{H_2H\alpha}$	3.9	3.3
$J_{H_2H\gamma}$	3.4	9.4

SCHEME IV-3



A (SR)

B (SS)

	A	B
H_1H_α	T	T
H_1H_γ	T	G
H_2H_α	G	G
H_2H_γ	G	T

T: trans

G: gauche

The proton coupling, however, does not indicate which proton is H_1 and which is H_2, and does not give any information regarding the predominant conformation of isomers. For example, if the synthesis started with the precursor L-C(α)-allylglycocyamine, two diastereomers SS and SR would be obtained as products. For each diastereomer (SS or SR), there would be 4 possible conformations which may be formed from the rotation of two C-C bonds between 2 chiral centers and four more possible conformations which may be formed by the changing positions of H_1 and H_2. Thus, for each diastereomer (SS or SR), four of these total 8 possible conformations fit with the proton couplings above and as shown in Schemes IV-4A ($S_\alpha R_\gamma$) and IV- 4B ($S_\alpha S_\gamma$). In order to determine the absolute configuration of chiral centers, an independent way is needed to identify the right conformer for each diastereomer. Proton-coupled ^{13}C NMR has then been introduced as a simple method to over-come the problem. The relationship between three-bonded carbon-proton

coupling constant and the geometry of involved atoms appears to be analogous to the above Karplus relationship. The three-bonded carbon-proton couplings ($^3J_{C-H}$) have a minimum value at the dihedral angle of 90°, a maximum at 0°, and a larger maximum at 180° (Lemieux et al., 1972; Wasylishen and Schaefer, 1972; Marshall et al., 1974). Rennekamp and Kingsbury (1973) suggested a $^3J_{C-H}$ (trans) value of 11 Hz and a $^3J_{C-H}$ (gauche) value of 2 Hz.

From the Newman projection in schemes IV-4A and IV-4B, it is of great interest to measure the long-range C-H coupling constants of methylene carbon (δ-C) of epoxide group and also carboxylate carbon. Proton-coupled ^{13}C NMR of B isomer is shown in Figure IV-26. The expanded regions of the methylene carbon (δ-C) of epoxide group and the carboxylate carbon are shown in Figures IV-27 and IV-28, respectively.

The carboxylate carbon, which had a doublet-triplet pattern, collapsed to a triplet (J = 2.4 Hz) by irradiation at the frequency of the transition at the proton H_α of α-carbon. The small coupling constant of 2.4 Hz indicates a gauche conformation between the carboxylate carbon and protons H_1 and H_2. The methylene carbon (δ-C) of epoxide group which had a quartet (J = 2.9 Hz) at one of its peaks as result of the coupling with H_γ, H_1, and H_2 also indicates a gauche relationship to both H_1 and H_2.

The findings from the three-bonded carbon-proton couplings have ruled out all the possible conformations which have a trans relationship between methylene carbon (δ-C) and H_1 and H_2 or between carboxylate carbon and H_1 and H_2. The right conformation for A isomer is SR (# 1) (Scheme IV-4A) and for B isomer is SS (# 2) (Scheme IV-4B), where methylene carbon (δ-C) and carboxylate carbon are in gauche relationships to H_1 and H_2.

If the natural amino acid L-C(α)-allylglycine was used as the precursor for the synthesis of isoepoxycreatine, then the A diastereoisomer, which was the first isomer to elute from HPLC column, is SR (S configuration at α-C), and the B diastereoisomer is SS (Scheme IV-5). If the precursor was D-C(α)-allylglycine, then the A diastereoisomer is RS (R configuration at α-C), and the B diastereoisomer is RR.

The result came out as predicted for the stable conformations of isoepoxycreatine isomers. Thus, the epoxy group prefers the least hindered position, that is trans to carboxylate group. The reason that the epoxide group is in gauche to the guanidinium group could be explained by the hydrogen bonding between the lone pair of oxygen and a hydrogen of guanidinium group.

Scheme IV-4A:

Conformers of $S_\alpha R_\gamma$-isoepoxycreatine which are fit with gauche-trans requirements in Table IV-3. In columns 1 and 2, H_1 and H_2 are shown in the schematic structure, whereas in columns 3 and 4, H_1 is in the place of H_2 and vice-versa. The first lines show the Newman projections of the bond C_α-C_β, while the second lines show those of the bond C_β-C_γ.

Column 1: Newman projection formulas of SR isomer (Fischer projection is shown in the scheme).

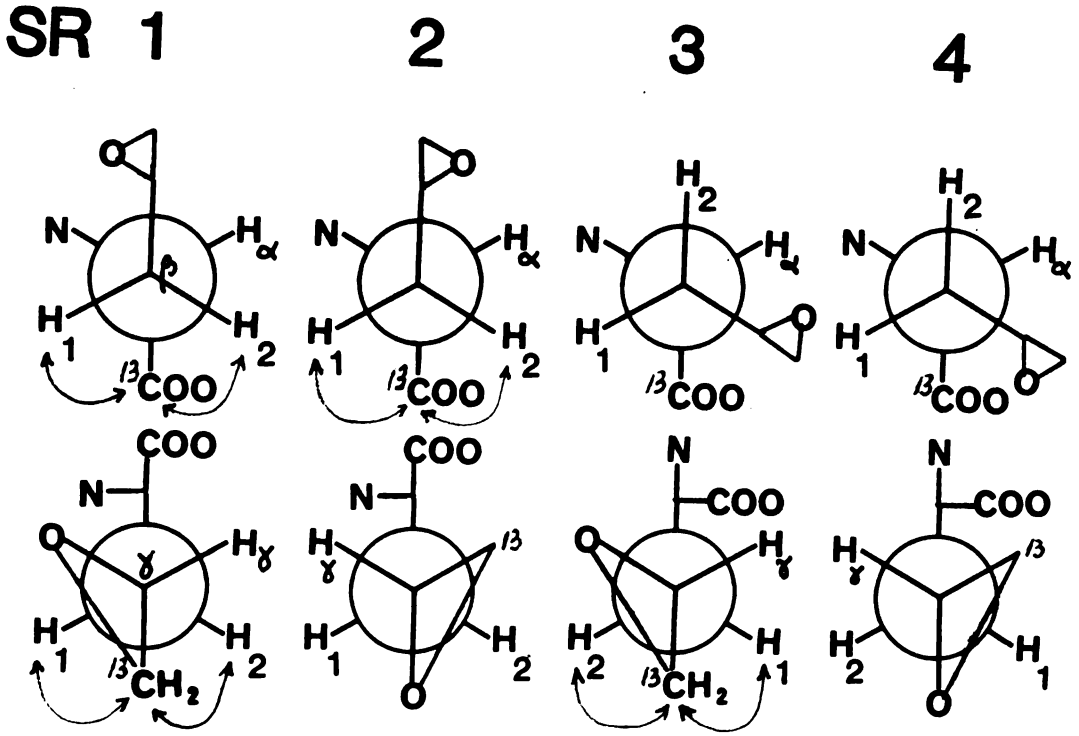
Column 2: 180° rotation of the C_γ-C_β bond of that Fischer structure.

