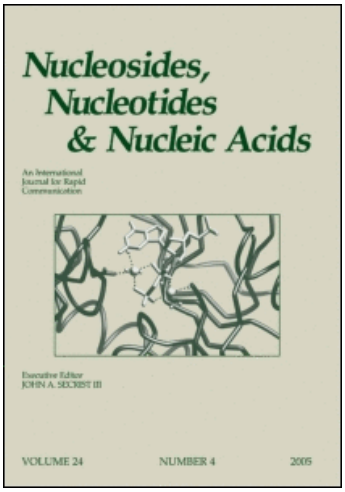

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Study of Creatinine and its 5-Alkoxy Analogs: Structure and Conformational Studies in the Solid and Solution States by X-Ray Crystallography, NMR, UV and Mass Spectrometry

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STUDY OF CREATININE AND ITS 5-ALKOXY ANALOGS: STRUCTURE AND CONFORMATIONAL STUDIES IN THE SOLID AND SOLUTION STATES BY X-RAY CRYSTALLOGRAPHY, NMR, UV AND MASS SPECTROMETRY

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ABSTRACT:

Creatinine (2-amino-1,5- dihydro-1-methyl-4-imidazolone) is a natural by-product of cellular metabolism related to muscular mass. It is excreted in human urine and is necessary for normal kidney function. Urinary secretion of creatinine serves as a bench mark for several clinical measurements. Recently, in our laboratories, during the course of an investigation of the urine of cancer patients for tumor markers, we found some new metabolites of creatinine. These were identified as 5-methoxy and 5-ethoxy creatinine by UV, NMR and Mass spectrometry and their tautomeric structures confirmed by crystal structural investigations of the metabolites. The x-ray crystallographic analysis confirmed the structures of the compound and showed that it exists in the 2-amino form. The spectral characteristics of these new compounds and the generality of the reaction are discussed in this paper. Creatinine, when allowed to react with methanol, ethanol and propanol respectively, in the presence of charcoal and air gave the 5-methoxy, 5-ethoxy and 5-propoxy creatinine derivatives respectively, suggesting a generality of a reaction. The reaction of creatinine with alcohols in the presence of charcoal and air takes place through a free radical reaction mechanism.

INTRODUCTION:

Creatinine (2-amino-1,5-dihydro-1-methyl-4-imidazolone) is a by-product of cellular metabolism related to muscle mass. It is excreted in human urine in the range of 1.2 to 1.8 grams/day for a normal kidney function. Urinary excretion of creatinine serves as a reference point

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** **Deceased**

for a number of clinical measurements. Creatinine is known to produce mutagens upon heating with sugars (1).

In our laboratory, investigation of the urine of cancer patients for tumor markers (2) led to the finding of two new creatinine derivatives; which were isolated and characterized as 5-(α-hydroxy)acetamido creatinine and 5-ethoxy-creatinine. The latter compound is formed by a novel reaction of creatinine with ethanol in the presence of charcoal and oxygen (3). These findings of 5-substituted creatinine led to the present study of this unusual chemical reaction of creatinine. Exhaustive literature search gave no insight into the origin or chemical formation of the above substances except in a publication by Dox and Yoder (4) who first reported the reaction of creatinine HCl with methyl-, ethyl- and butyl-alcohols and identified the product as creatinine methyl ester. Later, Kapfhammer (5) showed the compound to be a derivative of creatinine in which alcohol was bound neither as a solvent nor by an ester linkage, in "some uncertain manner". Thus the charcoal-celite column conditions of isolation from urine were simulated in the reaction flask to study the reactivity of creatinine with methanol, ethanol, propanol and butanol (see reaction scheme).

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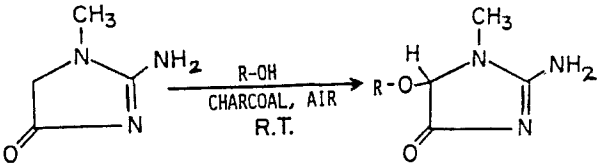


CREATININE 5-ALKOXYCREATININE

Reaction scheme showing the formation of the alkoxy derivative

Tijssen and co-workers (6) have shown that creatinine, adsorbed on activated carbon, undergoes catalytic oxidation in air. They concluded that during the oxidation, one of the protons at C-5 of the imidazole ring in creatinine is substituted by a group containing an oxygen atom. Further, Smith and co-workers (7) observed the same catalytic activity of activated carbons towards creatinine during dialysis. A preliminary results on the formation of 5-alkoxy derivative was previously reported (8-9).

