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THE SYNTHESIS AND INVESTIGATION OF CONFORMATIONALLY RESTRICTED ANALOGUES OF CREATINE

by

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B.S., University of Wisconsin Madison 1971
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DISSERTATION

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DOCTOR OF PHILOSOPHY

in

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in

GRADUATE DIVISION

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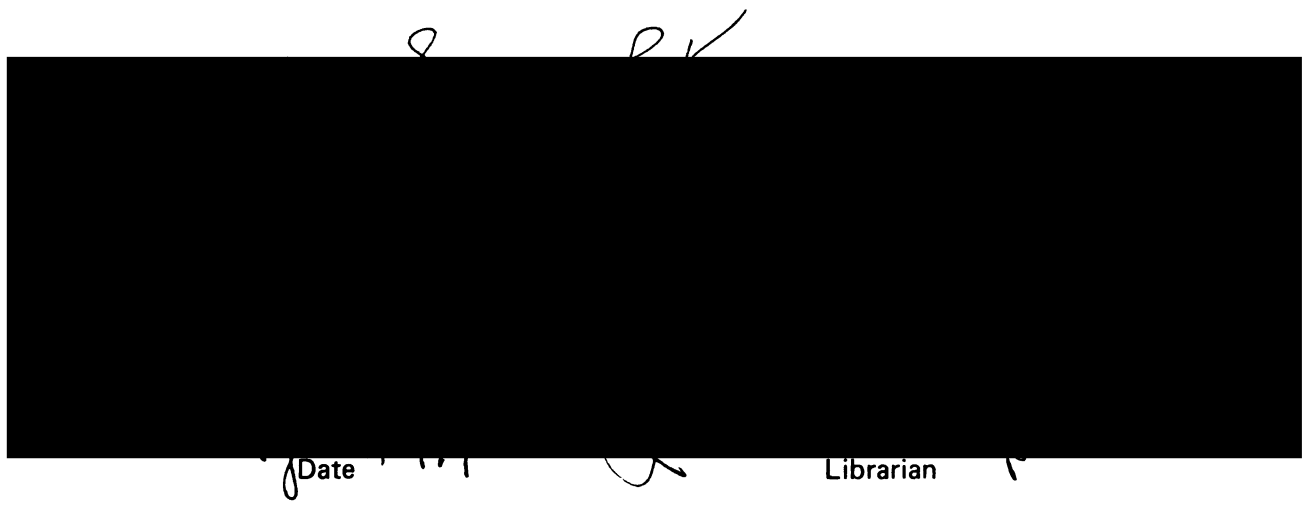
San Francisco



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TO NANCY

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ABSTRACT

Several conformationally restricted analogues of creatine have been both synthesized and examined as potential substrates or inhibitors of rabbit muscle creatine kinase (EC 2.7.3.2). When an asymmetric center was included in a creatine analogue in the position α- to the carboxyl group, the enzyme had a pronounced preference for the R-enantiomer. Thus, whereas R-N-amidinoazetidine-2-carboxylic acid has been shown to be a good substrate (K_s=72 m$\underline{M}$, K_m=39 m$\underline{M}$, V_{max}=29% relative to that of creatine) for creatine kinase, the corresponding S-enantiomer showed only barely detectable reactivity {V_{max} (rel.) << 1%}. When the corresponding ring-opened analogue, N-methyl-N-amidinoalanine, was examined as a substrate, creatine kinase again showed a strong preference for the R-enantiomer {K_s=94 m$\underline{M}$, K_m=82 m$\underline{M}$, V_{max} (rel.) ~10%}. The R-enantiomer was approximately 7 times as reactive as the S-enantiomer when they were both examined as substrates at 40 m$\underline{M}$ in the presence of 4 m$\underline{M}$ ATP. On the other hand, the monocyclic conformationally restricted creatine analogues N-{2-(4,5-dihydroimidazolyl)}sarcosine, 2-iminoimidazolidine-4-carboxylic acid, and 2-imino-3-methylimidazolidine-4-carboxylic acid, and a bicyclic creatine analogue, *trans*-2-imino-1,3-diazabicyclo{3.3.0} octane-8-carboxylic acid did not show detectable activity either as substrates or as inhibitors.

In conjunction with the characterization of these compounds, creatine (N-methyl-N-amidinoglycine), creatinine (1-methyl-2-aminoimidazolin-4-one), and a series of 38 of their close structural analogues have been examined using natural abundance carbon-13 NMR spectroscopy at 25.16 MHz. Both proton-coupled and proton noise-decoupled spectra were recorded. Unequivocal assignments of the carbon resonances could be made in the vast majority of cases. Both carbon-13 NMR chemical shifts and $^{1}J_{^{13}C-^{1}H}$ values can be used to characterize and to differentiate readily between analogues of creatine and analogues of creatinine. For example, the $^{1}J_{^{13}C-^{1}H}$ coupling constants for the α-carbons of the acyclic creatine analogues were all in the 140-142 Hz range, whereas the corresponding coupling constants for the related, cyclized creatinine analogues were all in the 150-152 Hz range.

Also, using both proton and carbon-13 nuclear magnetic resonance spectroscopy, the free energies of activation ($\Delta G_c^{\ddagger}$) values for barriers to rotation in 2-dimethylamino-2-imidazolin-4-one (an acylguanidine) and its hydrochloride salt (an acylguanidinium ion) have been determined to be 15.6 $\pm$ 0.1 kcal/mol and 17.6 $\pm$ 0.2 kcal/mol, respectively. In both cases, the rotational barrier about the exocyclic carbon-nitrogen bond was involved. Resonance arguments are used to rationalize these experimental results, and

ab initio theoretical calculations are presented that successfully reproduce the relative order of experimentally determined barriers to rotation in guanidinium ions, acylguanidines and acylguanidinium ions.

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CHAPTER 1

THE SYNTHESIS AND INVESTIGATION OF A BICYCLIC CREATINE ANALOGUE

INTRODUCTION

Creatine Kinase - Function and Properties. Creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2) catalyzes the reversible formation of adenosine 5'-triphosphate (ATP) from adenosine 5'-diphosphate (ADP) and phosphocreatine according to the reaction scheme shown below.

$$MgATP^{-2} + \underset{\text{Creatine}}{H_2N{=}\overset{\overset{CH_3}{|}}{\underset{\underset{NH_2}{\|}}{C^+}}{=}N{-}CH_2{-}CO_2^-} \rightleftharpoons MgADP^{-1} + \underset{\text{Phosphocreatine}}{{}^-O{-}\overset{\overset{O}{\|}}{\underset{\underset{-O}{|}}{P}}{-}NH{=}\overset{\overset{CH_3}{|}}{\underset{\underset{NH_2}{\|}}{C^+}}{=}N{-}CH_2{-}CO_2^-} + H^+$$

Phosphocreatine is thought to serve as an energy reserve from which the supply of ATP may be rapidly replenished. Creatine kinase is found at highest concentrations in the contractile and nervous tissues of vertebrates, representing as much as 10-20% (w/v) of the soluble sarcoplasmic proteins of muscle (Watts, 1973). It has been shown to exist in three electrophoretically different forms, a muscle type dimer (MM), a brain type dimer (BB), and a hybrid of the muscle and brain types (MB)(Watts, 1973). While there has been a good deal of interest in clinical assays of creatine kinase as an indicator of various human disease states, including muscular dystrophy (Roy, 1974) and myocardial

infarction (Sobel *et al.*, 1977), nearly all of the mechanistic investigations have been carried out on enzyme isolated from rabbit skeletal muscle. This is also the source of the enzyme used in this thesis work.

The rabbit skeletal muscle enzyme has been shown to be a dimer of apparently identical subunits, with an overall molecular weight of approximately 81,000 daltons (Noda *et al.*, 1954). Each subunit is a single polypeptide chain containing no disulfide bridges. Of the approximately 360 amino acids per subunit, only several short lengths representing about 15% of the total chain have so far been sequenced (Bickerstaff and Price, 1978).

By examination of the initial velocity pattern in the absence of products and the product inhibition patterns (Morrison and James, 1965), creatine kinase was shown to be an example of an enzyme that utilizes a rapid equilibrium, random, bimolecular, bimolecular kinetic mechanism according to the following scheme:

$$\text{E + M-ATP} \overset{K_s(\text{M-ATP})}{\rightleftharpoons} \text{E-M-ATP} \qquad \text{E-M-ADP} \overset{K_s(\text{M-ADP})}{\rightleftharpoons} \text{E + ADP}$$

$$\text{E-M-ATP} \overset{K_m(\text{Cr})}{\rightleftharpoons} \text{E-M-ATP-Cr} \qquad \text{E-M-ADP-PCr} \overset{K_m(\text{PCr})}{\rightleftharpoons} \text{E-M-ADP}$$

$$\text{E-M-ATP-Cr} \overset{K_{cat}}{\rightleftharpoons} \text{E-M-ADP-PCr}$$

$$\text{E + Cr} \overset{K_s(\text{Cr})}{\rightleftharpoons} \text{E-Cr} \overset{K_m(\text{ATP})}{\rightleftharpoons} \text{E-M-ATP-Cr}$$

$$\text{E-M-ADP-PCr} \overset{K_m(\text{M-ADP})}{\rightleftharpoons} \text{E-PCr} \overset{K_s(\text{PCr})}{\rightleftharpoons} \text{E + PCr}$$

where E = creatine kinase, M-ADP and M-ATP are the corresponding Mg(II) metal-nucleotide complexes, and Cr and PCr are creatine and phosphocreatine, respectively.

By means of a wide variety of approaches, a number of particular amino acid side chains have been indicated as being at or near the active site of the enzyme. The first of these side chain groups to be implicated in the active site region of creatine kinase was the thiol group of a cysteine residue. The reaction of one reactive sulfhydryl group per subunit of enzyme with classical thiol blocking reagents (e.g., iodoacetamide, iodoacetate, 1-fluoro-2,4-dinitrobenzene, 5,5'-dithiobis (2-nitrobenzoic acid) results in essentially complete inactivation of the enzyme (Watts, 1973). The formation of an CH_3S-S-enzyme derivative of creatine kinase from the reaction of methyl methanethiolsulfonate with the reactive sulfhydryl groups of the enzyme, on the other hand, leads to an enzyme with considerable residual activity (~20%)(Smith *et al.*, 1975). Also, the formation of an S-CN derivative of creatine kinase yields a derivative which retains up to 70% of the activity of the native enzyme (der Torrosian and Kassab, 1976). Thus, whereas these results indicate that a cysteinyl residue is in the vicinity of the active site of creatine kinase, they also clearly show that the sulfhydryl group is not essential for catalytic activity of the enzyme.

A specific residue for which suggestive evidence exists indicating a mechanistic role in the reaction catalyzed by creatine kinase is that of a lysine residue. Specific dansylation of a single lysine residue per active site was shown to result in complete inactivation of the enzyme (Kassab *et al.*, 1968). By utilizing a proton NMR double resonance technique and taking advantage of the existence of what is thought to be a transition-state analogue of the active creatine kinase complex (Reed and McLaughlin, 1974), James and Cohn (1974) were able to give further evidence for the existence of a lysine side chain in the region of the active site near the transferable phosphoryl group.

Nuclear magnetic resonance techniques have also been used to implicate an arginine residue near the nucleotide binding site of creatine kinase (James, 1976). These results are in agreement with the complete loss of enzymatic activity and nucleotide binding capability when one arginine group per subunit is modified with biacetyl (Borders and Riordan, 1975).

Two other groups, a histidine residue (Pradel and Kassab, 1968) and a tyrosine residue (Fattoum *et al.*, 1975) have been implicated as being at or near the active site by chemical modification studies, but less substantiating evidence concerning the role of these residues is available.

Conformationally Restricted Substrate Analogues. In conjunction with the wide variety of techniques available for the investigation of the interactions of enzymes and their particular substrates, the synthesis of substrate analogues has been shown to be very useful. Enzyme-substrate investigations using detailed kinetic studies, X-ray diffraction techniques, nuclear magnetic resonance and electron spin resonance methods all can benefit from the use of synthetic substrate analogues. Valuable information about the enzyme can be gained by careful, systematic modification of the structure of the normal substrate. These modifications, obviously, lead to alterations in the enzyme-substrate interactions. The subtle changes in substrate structure can result in compounds which a)inhibit the particular enzyme, b)still function as substrates for the enzyme, or c)fail to interact in any way with the enzyme; results corresponding to each one of these three possibilities can provide some insight into the interaction of the enzyme and its normal substrate.

The investigation of substrate analogues is also useful in the design of active-site-directed affinity labeling agents. An affinity label, by definition, must have not only the basic structural features necessary for selective binding to the enzyme, but also a highly reactive group that will covalently link it to the enzyme's active site.

The synthesis and investigation of a series of substrate analogues can assist in the design of an affinity label which is optimally "directed" toward the enzyme's active site.

In many compounds that function as substrates for enzymatic reactions, there are a number of energetically reasonable conformations. When a substrate is bound to the active site of an enzyme, it seems very likely that only one of these conformations is assumed. Therefore, in order to obtain a detailed mechanistic picture of the enzymatic reaction, the conformations of enzyme-bound substrates must be known (Kenyon and Fee, 1973).

Through the use of a particular kind of substrate analogue, one in which the molecule is already locked into a single conformation (or at least a limited number of conformations), one can begin to gain information about the enzyme-bound conformation.

These conformationally restricted analogues are formed by linking the various parts of the normal substrate molecule together into rings that limit molecular flexibility; but the structures must still retain features essential to binding to the enzyme (Kenyon and Fee, 1973).

In a classical study, Gass and Meister (1970) utilized conformationally restricted analogues of glutamic acid in their investigations of the enzyme glutamine synthetase. Glutamine synthetase catalyzes the formation of L-glutamine

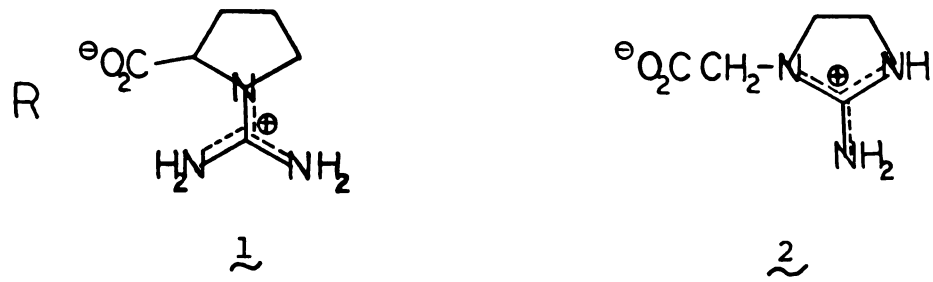
from L-glutamic acid, ammonia, and ATP. While *trans*-D,L-1-amino-1,3-cyclohexanedicarboxylic acid was unreactive in the reaction catalyzed by glutamine synthetase, the corresponding *cis*-isomer acted as a substrate with K_m and V_{max} values similar to those of L-glutamate. In both cases, the α- and γ- carbons are linked together in six-membered rings, but the *cis*- and *trans*- substitution differentiates between possible enzyme-bound conformations.

Benkovic *et al*. (1971) have used conformationally restricted analogues of fructose-1,6-biphosphate in an attempt to differentiate between the possibilities of the sugar binding to the enzyme fructose-1,6-biphosphatase in either a furanose configuration or the acyclic keto form. A series of substrate analogues that retained the 1,6-diphosphate groups, but that were locked into the furanose form, were shown to be effective competitive inhibitors with respect to fructose-1,6-biphosphate while several acyclic analogues had no effect. These results suggest, but do not prove, that the furanose form of the sugar biphosphate is the form bound to the enzyme.

The ultimate example of an effective conformationally restricted substrate analogue is not one synthesized by man, but instead is the natural product of a microorganism. The antibiotic penicillins apparently function by inhibiting bacterial cell-wall biosynthesis, acting as a conformationally restricted substrate analogue for the enzyme peptidoglycan

transpeptidase (Tipper and Strominger, 1965). The C-terminus of the normal substrate of this enzyme ends in a D-alanyl-D-alanine dipeptide residue. A comparison of the structural features of the penicillins and D-alanyl-D-alanine shows a great deal of similarity, especially when the peptide backbones are compared. The penicillins, however, contain reactive β-lactam groups, which presumably irreversibly acylate the enzyme.

In their investigations of creatine kinase, Rowley et al. (1971) and McLaughlin et al. (1972) synthesized a series of substrate analogues of creatine and examined the interactions of these compounds with the enzyme. Included in this series of analogues were two conformationally restricted analogues that showed substrate activity with creatine kinase, namely compounds 1 and 2.



Compound 1 was shown to have a V_{max} approximately 0.9% that of creatine, while compound 2 had a V_{max} 90% that of creatine (McLaughlin et al., 1972). It was proposed that by linking the main structural features of compounds 1 and 2 into a bicyclic compound, 2-imino-1,3-diazabicyclo{3.3.0}-octane-8-carboxylic acid (compound 3), one of the four

FIGURE 1

(5S),(8S)

(5R),(8R)

(5S),(8R)

(5R),(8S)

STEREOISOMERS OF 2-IMINO-1,3-DIAZABICYCLO{3.3.0}OCTANE-8-CARBOXYLIC ACID

stereoisomers shown in Figure 1 might serve as a template for the conformation of enzyme-bound creatine.
The synthesis of these bicyclic creatine analogues was thus one of the principal goals of this research. This work is described here in Chapter 1. Along the way, however, a number of monocyclic creatine analogues were synthesized, characterized and examined as substrates or inhibitors of creatine kinase. This latter work is described in the subsequent chapters.

RESULTS AND DISCUSSION

Synthesis. The synthetic route utilized in the synthesis of bicyclic creatine analogue 3 is outlined in Scheme I. Since a great deal of time was spent in the pursuit of a number of alternative approaches, a brief description of several of these will be presented.

The first synthetic approach considered involved the conversion of compound 8 (Cignarella *et al.*, 1961) to the corresponding thioamide which could then be converted to compound 12 in two steps as shown in Scheme II. Good precedents for both the formation of the thioamide (14) (Walter and Bode, 1966) and its subsequent Raney nickel reduction to compound 15 (Pettit and van Tamelen, 1962) had been reported in the literature. The troublesome step in this sequence, however, proved to be the thionation of the amide. A variety of solvents was used

SCHEME I

$HO_2C-(CH_2)_3-CO_2H$ (4) $\xrightarrow{SOCl_2}$ $\xrightarrow{Br_2}$ $\xrightarrow{EtOH}$ $EtO_2C-CHBr-(CH_2)_2-CHBr-CO_2Et$ (5)

5 → 3 $PhCH_2NH_2$ → 6 (N-benzyl pyrrolidine-2,5-diester, EtO_2C, CO_2Et, N–CH_2Ph)

6 → H_2/Pd → 7 (EtO_2C, CO_2Et, NH pyrrolidine)

7 → $PhCH_2NH_2$ → 8 (EtO_2C, $CONHCH_2Ph$, NH pyrrolidine)

8 → LAH → 9 (HO, NH, N(H)–Ph)

9 → 2 PhCOCl → 10 (HO, N–COPh, N(COPh)–CH_2Ph)

10 → $KMnO_4$ → 11 (HO_2C, N–COPh, N(COPh)–CH_2Ph)

11 → H_3O^+ →

SCHEME I (CONT.)

HO_2C–pyrrolidinium–$\overset{\oplus}{N}H_2$–CH_2–Ph (12) $\xrightarrow[\text{Isomers}]{\text{Separate}}$ $\xrightarrow{H_2/Pd}$ HO_2C–pyrrolidinium–CH_2–$\overset{\oplus}{N}H_3$ (13)

13 $\xrightarrow[\text{CNBr}]{\text{3NaOH}}$ 3 ($^{\ominus}O_2C$, $\oplus$, NH, H_2N)

SCHEME II

8 $\xrightarrow{P_2S_5}$ EtO_2C–pyrrolidine(NH)–C(=S)–NHCH_2Ph (14)

14 $\xrightarrow{\text{Raney Ni}}$ EtO_2C–pyrrolidine(NH)–CH_2–NH–CH_2–Ph (15)

15 $\xrightarrow{H_3O^{\oplus}}$ 12

(benzene, toluene, xylene, pyridine, and dioxane), none of which led to an isolable product. Chemical ionization mass spectral evidence indicated that the desired thioamide was being formed, but the product could not be separated from the unknown side products.

Another approach was proposed with the intention of taking advantage of the differential reactivity of diborane between amides and esters. The approach is shown in Scheme III:

SCHEME III

6 → EtO_2C–[N-CH_2Ph pyrrolidine]–$CONHCH_2Ph$ (16) → $^{-}O_2C$–[HN^{+}-CH_2Ph pyrrolidine]–$CONHCH_2Ph$ (17)

↓

13 $\xleftarrow{H_2}$ $\xleftarrow{H_3O^+}$ $\xleftarrow{B_2H_6}$ Cl_5PhO_2C–[N-CH_2Ph pyrrolidine]–$CONHCH_2Ph$ (18)

Russ and Caress (1976) reported an 85% yield for the reduction of a secondary amide in the presence of a pentachlorophenyl ester, which appeared to be the most questionable step in the sequence. The problematic step, however, proved to be the formation of the pentachlorophenyl ester, compound 18. The esterification was

attempted using N,N'-dicyclohexylcarbodiimide and also using trifluoroacetic anhydride, which had been shown to be particularly useful in the preparation of sterically hindered esters (Parish and Stock, 1965). In neither case was there any evidence of formation of desired product. Possibly steric repulsions prevent ester formation.

Scheme IV outlines another approach that showed promise.

SCHEME IV

8 —PhCOCl→ 19 (EtO_2C–pyrrolidine(N–COPh)–$CONHCH_2Ph$)

19 —1) $Et_3O^+BF_4^-$ 2) $NaBH_4$→ 20 (EtO_2C–pyrrolidine(N–CH_2Ph)–CH_2NHCH_2Ph)

20 —1) H_2 2) 6 N HCl→ 13

The attempted conversion of 19 to 20 using triethyloxonium fluoroborate was carried out under conditions identical to those described for the reduction of a series of amides by Borch (1968). The proton NMR spectrum of the crude product showed that the amide groups in compound 19 had not been fully reduced. It appeared from the NMR spectrum that the benzoyl group had not been reduced, while the other amide group had been reduced. This differential reactivity might be due to steric hindrance in the

ethylation of the N-benzoyl oxygen by triethyloxonium fluoroborate. While this particular product could have been utilized (acid hydrolysis would lead to compound **12**), this approach was abandoned in favor of the one outlined in Scheme I.