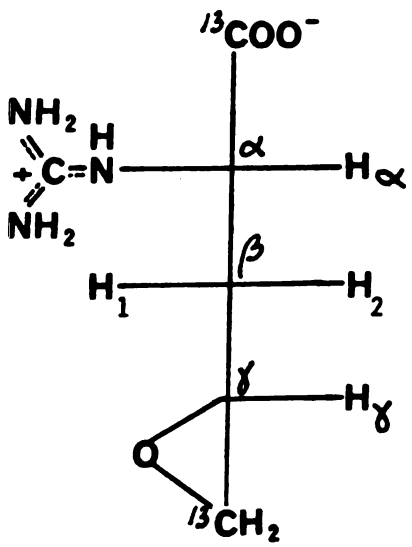
Column 3: 180° rotation of the C_α-C_β bond of the Fischer structure shown in the scheme with H_1 is in the place of H_2.

Column 4: 180° rotations of both C_α-C_β and C_β-C_γ bonds of Fischer structure shown in the scheme with H_1 is in the place of H_2.

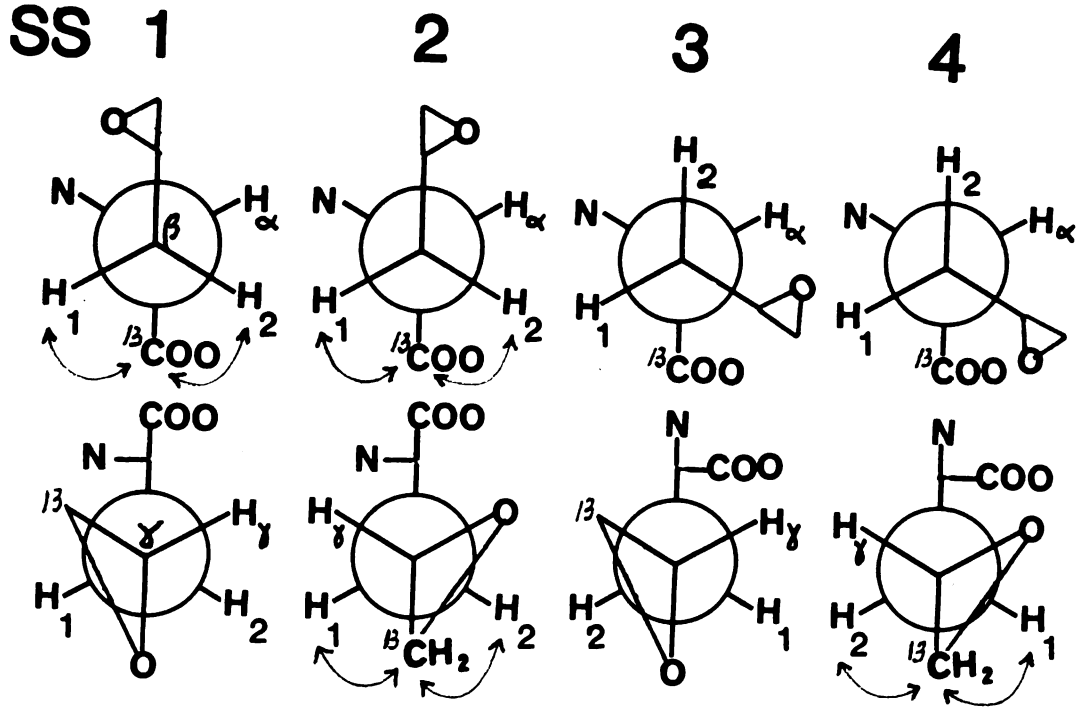
Scheme IV-4B:

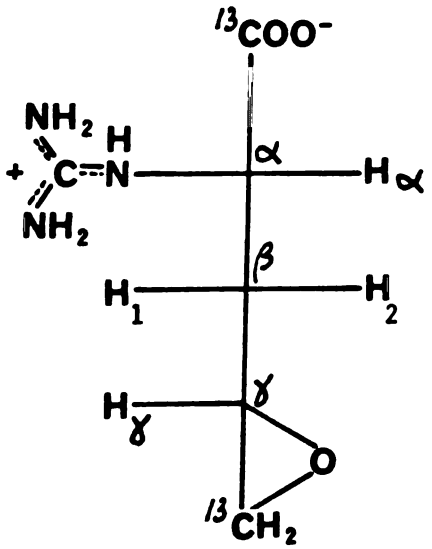
Conformers of $S_\alpha S_\gamma$-isoepoxycreatine which are satisfied the gauche-trans requirements in Table IV-3. The legends are the same as those in Scheme IV-4A.