EXPERIMENTAL METHODS:

Chemicals: The starting material, creatinine was purchased from Aldrich Chemical Co. Analytical grade alcohols were used for the reaction. Neutral charcoal was obtained from Fisher Scientific Co. All other chemicals were of reagent grade.

Instrumentation:

Preparative reverse phase high performance liquid chromatographic separation was done on a Zorbax ODS column using Altex pump (Model 256-07). The set-up was equipped with a UV detector for monitoring the eluting fractions at 254 nm wavelength.

The NMR spectra were recorded on a Bruker WP 200 spectrometer at 30°C. Deuterated dimethyl sulfoxide, DMSO-d_6 served as a solvent and as an internal reference. Chemical shifts were recorded relative to TMS.

The mass spectral data was obtained from a Finnigan 4000 GC-MS system. Samples were sent for elemental analysis to Robertson Laboratory, Inc., Florham Park, NJ. The reported melting points are uncorrected and are obtained from a Meltemp apparatus. Preparative thin layer chromatography was performed on 20 x 20 cm glass plates coated with 1 mm thick layer of silica gel GF (Analtech Inc.). The developed chromatogram was visualized with a 254 nm UV lamp. For the evaluation of biological activity, CV-1 cells were used for assay of antiviral activity and L1210 system for antitumor activity.

The crystal structural data on the creatinine derivatives were collected on a CAD-4 diffractometer fitted with a monochromator. The data were processed with Enraf Nonius package software (10) on a Microvax computer. The diagrams were plotted with ORTEP program (11).

Procedure

A typical reaction included dissolving 1.13 g of creatinine (0.01 M), in 120 ml of 50% ethanolic ammonia (2 N) solution. A mixture of 2 g of acid washed neutral charcoal and 2 g of celite was added to the stirred solution. The reaction mixture was kept stirring in a water bath at 37°C. The flask was equipped with a water condenser to prevent the loss of alcohol. The progress of reaction was monitored by analytical TLC on silica gel plates using isopropanol:conc. ammonium hydroxide:water (7:12) as eluant. At the end of a 48 h period, most of the creatinine was recovered into two major compounds. The R_f values were 0.58 and 0.38. Creatinine eluted in between these two derivatives (R_f = 0.48). The compound with higher R exhibited spectral properties characteristic of 5-ethoxycreatinine (Table 1). The one with lower R_f (0.38) appeared to be of a dimerized product. For samples of analytical purity, an aliquot of reaction mixture was chromatographed using reverse phase HPLC. Both the compounds were obtained as white powder (yield 45%) based on HPLC peak height. Like creatinine, both the derivatives did not give sharp melting points. 5-Ethoxycreatinine decomposed at 213°C and the dimerized product at 130°C. Elemental analysis for $C_6H_{11}N_3O_2$: calculated (%) C = 45.86; H = 7.01; N = 26.75; found (%) C = 45.68; H = 7.12; N = 26.67.

(a) Using the same procedure as above, the reaction with (a) 2 N 50% methanolic ammonia gave 2 compounds of R_f 0.53 and 0.37. This reaction was much faster compared to the reaction with ethanol and the conversion of creatinine was complete in 24 h. The compound with the lower R_f was identical to that obtained from the ethanol reaction. The compound with the higher R_f (0.53) had spectral properties corresponding to 5-methoxycreatinine. This compound was obtained as a white crystalline solid from HPLC purification (yield 50%). M.P. = 210 decomposed. Elemental analysis for $C_5H_9N_3O_2$: calculated (%) C = 41.96; H = 6.29; N = 29.38; found (%) C = 41.93; H = 6.20; N = 29.31.