In the execution of the synthetic sequence described in Scheme I, there exists the possibility a *cis*-product and a *trans*-product for compounds **6** through **13**. While, in theory, it would have been possible to separate the *cis*- and *trans*- isomers of each product, this was not considered practical or necessary. A sample of compound **10**, however, was separated into its *cis*- and *trans*- diastereoisomers by column chromatography on silica gel using 50/50 ethyl-acetate hexane as eluent. The conversion of compound **10** to compound **11**, however, was carried out on the mixture of *cis*- and *trans*- isomers.

It was also discovered that the *cis*- and *trans*- isomers of compound **12** could be easily separated. The *cis*- isomer of the dihydrochloride salt of **12** slowly cyclized to the corresponding lactam in aqueous solution, a process that can be monitored by proton NMR spectroscopy. The lactam could then be separated from the *trans*- diamino acid dihydrochloride by chromatography on anion exchange resin ($^{-}$OH form). When eluted with water, the lactam was washed from the column, while the diamino acid remained on the

column. The diamino acid could be removed from the column as the dihydrochloride salt by elution with 1.0 N HCl. The lactam could then be converted back to the cis-diamino acid by heating at reflux in 6 N HCl for several hours.

The cis- and trans- diamino acids were then separately converted to cis- and trans- **13**, and each was treated with cyanogen bromide in an attempt to prepare the two diastereomeric forms of compound **3**. The trans-diamino acid gave a product, which when isolated by preparative thin-layer chromatography, gave NMR spectra (proton and carbon-13) and an elemental analysis consistent with compound **3**; it evidently is the trans- isomer of **3**. The cis-diamino acid, when treated in the same manner, gave a guanidinium containing compound in low yield (<10%) which was isolated by thin-layer chromatography. The carbon-13 NMR spectrum and elemental analysis indicate that this is not the desired product, but instead a compound containing one less carbon atom. The proton-coupled carbon-13 spectrum showed what appeared to be a carbonyl carbon, a guanidinium carbon, three methylene carbons, and a methine carbon. Based on the data available, a structure could not be unambiguously assigned. No compound consistent with cis-compound **13** was isolated. Since these results showed no worthwhile benefit in the effort involved in the separation

of *cis*- and *trans*- compound **12**, subsequent syntheses of *trans*-3 were carried out on the *cis*- *trans*- mixtures.

Enzymatic Studies. When *trans*- compound **3** was investigated for substrate activity in the creatine kinase reaction, using the polyethylenimine-cellulose thin-layer chromatographic assay developed by Rowley and Kenyon (1974), no evidence of product formation (phosphocreatine analogue) could be detected. Since this assay has been shown to be sensitive enough to detect product formation in analogues that react 10,000 times more slowly than creatine, *trans*-**3** is at best an extremely poor substrate for creatine kinase, and more than likely, not a substrate at all.

In order to examine the possibility that *trans*-**3** might bind to the creatine binding site of creatine kinase, but not act as a substrate for the enzyme, *trans*-**3** was also examined as a competitive inhibitor for creatine binding. The initial rate of the enzyme catalyzed reaction of 15 m*M* creatine and 4 m*M* ATP was shown to be identical in the presence and absence of *trans*-3.

These results indicate that *trans*-3 not only does not act as a substrate for creatine kinase, but also does not bind to the enzyme to any appreciable extent. The bicyclic structure of *trans*-**3** apparently either forces the groups necessary for substrate binding into a conformation that does not allow for sufficient binding interactions, or

possibly one of the five-membered rings is twisted into position where steric bulk is not tolerated by the enzyme. Yet another possibility, which in fact is a more intriguing one, is that *trans*-3 is too rigid, and that some conformational flexibility is needed in the creatine analogue in the course of the enzyme-catalyzed phosphorylation reaction.

One way to begin to distinguish among these possibilities for the lack of reactivity of *trans*-3 is to prepare the corresponding bicyclic analogue that contains a four-membered ring in place of one of the five-membered rings; i.e.,

This bicyclic analogue is even more rigid than *trans*-3, but contains less bulk.

A synthesis of 21, based on the successful synthesis of *trans*-3, was initiated but not completed. A progress report on the synthesis of 21 is described in Appendix C.

EXPERIMENTAL

General. Proton NMR spectra were taken either at 60 MHz on a Varian A60A spectrometer or on a Varian XL-100 spectrometer operating in the pulse mode at 100.1 MHz as indicated. The carbon-13 spectra were also obtained on a Varian XL-100 spectrometer utilizing a Nicolet Multi Observe Nuclei Accessory (MONA) operating at 25.158 MHz. Chemical ionization mass spectra were obtained on an Associated Electronic Industries MS-902 spectrometer and electron impact mass spectra on an Associated Electronic Industries MS-12 spectrometer. Melting points were uncorrected, and microanalyses were obtained from the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, California.

Inhibition studies were carried out on a Radiometer TT2 pH-Stat, and the initial rate in the creatine kinase catalyzed reaction between 4 m*M* ATP and 15 m*M* creatine compared in the presence and absence of 30 m*M* *trans*- 3.

2-(N-Benzylaminomethyl)-5-hydroxymethyl)pyrrolidine (**9**). 2-Benzylcarbamyl-5-carbethoxypyrrolidine (**8**) (Cignarella *et al*., 1961) (26.0 g, 94.2 mmol), as a mixture of the diastereoisomers, was dissolved in 300 mL of anhydrous ether and added dropwise to an ice bath-cooled, stirred suspension of $LiAlH_4$ (20 g, 526 mmol)

in 300 mL of ether, under an N_2 atmosphere. After the addition was complete, the reaction mixture was heated at reflux for 32 hr. The reaction mixture was then cooled to 0°, and the excess hydride destroyed by careful sequential addition of 20 mL of H_2O, 20 mL of 15% NaOH, and 60 mL of H_2O. Stirring was continued for an additional hour, and the insoluble salts removed by filtration followed by thorough washing with ether. The filtrate was then dried over anhydrous potassium carbonate and concentrated at reduced pressure to a light yellow oil. The oil was distilled at high vacuum (140-145°/0.015 mm) to give 14.6 g (70% yield) of the desired diamino alcohol (9) as a diastereomeric mixture.

Anal. Calcd. for $C_{13}H_{20}N_2O$: C, 70.87; H, 9.15; N, 12.71. Found: C, 70.75; H, 9.08; N, 12.75.

The 100 MHz proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.0 - 2.0 (4H, complex multiplet), 2.4 - 2.8 (2H, multiplet), 3.04 (3H, singlet, D_2O exchangeable), 3.1 - 3.7 (4H, complex multiplet), 3.80 (2H, singlet), and 7.36 (5H, singlet).

N,N'-Dibenzoyl-2-(N-benzylaminomethyl)-5-hydroxymethylpyrrolidine (10). 2-(N-Benzylaminomethyl)-5-hydroxymethylpyrrolidine (9) (11.8 g, 53.6 mmol), as a mixture of its diastereomers, was dissolved in 200 mL of benzene with 26 g of anhydrous sodium carbonate and

the stirred suspension cooled to 8° in an ice bath. Benzoyl chloride (14.8 g, 12.2 mL, 105 mmol) in 50 mL of benzene was added dropwise to the stirred mixture, at such a rate that the temperature did not rise above 10°. After addition was complete, the reaction mixture was stirred and heated at reflux for an additional 2 hr. After cooling to room temperature, the insoluble salts were removed by filtration, and the filtrate was extracted first with 100 mL of 2% sodium hydroxide, followed by extraction with 100 mL of 5% HCl. The organic fraction was then dried over anhydrous potassium carbonate and concentrated at reduced pressure to a solid white foam. Thin layer chromatography on silica gel plates showed two spots, $R_f \sim 0$ and $R_f=0.1$, when developed with ethyl acetate-hexane (50:50 v/v) and visualized with iodine vapor. A 1.1 g sample of the crude product was applied to a 52 cm x 1.8 cm silica gel column (BIO-RAD, Bio-Sil A, 100 - 200 mesh) and eluted with 50:50 ethyl acetate-hexane. The fractions (50 mL) were monitored by thin-layer chromatography until the compound at $R_f=0.1$ had been completely eluted. The column was then washed with 300 mL of ethyl acetate to remove the compound of lower R_f value. While the proton NMR spectra of the two compounds were only similar, their electron impact mass spectra were nearly identical. This indicated a clean

separation of the two diastereomers. The crude mixture of the two diastereomers was converted from the solid foam to a white powdery solid by trituration in a small amount of hot ether followed by dropwise addition of absolute ethanol. The total yield of the two isomers of compound 10 was 18 g (78% yield), mp 120-130° C.

Anal. Calcd. for $C_{27}H_{28}N_2O_3$: C, 75.68; H, 6.59; N, 6.54. Found: C, 75.36; H, 6.53; N, 6.39.

The 100 MHz proton NMR spectrum ($CDCl_3$) showed a series of broad multiplets between δ 1.0 - 5.0 (13H) and 6.5 - 7.7 (15H).

The electron impact mass spectrum showed the molecular ion at m/e 428 (1.0% of base) and the following peaks greater than 5% of the base peak: m/e 323, 217, 204, 106, 105 (base), 91, 82, and 77.

N,N'-Dibenzoyl-2-(N-benzylaminomethyl)pyrrolidine-5-carboxylic Acid (11). An aqueous solution of potassium permanganate (5.3 g, 34 mmol in 100 mL of H_2O) was added dropwise to a heated (40-50°), stirred solution of N,N'-dibenzoyl-2-(N-benzylaminomethyl)-5-hydroxymethylpyrrolidine (10) (8.0 g, 19 mmol) in 75 mL of glacial acetic acid. After addition was complete (~2 hr), heating and stirring were continued for an additional 4 hr. At this point an additional 1.5 g of $KMnO_4$ in 50 mL of water was added and heating and stirring continued for 2 additional

hr. After treating the reaction mixture with about 10 mL of a saturated aqueous solution of sodium bisulfite to destroy the excess permanganate and convert the insoluble manganese dioxide to a water soluble salt, the crude product was concentrated to a slightly brown viscous oil. The residue was then taken up in $CHCl_3$ and extracted with 1% aqueous sodium bisulfite. The colorless chloroform solution was concentrated to a white solid foam under vacuum and thoroughly triturated with 300 mL of 0.5 N NaOH. The insoluble material was removed by filtration and the filtrate was then carefully acidified to pH ~1 with 2.4 N HCl. The crude precipitate, 6 g, was thoroughly dried and recrystallized from ethyl acetate-hexane to give 5.2 g (63% yield) of the desired product, mp 148-153°, as a mixture of the diastereoisomers.

Anal. Calcd. for $C_{27}H_{26}N_2O_4$: C, 73.28; H, 5.92; N, 6.33. Found: C, 72.91; H, 6.10; N, 6.42.

The 100 MHz proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.4 - 2.6 (4H, complex multiplet), 2.8 - 3.5 (2H, multiplet), 4.0 - 5.2 (4H, complex multiplet), 7.0 - 8.0 (15H, aromatic multiplets), 8.6 (1H, broad singlet).

The electron impact mass spectrum showed the molecular ion at m/e 442 (1.6%) and the following peaks greater than 5% of the base peak: m/e 224, 218, 106, 105 (base), 91, and 77.

2-(N-Benzylaminomethyl)pyrrolidine-5-carboxylic Acid (12). N,N'-Dibenzoyl-2-(N-benzylaminomethyl)pyrrolidine-5-carboxylic acid (11)(6.6 g, 15 mmol) was heated at reflux for 18 hr in 100 mL of 6.0 N HCl. After cooling to room temperature, the reaction mixture was extracted three times with 100 mL portions of ether to remove benzoic acid. Removal of the aqueous acid *in vacuo* left 4.6 g (quantitative yield) of *cis*- and *trans*- compound 12 dihydrochloride salt as a slightly yellow glass.

The chemical ionization mass spectrum (isobutane) gave an M + 1 peak, accurately mass measured at m/e 235.142 (calculated for $C_{13}H_{19}N_2O_2$; 235.144). Other significant peaks in spectrum were m/e 217, 145, 115, and 91.

The 100 MHz proton NMR spectrum (D_2O) showed peaks at δ 1.9 - 2.9 (4H, multiplet), 3.0 - 4.25 (4H, multiplet), 4.38 (2H, singlet), and 7.56 (5H, singlet).

A small amount of the *cis*- *trans*- mixture was separated as described in the "Result and Discussion" Section.

2-Aminomethylpyrrolidine-5-carboxylic Acid (13). 2-(N-Benzylaminomethyl)pyrrolidine-5-carboxylic acid dihydrochloride (12)(2.79 g, 9.05 mmol) was dissolved in 200 mL of 90% aqueous ethanol and hydrogenated at 50 psi in the presence of 1.5 g of 10% palladium on charcoal on a Paar hydrogenation apparatus. After 18 hr, the catalyst was

removed by filtration and the solvent was evaporated *in vacuo*. The crude product was shown by proton NMR spectroscopy to be a mixture of *cis*- and *trans*- **13** along with ~10% of the lactam formed from the cyclization of the starting material, compound **12**. This lactam side product was removed by anion exchange chromatography (^{-}OH form); when the mixture was applied to an AG-1-X8 Bio-Rad column, the lactam was eluted from the column with water. The diamino acid was then removed from the column by elution with 1.0 *N* HCl to give an 85% yield of the *cis*- and *trans*- mixture of **13** dihydrochloride as a slightly yellow glass.

The chemical ionization mass spectrum (isobutane) gave an M + 1 peak, accurately mass measured at m/e 145.0987 (calculated for $C_6H_{12}N_2O_2$; 145.0977). Other significant peaks in the chemical ionization mass spectrum included m/e 127 and 115.

The 100 MHz proton NMR (D_2O) showed peaks at δ 1.6 - 2.8 (4H, complex multiplet) and 3.3 - 4.8 (4H, overlapping multiplets).

trans-2-Imino-1,3-diazabicyclo{3.3.0}octane-8-carboxylic Acid (**3**). 2-Aminomethylpyrrolidine-5-carboxylic acid dihydrochloride (**13**) (230 mg, 1.1 mmol) was dissolved in 3 mL of 1.1 *N* NaOH. To this stirred solution, cyanogen bromide (128 mg, 1.2 mmol) in 10 mL of methanol was added

slowly over a period of 4 hr. The reaction mixture was stirred an additional 2 hr, and the solvents were removed _in vacuo_. Analytical thin-layer chromatography on Merck silica gel plates eluted with $CHCl_3$, MeOH, and aqueous NH_3 (40:40:10, v/v/v) showed two spots, one pinkish-violet (R_f=0.12) and the other blue (R_f=0.28), when visualized with sodium nitroprusside-potassium ferricyanide solution (von Arx, E. and Neher, R., 1963). The product mixture was dissolved in approximately 1 mL of methanol, the insoluble salts removed by filtration, and 0.5 mL of the methanol solution applied to a 2000 μ thick Merck preparative silica gel plate that had been pre-run with methanol. The two bands were visualized by blocking the majority of the plate, and spraying with the sodium nitroprusside-potassium ferricyanide solution, and then were scraped from the plate and extracted with methanol. The compound at R_f=0.28, shown to be _trans_- compound **3** (See Results and Discussion), was isolated in ~50% yield by dissolving the methanol extracted compound in a small amount of water followed by careful precipitation with acetone, to give _trans_- **3** as a partial hydrate, mp = 260-265° C (dec.).

Anal. Calcd. for $C_7H_{11}N_3O_2 \cdot 0.75H_2O$: C, 46.02; H, 6.90; N, 23.00. Found: C, 46.47; H, 6.66; N, 23.02.

The 60 MHz proton NMR spectrum (D_2O) showed peaks at δ 1.3 - 2.8 (4H, complex multiplet) and 3.0 - 4.8 (4H, overlappling multiplets).

The 25.2 MHz carbon-13 NMR spectrum showed peaks at δ 178.4 (CO_2^-), 160.2 (guanidinium carbon), 64.7 (-CH-, J=145 Hz), 61.9 (-CH-, J=151 Hz), 49.3 ($-CH_2-$, J=144 Hz), 36.3 ($-CH_2-$, J=135 Hz), and 29.1 (J=135 Hz).

The carbon-13 NMR spectrum of the hydrochloride salt of trans- 3 showed peaks at δ 175.0 (CO_2H), 159.9 (guanidinium carbon), 64.4 (-CH-, J=145 Hz), 59.2 (-CH-, J= 151 Hz), 49.2 ($-CH_2-$, J=147 Hz), 36.0 ($-CH_2-$, J=135 Hz), and 28.9 ($-CH_2-$, J=135 Hz).

The unknown side product, R_f=0.12, had the following properties:

Anal. Calcd. for possible formula $C_6H_{10}N_3O_2 \cdot 0.45H_2O$: C, 43.87; H, 6.69; N, 25.58). Found: C, 44.45; H, 6.83; N, 25.11.

The 60 MHz proton NMR spectrum (D_2O) showed peaks at δ 1.4 - 2.4 (4H, multiplet) and 3.0 - 4.2 (~3H, multiplet).

The carbon-13 NMR spectrum (D_2O) of the unknown showed peaks at δ 182.8 (carbonyl), 160.3 (guanidinium carbon), 56.1 (-CH-, J=145 Hz), 48.6 ($-CH_2-$, J=145 Hz), 33.8 ($-CH_2-$, J=145 Hz), 33.8 ($-CH_2-$, J=120 Hz), and 31.7 ($-CH_2-$, J=130 Hz).

1-Benzoyl-2-benzylcarbamyl-5-carbethoxypyrrolidine (19). 2-Benzylcarbamyl-5-carbethoxypyrrolidine (8) (5 g, 18.1 mmol) was dissolved 70 mL of benzene to which 1.25 g of anhydrous sodium carbonate had been added. The stirred suspension

was cooled in an ice bath and benzoyl chloride (2.81 g, 2.32 mL, 20.0 mmol) in 50 mL benzene was added dropwise. After addition was complete (1 hr), the reaction mixture was heated at reflux for 2 hr. After cooling to room temperature the reaction mixture was extracted with 0.5 N NaOH, 1.0 N HCl, and finally water. The benzene solution was dried over magnesium sulfate, and after removal of the solvent *in vacuo* was crystallized by trituration and cooling in ether to give 6.0 g (87% yield) of **19**, mp = 91-97° C.

Anal. Calcd. for $C_{22}H_{24}N_2O_4$: C, 69.46; H, 6.36; N, 7.36. Found: C, 69.65; H, 6.37; N, 7.40.

The 100 MHz proton NMR showed peaks at δ 1.25 (3H, broad triplet, J=7 Hz), 1.6 - 2.5 (4H, multiplet), 3.9 - 4.8 (6H, overlapping multiplets), 7.35 (10H, broad singlet) and 8.9 (broad singlet).

1-Benzyl-2-benzylcarbamyl-5-carbethoxypyrrolidine (**16**). 1-Benzyl-2,5-dicarbethoxypyrrolidine (**6**) (17.7 g, 58.0 mmol) as a 50/50 mixture of its *cis*- and *trans*-isomers was dissolved in 100 mL of xylene and treated with benzylamine (6.20 g, 57.8 mmol). The solution was heated at reflux for 22 hr, and then placed in the refrigerator for 2 hr. A white crystalline solid precipitated (diamide side product) and was removed by filtration. The proton NMR spectrum of the crude reaction

mixture, after evaporation of the solvent and unreacted benzylamine, showed incomplete reaction, with one of the isomers of starting material more completely converted to product than the other. The unreacted diester starting material was removed by distillation (0.1 mm/ 130° C) leaving 7.0 g (33% yield) of (16), which was used without further purification.

The 60 MHz proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.05 (3H, triplet, J=7 Hz), 1.5 - 2.5 (4H, multiplet), 3.3 - 4.2 (4H, overlapping multiplets), 3.78 (2H, singlet), 4.35 (2H, doublet, J=6 Hz), 7.15 (5H, singlet), 7.25 (5H, singlet), and 8.35 (1H, triplet, 6 Hz).

1-Benzyl-2-benzylcarbamylpyrrolidine-5-carboxylic Acid (17). 1-Benzyl-2-benzylcarbamyl-5-carbethoxypyrrolidine (16)(5.0 g, 13.7 mmol) was suspended in 13.6 mL of 1.0 N NaOH and heated at reflux with stirring 36 hr. After cooling to room temperature, the reaction mixture was extracted several times with ether. The pH of the aqueous portion, after removal of dissolved ether *in vacuo*, was carefully adjusted to pH 7 with 1.0 N HCl and the zwitterionic product that precipitated removed by filtration. The white solid was recrystallized from acetonitrile to give 3.0 g (65% yield) of compound 17, mp 188-190° C.

Anal. Calcd. for $C_{20}H_{22}N_2O_3$: C, 70.98; H, 6.55; N, 8.28. Found: C, 70.75; H, 6.45; N, 8.12.

No proton NMR spectrum was available because of solubility problems.

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CHAPTER 2

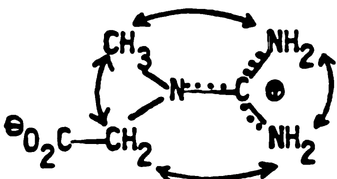
CONFORMATIONALLY RESTRICTED CREATINE ANALOGUES AND THE SUBSTRATE SPECIFICITY OF RABBIT MUSCLE CREATINE KINASE

INTRODUCTION

Creatine kinase (adenosine-5'-triphosphate-creatine phosphotransferase, E.C. 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from ATP to creatine. An important physiological function of the enzyme is the regeneration of ATP from phosphocreatine as the ATP is utilized during muscular contraction. It exists as a dimer of two apparently identical subunits with a total molecular weight of 81,000 daltons (Noda *et al.*, 1954). Kinetic analyses have indicated that the reaction follows a rapid equilibrium, random, bimolecular, bimolecular scheme with phosphoryl transfer as the rate-limiting step (Morrison and James, 1965; Morrison and Cleland, 1966).

In an attempt to provide information about the mechanism of action of creatine kinase at a molecular level, a series of analogues of creatine have been synthesized and examined as potential substrates for the creatine kinase-catalyzed reaction (Rowley *et al.*, 1971; McLaughlin *et al.*, 1972). Substrate analogues can serve not only as probes of the active site to determine where steric bulk may or may not be tolerated, but also, by use of conformationally restricted analogues, information may be gained about the preferred geometry of the substrate when bound to the active site of the enzyme. In conformationally restricted analogues, the different parts of

the normal substrate molecule may be tied together in rings, locking the molecules into particular conformations (Kenyon and Fee, 1973). In this study, we report the synthesis and the investigation of the interactions with creatine kinase of several conformationally restricted analogues of creatine. The four arrows in the structure shown below represent potential positions for the formation of rings to lock the basic creatine structure into particular conformations:



Results of kinetic studies with representatives of each of these four types of conformationally restricted analogues are presented.