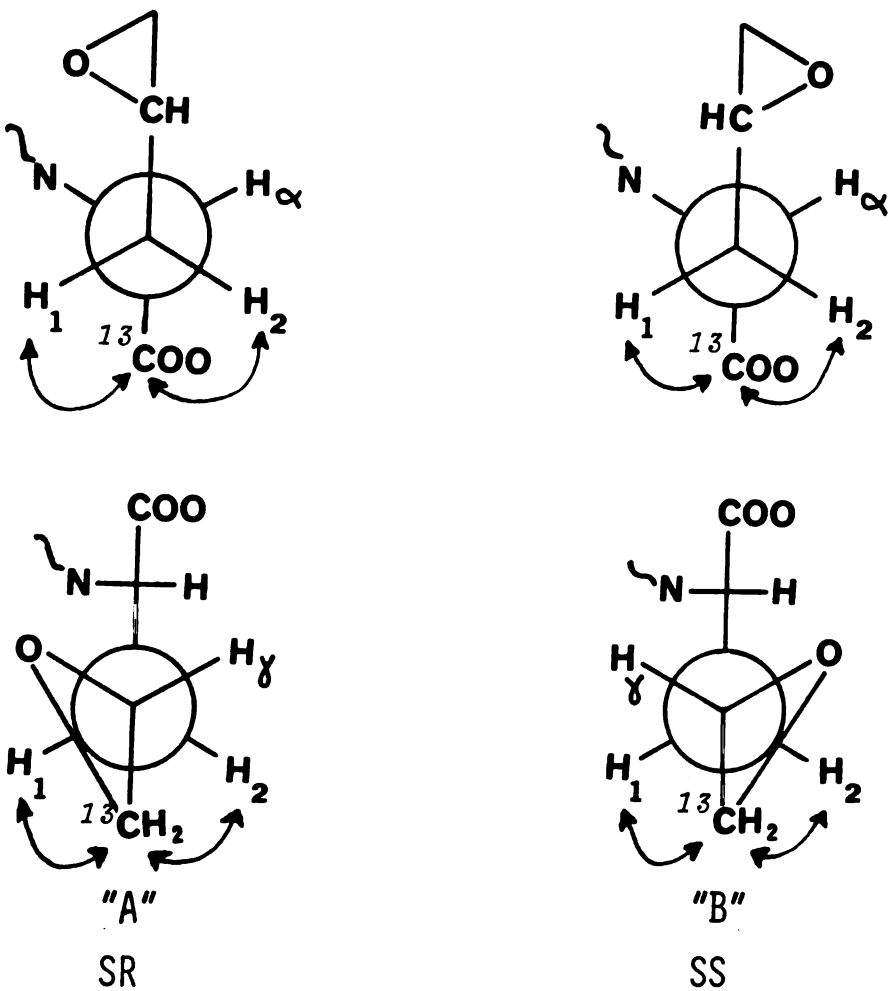


SCHEME IV-4A: $S_{\alpha}R_{\gamma}$-ISOEPOXYCREATINE





SCHEME IV-4B: $S_{\alpha}S_{\gamma}$-ISOEPOXYCREATINE


O
CH
N
Hα
H1
H2
13
COO
O
HC
N
Hα
H1
H2
13
COO
COO
N
H
O
Hγ
H1
H2
13
CH2
"A"
SR
COO
N
H
H
γ
O
H1
H2
13
CH2
"B"
SS

SCHEME IV-5

Figure IV-24:

Expanded ^{1}H NMR spectrum of the methylene protons of $S_{\alpha}R_{\gamma}$-isoepoxycreatine ("A" isomer). A full spectrum of this isomer is shown in Figure IV-16. The legends are those of that figure.

Figure IV-25:

Expanded ^{1}H NMR spectrum of the methylene protons of $S_{\alpha}S_{\gamma}$-isoepoxycreatine ("B" isomer). A full spectrum of this isomer is shown in Figure IV-17 and the legends are those of that figure.

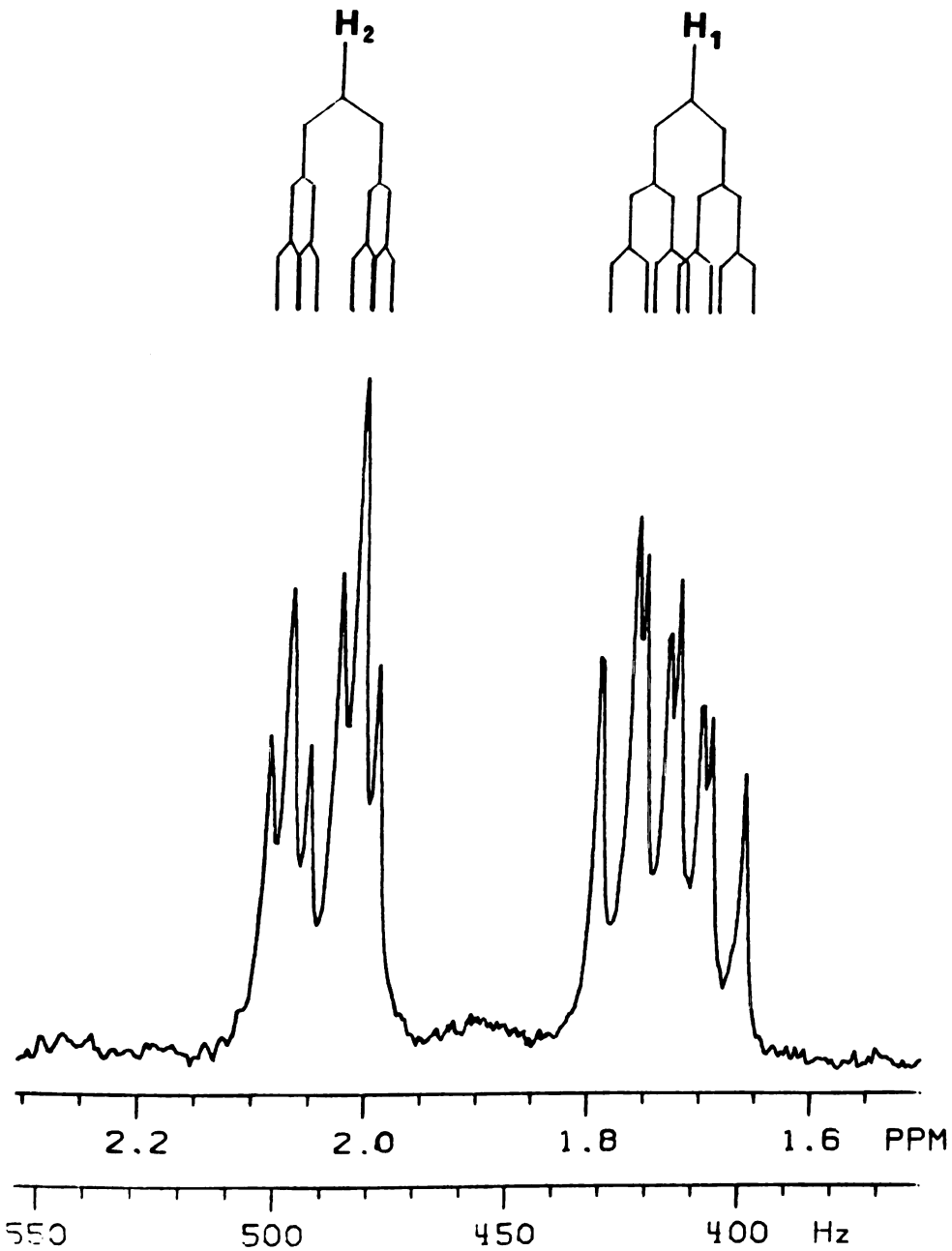


FIGURE IV-24:

^1H NMR OF METHYLENE PROTONS (H_1 AND H_2) OF "A" ISOMER

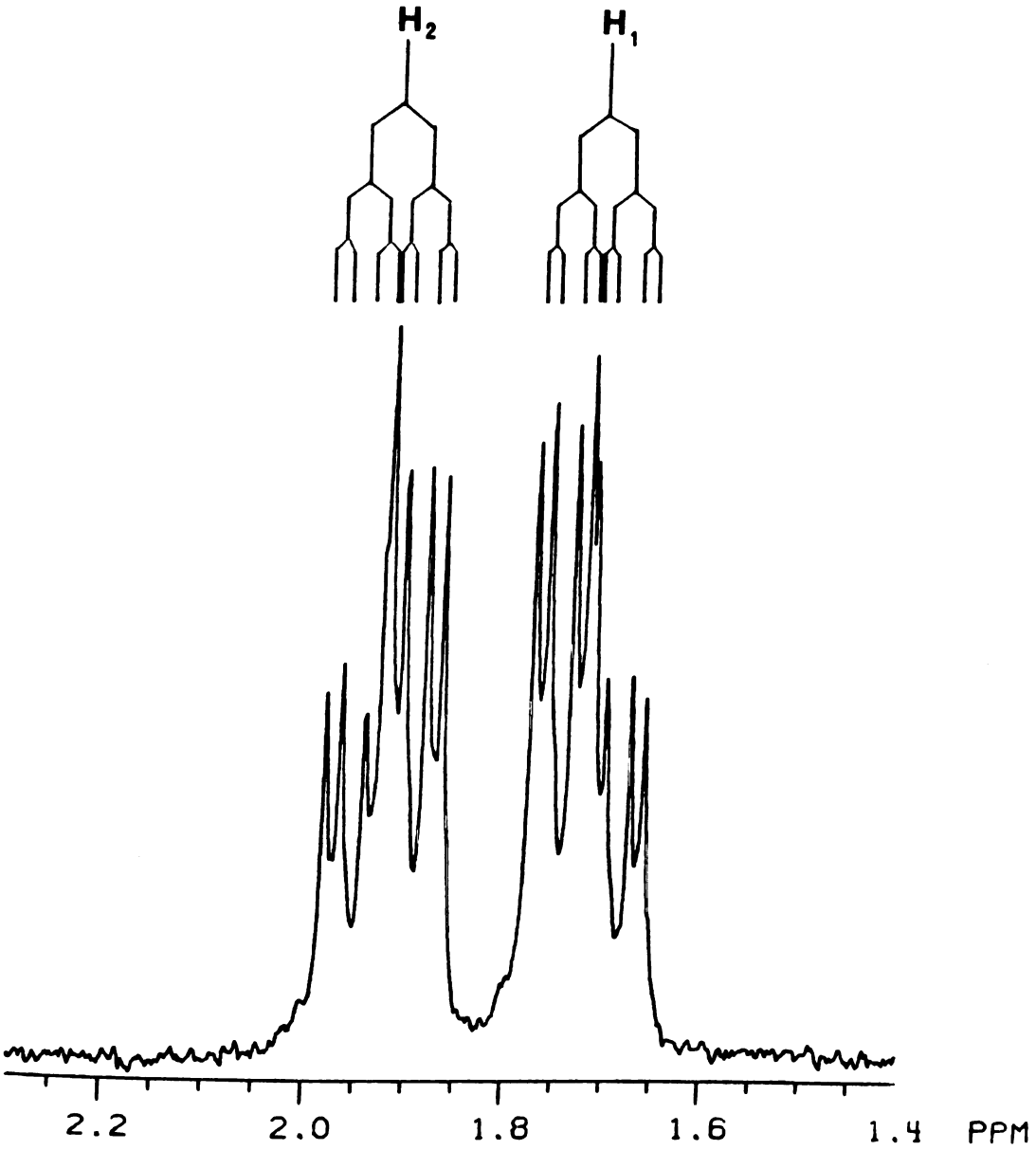


FIGURE IV-25:

^1H NMR OF METHYLENE PROTONS (H_1 AND H_2) OF "B" ISOMER

Figure IV-26:

Proton fully-coupled ^{13}C NMR of $S_{\alpha}S_{\gamma}$-isoepoxycreatine ("B" isomer). The legends are shown in page 124 and in the figure. The peaks are shown below:

PP

LINE#	HEIGHT	FREQ(HZ)	PPM
1	386.97	11735.16	194.094
2	615.64	11732.80	194.055
3	668.87	11730.42	194.016
4	493.61	11728.05	193.977
5	298.22	11725.87	193.941

>
PP

LINE#	HEIGHT	FREQ(HZ)	PPM
1	260.33	10352.19	171.221
2	743.39	10345.77	171.114
3	653.81	10343.68	171.080
4	213.99	10339.92	171.018

>
PP

LINE#	HEIGHT	FREQ(HZ)	PPM
1	239.69	4290.15	70.957
2	415.27	4286.56	70.898
3	415.55	4282.92	70.837
4	435.02	4281.90	70.821
5	304.75	4279.27	70.777
6	204.36	4154.75	68.717
7	355.19	4151.64	68.666
8	458.22	4149.33	68.628
9	436.60	4146.42	68.580
10	420.77	4144.98	68.556
11	463.11	4142.40	68.513
12	453.24	4141.18	68.493
13	398.34	4138.23	68.444
14	318.72	4136.62	68.418
15	219.45	4134.32	68.380
16	161.97	4132.69	68.353

>PP

LINE#	HEIGHT	FREQ(HZ)	PPM
1	222.11	4014.17	66.392
2	648.94	4009.43	66.314
3	315.91	4004.05	66.225
4	223.66	3869.75	64.004
5	324.45	3866.97	63.958
6	323.07	3863.70	63.904
7	163.62	3860.99	63.859