(b) The reaction with 2 N 50% n-propanolic ammonium gave two compounds of similar R_f , 0.63 and 0.37. This was a much slower reaction compared to the above. The amount of unchanged creatinine after a week was about one-fourth. The compound with R_f 0.63 had spectral properties indicating the formation of 5-n-propoxycreatinine. This compound decomposed at 220°C and was obtained as a white solid from HPLC purification (yield 30%). The lower R_f product was identical to that obtained from previous reactions. Elemental analysis for $C_7H_{13}N_3O_2$: calculated (%) C = 49.12; H = 7.60; N = 24.56; found (%) C = 48.91; H = 7.89; N = 24.18.

(c) Similarly, two compounds were obtained with 2 n-butanolic ammonia reaction. The R_f values were 0.65 and 0.37. Again, this reaction was slower than the n-propanol reaction. About a third of creatinine remained unchanged after a week. Spectral properties of the product with the higher R_f (0.65) identified this compound as 5-n-butoxycreatinine. This compound was obtained as a white solid after HPLC purification (M.P. = 204°C decomposed; yield 25%). Again, the compound with the lower R_f appeared to be a dimerized product similar to those obtained in the

Table 1. Chromatographic Data

Compound	TLC - R_f Values A[1]	B[2]	RP- HPLC[3] Retention Time (Min.)
creatinine	0.48	0.38	11.00
5-Methoxycreatinine	0.53	0.53	18.00
5-Ethoxycreatinine	0.58	0.68	21.00
5-n-Propoxycreatinine	0.63	0.80	24.00
5-n-Butoxycreatinine	0.65	0.83	33.00

1. Anal. cellulose plates, cel. mn 300 UV_{254}; eluant: 85% isopropyl alcohol.
2. Anal. silica gel plates, sil. G/UV_{254}; eluant: isopropyl alcohol: conc. ammonium hydroxide: water (7:1:2 v/v).
3. Zorbak-ODS preparative column; 0-100% water-acetonitrile, 1 hr. linear gradient elution.

other reactions. Elemental analysis for $C_8H_{15}N_3O_2$: calculated (%) C = 51.80; H = 8.11; N = 22.70; found (%) C = 51.59; H = 8.09; N = 22.70.

(d) Crystals of creatinine and its ethoxy and methoxy derivatives were obtained by controlled vapor diffusion techniques. Crystals of 5-methoxycreatinine were obtained by a slow evaporation of a super saturated solution from ethanol/methanol/water. 5-ethoxycreatinine crystals crystals were obtained by the method of seeding small crystals obtained from a mixture of trifluroethanol/water. Creatine monohydrate crystals were obtained by vapor diffusion of propanol/butanol mixture into an aqueous solution of the compound. The unit cell dimensions for these compounds were determined by measuring 25 reflections with θ in the range from 25 to 50 degrees on a CAD4 diffractometer (see Table 2).

Complete three dimensional intensity data were collected by the $\omega/2\theta$ technique using CuKα radiation (λ = 1.5418 Å). The scan widths were calculated using the relation (A + B tan θ) with values of 0.5 and 0.15 for A and B respectively. Aperture widths were determined using the equation (3.0 + 1.2 tan θ)mm. The maximum time spent on any reflection measurement was 100 seconds and the background count time was half the scan time. A faster scan was used for strong reflections. The intensities were monitored by measuring three reflections after every hour of x-ray exposure, and the variation of intensities was less than 3% during the complete data collection. The orientation matrix was checked every 100 reflections. The intensities of three reflections at or near $\chi = 90°$ was measured for all values of ϕ from 0 to 360° and the resultant curve of transmission factors as a function of ϕ was used to calculate the absorption for all the reflections.

The structures of the different creatinine derivatives were established by the application of multi solution techniques (12) which readily yielded all the non-hydrogen atoms in the molecule. These structures were refined by the full matrix least squares method, the function minimized being $\sum w (| k F_o| - | F_c|)^2$. Difference Fourier maps were used to locate the atomic positions of all hydrogen atoms in the molecule. The structure was further refined using isotropic temperature factors for the hydrogen atoms and anisotropic thermal parameters for the non-hydrogen atoms. The final reliability index R defined as