EXPERIMENTAL PROCEDURES

Materials. ATP, creatine, and bovine serum albumin were all obtained from Sigma Chemical Co. Creatine kinase used in the analyses of compounds **7**, **11**, **12**, and **13** was isolated from fresh rabbit skeletal muscle as described

by Kuby et al. (1954) and had a specific activity of ~110 μmoles/min/mg protein in the forward direction; the enzyme used in the analysis of compound 2 was purchased from Calbiochem, with a corresponding specific activity of ~60 μmoles/min/mg protein.

The following synthetic procedures were used in the preparation of compounds utilized in this study. A carbon-13 NMR spectrum for each analogue, consistent with its proposed structure, is reported elsewhere (See Chapter 4).

R- and S-N-Amidinoazetidine-2-carboxylic Acid (6 and 7). R-Azetidine-2-carboxylic acid (0.5 g, 5 mmol) (Rodebaugh and Cromwell, 1969), and the corresponding S-enantiomer (0.5 g, 5 mmol) (Sigma Chemical Co.) were each added to separate solutions of cyanamide (0.25 g, 6 mmol) dissolved in 0.4 mL of water followed by the addition of 2 drops of concentrated aqueous ammonia. After standing for 48 hr at room temperature, the crystals that had formed were filtered to give 0.6 g (85% yield) of each enantiomeric product, mp 275-285° C (dec.) and $\{\alpha\}_{D}^{28.5^{o}}$ = -321° (S-enantiomer) and -318° (R-enantiomer) (C=2, water).

Anal. Calcd. for $C_5H_9N_3O_2$: C, 41.95; H, 6.34; N, 29.35. Found: (S-enantiomer) C, 41.68; H, 6.27; N, 29.14; (R-enantiomer) C, 41.96; H, 6.31; N, 29.22.

The 100 MHz proton NMR spectrum (D_2O) showed peaks at δ 2.1 - 2.9 (2H, complex multiplet), 4.04 (2H, triplet,

J = 7 Hz), and 4.81 (1H, doublet of doublets, J = 5 Hz and 6 Hz).

R- and S-N-Methyl-N-amidinoalanine (**8** and **9**). R- and S-N-Methylalanine (Quitt *et al.*, 1963) were treated exactly as described by Rowley *et al.* (1971) in the preparation of the racemic analogue. Recrystallization, however, was accomplished by careful precipitation of the final product from water by the addition of acetone. Each enantiomer was shown to have a 60 MHz proton NMR spectrum identical to that reported for the racemic compound, $\{\alpha\}_D^{28.5^o}$ = +103° (R-enantiomer) and -105° (S-enantiomer)(C=1, water). It should be noted that unless great care is taken in the purification of **8** and **9**, they will cyclize to the corresponding creatinine analogues (Rowley *et al.*, 1971).

N-{2-(4,5-Dihydroimidazolyl)}sarcosine (**10**). Sarcosine (1.1 g, 12.4 mmol, Aldrich Chemical Co.) was ground to a fine powder with 2-methylmercapto-4,5-dihydroimidazole (2.9 g, 25 mmol), which was prepared from the hydroiodide salt (Aspinall and Bianco, 1951) by neutralization with sodium hydroxide and extraction of the free base into ethyl acetate. The mixture was then heated to 120°, and heating was continued for 0.5 hr. The solid residue was triturated with acetone and filtered. The white solid was then dissolved in hot water, and acetone was added until turbidity was permanent. The white crystalline solid that formed was filtered to give 1.6 g (84% yield) of N-{2-(4,5-

dihydroimidazolyl)}sarcosine monohydrate, mp 240-250° C (dec., sinters 190-195° C).

Anal. Calcd. for $C_6H_{11}N_3O_2 \cdot H_2O$: C, 41.13; H, 7.48; N, 23.99. Found: C, 41.15; H, 7.30; N, 23.98.

The 60 MHz proton NMR spectrum (D_2O) showed peaks at δ 3.06 (3H, singlet), 3.77 (4H, singlet), and 3.91 (2H, singlet).

2-Iminoimidazolidine-4-carboxylic Acid (11). 2,3-Diaminopropanoic acid hydrochloride (1.5 g, 10.6 mmol) (See Chapter 3) was dissolved in 3 mL of 7N NaOH, and cyanogen bromide (1.3 g, 12.3 mmol) in 1.5 mL of absolute methanol was added dropwise to the stirred solution using a 2 mL syringe and a septum over a period of one hr. After stirring for an additional 8 hr, the solvent was removed *in vacuo*, and the solid residue was suspended in cold, concentrated ammonia. The white solid was filtered and crystallized by dissolving in the minimum amount of water, followed by the addition of two volumes of absolute ethanol. Filtration of the white crystalline solid gave 705 mg (52% yield) of 2-iminoimidazolidine-4-carboxylic acid, mp 275-285° C (dec.).

Anal. Calcd. for $C_4H_7N_3O_2$: C, 37.21; H, 5.46; N, 32.54. Found: C, 37.27; H, 5.44; N, 32.63.

The 60 MHz proton NMR spectrum (D_2O) showed an ABC pattern (δ 3.5 - 4.6) with a doublet of doublets centered at δ 4.48 (J = 6.5 Hz and 10 Hz).

2-Imino-3-methylimidazolidine-4-carboxylic Acid (12). 3-Amino-2-methylaminopropanoic acid monohydrochloride (1.0 g, 6.5 mmol) (See Chapter 3) was stirred in 2.0 mL of 6.5 N NaOH, and cyanogen bromide (0.7 g, 6.6 mmol) in one mL of methanol was added to the stirred suspension with a 2 mL glass syringe through a septum over a period of one hr. Immediately after the cyanogen bromide addition was initiated, the stirred suspension was transformed into a yellow solution. As the addition was continued a precipitate appeared, and the suspension was stirred an additional 0.5 hr after the last of the cyanogen bromide had been added. The precipitate was filtered, washed with methanol, cold concentrated ammonia, and again with methanol. The filtrate was concentrated *in vacuo*, suspended in cold concentrated ammonia, filtered and washed with methanol. The two solids, identical according to their proton NMR spectra, were combined to give 573 mg (62% yield) of 2-imino-3-methylimidazolidine-4-carboxylic acid, mp 345-350° C (dec.).

Anal. Calcd. for $C_5H_9N_3O_2$: C, 41.96; H, 6.34; N, 29.36. Found: C, 41.85; H, 6.30; N, 29.46.

The 60 MHz proton NMR spectrum (D_2O) showed peaks at δ 3.0 (3H, singlet) and a multiplet from δ 3.4 to 4.55 typical of an ABC pattern including a doublet of doublets centered at δ 4.37 (J = 7.0 Hz, 10.5 Hz).

N,N'-Dibenzoyl-2-(2-aminoethylamino)ethanol. 2-(2-Aminoethylamino)ethanol (10 g, 96 mmol, Eastman Organic Chemicals) and anhydrous sodium carbonate (25 g) in 250 mL of benzene were stirred in a three-necked flask equipped with a dropping funnel, a condenser, and a thermometer. The stirred suspension was cooled to 8° and benzoyl chloride (25 mL, 30.3 g, 215 mmol) in 100 mL of benzene was added sufficiently slowly that the temperature did not rise above 10° (~2 hr). The stirred suspension was warmed to room temperature, stirred 2 hr, and then heated at reflux an additional 2 hr. The warm suspension was filtered and the white precipitate washed with three 100 mL-portions of $CHCl_3$. The combined filtrates were taken to dryness, and the white solid residue was recrystallized from benzene to give 26 g (87% of theoretical) of N,N'-dibenzoyl-2-(2-aminoethylamino)ethanol, mp 133-134°.

Anal. Calcd. for $C_{18}H_{20}N_2O_3$: C, 69.21; H, 6.45; N, 8.97. Found: C, 69.34; H, 6.50; N, 8.94.

The 100 MHz proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.84 (1H, broad singlet), δ 3.2 - 4.1 (8H, broad multiplet), and δ 7.2 - 7.9 (11H, aromatic multiplets and amide-NH).

The chemical ionization mass spectrum (isobutane reagent gas) showed the following peaks greater than 10% of the base peak: m/e 313 (M + 1, base), 295, 269, 191, 123, 105.

N,N'-Dibenzoyl-2-(2-aminoethylamino)ethanoic Acid. Potassium permanganate (2.2 g, 13.9 mmol) in 60 mL of water was added dropwise over a period of about 1 hr to a stirred solution of N,N'-dibenzoyl-2-(2-aminoethylamino)-ethanol (2.5 g, 8.01 mmol) in 25 mL of glacial acetic acid. The reaction temperature was maintained at 40° during the addition by gentle heating, and heating was continued for 1 hr after addition was complete. At this point the excess permanganate and MnO_2 were converted to soluble salts by the addition of about 2 g of sodium bisulfite. The reaction mixture was concentrated *in vacuo* to dryness, 200 mL of water added, and the pH adjusted to approximately 8. Chloroform extraction of the crude mixture was followed by acidification of the aqueous fraction with 3 N HCl. The aqueous portion was cooled and the crystalline product that precipitated was filtered to give 1.7 g (65% yield) of N,N'-dibenzoyl-2-(2-aminoethylamino)acetic acid, mp 137-138° C.

Anal. Calcd. for $C_{18}H_{18}N_2O_4$: C, 66.25; H, 5.56; N, 8.58. Found: C, 66.09; H, 5.57; N, 8.57.

The 100 MHz proton NMR spectrum showed peaks at δ 3.2 (6H, broad multiplet) and δ 7.0 - 8.1 (12H, aromatic multiplet, amide-NH, and -COOH).

The chemical ionization mass spectrum (isobutane reagent gas) showed the following peaks greater than 10% of the base: m/e 310, 309 (M + 1 - 18, base), 205, 161, 123, 105.

2-(2-Aminoethylamino)ethanoic Acid Dihydrochloride. N,N'-Dibenzoyl-2-(2-aminoethylamino)acetic acid (800 mg, 2.45 mmol) was suspended in 15 mL of 6 N HCl and heated at reflux for 18 hr. After cooling to room temperature, the aqueous solution was extracted with three 25 mL portions of ether to remove benzoic acid, and the aqueous portion was concentrated to dryness *in vacuo*. The residual white solid product, obtained in quantitative yield, was identical to 2-(2-aminoethylamino)acetic acid dihydrochloride prepared by an alternative method (Rowley *et al.*, 1971) which is used in the preparation of the creatine analogue 1-carboxymethyl-2-iminoimidazolidine (**2**).

METHODS

Proton NMR spectra were determined on either a Varian A60A spectrometer or a Varian XL-100 spectrometer operating in the pulse mode at 100.1 MHz (as indicated), in 2-10% solutions in D_2O or $CDCl_3$, and are reported relative to internal sodium 2,2-dimethyl-2-silapentane-5-sufonate (DSS) for the D_2O solutions and relative to internal tetramethylsilane (TMS) for the $CDCl_3$ solutions. Mass spectral measurements were made on an Associated Electronic Industries MS-902 spectrometer. Melting points are uncorrected, and microanalyses were obtained from the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, California.

Creatine kinase assays were performed on a Radiometer TT2 pH-stat using a modification of the procedure of Mahowald *et al*. (1962) at 30° C. The pH was maintained at pH 9.00 by the addition of 2.50 m*M* NaOH. The magnesium ion concentration in the assay solution was so adjusted that free Mg(II) was held constant at 1.0 m*M*, and the total ionic strength of the assay solution was held constant at 50 m*M* by addition of the appropriate amounts of sodium acetate. The creatine kinase concentration was determined spectrophotometrically at 280 nm by using the extinction coefficient $\varepsilon_{280} = 7.1 \times 10^4 M^{-1} cm^{-1}$ (Noda *et al*., 1954).

The initial velocity data for the creatine analogues were analyzed by using the computer program SEQUEN generously supplied by Dr. W. W. Cleland and modified as described previously (Maggio *et al*., 1977).

The inhibition studies were carried out by comparing the observed initial rate for 15 m*M* creatine and 4 m*M* ATP in the presence and absence of 30 m*M* analogue.

RESULTS

The kinetic parameters for a series of conformationally restricted creatine analogues are summarized in Table I. For those analogues in which an asymmetric center has been included in the molecule, and substrate activity has been

TABLE I

COMPARISON OF THE KINETIC PARAMETERS OF CONFORMATIONALLY RESTRICTED CREATINE ANALOGUES

	Substrate	Structure	Relative V_{max}	K_m (mM)	K_s (mM)	Relative V_{max}/K_m
1	Creatine	$^{-}O_2C-CH_2-N(CH_3)-C(NH_2)_2^{+}$	100[a]	8.61 ± .57	24.4 ± 5.5	11.6
2	1-Carboxymethyl-2-imimoimidazolidine	$^{-}O_2C-CH_2-N$ (ring) NH, NH_2, +	90[b]	25		3.6
3	1-Carboxymethyl-2-imino-hexahydropyrimidine	$^{-}O_2C-CH_2-N$ (ring) NH, NH_2, +	n.d.[b,c]			
4	S-N-Amidinoproline	$^{-}O_2C$ (pyrrolidine ring) N, NH_2, NH_2, +	n.d.[b]			

(Table I, Cont.)

	Substrate	Structure	Relative V_{max}	K_m (mM)	K_s (mM)	Relative V_{max}/K_m
5	R-N-Amidinoproline	$^{-}O_2C$, N, NH_2, +, NH_2	0.9[b]	100		0.009
6	S-N-Amidinoazetidine-2-carboxylic Acid	$^{-}O_2C$, N, NH_2, +, NH_2	n.d.			
7	R-N-Amidinoazetidine-2-carboxylic Acid	$^{-}O_2C$, N, NH_2, +, NH_2	29	39.0 ± 5.8	71.6 ± 25.1	0.74
8	S-N-Methyl-N-amidinoalanine	$^{-}O_2C$-C-N, H, CH_3, CH_3, NH_2, NH_2	-[d]			

(Table I, Cont.)

	Substrate	Structure	Relative V_{max}	K_m (mM)	K_s (mM)	Relative V_{max}/K_m
9	R-N-Methyl-N-amidinoalanine		10	82.2 ± 32.8	93.5 ± 18.4	0.12
10	N-{2-(4,5-Dihydroimidazolyl)}sarcosine		n.d.			
11	R,S-2-Iminoimidazolidine-4-carboxylic Acid		n.d.			
12	R,S-2-Imino-3-methylimidazolidine-4-carboxylic Acid		n.d.			

FOOTNOTES TO TABLE I

[a]From Maggio *et al*., 1977, pH 9.00 at 30° C.

[b]From McLaughlin *et al*., 1972, pH 9.00 at 1.0° C.

[c]n.d. = Non-detectable

[d]Compound 8 was shown to be a substrate, but of such low reactivity, kinetic parameters were not determined.

retained, creatine kinase shows a strong preference for the R-enantiomer. Compounds 4 and 6 both react more than 100 times slower than their corresponding R-enantiomers, whereas 9, which does not contain a ring but which does include an asymmetric center, was shown to react approximately 7 times faster than its S-enantiomer (8) under identical conditions. Two other analogues including asymmetric centers (11 and 12) were shown to be ineffective as either substrates or inhibitors of rabbit muscle creatine kinase when examined as racemic mixtures. Compound 10 also acted neither as a substrate nor an inhibitor.

The values of K_m reported for compounds 7 and 9 were determined using the computer program SEQUEN as described in the Materials and Methods section. While the errors reported for the K_m values of 7 and 9 calculated from the computer program were quite large, $\pm$ 39% and $\pm$ 15%, respectively, a least-squares graphical approach yielded K_m values identical to those determined by the computer program.

We also report a new high yield synthesis of 2-(2-aminoethylamino)ethanoic acid, the synthetic precursor of the highly reactive creatine analogue, 1-carboxymethyl-2-iminoimidazolidine (2).

DISCUSSION

As can be seen in Table I, a wide variety of substrate analogue activities is observed from relatively minor changes in the structure of the normal substrate, creatine. This wide range of activities can not only permit predictions about the three-dimensional structure of creatine bound to the active site of creatine kinase, but also can give information about bulk tolerances of various regions of the creatine binding site.

Compound 2 was shown by McLaughlin et al. (1972) to be a very good substrate for creatine kinase, with a V_{max} 90% that of creatine and a relative V_{max}/K_m about 3 times lower than that of creatine. The addition of one methylene group expanding the five- to a six-membered ring (3), almost completely destroys activity. Rowley et al. (1971) indicate that 3 reacts approximately 400 times slower than 2 in initial rate studies, indicating very little tolerance for extra bulk about the planar guanidinium group.

Comparison of the R-enantiomers of the four- and five-membered ring analogues shows a similar sensitivity to the addition of steric bulk in the region of the creatine molecule between the α-carbon and the N-methyl group. R-N-Amidinoazetidine-2-carboxylic acid (7) was shown to have a relative V_{max}/K_m about 15 times smaller than that of creatine, but approximately 80 times larger than that of the corresponding five-membered ring analogue, compound 5.

Even more interesting is the stereospecificity observed for the R-enantiomers of 5 and 7. Rowley et al. (1971) report that 5 reacts about 100 times faster than S-enantiomer (4), and we found the selectivity for the R-enantiomer of the azetidine analogue to be even greater. Detection of product formation for 8 was possible only by using the extremely sensitive polyethylenimine-cellulose thin-layer chromatographic assay devised by Rowley and Kenyon (1974). In both cases, the low reactivity observed for the S-enantiomer might be due to a small amount of contamination (< 1%) by the corresponding R-enantiomer. This pronounced selectivity for the R-enantiomer of these two analogues considerably limits the number of potential conformations for creatine when bound to creatine kinase.

While compound 9, R-N-methyl-N-amidinoalanine, is not a conformationally restricted analogue of creatine in the usual sense since it contains no rings, it is interesting to compare it to the four-membered ring analogues 6 and 7, which contain the same number of carbon atoms. Once again the R-enantiomer is more reactive than the S- counterpart but, in this case, the difference is not nearly as great as in the case of the azetidines. At 40 mM analogue concentration, the R-enantiomer of 9 was shown to react approximately seven times faster than its S- counterpart as compared to the factor of over 100 for the selectivity

of the R-enantiomers of both **5** and **7**. When the relative V_{max}/K_m values of R-isomers of **7** and **9** are compared, the rigid, four-membered ring analogue was shown to be a better substrate by a factor of about six. The added flexibility of **8** and **9** decreases the relative selectivity for the R-isomer, as compared to the other two active analogues with asymmetric centers. The higher K_m value of **9** (82.2 m*M* compared to 39.0 m*M* for **7**) may be a reflection of the higher energy of the conformer of **9** in which the two methyl groups are eclipsed. This particular conformer would have a three-dimensional structure closest to that of R-N-amidinoazetidine-2-carboxylic acid (**7**), but might represent only a small percentage of the total conformational population present in solution.

It is also interesting to compare the synergistic effects of metal-nucleotide binding for the normal substrate, creatine, and analogues **7** and **9**. This synergism is reflected by the ratios of the K_s and K_m values, the binding constants in the absence and presence of nucleotide (ATP), respectively (Morrison and James, 1965). For creatine binding, K_s/K_m is 2.8, indicating a strong synergistic effect. The K_s/K_m values for **7** and **9**, respectively, are 1.8 and 1.1. While compound **7** is still strongly affected by ATP binding, and *vice versa*, compound **9** is nearly unaffected. Presumably, the conformational

change produced upon substrate binding (Reed and Cohn, 1972) is not so effectively produced by the poorer substrate. It should also be noted that a similar loss of synergism in substrate binding was observed when the kinetic consequences of the presence of a CH_3S-blocking group on the active sulfhydryl group of the enzyme was examined (Maggio *et al.*, 1977).

Compounds 10, 11, and 12 showed no detectable activities as substrates for creatine kinase when examined using the highly sensitive polyethylenimine cellulose thin-layer chromatography assay, which is able to detect product formation for analogues that react ~10,000 times more slowly than creatine (Rowley and Kenyon, 1974). The fact that compound 10 is neither a substrate nor an inhibitor of creatine kinase is in agreement with similar findings (Rowley *et al.*, 1971) for N-methylcreatine (N-methylamidino-N-methylglycine). In this compound, the methyl group was shown (Kenyon *et al.*, 1976) to occupy the positions analogous to the two ring methylenes of 10.

10

The other two conformationally restricted analogues that function as neither substrates nor inhibitors, 11 and 12, indicate an additional region around the normal substrate where steric bulk is not tolerated. Addition of a single carbon atom and the formation of a five-membered ring on the side of the molecule opposite the N-methyl group of creatine completely eliminates observable binding to creatine kinase.

From this data, we are able to propose what we believe to be a reasonable three-dimensional picture of creatine as it is bound to creatine kinase, and, moreover, we can pinpoint regions where steric bulk, in the form of methylene or methyl groups, can and cannot be tolerated.

When single methylene groups are added to regions 1 and 2 and four- and five-membered rings are formed, good substrate activity is retained. The addition of methylene groups to regions 3 and 4 destroys all detectable substrate binding.

These results are in agreement with the very tight steric requirements in the region of the active site for

the binding of creatine proposed by Rowley *et al*. (1971). Once the crystal structure of creatine kinase is determined, these analogues should be valuable in the investigation of the binding of creatine to the enzyme, and in elucidating the mechanism of the transfer of the phosphoryl group from ATP.

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CHAPTER 3

THE PREPARATION OF α-SUBSTITUTED β-ALANINE DERIVATIVES FROM 5-SUBSTITUTED URACILS

While there have been many reports of a wide variety of studies involving 2,3-diaminopropanoic acid (1) in the chemical and biological literature (Prados *et al.*, 1974; Hay and Morris, 1972; Preston *et al.*, 1974; Tyankova, 1972), it is available only at prohibitively high cost commercially, or by somewhat tedious, low yield synthetic procedures. The dihydrobromide salt of 1 can be prepared from 2,3-dibromopropanoic acid and ammonia, but the yield is only 40 - 55% and high pressures and temperatures are required (Poduska *et al.*, 1955).

2-Methylamino-3-aminopropanoic acid (2) was first reported in the literature (Tafel and Frankland, 1909) as one of the barium hydroxide hydrolysis products of desoxytheobromine (3,7-dimethyl-2-oxo-1,6-dihydropurine) (Tafel, 1899). This synthetic procedure first requires the reduction of theobromine to desoxytheobromine (Tafel, 1899), and is not a desirable synthesis due to the fact that yields are low and difficult to reproduce.

Martin *et al.* (1968) also report the synthesis of 2, in this case from the condensation of diethyl N-methylacetaminomalonate (Uhle and Harris, 1956) and N-bromomethylphthalimide (Mancera and Wemberger, 1950), followed by acid hydrolysis of the condensation product. A 30% yield is reported for these final two steps (Martin *et al.*, 1968).