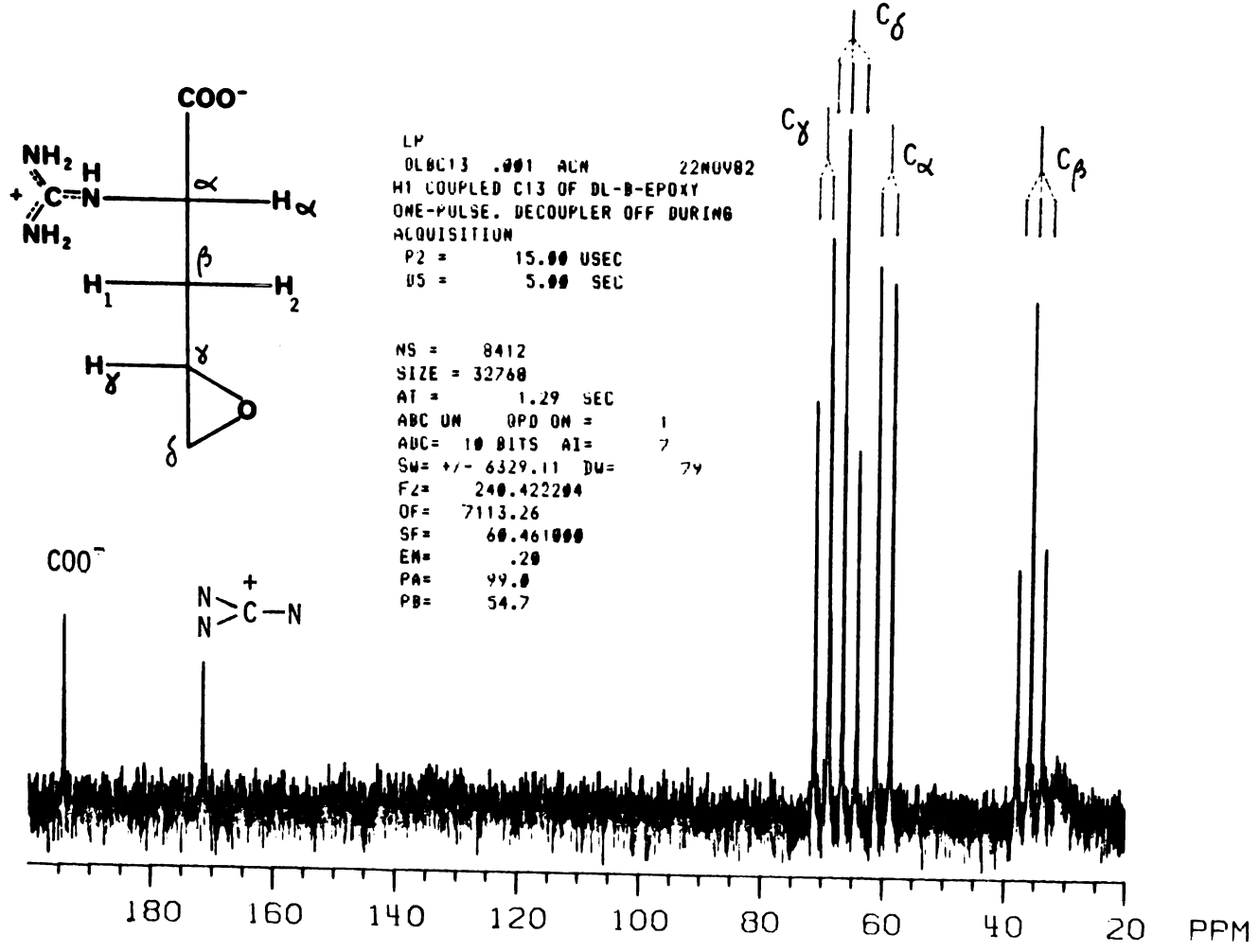
>
>PP

LINE#	HEIGHT	FREQ(HZ)	PPM
1	288.67	3678.86	60.846
2	418.93	3676.59	60.809
3	415.27	3674.06	60.767
4	407.04	3530.63	58.395
5	414.09	3528.24	58.355

>
>PP

LINE#	HEIGHT	FREQ(HZ)	PPM
1	272.70	2276.77	37.656
2	281.48	2274.32	37.616
3	292.23	2273.01	37.594
4	231.64	2270.05	37.545
5	452.40	2148.07	35.528
6	499.20	2145.58	35.487
7	361.76	2141.75	35.423
8	233.84	2019.49	33.401
9	245.59	2016.17	33.346
10	215.94	2013.37	33.300

FIGURE IV-26: ^{13}C NMR (PROTON-COUPLED) OF ISOEPOXYCREATINE



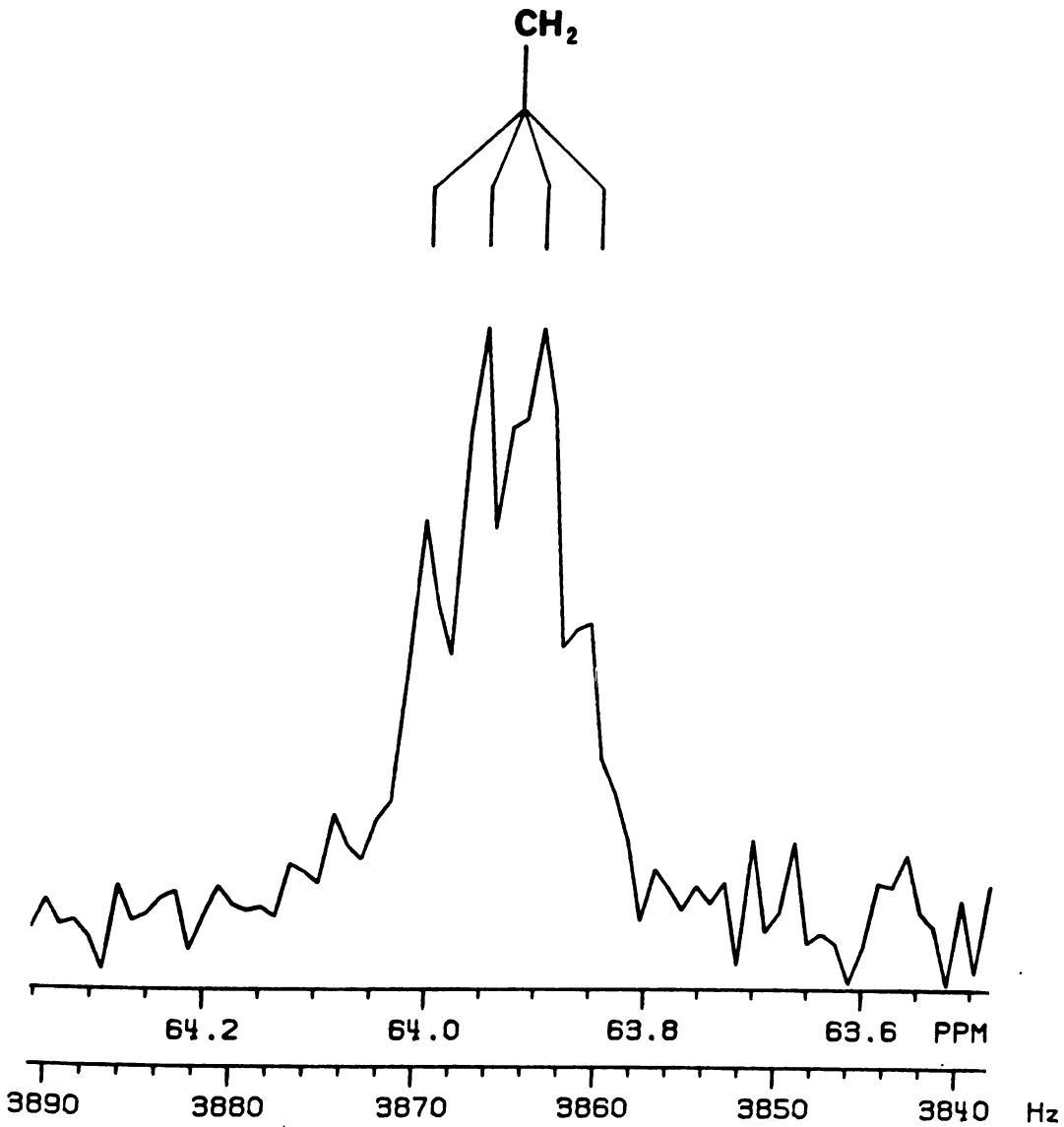


FIGURE IV-27:

^{13}C NMR OF METHYLENE CARBON (6-C) OF "B" ISOMER

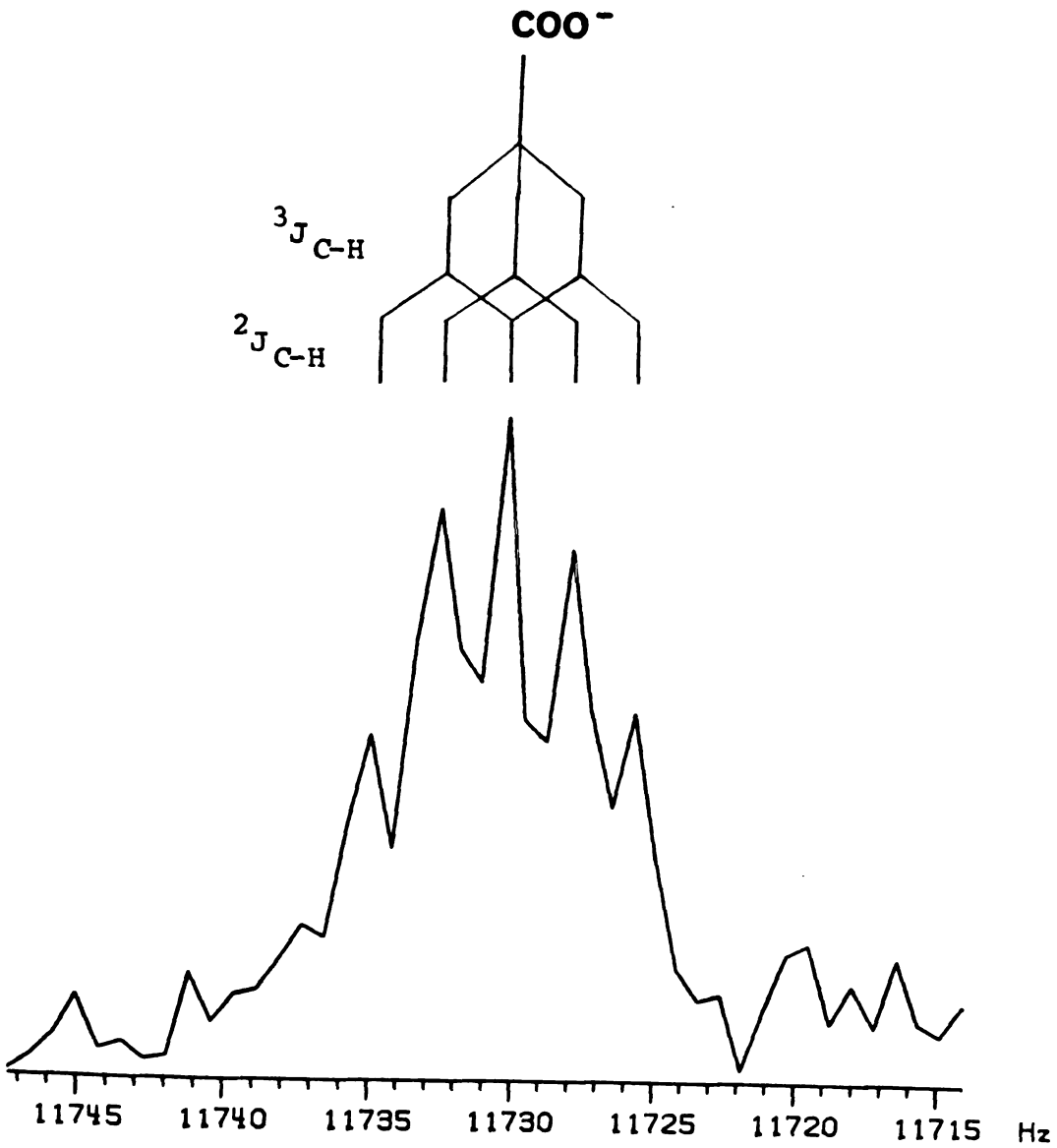


FIGURE IV-28:

^{13}C NMR OF CARBOXYLATE CARBON OF "B" ISOMER

PRELIMINARY ENZYME STUDIES

Isoepoxycreatine has been designed because of its structure resemblance both to that of creatine, the natural substrate of creatine kinase, and that of epoxycreatine, a synthetic analog which has been found to satisfy all requirements of an affinity label (Marletta and Kenyon, 1979). As expected, preliminary incubation of creatine kinase with isoepoxycreatine caused a complete inactivation of the enzyme activity. The inhibitor exhibited a time-dependent inhibition which followed pseudo-first-order kinetics. These criteria are minimal requirements for an active-site-directed reagent.

The inhibition of creatine kinase by isoepoxycreatine was performed at 0° C. A given amount of isoepoxycreatine was dissolved in 0.498 mL of 0.01 M HEPES (pH 7.5) and immersed in ice. At time 0, 12 μL of stock enzyme (1 mg/mL of 0.01 M HEPES) was added into the incubation cell and quickly withdrawn (0.05 mL) to the reaction cell to initiate the enzymatic reaction. At time t, an equal amount of incubated enzyme was withdrawn to another reaction cell to measure the remaining enzymatic activity. The control reactions were performed under the same conditions without isoepoxycreatine. The control enzyme was stable for at least several hours.

The percentage of remaining enzymatic activity was

measured as the percentage of the slope at time t over slope at time 0. The slope of enzymatic activity at time 0 was equal to the slope of the control.

Typical semilog plots of remaining enzymatic activity *vs.* time of incubation are given in Figure IV-29 (S-configuration in the position of isoepoxycreatine) and in Figure IV-30 (for R-configuration in the α-position of isoepoxycreatine).

Incubation of enzyme with different isomers of isoepoxycreatine indicates that the enzyme shows a preference for the R-configuration in the α-position of isoepoxycreatine. Dietrich *et al.* (1980) also reported the preference of creatine kinase for the R-isomers.

The straight line in Figure IV-29 presented pseudo-first-order kinetics. In Figure IV-30, at high concentration of R-C(α)-isomers, a straight line was obtained, while at the lower concentration of inhibitor, the percentage of remaining activity of enzyme presented curved lines which can be explained by the facile decomposition of isoepoxycreatine.

This facile cyclization of isoepoxycreatine can be shown by using different solvents for the incubation. The inactivations of enzyme by R-C(α)-isoepoxycreatine (3.4 *mM*) in 1 mL H_2O and in 1 mL HEPES (0.01 *M*, pH 7.5) gave different curves as shown in Figure IV-31. The inhibitor decomposed faster in H_2O than in HEPES buffer. Fee *et al.*,

1974, explained that an enzyme might have different reactivity with different isomers of an affinity lable, so the less active form can protect the enzyme from being inhibited by another isomer. Although the facile cyclization is more likely to be the reason to explain the curved lines, the above idea cannot be ruled out.

Figure IV-29:

Time-dependent inactivation of creatine kinase by R_{α}-isoepoxycreatine. The loss of percentage original enzymatic activity was plotted vs time of incubation in 0.01 M HEPES (pH 7.5) and at 0° C. Conditions were as follows: creatine kinase 1.2 μg/mL, creatine 5 mM. The isoepoxycreatine concentration are stated on the figure. Concentrations of other substrates and of auxiliary system are the same as those in Table III-1. The samples 4.6 mM, 5.8 mM, 6.9 mM, and 8.7 mM each were dissolved in 0.5 mL of 0.01 M HEPES at 0° C just belore the addition of creatine kinase. The samples of 9.8 mM and 11.5 mM each were dissolved in 0.7 mL of 0.01 M HEPES due to solubility problems. The kinetic run was at 30° C in glycine buffer (0.1 M, pH 9).