An alternative method for the synthesis of compounds 1 and 2 is presented in Scheme I:

SCHEME I

·HCl

3 $R_1=R_2=H$
4 $R_1=CH_3, R_2=CH_2Ph$

H_2
Rd/Alumina

·HCl

5 $R_1=R_2=H$
6 $R_1=CH_3, R_2=H$

6 N HCl

CO_2H
H_2N
·2HCl

1 $R_1=R_2=H$
2 $R_1=CH_3, R_2=H$

Compound 3, 5-aminouracil, is commercially available, and 4, 5-benzylmethylaminouracil (Philips, 1951) is easily prepared in high yield from commercially available 5-bromouracil. The hydrogenation step is clean and nearly quantitative for both 3 and 4. The acid hydrolysis of both 5 and 6 gives one equivalent of ammonium chloride as a product, along with the dihydrochloride of the corresponding

diamino acid. The two products are easily separated by means of ion exchange chromatography on a strongly basic (hydroxide) anion exchange resin. The amino acid and chloride ion bind tightly to the column and the ammonia is eluted with water. The amino acid can then be removed from the column by elution with an acidic solution, e.g., 1.0 N HCl or 1.0 N HCO_2H.

The overall method should be directly applicable to the preparation of a variety of 2-alkylamino-3-amino-propanoic acids from other 5-substituted aminouracils, which are easily prepared from 5-bromouracil and the appropriate amine (Philips, 1951). Also, any 5-substituted uracil derivative, in which the 5-substituent is stable to the hydrogenation and acid hydrolysis conditions, could be readily converted to the corresponding α-substituted β-alanine derivative as depicted in Scheme II:

SCHEME II

H_2, Pd/Alumina → 6 N HCl → H_2N(·HCl)–CH_2–CH(X)–CO_2H

EXPERIMENTAL

General. Proton NMR spectra were determined on a Varian A-60A spectrometer in D_2O, and are reported relative to the internal standard, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Melting points are uncorrected, and microanalyses were obtained from the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, California.

5-Amino-5,6-dihydrouracil Hydrochloride (**5**). 5-Aminouracil (**3**) (7.7 g, 60 mmol, Aldrich Chemical Co.) was converted to its hydrochloride salt by treatment with 6 *N* HCl followed by removal of the excess aqueous acid *in vacuo*. The pale yellow solid was then suspended in 200 mL of water and hydrogenated under 45 psi of hydrogen in the presence of 1 g of 5% rhodium on powdered alumina (Matheson Coleman and Bell). After the theoretical amount of hydrogen had been absorbed (24 hr.), the catalyst was separated by filtration and the water removed in high vacuum. The product was purified by recrystallization from ethanol-water to give 9.6 g (97% yield) of colorless crystals, mp 239-241° C (dec.).

Anal. Calcd. for $C_4H_8ClN_3O_2$: C, 29.02; H, 4.87; Cl, 21.41; N, 25.38. Found: C, 28.93; H, 4.92; Cl, 21.16; N, 25.11.

The proton NMR spectrum (D_2O) showed peaks at δ 3.3-4.1 (2H, multiplet, ABC pattern) and 4.54 (1H, doublet of doublets, J = 7 Hz and 13 Hz).

2,3-Diaminopropanoic Acid Monohydrochloride (1). 5-Amino-5,6-dihydrouracil hydrochloride (5) (6.0 g, 36 mmol) was heated at reflux for 72 hr in 150 mL of 6 N HCl. The aqueous acid was removed at reduced pressure and the residue taken up in water several times and concentrated *in vacuo* to remove residual HCl, leaving the dihydrochloride of 2,3-diaminopropanoic acid (1) and one equivalent of ammonium chloride. The product was dissolved in 5 mL of water and applied to a 50 g column of Bio-Rad AG-1-X8, 20-50 mesh, hydroxide form anion exchange resin. The column was eluted with water until the effluent was neutral to pH paper. The diamino acid was then eluted with 1 N HCl as the dihydrochloride salt until the column effluent was no longer ninhydrin positive. The product, after concentration *in vacuo*, was dissolved in the minimum amount of warm methanol and pyridine added to the solution until the pH rose to a value of about 4. The precipitate was recrystallized from aqueous ethanol to give 4.6 g (90% yield) of 2,3-diaminopropanoic acid monohydrochloride, mp 225-226° C; lit. (McCord, Ravel, Skinner, and Shive, 1957) mp 226-227° C. The proton NMR was identical to the commercially available material.

5,6-Dihydro-5-methylaminouracil Hydrochloride (6). 5-Methylbenzylaminouracil hydrochloride (4) (Philips, 1961) (4.0 g, 15 mmol) was suspended in 200 mL of water with 1 g of 5% rhodium on powdered alumina and hydrogenated at 30 psi hydrogen on a Parr hydrogenation apparatus for 24 hr. The catalyst was removed by filtration and the solvent evaporated at reduced pressure to give a quantitative yield of 5,6-dihydro-5-methylaminouracil hydrochloride (6) (2.65 g). A sample was recrystallized for elemental analysis from aqueous ethanol, mp 239-240° C.

Anal. Calcd. for $C_5H_{10}N_3O_2Cl$: C, 33.44; H, 5.61; N, 23.40. Found: C, 33.15; H, 5.50; N, 23.13.

The proton NMR spectrum (D_2O) showed peaks at δ 2.91 (3H, singlet) and an ABC pattern δ 3.4-5.7 with a doublet of doublets centered at δ 4.53 (J = 6.5 Hz and 12.5 Hz).

2-Methylamino-3-aminopropanoic Acid Monohydrochloride (2). 5,6-Dihydro-5-methylaminouracil (6) (2 g, 11.1 mmol) was heated at reflux 72 hr in 50 mL of 6 N HCl after which the aqueous HCl was removed *in vacuo*. The crude product was dissolved in a small amount of water and applied to a 30 g column of Bio-Rad AG-1-X8, 20-50 mesh hydroxide form anion exchange resin. Elution with water was continued until the column effluent was no longer basic to pH paper (~600 mL) and this first fraction discarded. The column was then washed with 1 N HCl and the

effluent monitored for ninhydrin reactivity on filter paper. When the column wash was no longer ninhydrin positive, the acid eluted fraction was concentrated to dryness at reduced pressure and dissolved in the minimum amount of absolute methanol. The pH of the methanol solution was adjusted to approximately pH 5 with pyridine and the white crystalline solid that formed was collected by filtration to give 1.3 g of **2** (76% yield). A sample was recrystallized from aqueous ethanol for elemental analysis, mp 200-213° C (dec).

Anal. Calcd. for $C_4H_{11}N_2O_2Cl$: C, 31.08; H, 7.17; N, 18.12; Cl, 22.93. Found: C, 31.21; H, 7.17; N, 18.18; Cl, 22.88.

The proton NMR (D_2O) showed peaks at δ 3.85 (3H, singlet) and an ABC pattern, δ 3.4-4.2, with a doublet of doublets centered at 4.0 (J = 5 Hz and 8.5 Hz).

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CHAPTER 4

CARBON-13 NUCLEAR MAGNETIC RESONANCE STUDIES OF CREATINE, CREATININE, AND SOME OF THEIR ANALOGUES

INTRODUCTION

Creatine (1) and its phosphorylated derivative, phosphocreatine (N-phosphonoamidinosarcosine), are both found in significant quantities in muscle and brain tissues of vertebrates (Kuby and Noltmann, 1962). Phosphocreatine has been shown to be involved intimately in muscular contraction (Cain and Davies, 1962; Mommaerts, 1962) by its reversible reaction with adenosine 5'-diphosphate (ADP) to form adenosine 5'-triphosphate (ATP) and creatine in the presence of the enzyme, creatine kinase. The cyclization product of creatine, creatinine (2), is an important end-product of nitrogen metabolism in vertebrates, and its levels in urine are also important clinically as indicators of a variety of disease states (Brainerd *et al.*, 1968).

1

2

In our studies of the mechanism of action of creatine kinase, which utilize analogues of creatine to probe the active site of the enzyme (Rowley *et al.*, 1971; McLaughlin *et al.*, 1972), it has often been difficult to differentiate between the creatine analogues and their respective cyclization products, the creatinines. This problem is

exacerbated by the fact that a number of creatine analogues rather readily cyclize to the corresponding creatinine analogues under very mild conditions (Rowley *et al.*, 1971). Proton NMR spectroscopy, one of the primary tools available to the organic chemist, for example, cannot reliably differentiate between the two types of structures *a priori*, although once a given analogue has been assigned to one class or the other, proton NMR can be used to identify it. Even elemental microanalyses are often not conclusive in the initial structural assignments, since creatinines, including creatinine itself, commonly crystallize as integral hydrates. Faced with this problem, Kenyon and Rowley (1971) developed a series of criteria by which these two types of structures may be differentiated. These included measurement of pKa values, hydrolytic stabilities, deuterium exchange rates, and ultraviolet spectral properties; but straightforward differentiation of the creatinines from the corresponding acyclic creatine analogues using these techniques often remained both difficult and rather time-consuming. In this paper, we describe how carbon-13 NMR spectroscopy appears to allow for the reliable and relatively straightforward distinction between the creatine- and creatinine-types of structures.

RESULTS AND DISCUSSION

In Table I are shown the observed carbon-13 NMR chemical shifts and coupling constants for creatine, creatinine and some of their structural analogues. All compounds, with the exception of acid-labile 9, were examined as their hydrochloride salts because of the low water solubilities of some of the corresponding neutral compounds.

Chemical shifts. In order to rule out major concentration effects on the carbon-13 chemical shifts of the various analogues, the chemical shifts of both creatine hydrochloride and creatinine hydrochloride were measured as a function of concentration between 0.25 *M* and 2.0 *M*. All measured chemical shifts were found to be consistent within a range of ±0.1 ppm with the exception of the carbonyl carbon in both creatine hydrochloride and creatinine hydrochloride; both of these carbons showed a downfield shift of approximately 0.5 ppm on going from the spectrum at low concentration to the spectrum at high concentration.

Even with this small concentration-dependence, the chemical shifts for the carboxylate carbons are quite consistent throughout the series of creatine analogues. The analogues without substitution on the *alpha* carbon, for example, all have chemical shifts in the range of 171.1 ppm to 173.6 ppm. *Alpha* substitution causes a slight downfield shift; the analogues with *alpha* substituents have

TABLE I. CARBON-13 CHEMICAL SHIFTS[a,b] AND COUPLING CONSTANTS[c] FOR CREATINE, CREATININE, AND SOME OF THEIR ANALOGUES.

CREATINE ANALOGUES

Compound	C_0	C_g	C_α	C_1	C_2	C_3	C_{α_1}	C_{α_2}	C_N
3: C_0–C_α–N–C_g(N)(N)	172.6	158.1	43.6 t(141)[c]						
1: C_0–C_α–N(C_1)–C_g(N)(N)	172.3	158.3	52.6 t(142)	38.1 q(141)					
4: C_0–C_α–N(C_1–C_2)–C_g(N)(N)	172.5	157.6	50.4 t(141)	46.1 t(140)	12.3 q(128)				
5: C_0–C_α–N(C_1–C_2–C_3)–C_g(N)(N)	172.7	157.7	50.8 t(140)	52.5 t(140)	20.7 t(125)	10.9 q(124)			
6: C_0–C_α–N(C_1–C_2OH)–C_g(N)(N)	172.6	159.0	51.2[d] t(141)	53.1[d] t(140)	59.7 t(144)				

	C_0	C_g	C_α	C_1	C_2	C_3	C_{α_1}	C_{α_2}	C_N
7 C_0–C_α–N(–C_g(N)N)–C_1–C_2–SH	171.9	158.1	51.2 t(141)	53.5 t(143)	21.8 t(141)				
8 C_0–C_α–N(–C_g(N)N)–C_1–C_2(OH)–C_3(OH)	172.5	159.2	51.5 t(141)	53.6 t(144)	70.0 d(145)	63.4 t(144)			
9 C_0–C_α–N(–C_g(N)N)–C_1–C_2–C_3 (epoxide, O bridging C_2 and C_3) [e,f]	175.1	158.6	53.8[d]	52.4[d]	51.4 d(~175)	46.5 t(175)			
10 C_0–C_α–N(–C_g(N)N)–C_1–C_2=C_3	173.6	158.0	51.5 t(144)	52.9 t(147)	130.9 d(162)	119.0 t(165)			
11 C_0–C_α–N(–C_g(N)N)–C_1–C_2≡C_3	172.2	158.1	50.9 t(141)	40.5 t(143)	70.6 d(50)[g] t(9)	76.8 d(254)			

	C_0	C_g	C_α	C_1	C_2	C_3	C_{α_1}	C_{α_2}	C_N
12 (C_0–C_α–N(C_1–C_2 phenyl)–C_g(N)(N))	171.8	158.5	50.7 t(140)	53.9 t(141)	134.5	130.0[d] d(165) 129.3[d] d(161) 128.4[d] d(161)			
13 (C_0–C_α–N(C_1)–C_g(N)(N–C_N))	172.9	158.1	52.5 t(142)	37.8 q(137)					29.2 q(139)
14 (C_0–C_α–N–C_g(N)(N–C_N))	173.3	157.8	43.5 t(141)						28.5 q(140)
15 (C_0–C_α(C_{α_1})–N–C_g(N)(N))	175.6	157.4	50.9 t(142)				18.0 q(131)		

	C_0	C_g	C_α	C_1	C_2	C_3	C_{α_1}	C_{α_2}	C_N
16 (C_0–C_α(–C_{α_1}–C_{α_2})–N–C_g(N)N)	174.9	157.7	56.4 d(141)				25.6 t(129)	9.8 q(126)	
17 (C_0–C_α(–C_{α_1})–N(C_1)–C_g(N)N)	174.7	158.5	56.6 d(140)	32.4 q(142)			15.0 q(128)		
18 (C_0–C_α–C_{α_1}(C_{α_2})(C_{α_2}); C_α–N–C_g(N)N)	174.3	158.0	60.6 d(141)				31.4 d(134)	19.0 q(127) 17.5 q(127)	
19 (C_0–C_α–N(C_1)–C_g; ring N–C_N–C_N–N)	172.5	161.6	52.9 t(140)	38.4 q(141)					44.0 t(148)

	C_0	C_g	C_α	C_1	C_2	C_3	C_{α_1}	C_{α_2}	C_N
20	172.6	155.1	51.8 t(141)	48.3 t(144)	21.1 t(133)				39.0 t(143)
21	172.0	160.1	46.7 t(141)	49.7 t(150)	41.8 t(149)				
22	172.2	160.1	46.7 t(141)	50.0 t(148)	42.0 t(148)				29.8 t(141)
23	174.9	155.9	60.9 d(148)	48.9 t(143)	23.9 t(137)		31.2 t(135)		

	C_0	C_g	C_α	C_1	C_2	C_3	C_{α_1}	C_{α_2}	C_N
24	174.7	157.4	62.5 d(150)	49.4 t(152)			21.2 t(143)		
25	174.7	160.5	56.6 d(149)				46.8 t(148)		
26	173.9	160.0	62.6 d(150)	31.6 q(141)			45.5 t(148)		
27	171.1	147.5	47.4 t(144)	118.0 d(203) d(11)[h]	113.7 d(203) d(11)[h]				

	C_0	C_g	C_α	C_1	C_2	C_3	C_{α_1}	C_{α_2}	C_N
28	172.0	163.2	47.3 t(142)	49.7 t(148)	70.1 t(157)				
29	173.2	170.2	48.9 t(143)	57.1 t(147)	28.8 t(147)				
30		158.1	51.1 d(92)[i] t(139)	38.9 q(141)					
31	175.8	157.8	34.1 t(126)				37.9 t(138)		

	C_0	C_g	C_α	C_1	C_2	C_3	C_{α_1}	C_{α_2}	C_N
32 (C_0–C_α–C_{α_1}–N(C_1)–C_g(N)N)	176.1	157.6	32.3 t(129)	36.5 q(140)			46.8 t(139)		

CREATININE ANALOGUES

	C_0	C_g	C_α	C_1	C_2	C_{α_1}	C_N
33	175.7	159.5	49.5 t(150)				
2	173.3	157.8	55.1 t(150)	32.1 q(142)			
34	173.0	156.9	52.7 t(150)	40.8 t(141)	12.5 q(131)		
35	172.3	158.8	54.5 t(151)	32.5 q(142)			27.1 q(143)

	C_0	C_g	C_α	C_1	C_2	C_{α_1}	C_N
36	173.9	158.1	55.1 t(150)	32.0[d] q(142)			30.5[d] q(142)
37	177.0	157.0	61.1 d(150)	29.7 q(142)		14.1 q(131)	
38	175.9	158.5	49.8 t(150)				39.9 t(142) 38.6 t(143)

	C_0	C_g	C_α	C_1	C_2	C_{α_1}	C_N
39	173.5	154.9	53.5 t(150)	42.0 t(147)	19.7 t(130)		39.1 t(147)
40	173.6	156.9	53.1 t(150)	47.1 t(142)	20.5 t(125)	C_3 10.9 q(124)	

FOOTNOTES TO TABLE I

[a]Chemical shifts are reported for the hydrochloride salts, unless otherwise specified, dissolved in D_2O/H_2O, and in ppm relative to TMS. Internal dioxane was used as a secondary reference and chemical shifts were converted to the TMS scale using the dioxane-TMS shift difference of 67.4 ppm (Johnson and Jankowski, 1972).

[b]Chemical shift assignments are based on signal multiplicities in the coupled spectra, coupling constant magnitude, comparison of the shift assignments in similar compounds (Horsley *et al.*, 1970), and utilization of shift parameters as described in text.

[c]Carbon-hydrogen coupling constants (Hz), unless otherwise specified, and multiplicities, are reported directly below the corresponding carbon chemical shift.

[d]The specific carbon assignment is uncertain.

[e]Due to the instability of **9** at acidic pH values, the spectrum of the zwitterionic structure was obtained, rather than the hydrochloride salt.

[f]Because of the lack of sufficient quantities of pure **9**, the proton-coupled spectrum was obtained as a mixture of **9** and its synthetic precursor **10**; only the $^1J_{^{13}C-^1H}$ values for C_2 and C_3 could be obtained.

[g]The coupled signal was observed as a doublet of triplets (long range coupling to C_1 protons).

[h]Long range coupling was observed.

[i]The proton-decoupled spectrum showed the *alpha* carbon to be a doublet due to carbon-13-phosphorus-31 coupling.

carboxylate chemical shifts between 173.9 ppm and 175.6 ppm. The carbonyls of the creatinine analogues show similar trends, with the only analogue available with an *alpha* substituent (37) having the chemical shift farthest downfield, namely 177.0 ppm. The chemical shifts of the carbonyl carbons of the other creatinine analogues ranged between 172.3 ppm (35) and 175.9 ppm (38).

While observations of the magnitudes of chemical shift values of the guanidinium carbons for creatines and creatinines are not sufficient to differentiate between the two types of structures, the narrow shift range for these carbons makes the creatines and creatinines readily distinguishable from their precursor amino acids; this simple differentiation, which is useful in syntheses of the creatine analogues, is often not possible with proton NMR spectroscopy.

The most notable trend observed in the chemical shifts of the guanidinium carbons of the creatine analogues was the difference between the cyclic and acyclic analogues. The analogues in which the guanidinium carbon was not in a ring had chemical shifts for this carbon between 155.9 ppm (23) and 159.2 ppm (8). When the guanidinium carbon was part of a five-membered ring, the observed shifts for these carbons ranged from 160.0 ppm (26) to 161.6 ppm (19). In the one creatine analogue where the guanidinium carbon is included in a six-membered ring (20), the chemical shift fell to 155.1 ppm.

In all of the creatinine analogues examined, the chemical shift of the guanidinium carbon had a narrower range, from a low of 154.9 ppm in **39**, where it is part of a six-membered ring, to a high of 159.5 ppm in **33**.

The chemical shifts of the two carbons that have proven to be most useful in the differentiation between the creatines and creatinines have been the carbons α- to the carbonyl and the carbons in the position analogous to the N-methyl groups of creatine (**1**) and creatinine (**2**) (designated C-1 in Table I). Both of these types of carbons were quite sensitive to the conversion of a creatine analogue into the corresponding creatinine analogue. In the eight creatine-creatinine analogue pairs available, the chemical shift of the α-carbon of the creatinine analogue was consistently downfield from that of the corresponding creatine analogue. The observed downfield shifts obtained were rather substantial, ranging from a low of 1.7 ppm in the conversion of **20** to **39** to a high of 6.0 ppm in the conversion of **3** to **33**.

The carbon atoms in the position analogous to the N-methyl group of creatine (C-1) also showed a characteristic change in chemical shift upon conversion to the corresponding creatinine analogue. For these particular carbons, however, the change in chemical shift was consistently upfield rather than downfield. Seven sets of compounds were available for a direct comparison of this parameter

between pairs of creatine and creatinine analogues. Again, the observed shifts were rather impressive. The smallest upfield shift observed was for the conversion of 17 to 37 (2.7 ppm), while the largest was for the conversion of 20 to 39 (6.2 ppm). It is interesting to note that the smallest downfield shift for the α-carbon upon conversion of a creatine analogue to a creatinine analogue, 1.7 ppm for the conversion of 20 to 39, is accompanied by the largest upfield shift, 6.2 ppm, for the C-1 carbon in the position analogous to the methyl group of creatine. Also, in the compound with the largest observed downfield shift (excluding glycocyamine, 3), 4.5 ppm, for the alpha carbon in the conversion of 17 to 37, there is the smallest observed upfield shift, 2.7 ppm, for the C-1 carbon.

In two compounds in our study, carbon-13 NMR spectroscopy showed nonequivalence of two methyl groups attached to the same atom. In compound 18, the fact that the two diastereotopic methyl groups were distinguishable was not surprising since they had earlier been resolved using proton NMR spectroscopy at 60 MHz (Rowley *et al.*, 1971). In compound 38, however, the proton NMR spectrum at 60 MHz of the free base had been reported to show only a single absorption for the two methyl groups (Kenyon and Rowley, 1971). The carbon-13 NMR spectrum of both the free base and hydrochloride salt of 38, however, showed the two methyl groups to be nonequivalent at room temperature due to

restricted rotation about the carbon-nitrogen bond. In a study reported elsewhere using variable temperature NMR spectroscopy, we have shown the rotational barrier ($\Delta G^{\ddagger}$ at the coalescence temperature) for the hydrochloride salt of 38 to be 17.6 $\pm$ 0.2 kcal/mol and that for the corresponding free base to be 15.6 $\pm$ 0.2 kcal/mol (See Chapter 5).

In a systematic study of the carbon-13 NMR spectra of the common amino acids, Horsley *et al*. (1970) were able to predict the resonance positions of the various carbon atoms with considerable accuracy. Using a suitable set of chemical shift parameters and refinement corrective terms (Horsley *et al*., 1970; Grant and Paul, 1964), they were able to obtain good estimates for the chemical shifts of many of the carbons of these amino acids. For the creatine and creatinine analogues, however, we were unable to find suitable parameters to predict the effect of substitution on a guanidinium nitrogen, through that nitrogen, to other carbons attached to the same nitrogen. The shift parameters described by Horsley *et al*. (1970), nevertheless, were useful in assigning chemical shifts to particular carbon atoms. The chemical shift parameters could be used to predict the shifts of particular carbon atoms from the corresponding parent molecules where a hydrogen had been replaced by a substituent (e.g., $-CH_3$, -SH, -OH). For example, in the

assignment of the methylene carbons of compound 6, the three carbons show chemical shifts between 51.2 ppm and 59.7 ppm, a rather narrow range, which makes assignments difficult. However, if one uses the alpha and beta shift parameters for an -OH substituent (-48.5 and -10 ppm, respectively) on compound 4, the predicted value for C_1 is 56.1 and for C_2, 60.8 ppm. The observed shifts are 51.2 ppm (assigned to C_α), 53.1 ppm (C_1) and 59.7 ppm (C_2).

Coupling Constants. The proton-coupled spectra of organic molecules are generally more complex and often much more difficult to interpret than the corresponding proton noise-decoupled spectra due to the large coupling constants and resultant peak overlapping. For relatively simple molecules like the creatines and creatinines, however, the coupled spectra are extremely useful in the characterization and differentiation of these types of structures. The most valuable in this study were $^1J_{^{13}C-^1H}$ values for the carbons in the position α- to the carbonyl carbons in the various analogues. In acyclic creatine analogues, for example, the α-carbons exhibit $^1J_{^{13}C-^1H}$ values in a very narrow range between 140 and 142 Hz, with the exception of compound 10 which shows a $^1J_{^{13}C-^1H}$ value of 144 Hz. This slightly higher value for compound 10 might be attributable to a long-range substituent effect of the vinyl group. The values in the cyclic creatine analogues (i.e., 23, 24, 25 and 26) are somewhat larger,

reaching a maximum of 152 Hz for the α-carbon of the four-membered ring analogue, 24.

In the analogue 30, where a phosphinic acid group has replaced the carboxylic acid group of creatine, a $^{1}J_{^{13}C-^{31}P}$ value of 92 Hz was measured, which is in line with similar such values reported in the literature (Gray and Cremer, 1972).

The $^{1}J_{^{13}C-^{1}H}$ values for the α-carbons of the creatinine analogues also showed a very narrow range. The point of interest, however, is that the magnitude of the values are between 150 and 152 Hz, approximately 10 Hz greater than those of the corresponding creatine analogues. This parameter alone, then, apparently allows for direct distinction between acyclic creatines and their corresponding creatinines.

Whereas $^{1}J_{^{13}C-^{1}H}$ values have been shown directly to correlate with carbon hybridization states for simple hydrocarbons (i.e., the observed coupling constant is directly proportional to the amount of S-character) (Muller and Pritchard, 1959; Shoolery, 1959), other factors have also been shown strongly to influence $^{1}J_{^{13}C-^{1}H}$ values. Electronegative substituents induce large increases in coupling constants (e.g., CH_4, $^{1}J_{^{13}C-^{1}H}$ = 125 Hz; CH_3Cl, $^{1}J_{^{13}C-^{1}H}$ = 150 Hz; CH_2Cl_2, $^{1}J_{^{13}C-^{1}H}$ = 178 Hz; $CHCl_3$, $^{1}J_{^{13}C-^{1}H}$ = 209 Hz) (Malinowski, 1961) and changes in molecular geometry also can affect coupling constant values (Gill and Geraldes, 1974). The observed 10 Hz difference in the $^{1}J_{^{13}C-^{1}H}$ values

of the creatines and the creatinines could be attributed to any one or all of these factors; the molecular geometry is obviously changed, the substituent has changed from a carboxylic acid group to an acylguanidinium group, and there also probably is a small hybridizational change at the carbon atom in question owing to the compression accompanying the five-membered ring formation.

In two cases, we were able to observe $^{1}J_{^{13}C-^{13}C}$ coupling constants. Such coupling between carbon-13 atoms is generally not observed in natural abundance carbon-13 NMR spectra because of the relatively low isotopic abundance of carbon-13 atoms (1.1%). By observing the spectra of creatine and creatinine 50% enriched at the carbonyl position (Rowley and Kenyon, 1972), however, we were able to obtain the $^{1}J_{^{13}C-^{13}C}$ values between the carbonyl carbon and the α-carbon. The value obtained for creatine, 59 Hz, is reasonably close to the analogous $^{1}J_{^{13}C-^{13}C}$ value reported for acetic acid (56.7 Hz)(Gray _et al._, 1969). Creatinine, on the other hand, showed a corresponding value of only 50 Hz. Since the $^{1}J_{^{13}C-^{13}C}$ coupling constants between the carbonyl carbon and the α-carbon of acetyl compounds have been shown to be quite sensitive to substituent effects (Gray _et al._, 1969), it is not surprising to see a fairly large change in this parameter between creatine and creatinine. The decreased $^{1}J_{^{13}C-^{13}C}$ value of dimethylacetamide (52.2 Hz)(Gray _et al._, 1969) when compared to

that for acetic acid (56.7 Hz)(Gray et al., 1969) is in line with the direction of the corresponding change observed between creatine and creatinine.

Conclusions. Carbon-13 NMR chemical shifts and $^{1}J_{^{13}C-^{1}H}$ values (especially for the carbons α- to the carbonyl group) can be used to characterize and to differentiate readily between analogues of creatine and creatinine. The method actually proved itself in the course of examining the data collected during this study, when an unexpectedly high $^{1}J_{^{13}C-^{1}H}$ value (150 Hz) was observed for the α-carbon of what was thought to be creatine analogue 4. At that point, it was suspected that 4 might have cyclized to the corresponding creatinine analogue in the course of inadvertently allowing the sample to sit in acidic solution for a period of days prior to measurement. The carbon-13 spectrum of a freshly prepared sample of 4 showed this indeed to have been the case, since a $^{1}J_{^{13}C-^{1}H}$ value of 141 Hz was found for its α-carbon.

Finally, the compilation of coupling constants and chemical shift data in Table I should also be useful in the characterization of other acylguanidine and guanidinium compounds found in nature.

EXPERIMENTAL SECTION

The following chemicals were purchased and used without further purification: creatine (1) (Matheson Coleman and

Bell), creatinine (2) (Calbiochem Corp.), glycocyamine (3) (J. T. Baker), N-amidinoalanine (15) (Eastman Organic Chemicals), and N-amidino-β-alanine (31) (Sigma Chemical Comp.).

Compounds prepared as described by Rowley et al. (1971) included N-amidino-N-propylglycine (5), N-amidino-N-(2-thioethyl)glycine (7), N-amidino-N-benzylglycine (12), N-methylamidino-N-methylglycine (13), N-methylamidinoglycine (14), D,L-N-methyl-N-amidinoalanine (17), D,L-N-amidinovaline (18), 1-carboxymethyl-2-iminohexahydropyrimidine (20), 1-carboxymethyl-2-iminoimidazolidine (21), D,L-N-amidinoproline (23), 2-imino-3-carboxymethylthiazolidine (29), N-methyl-N-amidinoaminomethylphosphinic acid (30), and N-methyl-N-amidino-β-alanine (32).

1,3-Dimethyl-2-iminoimidazolin-4-one (35), 1-methyl-2-methylamino-2-imidazolin-4-one (36), 2-dimethylamino-2-imiazolin-4-one (38), and 2-oxo-2,3,5,6,7,8-hexahydroimidazo-{1,2-a}pyrimidine (39) were prepared according to the methods described by Kenyon and Rowley (1971).

N-Ethylglycocyamine (4) and N-ethylglycocyamidine (34) were prepared using the method described by Armstrong (1956).

α-Guanidinobutyric acid (16) was prepared as described by Lopez and Monteoliva (1967).

Glycocyamidine (30) was prepared according to the method of Bengelsdorf (1953).

4,5-Dihydro-1-carboxymethyl-2-methylaminoimidazole (22) was a gift of Dr. T. Sakurai (unpublished synthesis).

N-Amidino-N-(2,3-dihydroxypropyl)glycine (8), N-amidino-N-(2,3-epoxypropylglycine) (9), and N-amidino-N-(2-propenyl)glycine (10), N-amidino-N-(2-propynyl)glycine (11), 1-carboxymethyl-2-aminoimidazole (27), and 2-amino-3-carboxymethyloxazolidine (28) were prepared by Marletta (1978).

N-{2-(4,5-dihydroimidazolyl)}sarcosine (19), N-amidino-azetidine-2-carboxylic acid (24), 2-iminoimidazolidine-4-carboxylic acid (25), and 2-imino-3-methylimidazolidine-4-carboxylic acid (26) were prepared as described in Chapter 2.

N-(2-Hydroxyethyl)glycine was prepared from a solution of iodoacetic acid (10.0 g, 0.054 mol) in 25 mL of water which was added dropwise to ice-cold, rapidly stirred ethanolamine (26.39 g, 0.43 mol). The reaction mixture was allowed to stand for 20 hr after which time the water and excess ethanolamine were both removed *in vacuo* leaving a viscous yellow oil. The oil was dissolved in 25 mL of absolute ethanol and 310 mL acetone. The solution was allowed to stand at 4° C for 12 hr during which time a white precipitate formed. The white powder (5.6 g) was filtered, washed with acetone, dried and used without further purification (87.5% yield), mp 170-172° C (dec.); lit. (Stewart, 1962) 174-175° C.

^{1}H NMR δ 3.22 (2H, unsymmetrical quartet, 3.68 (2H, singlet), 3.87 (2H, unsymmetrical quartet).

N-Amidino-N-(2-hydroxyethyl)glycine (6) was prepared by dissolving cyanamide (0.84 g, 20 mmol) in a solution of 1 mL of water followed by the addition of N-(2-hydroxyethyl)glycine (1.9 g, 16 mmol). After the addition of 2 drops of concentrated aqueous NH_3, the solution was allowed to stand for 5 days. The white crystalline solid that had formed was filtered, washed with acetone and recrystallized from ethanol/water to give 1.15 g (45% yield), mp 203-205° C (dec.).

Anal. Calcd. for $C_5H_{11}N_3O_3$: C, 37.26; H, 6.88; N, 26.07. Found: C, 37.52; H, 6.87; N, 25.83.

^{1}H NMR - D_2O/DCl - δ 4.35 (2H,S), δ 3.5-4.0 (4H, A_2B_2 multiplet).

1,5-Dimethyl-2-amino-2-imidazolin-4-one (37) was prepared by the spontaneous quantitative cyclization of N-methyl-N-amidinoalanine in aqueous solution, mp 260-265° C (dec.).

^{1}H NMR δ 1.37 (3H, doublet, J = 7 Hz), 3.04 (3H, singlet), 4.05 (1H, quartet, J = 7 Hz).

Anal. Calcd. for $C_5H_9N_3O$: C, 47.23; H, 7.14; N, 33.05. Found: C, 47.08; H, 7.14; N, 33.21.

N-Propylglycocyamidine (40) was prepared from N-propylglycocyamine (N-amidino-N-propylglycine) (5) by the method used in the preparation of N-ethylglycocyamidine (34) described above. The residue was recrystallized from ethanol to give product of mp 225-230° C (dec.).

^{1}H NMR δ 4.09 (2H, S), 3.36 (2H, t, J = 7 Hz), 1.63 (2H, m, J = 7 Hz), 0.90 (3H, t, J = 7 Hz).

Anal. Calcd. for $C_6H_{11}N_3O$: C, 51.04; H, 7.83; N, 29.77. Found: C, 51.06; H, 7.83; N, 29.68.

Methods. Carbon-13 NMR spectra were obtained at ambient temperature using a Varian XL-100 spectrometer operating at a frequency of 25.158 MHz, interfaced with a Nicolet Instrument Corporation Model NIC-80 data processer, and modified with a Nicolet "Multi Observe Nuclei Accessory" (MONA). Deuterium of the solvent was used as an internal lock signal. Samples (0.20-1.5 *M*) were spun in 12 mm tubes in 60-70% D_2O/H_2O, and the pH of the sample adjusted with approximately 1.5 molar equivalents of HCl (using 12 *M* HCl) per molar equivalent of sample. Chemical shifts for all carbons, with the exception of the carbonyl carbons (See Text) were reproducible to within $\pm$ 0.1 ppm, even when samples were examined over a rather wide concentration range. The coupled and proton noise-decoupled spectra were obtained under identical conditions using the computer controlled link GAWTGOSAGBDFGODNSB, in which with the decoupler on, the decoupled spectrum is collected and saved, and in which with the decoupler off, the proton-coupled spectrum is collected and saved. Typical runs to obtain both coupled and decoupled spectra took 12 hr.

Proton NMR spectra were determined on a Varian A 60A spectrometer in 5-10% solutions in D_2O, and are reported

relative to the internal standard, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Ultraviolet spectra were recorded using a Cary Model 14 Spectrometer. Melting points are uncorrected, and microanalyses were obtained from the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, California.

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CHAPTER 5

BARRIERS TO ROTATION IN ACYLGUANIDINES AND ACYLGUANIDINIUM IONS

While extensive investigations of both rotational barriers and barriers to inversion have been carried out on guanidines and guanidinium ions (Kessler and Leibfritz, 1969a, 1969b, 1970, and 1971), acylguanidines and acylguanidinium ions have received relatively little attention. This is in spite of the fact that there are numerous examples of biologically important molecules containing acylguanidine moieties (e.g., creatinine, some purines, some pyrimidines, biopterin and tetrahydrofolate).

The specific molecules we have chosen to examine, 2-dimethylamino-2-imidazolin-4-one (**1a**) and its hydrochloride salt (**1b**), provide ideal models for examining rotational

1a **1b**

barriers about a particular carbon-nitrogen bond in an acylguanidine molecule and the corresponding acylguanidinium ion. Although Kessler and Leibfritz (1969a, 1969b, 1970, and 1971) have compared "isomerization" barriers in guanidines and guanidinium ions using NMR spectroscopy, they concluded that the particular guanidines they examined "isomerize" by an inversion mechanism, while the guanidinium ions "isomerize" by a rotational mechanism (Kessler and

Leibfritz, 1971). Since an inversion mechanism is not possible for either **1a** or **1b**, we can be assured that we are observing only the barrier to rotation about one particular bond in each case, namely the bond between the ring C-2 carbon and the nitrogen of the dimethylamino substituent.

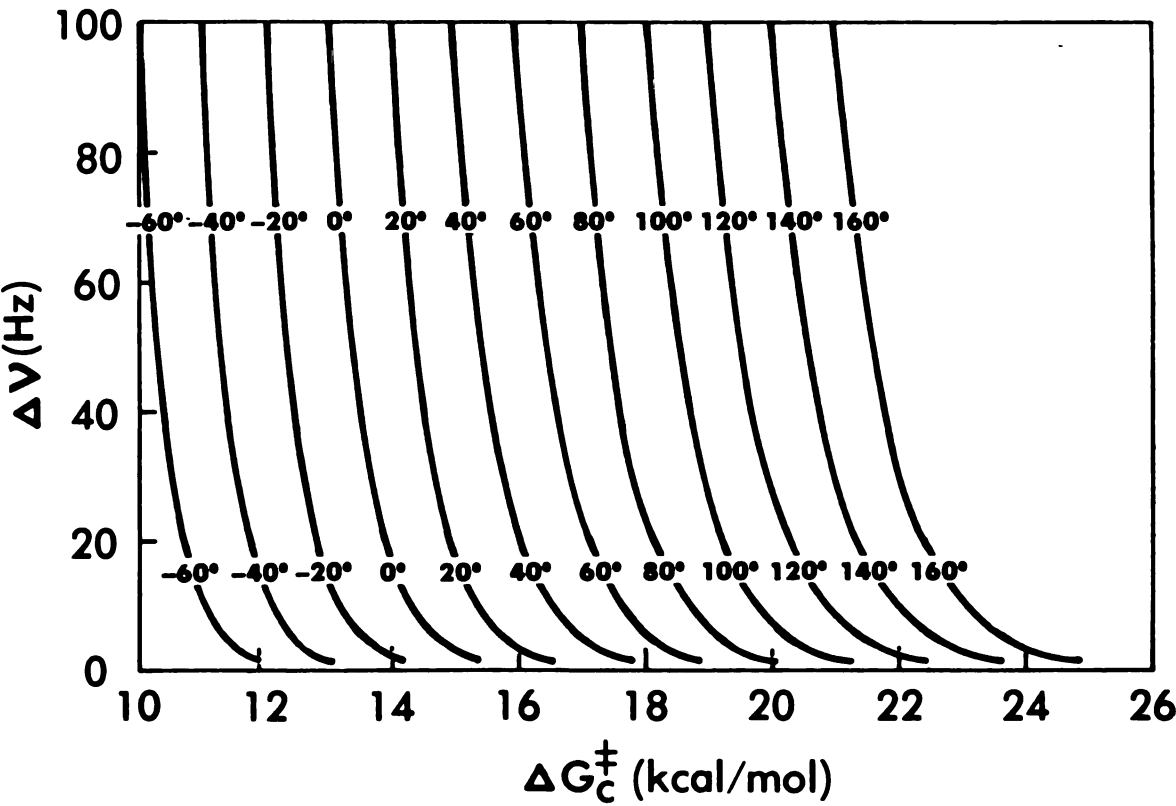
The proton-decoupled carbon-13 NMR spectrum at $30^{o}C$ of the acylguanidine **1a** showed the two methyl peaks of the dimethylamino group to be nonequivalent and to be separated by 46 Hz, and so, in principle, the barrier to rotation could have been examined by carbon-13 NMR spectroscopy. For reasons of convenience, however, we chose variable temperature proton NMR spectroscopy to evaluate the barrier to rotation about the bond in question.

Figure 1 is a representation of the relationships among $\Delta\nu$ {the difference in frequency (in Hertz) in an NMR spectrum of the signals due to two exchangeable species}, T_c {the coalescence temperature}, and $\Delta G_c^{\ddagger}$ {the free energy of activation (in kcal/mol) for the coalescence process}. It was constructed by substituting various values of $\Delta\nu$ and T_c into equation (1) and the Eyring equation (2):

$$k_c = \pi\ (\nu_a - \nu_b)/\sqrt{2} \qquad (1)$$

where k_c is the rate constant for exchange at coalescence and

FIGURE 1



Plot of the free energy of activation at the coalescence temperature ($\Delta G_c^{\ddagger}$) vs. the difference in frequencies of the NMR absorption signals for two exchangeable species ($\Delta\nu$) for various coalescence temperatures T_c.

$$k_c = KT/h \cdot e^{-\Delta G^{\ddagger}/RT} \qquad (2)$$

where K = Boltzmann's constant and h = Planck's constant.

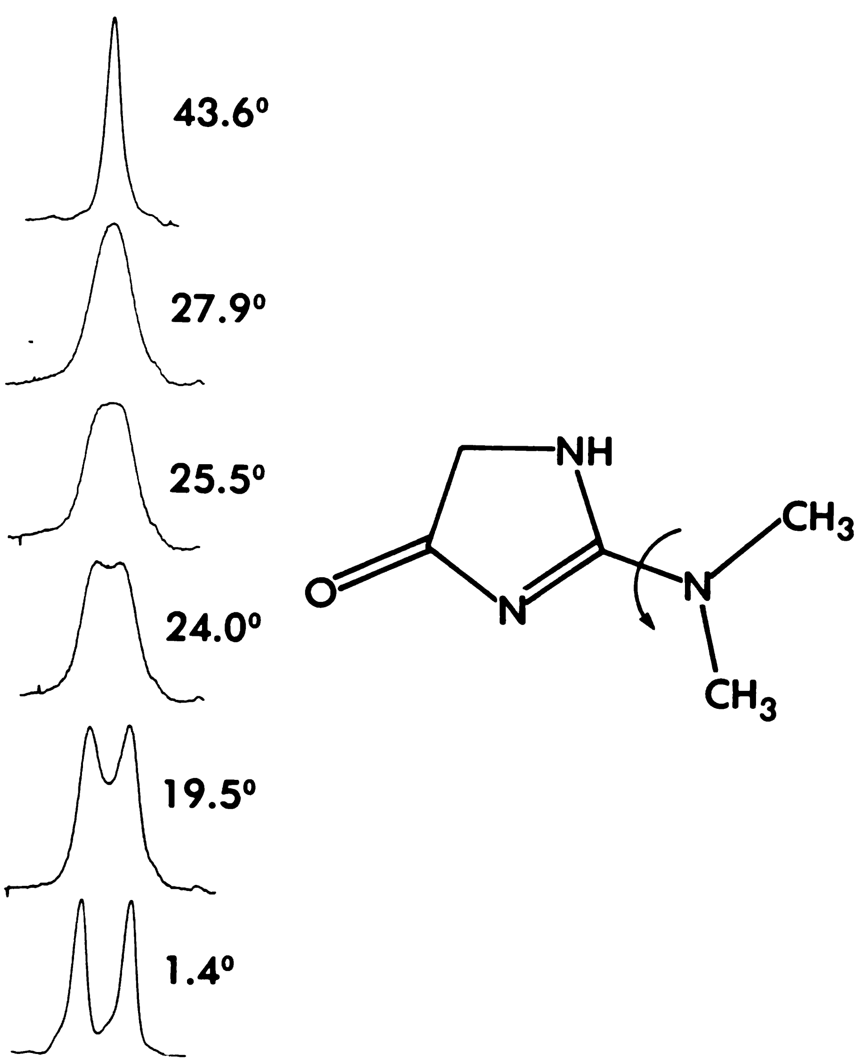
Figure 1 serves as a simple graphic device for quickly determining any one of the three parameters when the other two are known, and is applicable to many exchange phenomena observable by NMR spectroscopy. Even though equations (1) and (2) are both well-known and have been widely applied, such a graphical representation as shown in Figure 1 has not to our knowledge been presented before.

At 1.4°C, the proton NMR spectrum of acylguanidine **1a** showed two sharp singlets separated by 11 ± 1 Hz, indicating a rate constant at coalescence of 24.4 sec^{-1}. Substitution of both this value and the observed coalescence temperature of 25.5 ± 0.5°C as shown in Figure 2 into the Eyring equation yields a free energy for the barrier to rotation ($\Delta G_c^{\ddagger}$) of 15.6 ± 0.1 kcal/mol.

The rotational barrier in the corresponding acylguanidium ion (**1b**) was obtained utilizing both proton NMR and carbon-13 spectroscopy. The proton NMR spectrum (25.4°C) showed a pair of well-resolved singlets ($\Delta\nu$= 2.6 ± 0.2 Hz) which coalesce at 47 ± 0.5°C, indicating a $\Delta G_c^{\ddagger}$ of 17.7 ± 0.1 kcal/mol. The data used in the analysis are shown in Figure 3.