Figure IV-30:

Inactivation of creatine kinase by S_{α}-isoepoxycreatine. The legends are the same as those of the Figure IV-29 except that each sample, which are stated on the figure, was dissolved in 1 mL of 0.01 M HEPES (pH 7.5) at 0° C just before the addition of creatine kinase.

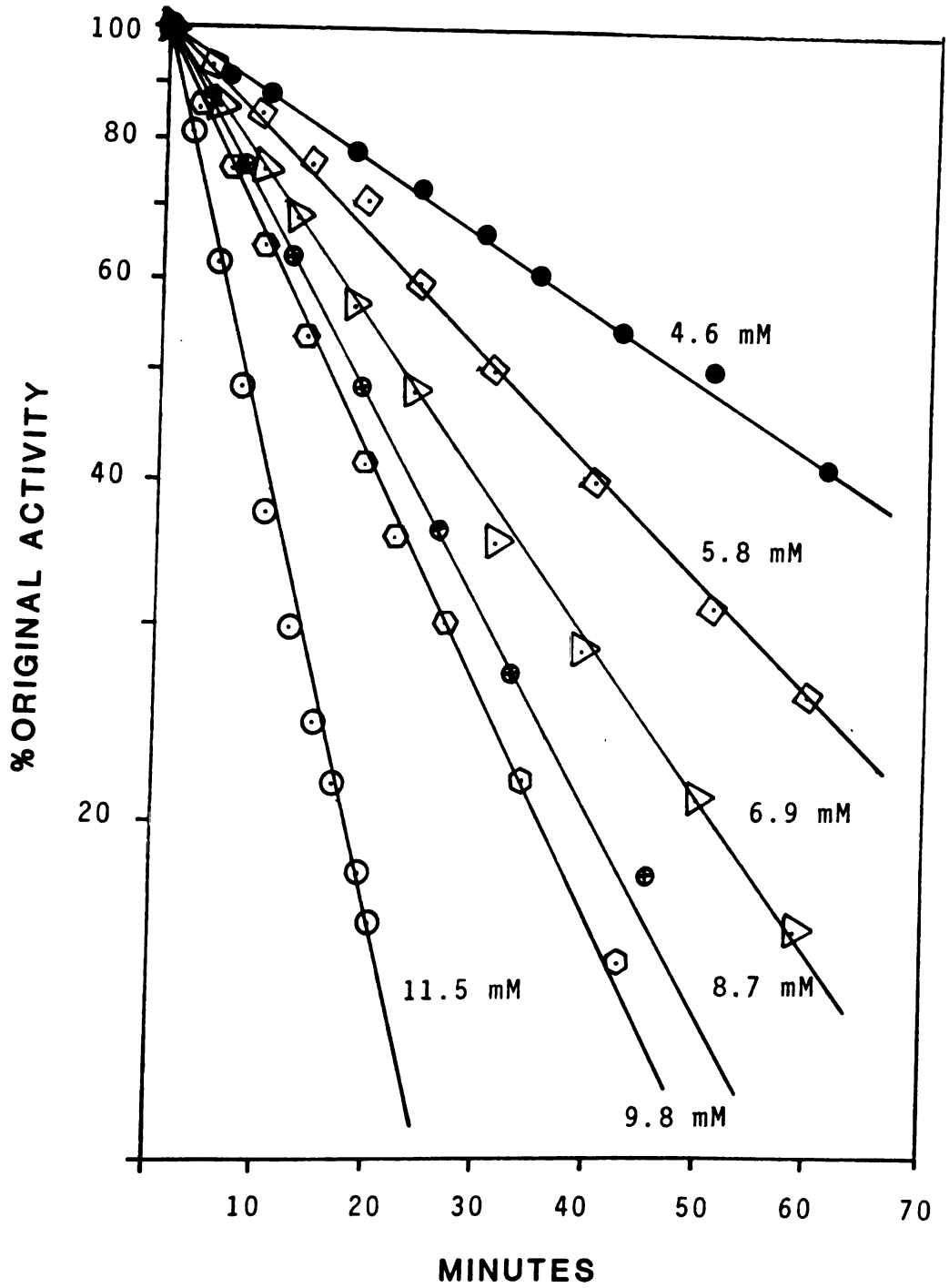


FIGURE IV-29: INACTIVATION OF CREATINE KINASE BY R-C(α)-ISOEPOXYCREATINE

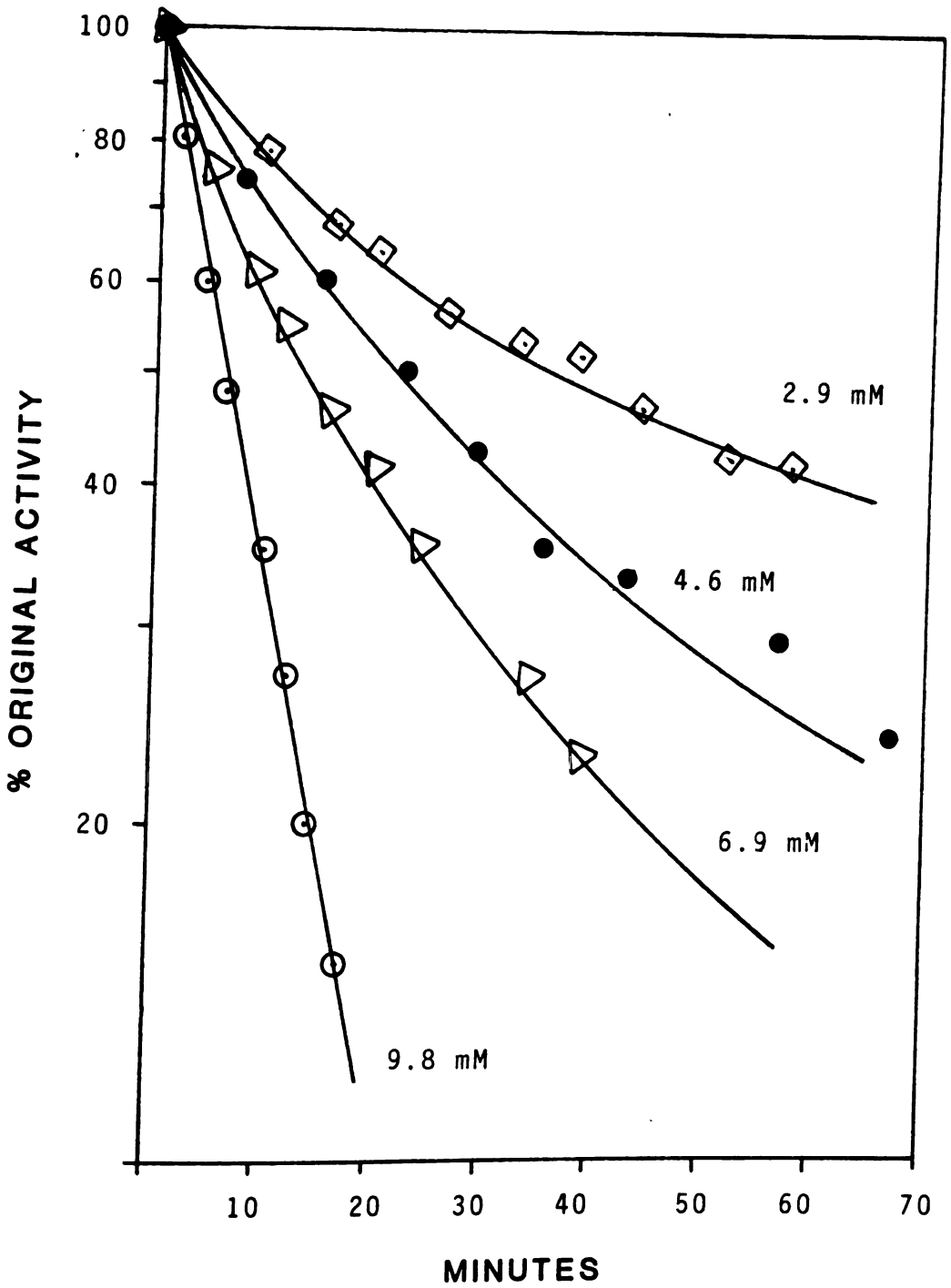


FIGURE IV-30: INACTIVATION OF CREATINE KINASE BY S-C(α)-ISOEPOXYCREATINE

Figure IV-31:

Solvent-dependence of inactivation of creatine kinase by S_{α}-isoepoxycreatine. One sample was dissolved in H_2O (1 mL) as a solvent for the incubation, whereas the other sample was in 1 mL of HEPES (pH 7.5, 0.01 M). The legends are the same as those of Figure IV-29.

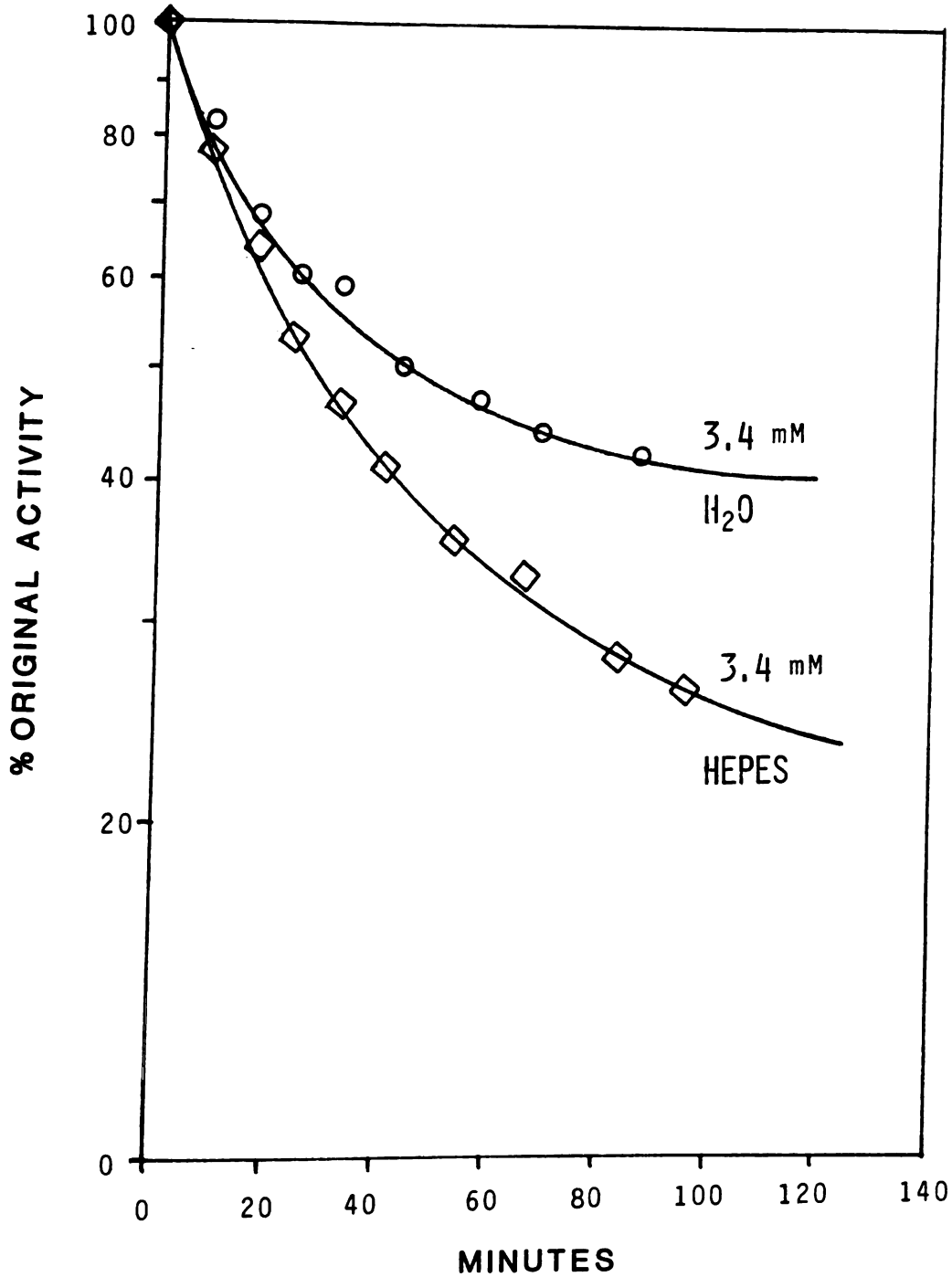


FIGURE IV-31: COMPARISON OF REMAINING ENZYMATIC ACTIVITY IN DIFFERENT SOLVENTS

CONCLUSIONS

Isoepoxyecreatine has been synthesized because of its structural resemblance to that of both creatine and epoxycreatine. Its structure has been confirmed by microanalysis, NMR, IR, GC-MS, LSIMS, and HPLC retention times.

Selective homonuclear and heteronuclear decouplings allow the determination of coupling constants and coupling patterns.

Karplus relationships of vicinal methylene protons (^{1}H NMR) and of three-bonded carbon-proton (^{13}C NMR) may be used to determine configurations of chiral centers.

Preliminary enzyme kinetic studies indicate pseudo-first-order kinetics of the inactivations of creatine kinase by isoepoxycreatine.

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