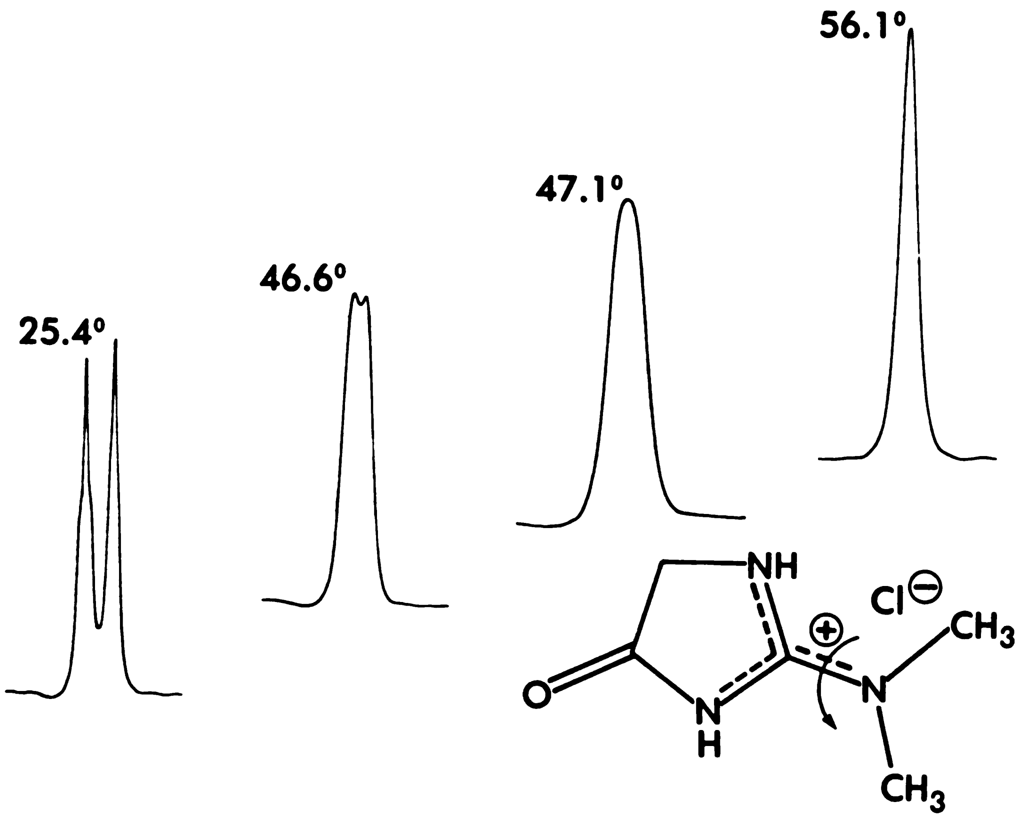
This barrier was confirmed using proton-decoupled carbon-13 NMR spectroscopy. The two methyl peaks were

FIGURE 2



Variable-temperature proton NMR spectra of 2-dimethylamino-2-imidazolin-4-one (1a), focusing only on the N-CH_3 absorptions.

FIGURE 3



Variable-temperature proton NMR spectra of 2-dimethylamino-2-imidazolin-4-one hydrochloride (1b), focusing only on the N-CH_3 absorptions.

found to be separated by 34 ± 2 Hz at 30°C, and these peaks coalesced at 75 ± 2°C, indicating a $\Delta G_c^{\ddagger}$ value of 17.5 ± 0.2 kcal/mol. This rotational barrier is significantly higher than the barrier for a guanidinium ion reported previously by Kenyon *et al.* (1976) (See Table I). Much closer agreement is found by comparing this value to the rotational barrier in pentamethyl-4-acetylphenylguanidinium iodide (**3**), which can be considered a phenylogous analog of an acylguanidinium ion. The barrier in this molecule was found to be 16.6 kcal/mol by Kessler and Leibfritz (1971).

The unusually high barrier in **1b** can be rationalized using arguments similar to those of Kessler and Leibfritz (1971) for 4-substituted aromatic guanidinium ions. Comparison of the three potential resonance structures for guanidinium ion show them all to be equivalent, implying equal delocalization of the double bond over the three carbon-nitrogen bonds:

A comparison of the three similar resonance structures for an acylguanidinium ion shows two equivalent resonance structures (**11a** and **11b**) plus a more energetically unfavorable structure (**11c**) in which a formal positive charge is on the nitrogen atom adjacent to the partially positively charged carbonyl carbon.

Table 1. Barriers to Rotation in Guanidines, Guanidinium Ions, Acylguanidines, Acylguanidinium Ions and Related Compounds.

Structure	Barriers to Rotation (Kcal/mol)		
	Experimental	Calculated (ΔE)[a]	
		STO-3G	4-31G
1a	$15.6\pm0.1(\Delta G_c^\ddagger)$	15.5	
1b	$17.6\pm0.2(\Delta G_c^\ddagger)$	23.7	
2	$13.6\pm1.9(\Delta H_c^\ddagger)$[b]		
3	$16.5\pm0.2(\Delta G_c^\ddagger)$[c]		
4	$15.5\pm0.2(\Delta G_c^\ddagger)$[c]		

Table 1, Cont'd.

Structure	Barriers to Rotation (Kcal/mol)		
	Experimental	Calculated (ΔE)[a]	
		STO-3G	4-31G
H_2N–C(=NH_2)(NH_2)$^{\oplus}$ **5a**		22.1	15.5[d]
H_2N–C(NH_2)=NH **5b**			12.2
H_2N–C(NH_2)=N–CHO **6**			17.0
H_2N–C(NH_2)=N(H)–CHO$^{\oplus}$ **7**		23.4	17.7
$(CH_3)_2N$–CHO **8**	20.9±0.2,[e] 21.0±0.4[f] ($\Delta G_c^{\ddagger}$) 20.2±0.2;[e] 20.5±0.1[f] ($\Delta H_c^{\ddagger}$) -1.7 e.u.[e]; -1.4±1 e.u.[f] ($\Delta S^{\ddagger}$)		

Table 1. Cont'd.

Structure	Barriers to Rotation (Kcal/mol)		
	Experimental	Calculated (ΔE)[a]	
		STO-3G	4-31G
9	$15.6 \pm 0.2 (\Delta G_c^{\ddagger})$[g]		
10	$18\text{-}19 (\Delta G_c^{\ddagger})$[h]		

FOOTNOTES TO TABLE I

[a]The calculated ΔE between the planar and perpendicular conformations.

[b]Kenyon *et al*. (1976).

[c]Kessler and Leibfritz (1971).

[d]Kollman *et al*. (1975); the calculated barrier when the carbon-nitrogen bonds were both optimized in planar and perpendicular geometries was 14.1 kcal/mol.

[e]Rabinovitz and Pines (1969).

[f]Drakenberg *et al*. (1972).

[g]Blanchard *et al*. (1967).

[h]Hammond and Neuman (1963).

11a 11b 11c

These considerations lead to the prediction of a higher contribution from resonance structures **11a** and **11b**, with more double-bond character in those carbon-nitrogen bonds and a higher rotational barrier about those bonds. The slightly lower barrier in the phenylogous compound can be explained by the insulating effect of the phenylene ring.

While the comparison between **1b** and the acylphenylguanidinium ion (**3**) shows fairly good agreement, there is more than likely a larger steric contribution to the energy barrier in the phenyl-substituted compound. Pentamethylphenylguanidinium ion (**4**) has a rotational barrier of 15.5 kcal/mol, only 1.1 kcal/mol lower than the 4-acetylphenyl compound. Compound **1b**, on the other hand, has a barrier about 4 kcal/mol higher than a similar guanidinium ion (Kenyon *et al.*, 1976). Thus, the effect of the acetyl group on the phenylguanidinium ion may be dampened not only by the insulating property of the phenylene group, but also by steric effects present in both **3** and **4**.

The resonance arguments for the relatively high rotational barrier in 1b indicate that the barrier to rotation in acylguanidinium ions (17.6 ± 0.2 kcal/mol) might more closely agree with amidinium ions than with similar guanidinium ions. This indeed appears to be the case. Using the data of Hammond and Neuman (1963), one can estimate a $\Delta G_c^{\ddagger}$ value for acetamidinium chloride (10) in dimethyl sulfoxide of 18 to 19 kcal/mol.

The rotational barrier of 15.6 kcal/mol in 1a can be put into perspective by considering it as being analogous to a vinylogous amide. The well-studied amide, dimethylformamide (8), has been shown to have a rotational barrier ($\Delta G_c^{\ddagger}$) of 21.0 kcal/mol (Rabinovitz and Pines, 1969; Drakenberg *et al.*, 1972), while the corresponding vinylogous compound 9 has a $\Delta G_c^{\ddagger}$ value of 15.6 kcal/mol (Blanchard *et al.*, 1967). Thus, just as the resonance hybrid of compound 9 has an important contribution from resonance structure 9a, so the resonance hybrid of compound 1a has an important contribution from resonance structure 1c:

9a

1c

The possibility exists that rotation about the carbon-nitrogen bond in **1b** might occur by a mechanism involving deprotonation, rotation, followed by reprotonation. In order to explore this possibility, the barrier to rotation was determined at two different acidic pD values. If deprotonation is necessary for rotation to occur, then a higher pD value, with a correspondingly higher proportion of the unprotonated species, should lead to a lower observed rotational barrier. In DCl (pD$\sim$-1), the observed barrier was 17.5 $\pm$ 0.1 kcal/mol, while at pD = 2.0, the barrier was found to be 17.4 $\pm$ 0.1 kcal/mol. {The value of pD = 2.0 was chosen to be within approximately two pK units of the known pK_a value of **1b** (4.45)(Kenyon and Rowley, 1971).} These results imply that it is not likely that the unprotonated species is necessary for the rotational process to occur.

In conjunction with these experiments, we carried out *ab initio* calculations on the barriers to rotation about the exocyclic carbon-nitrogen bonds of compounds **1a** and **1b** as well as calculations on the simpler guanidinium ions **5a**, **5b**, **6** and **7** (See Table I).

With the STO-3G basis set, the acylguanidinium ion **6** has a calculated barrier that is 1.3 kcal/mol higher than that for guanidinium ion itself (**5a**); with the 4-31G basis set this difference was calculated to be 2.2 kcal/mol. This result with the more extended basis set is in reasonably

good agreement with the experimentally determined difference in barriers of ∿ 2.1 kcal/mol between a highly substituted guanidinium ion (4) and acylguanidinium ion (1b). It was considered to be too expensive to carry out calculations at the 4-31G level on 1b itself, but calculations at the STO-3G level on this molecule led to a "predicted" barrier 1.6 kcal/mol higher than that for guanidinium ion (5a).

The *ab initio* calculated bond orders in compound 1b for both the exocyclic carbon-nitrogen bond and the carbon-nitrogen bond not conjugated with the carbonyl (0.446 and 0.441, respectively) were significantly higher than that of the carbon-nitrogen bond which is conjugated with the carbonyl (0.415). Similar relative bond orders were also found in model compound 6. These results are in accord with the resonance arguments presented above in which resonance forms 11a and 11b were favored over resonance form 11c.

For the corresponding acylguanidine 1a, the *ab initio* calculations find a lower barrier than that for the acylguanidinium ion 1b, again consistent with the experimental results, although the calculated difference in the barriers is exaggerated. As expected, the calculated bond orders for the exocyclic and endocyclic C-N bonds (both 0.394) are significantly smaller than that for the conjugated C=N bond (0.438).

The calculations at the 4-31G level on model compounds **5b** and **6** illustrate the important role of the acyl group in raising the rotational barrier of the guanidine. Assuming that compounds **6** and **7** are faithful models of **1a** and **1b**, respectively, the 4-31G calculations are successful in reproducing the relative experimental barriers to rotation in **1a** and **1b**, although the observed difference ($\Delta\Delta G_c^{\ddagger}$) is 2 kcal/mol and the calculated difference between **6** and **7** is only 0.7 kcal/mol. The barriers are, of course, quite sensitive to the carbon-nitrogen bond distances employed, and the use of a somewhat longer C-N distance in **7** would improve the agreement (calculations on compound **5b** suggest, for example, that the barrier to rotation decreases by approximately 0.5 kcal/mol for every 0.01 Å the rotatable C-N bond is lengthened). However, complete geometry optimizations of these structures were considered to be too expensive and no sufficiently accurate x-ray crystal structures of related compounds are available. In view of these uncertainties in the bond lengths involved, we feel that the calculations using the extended 4-31G basis set adequately represent the rotational barriers in these molecules.

EXPERIMENTAL

Materials. Compound **1a** was prepared according to the procedure of Kenyon and Rowley (1971).

The pD = 2.0 buffer solution was prepared by exchanging 85% phosphoric acid three times with 99.8% D_2O, diluting with 99.8% D_2O to 1.0 M D_3PO_4, and adjusting the pD to 2.0 by the addition of anhydrous, powdered K_2CO_3.

NMR Spectroscopy. Both the proton and carbon-13 spectra were obtained with a Varian XL-100 spectrometer in the pulse mode interfaced with a Nicolet Instrument Corporation Model NIC-80 data processer. For the carbon-13 spectra, a Nicolet Multi Observe Nuclei Accessory (MONA) was utilized, and the spectra obtained at 25.158 MHz with broadband proton decoupling on a ∿ 1.2 M solution in 70% D_2O/H_2O. The proton NMR spectra were obtained from ∿ 0.1 M solutions in 99.8% D_2O at 100.1 Hz. Temperatures were measured by direct insertion of a Doric Trendicator 412-A copper-constantan thermocouple into the sample.

Computational Details. We used the program GAUSSIAN 70 (Hehre *et al.*) in the *ab initio* calculations with STO-3G (Hehre *et al.*, 1969) and 4-31G (Ditchfield *et al.*, 1971) basis sets. In the STO-3G calculations on guanidinium ion (**5a**) and acylguanidinium ion (**6**) standard geometries were used and the energies evaluated at planar and perpendicular structures.

Since no x-ray crystal structures were available for either compounds **1a** or **1b**, we used the QCFF-PI (Warshel and Levitt) program to calculate reasonable geometries for **1b**. For **1a**, we used the same geometry as for **1b**, except that the proton was removed. In order to simplify comparisons among

calculations of barriers using the STO-3G basis set, the exocyclic carbon-nitrogen bond around which rotation occurs was kept at bond distance R = 1.37 Å for all calculations at this level. This value was the STO-3G optimized value found earlier for guanidinium ion (Kollman et al., 1975).

For the 4-31G calculations on **6**, we used the minimum energy C-N distance (1.33 Å) found for compound **5a** (Kollman et al., 1975). In the 4-31G calculations on **5b** and **7** we used R (C=N) = 1.26 Å and R (C-N) = 1.365 Å; the former was taken from the calculated C=N bond length for methylenimine (Lehn, 1970); the latter was chosen so that the sum of the three carbon-nitrogen bond lengths would be the same as the sum of the three carbon-nitrogen bond lengths in the guanidinium ion (**5a**).

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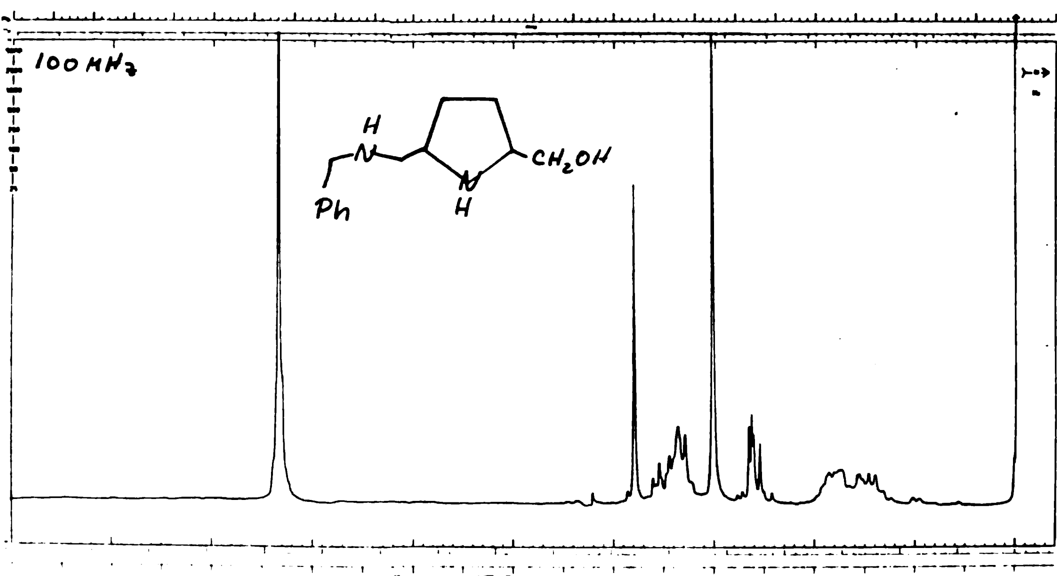
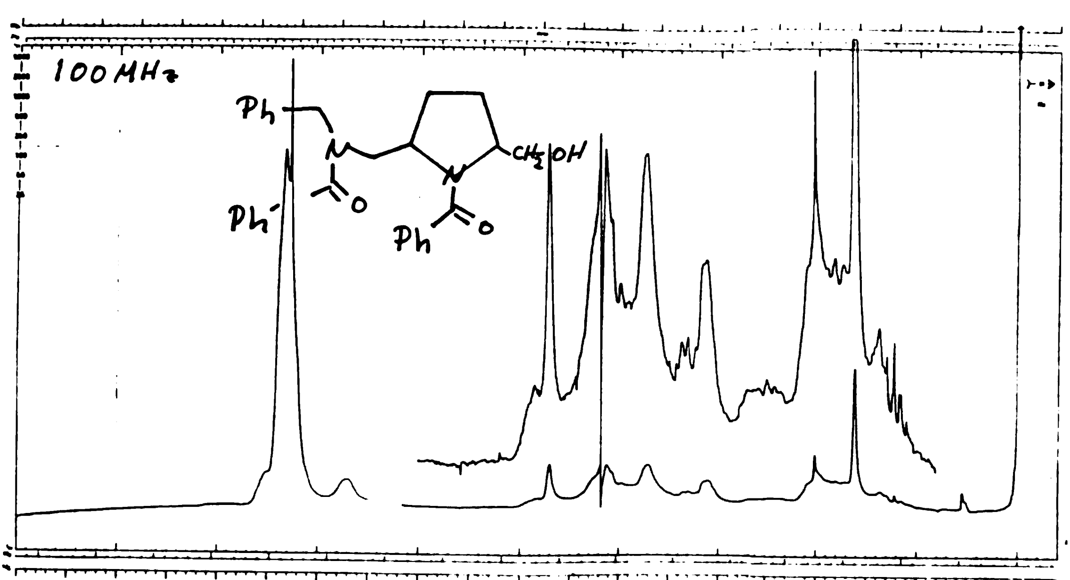
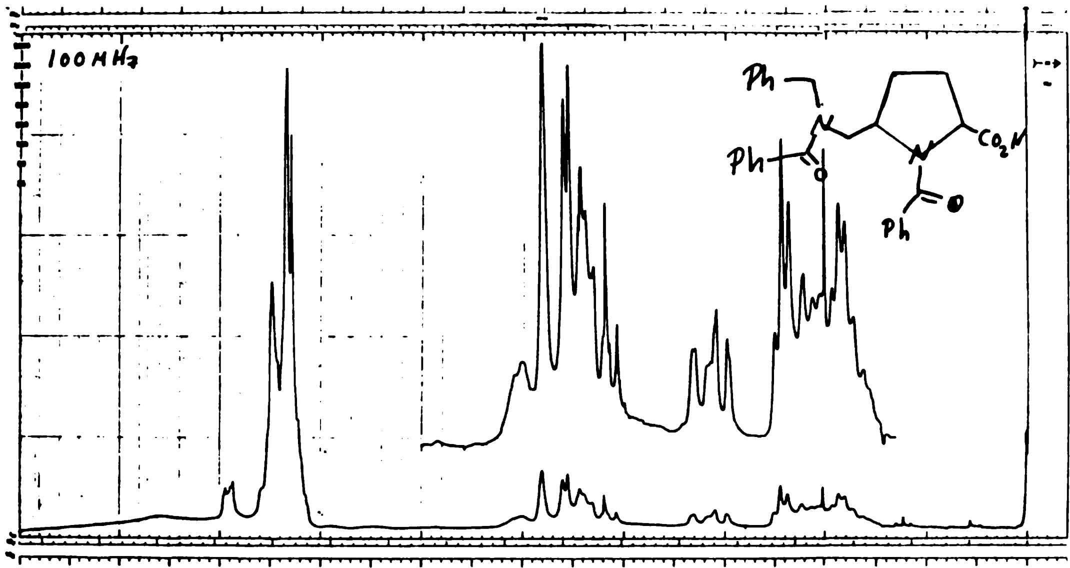
APPENDIX A

PROTON NMR SPECTRA



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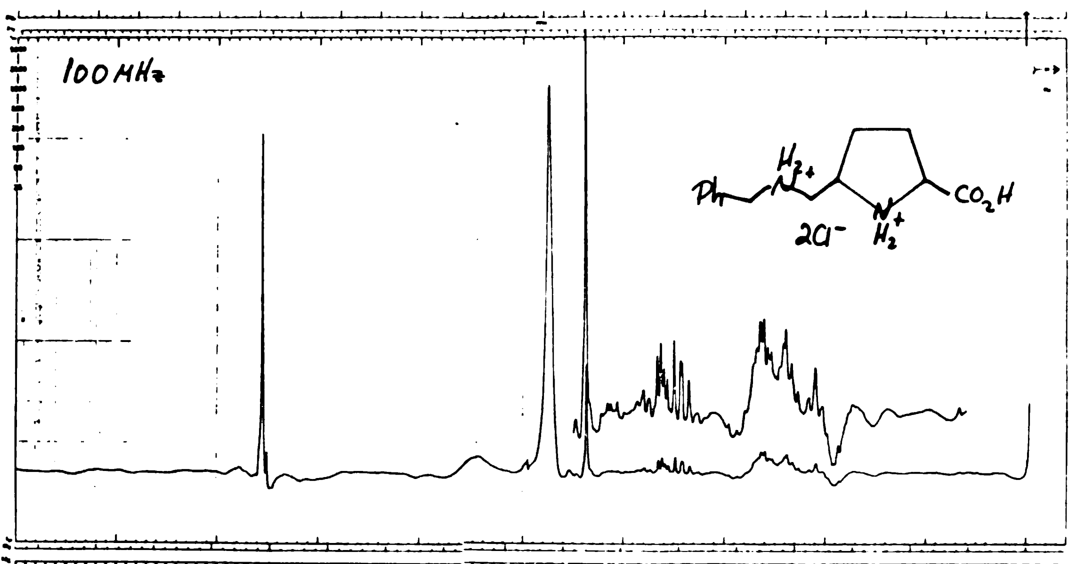
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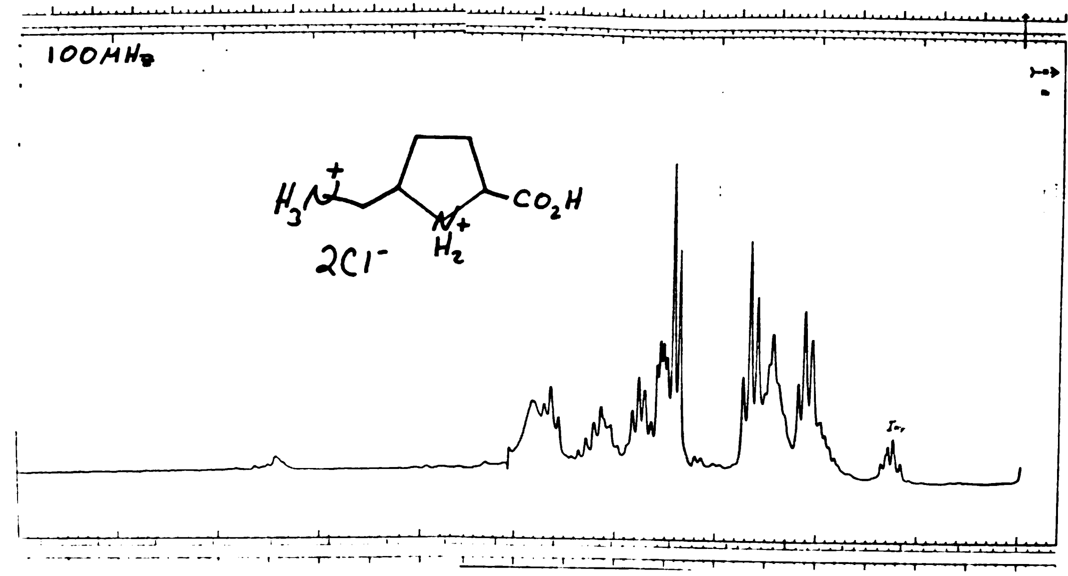

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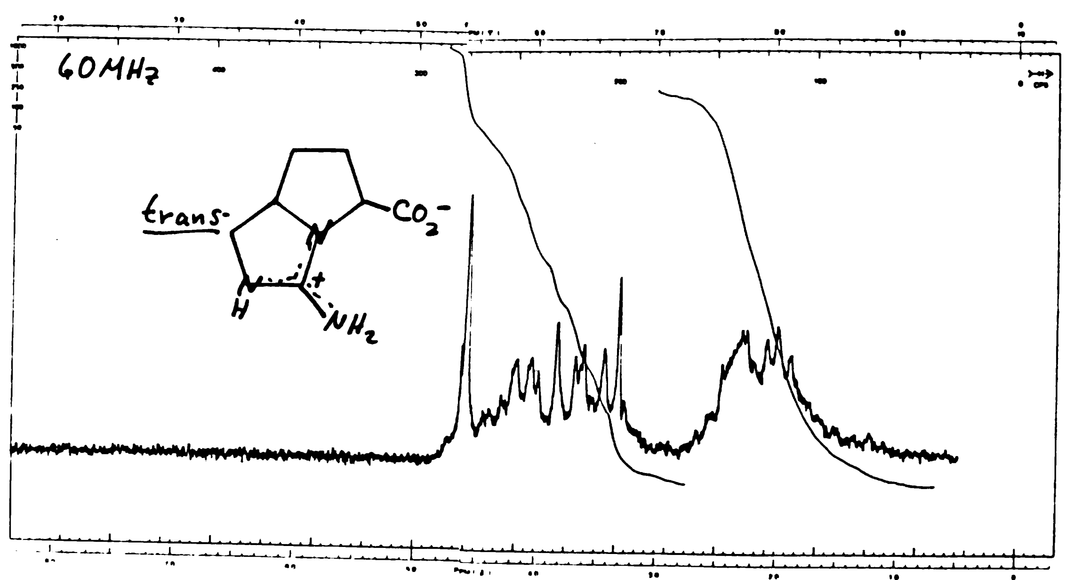

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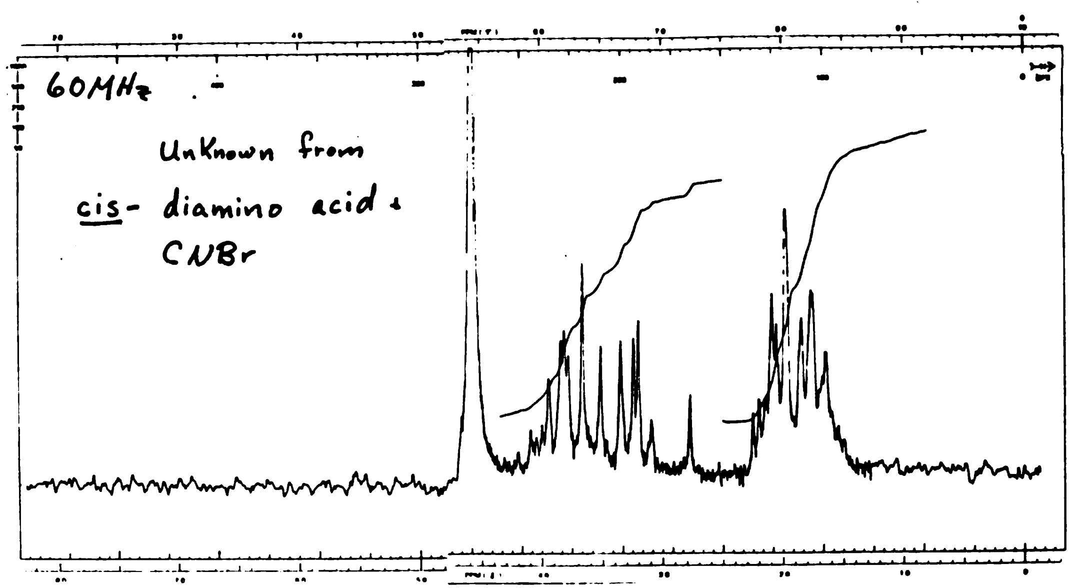
NH_2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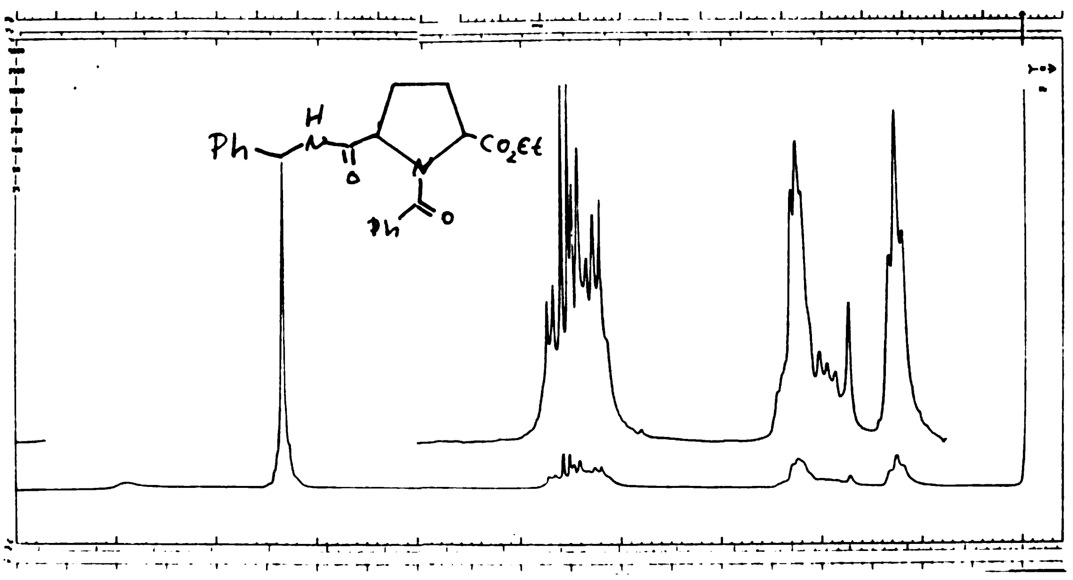
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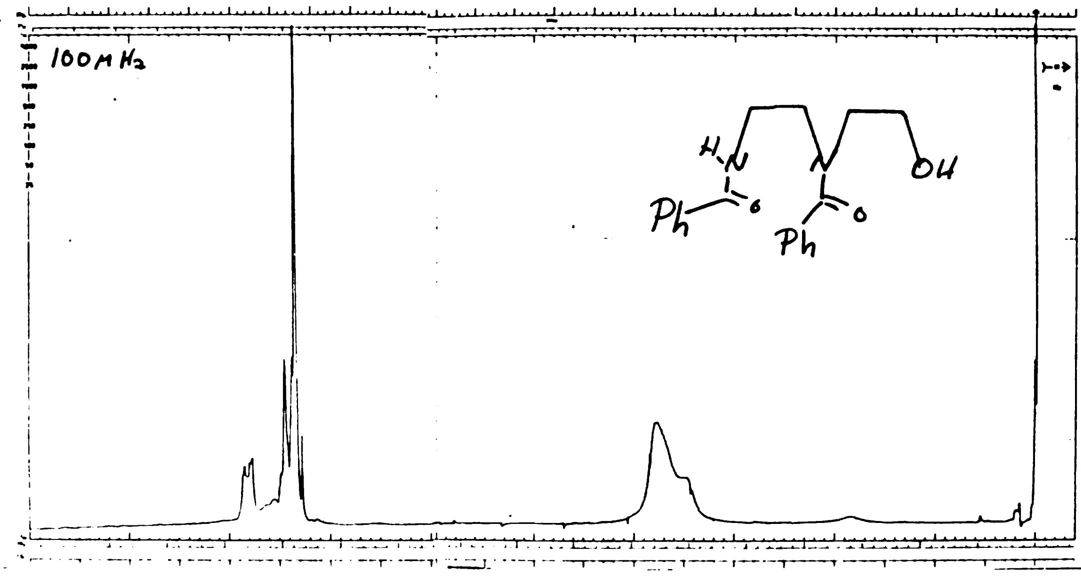
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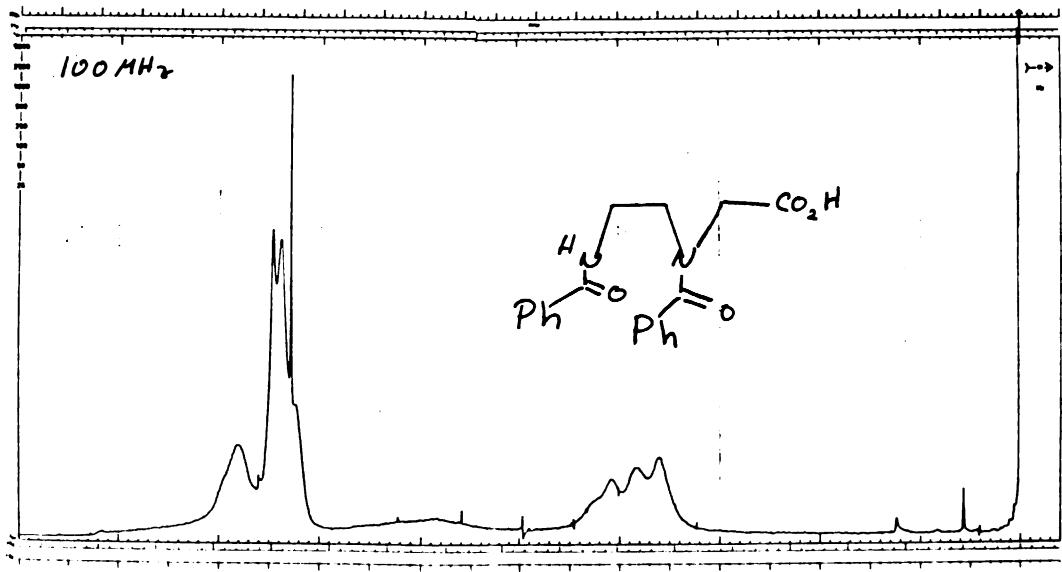
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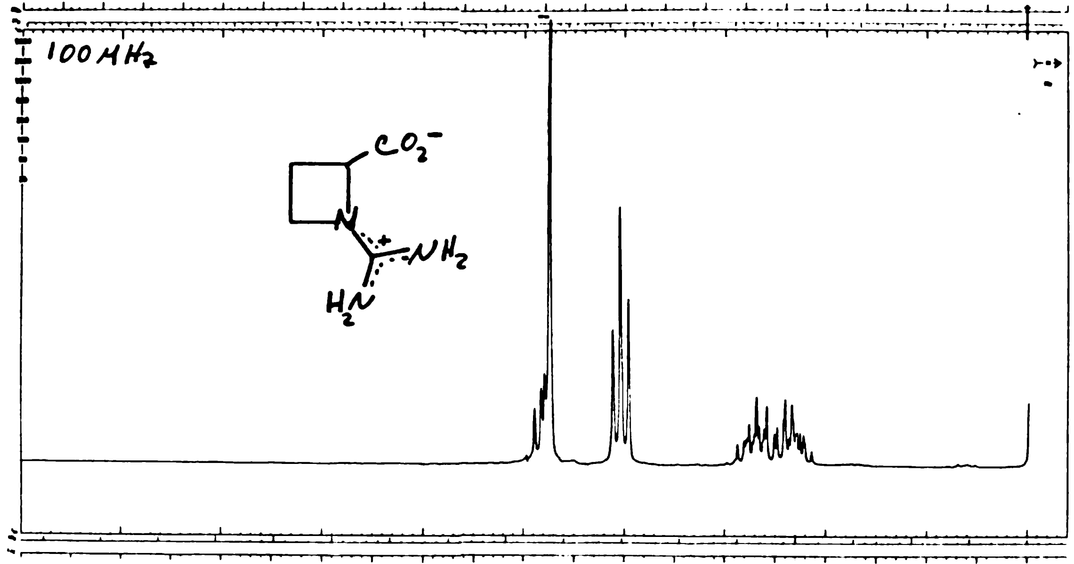
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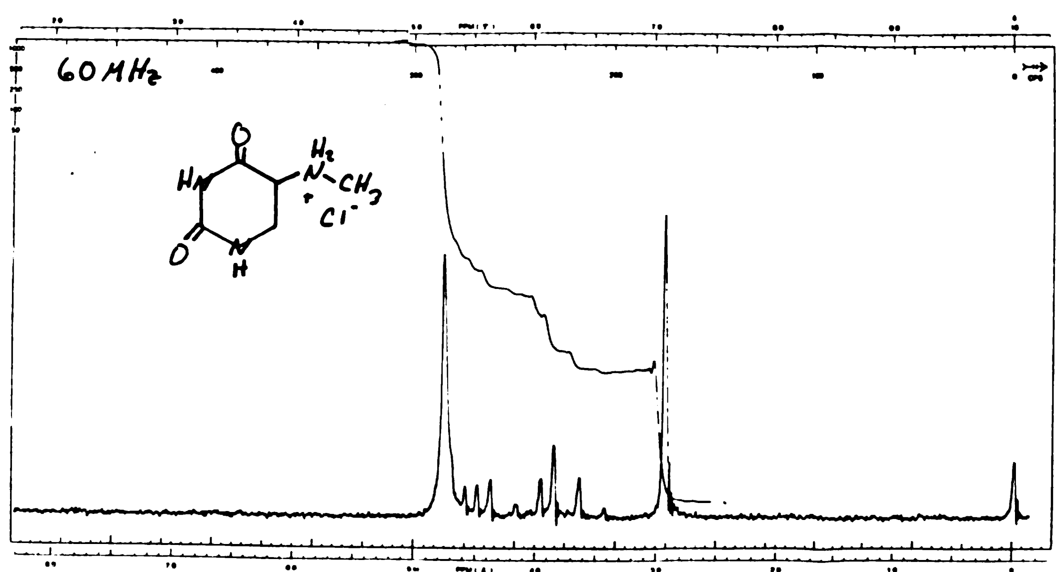
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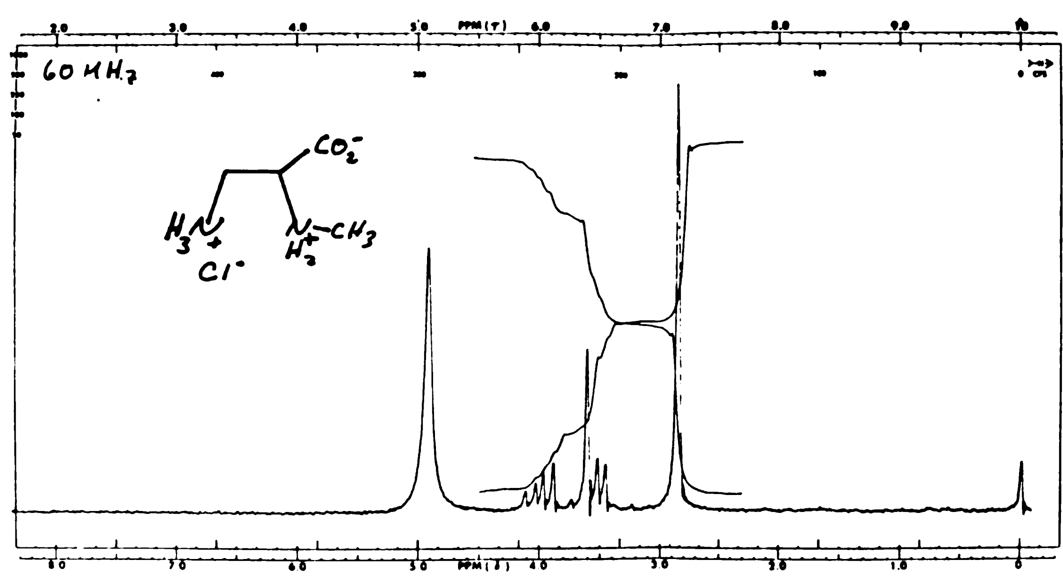
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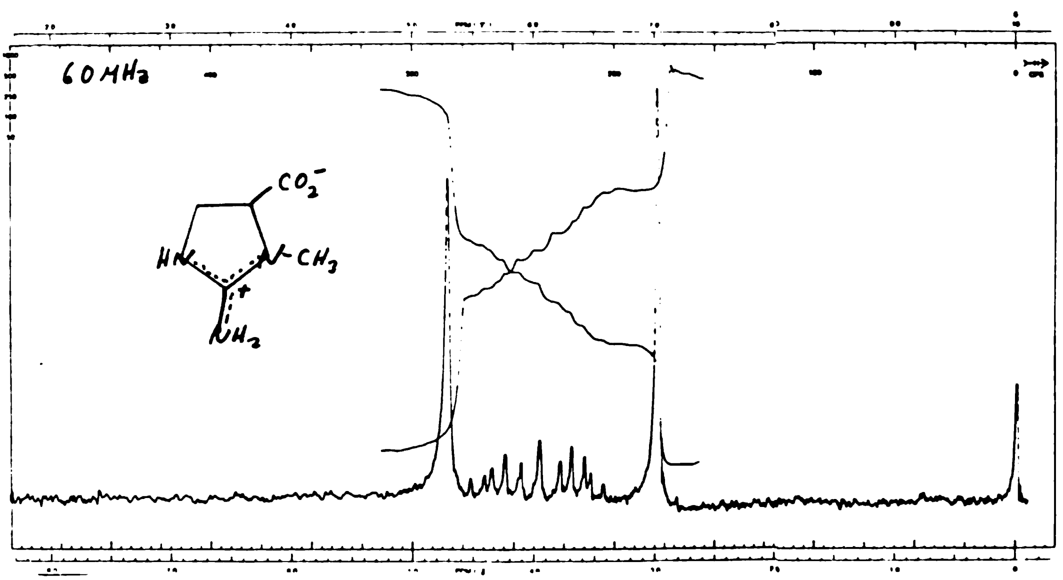
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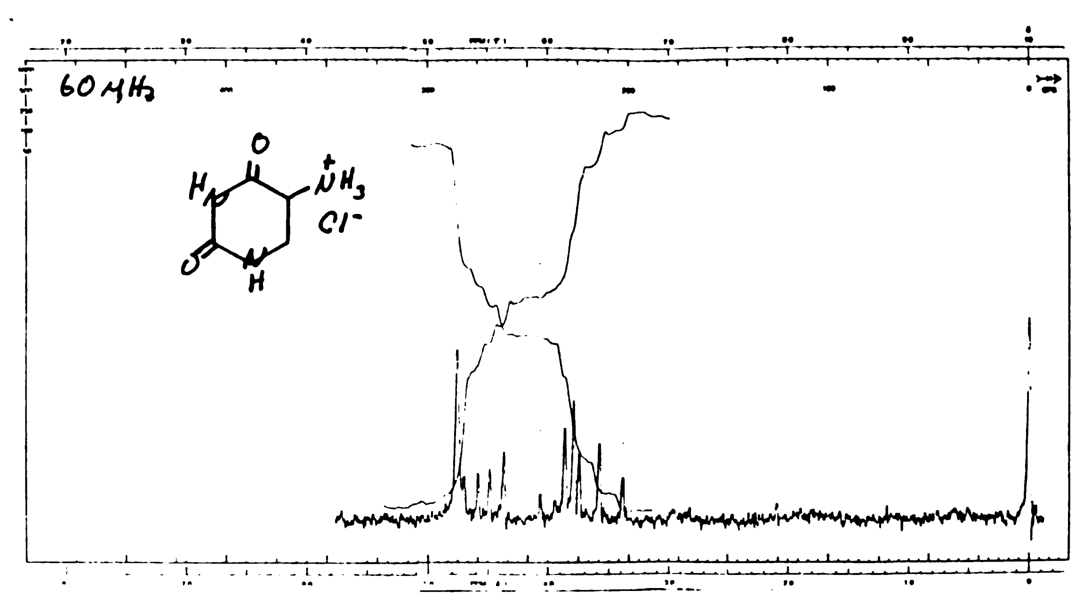
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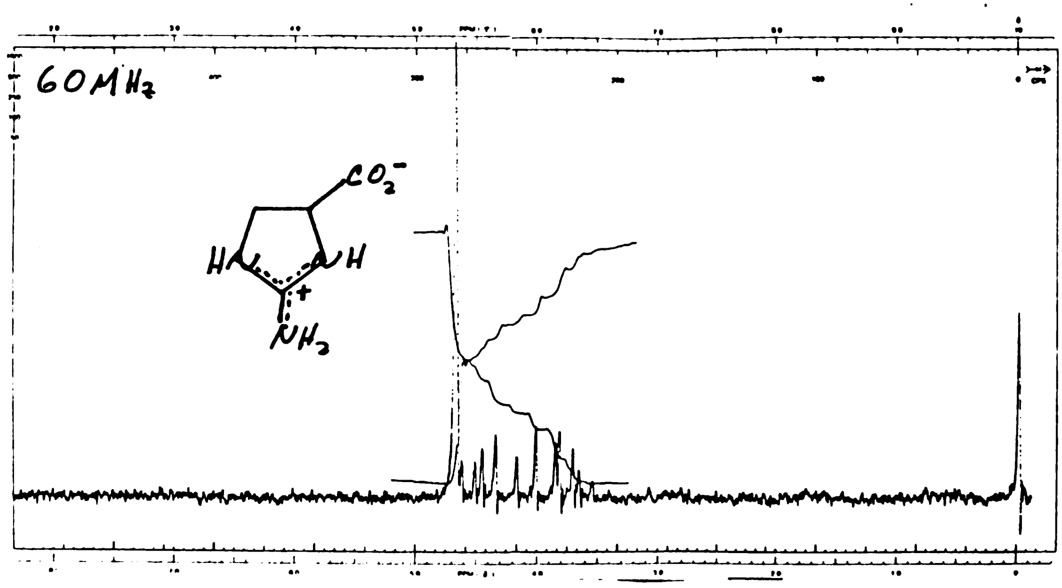
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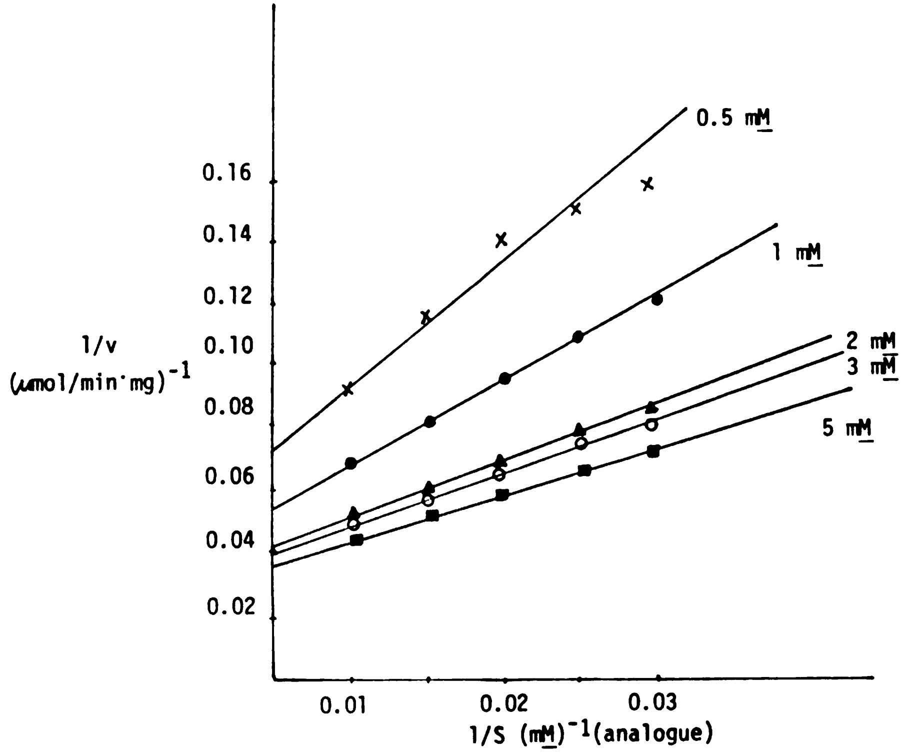
APPENDIX B

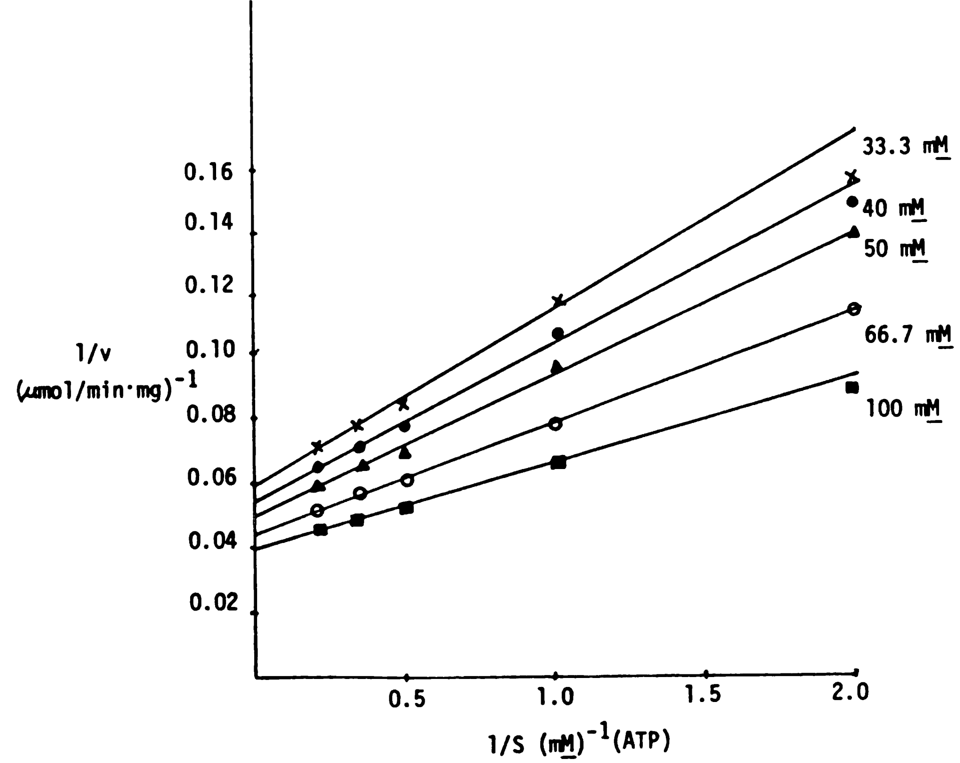
CREATINE KINASE ASSAY COMPONENTS AND DOUBLE RECIPROCAL PLOTS FOR R-N-AMIDINOAZETIDINE-2-CARBOXYLIC ACID AND R-N-METHYL-N-AMIDINOALANINE

The creatine kinase assays briefly described in Chapter 2 utilized a 2 mL assay mixture made up of the following components.

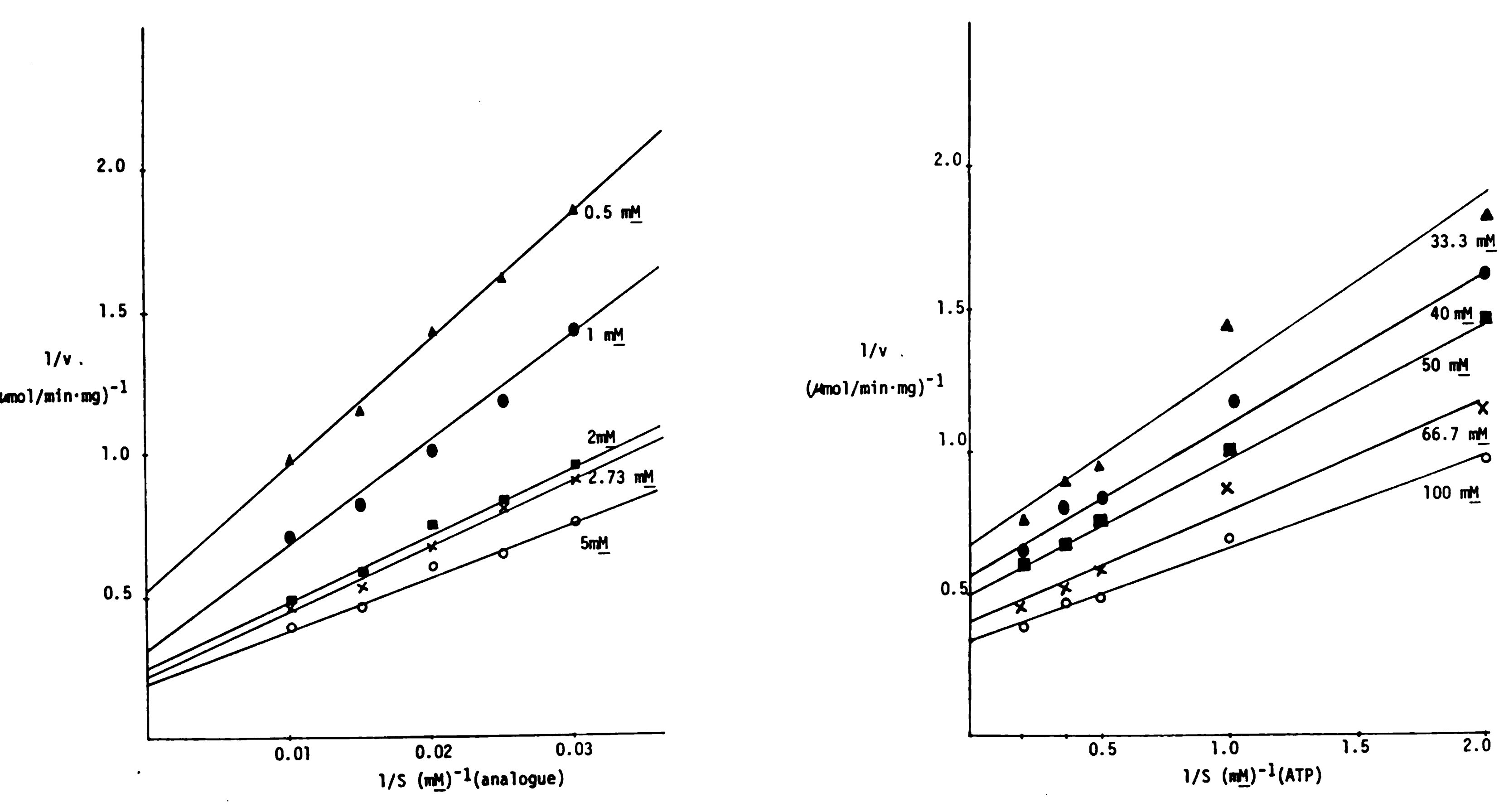
1. 0.1 mL of a 2% BSA solution which had been adjusted to pH 9.0 with 1.0 N and 0.1 N NaOH (final concentration = 0.1%).
2. 0.1 mL of the appropriate ATP stock solution which had been adjusted to pH 9.0 with 1.0 N and 0.1 N NaOH; final ATP concentrations used were generally 5 mM, 3 mM, 2 mM, 1 mM, and 0.5 mM and each stock solution used was adjusted with $Mg(OAc)_2$-NaOAc solutions to give a free Mg(II) concentration of 1.0 mM and an ionic strength (μ) of ~50 mM.
3. 1.0 mL of appropriate creatine or creatine analogue solution.
4. 0.8 mL distilled water minus the amount of titrant (2.5 mM NaOH) needed to bring the assay mixture to pH 9.0.
5. Creatine kinase, the amount added being dependent on the reactivity of the particular substrate and the specific activity of the enzyme used.

KINETIC DATA FOR R-N-AMIDINOAZETIDINE-2-CARBOXYLIC ACID





KINETIC DATA FOR R-N-METHYL-N-AMIDINOALANINE



APPENDIX C

INITIAL STEPS IN THE SYNTHESIS OF 2-IMINO-1,3-DIAZABICYCLO {3.2.0}HEPTANE-7-CARBOXYLIC ACID (21)

A logical follow-up of the synthesis and investigation of the enzyme-substrate interactions of the *trans*- bicyclic creatine analogue with two five-membered rings described in Chapter 1, is the synthesis of a bicylic analogue containing a four- and a five-membered ring.

This analogue contains an azetidine ring instead of the pyrrolidine ring of the bicyclic creatine analogue discussed in Chapter 1. When the monocyclic creatine analogues are compared, the R-azetidine analogue has a V_{max} (rel.)/K_m approximately 80 times larger than the corresponding R-pyrrolidine analogue. Based on these results, it would be interesting to compare the two types of bicyclic creatine analogues.

A synthesis of compound 21, based on the successful synthesis of the *trans*- bicyclic creatine analogue described in Chapter 1 has been initiated and the following compounds have been prepared.

<u>Diethyl α,α'-Dibromoglutarate</u>. In a one liter round-bottom flask equipped with a magnetic stirrer and a reflux condenser was placed 266 g (2.0 mol) glutaric acid to which was added 440 mL (717 g, 6.0 mol) thionyl chloride in one portion. The mixture was stirred and heated to 60° C for 4 hr. After rotary evaporation to remove excess thionyl chloride, the crude product was distilled to yield 262.8 g (1.56 mol, 78%) glutaryl chloride, bp 65-71°/0.4 mm. The acid chloride was then stirred in a 1 L round-bottom flask fitted with a high efficiency condenser and heated to 70° C. Using a constant addition funnel, 192 mL (561.6 g, 3.51 mol) bromine was added at such a rate that the loss of Br_2 through the condenser was kept to a minimum. At this scale, addition took 40 hr, and the mixture was stirred for another 12 hr. After cooling, the reaction mixture was transferred to an addition funnel and added in a small stream to an ice-cooled 2 L flask containing 1200 mL anhydrous ethanol. This was stirred at room temperature for 1 - 2 days. After evaporation to ~800 mL and addition of an equal volume of water, the product was extracted with ether. If necessary, salt (NaCl) was added to break emulsions. The ether extract was washed with 5% $NaHSO_3$ (4 x 100 mL), 5% $NaHCO_3$ (until evolution of CO_2 ceases), and finally water or saturated NaCl. The solution was dried over $MgSO_4$, filtered, and evaporated to give an almost

colorless residue pure enough for further use. The yield of the crude residue was 501.5 g (93% from acid chloride, 73% overall). After distillation, the recovery was 493 g (1.43 mol) bp 95-105°/0.01 mm.

The proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.32 (6H, triplet, J=7 Hz), 2.70 (2H, triplet, J=7 Hz), 4.30 (4H, quartet, J=7 Hz), 4.43 (1H, triplet, J=6.5 Hz), and 4.57 (1H, triplet, J=6.5 Hz).

N-Benzhydryl-2,4-dicarbethoxyazetidine. A 500 mL round-bottom flask was charged with 28.7 g (83 mmol) diethyl α,α'-dibromoglutarate in 150 mL acetonitrile and the mixture was heated to reflux. Through the condenser was added 45.3 g (0.247 mol) benzhydrylamine in another 50 mL acetonitrile. The mixture was held at reflux for 90 hr. As benzhydrylamine hydrobromide precipitated in large quantities during the course of the reaction, smooth boiling was facilitated with the use of magnetic stirring. After cooling and filtering the bulk of the salt, the solvent was removed *in vacuo*. Ether was added to the residue (150 mL), the remaining salt was filtered, and the filtrate evaporated to dryness to give 31.8 g of crude product. Purification was achieved using column chromatography with Florisil (100-200 mesh) as the adsorbent. The crude product (20.9 g) was dissolved in ethyl acetate:hexanes (25:75, v/v), applied to 250 g Florisil in the same solvent system, and eluted. Fractions

(100 mL) were collected and the contents were monitored by TLC on EM(Merck) silica with F_{254} U V indicator using ethyl acetate:hexanes (50:50, v/v) as eluent. The R_f values for trans- and cis- product were 0.54 and 0.47, respectively. This was sufficient separation to allow for the isolation of the two products on the column and NMR spectra to be taken. The isomer assignment was made on the basis of these spectra. Including some contamination of the trans- isomer by starting material, the total recovery of both isomers was 18.95 g.

The cis- compound, mp 78-79° C had the following properties:

Anal. Calcd. for $C_{22}H_{25}NO_4$: C, 71.91; H, 6.86; N, 3.81. Found: C, 71.84; H, 6.85; N, 3.75.

The proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.03 (6H, triplet, J=8 Hz), 2.42 (2H, overlapping triplets, J= 8 Hz), 3.66 (2H, triplet, J=8 Hz), 3.93 (4H, quartet, J= 8 Hz), 4.67 (1H, singlet), and 7.33 (10H, broad multiplet).

The proton NMR spectrum ($CDCl_3$) of the trans- compound showed peaks at δ 0.95 (6H, triplet, J=7 Hz), 2.43 (2H, triplet, J=7 Hz), 3.85 (4H, quartet, J=7 Hz), 4.32 (2H, triplet, J=7 Hz), 5.13 (1H, singlet), and 7.10 - 7.70 (10H, broad multiplet).

Diethyl γ-Chloro-N-benzhydrylglutamate Hydrochloride. The synthesis for N-benzhydryl-2,4-dicarbethoxyazetidine was followed up to the point at which the ether solution

of the crude product was filtered. The clear solution was treated with a stream of HCl gas, generating at first a white precipitate of benzhydrylamine hydrobromide from the small quantity of unreacted amine. Further bubbling of HCl into the solution yielded an off-colored flocculent solid which clumped into an oily amorphous mass from which the ether was decanted. The semi-solid was dissolved in $CHCl_3$, dried over K_2CO_3, and evaporated to give a glassy solid which was slightly hygroscopic. The decanted ether was allowed to stand at room temperature for 1 - 2 days while white crystals formed. These were filtered and dried. Requiring no recrystallization, the mp 147-148° C and the NMR was almost identical to that of the glassy solid. Elemental analysis indicated that the ring had been opened to give the _gamma_-chloroglutamate derivative.

Anal. Calcd. for $C_{22}H_{27}NO_4Cl_2$: C, 60.00; H, 6.18; N, 3.18. Found: C, 60.21; H, 6.17; N, 3.18.

The proton NMR spectrum showed peaks at δ 1.27 (6H, overlapping triplets, J=7 Hz), 2.87 (2H, multiplet), 4.17 (5H, multiplet), 4.75 (1H, triplet, J=8 Hz), 5.67 (1H, singlet), 7.42 (5H, multiplet), and 7.80 (5H, multiplet).

N-Benzyl-2,4-dicarbethoxyazetidine. In a 1 L round-bottom flask with powerful magnetic stirring and a reflux condenser was placed 135.5 g (0.392 mol) diethyl α,α'-dibromoglutarate with 400 mL of benzene and the solution was heated to reflux. An addition funnel with a pressure-

equalizing side arm was fitted to the top of the condenser and 126.0 g (1.17 mol) benzylamine in 100 mL benzene was added dropwise. The addition of the amine and the subsequent 60 hr of reflux were accompanied by precipitation of benzylamine hydrobromide. After cooling and filtering the bulk of the salt, the solvent was evaporated *in vacuo*. Ether was added to the residue and after the last salt was filtered, was removed by evaporation. Distillation at high vacuum yielded a fraction with bp 117-127°/0.01 mm which weighed 70.85 g (0.244 mol; 62%). The wide boiling range is most likely due to the presence of the two isomers.

Anal. Calcd. for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.27; N, 4.81. Found: C, 65.70; H, 7.15; N, 4.70.

The proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.17 (6H, overlapping triplets, J=7 Hz), 2.50 (2H, triplet, J= 7 Hz), 3.62 (1H, triplet, J= 8.5 Hz), 3.92 (2H, singlet), 4.09 (4H, quartet, J=7 Hz), 4.2 (1H, triplet, J=7 Hz), and 7.35 (5H, singlet).

2,4-Dicarbethoxyazetidine. A solution of 18.6 g (0.0639 mol) of N-benzyl-2,4-dicarbethoxyazetidine in 150 mL ethanol was cautiously added to 2.0 g 10% palladium on charcoal in a 500 mL hydrogenation bottle. After hydrogenation at 3 atmospheres for 50 hr, the theoretical quantity of H_2 had been taken up. The catalyst was filtered and the

solvent evaporated to give 12.3 g (.0612 mol; 96%) product. The reaction could be monitored by TLC using EM (Merck) silica gel plates with ethyl acetate:hexanes (50:50, v/v) as solvent. When the starting material spot at R_f=0.6 had faded, leaving only the spot at R_f=0.15, the reaction was complete. Impure starting material (contaminated with bromo compounds) poisoned the catalyst and required secondary hydrogenations. The final product was generally quite pure by NMR without distillation. Any attempt at preparative distillation, even with diffusion pump vacuum, resulted in no more than 30% recovery of product with the remainder converted to a black tar in the still pot. A small sample was distilled at 76-77°/0.02 mm and submitted for elemental analysis.

Anal. Calcd. for $C_9H_{15}NO_4$: C, 53.72; H, 7.51; N, 6.96. Found: C, 53.68; H, 7.49; N, 6.82.

The proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.30 (6H, triplet, J=7 Hz), 2.73 (2H, triplet, J=8 Hz), 3.20 (1H, singlet), 4.27 (4H, quartet, J=7 Hz), and ~4.3 (2H, triplet, J=8 Hz).

N-Benzyl-2-benzylcarbamyl-4-carbethoxyazetidine. In a 15 mL pear-shaped flask were placed 4.92 g (0.0169 mol) N-benzyl-2,4-dicarbethoxyazetidine and 1.81 g (0.0169 mol) benzylamine as neat liquids. Under a nitrogen atmosphere, the flask was immersed in a 100° C oil bath for 50 hr (25

hr is probably sufficient). After suspending in ethyl acetate:hexanes (50:50, v/v), the flask is heated on a steam bath to reflux, and ethyl acetate is added until all the oil is dissolved. Upon cooling slowly, the dibenzylation product (N-benzyl-2,4-dibenzylcarbamyl-azetidine) crystallizes and can be filtered. After recrystallization from the same solvent system, analytically pure crystals are obtained, mp 134.0-135.5^{o} C.

Anal. Calcd. for $C_{26}H_{27}N_3O_2$: C, 75.52; H, 6.58; N, 10.16. Found: C, 75.32; H, 6.53; N, 10.05.

The proton NMR spectrum ($CDCl_3$) showed peaks at δ 2.17 (1H, triplet, J=8 Hz), 2.85 (1H, multiplet), 3.62 (2H, singlet), 3.68 (2H, triplet, J=8 Hz), 4.18 (4H, doublet, J=5.5 Hz), and 6.80 - 7.35 (17H, multiplet).

The monoamide can be further purified by column chromatography on silica gel using ethyl acetate:hexanes (50:50, v/v) as the solvent. Application of the material dissolved in a small quantity of the same solvent is possible only if all the diamide has been removed. Fractions were monitored by TLC (same adsorbent and solvent) with the spots of starting material at R_f=0.38, 0.48 (two isomers), monoamide at R_f=0.13, 0.20 (two isomers), and diamide at R_f<0.04 being visualized either by UV light or immersion in an I_2 chamber.

The proton NMR spectrum ($CDCl_3$) showed peaks at δ 1.20 (3H, overlapping triplets, J=7 Hz), 2.0 - 3.0 (2H, multiplet),

3.6 - 4.4 (8H, multiplet), and 7.2 (10H, multiplet).

The chemical ionization mass spectrum showed a strong molecular ion at m/e 353 with fragments at 248, 218, and 91.

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