Table 2

CRYSTAL DATA FOR ETHOXY, METHOXY CREATININE AND CREATINE MONOHYDRATE

	Ethoxy creatinine	Methoxy creatinine	creatine H_2O
Empirical Formula	$C_6N_3O_2H_{11}$	$C_5N_3O_2H_9$	$C_4N_3O_3H_{11}$
F.W.	157	143	138
Crystal System	Monoclinic	Monoclinic	Monoclinic
Space group	$P2_1/c$	$P2_1/a$	$_1P2$ /a
a	10.869 (2) Å	12.928 (3) Å	12.159 (1)Å
b	5.756 (4)	5.928 (1)	5.038 (2) c
	13.284 (4)	10.091 (2)	12.491 (2)
α	90.00°	90.00°	90.00°
β	102.37 (2)	112.83(1)	108.87(1)
γ	90.00	90.00	90.00
Volume	811.7 Å^3	713.2 Å^3	724.0 Å^3
Z	4	4	4
D_o (g/c.c) (flotation in Bromoform and Benzene)	1.29	1.29	1.27
D_C (g/c.c)	1.286	1.287	1.266
μ (cm^{-1})	7.87	7.86	8.48
λCuKα	1.5418 Å	1.5418 Å	1.5418Å
Total no. of reflections	1899 (1133≥3σ)	1744 (788 ≥3σ)	1532 (976 ≥3σ)
Final R value	0.080	0.065	0.042
Reference	This Work	This Work	This Work

$$\frac{\sum ||kF_o|-|F_c||}{\sum |F_o|}$$

were 0.080 for 5-methoxycreatinine, 0.062 for 5-ethoxycreatinine and 0.032 for creatine monohydrate. The final fractional atomic parameters are given in Table 3 for the 5-methoxy creatinine, Table 4 for the 5-ethoxycreatinine and Table 5 for creatine monohydrate. The atomic scattering factors and the dispersion corrections for carbon, nitrogen, oxygen and hydrogen were taken from the " International Tables for X-ray Crystallography" (13).

RESULTS AND DISCUSSION:

The reactions of creatinine and lower aliphatic alcohols were carried out in the presence of acid washed neutral charcoal and alcoholic ammonia solution. The progress of the reaction was

TABLE 3

5-METHOXYCREATININE

Final Fractional Positional and thermal Parameters with estimated standard deviations given in Parentheses

ATOM	X	Y	Z	B(Å^2)†
O4	0.1117(2)	0.0026(5)	0.3313(3)	4.34(7)
O5	-0.1149(2)	-0.1046(5)	0.1083(3)	4.43(6)
N1	-0.1666(2)	0.0776(5)	0.2846(3)	3.35(7)
N2	-0.1562(2)	0.4094(5)	0.4161(3)	3.54(7)
N3	0.0022(2)	0.2508(5)	0.3968(3)	3.26(7)
C1	-0.2835(3)	0.0197(8)	0.2484(5)	5.10(1)
C2	-0.1106(3)	0.2524(7)	0.3669(4)	3.14(8)
C4	0.0204(3)	0.0649(7)	0.3320(4)	3.31(8)
C5	-0.0883(3)	-0.0661(6)	0.2530(4)	3.23(8)
C6	-0.1240(4)	0.0934(9)	0.0255(5)	5.9(1)
H1N2	-0.229(3)	0.411(6)	0.396(4)	3.9(9)*
H2N2	-0.102(3)	0.516(7)	0.485(4)	6(1)*
H1C1	-0.320(5)	0.02(1)	0.129(7)	11(20*
H2C1	-0.295(4)	-0.14(1)	0.249(5)	9(2)*
H3C1	-0.309(4)	0.089(8)	0.296(5)	8(1)*
H1C6	-0.135(3)	0.057(7)	-0.079(4)	6(1)*
H2C6	-0.172(4)	0.194(9)	0.035(6)	11(2)*
H3C6	-0.058(5)	0.19(1)	0.035(6)	11(2)*
HC5	-0.088(3)	-0.219(6)	0.292(4)	4(9)

†Starred values were refined isotropically. Anisotropically refined atoms are given in the form of the isotropic equivalent dispacement parameter defined as : (4/3) * [A2* B(1,1) + B2* B(2,2)+ C2* B(3,3)+ AB (Cos Gamma)* B(1,2)+ AC (Cos Beta)* B (1.3) + BC (Cos Alpha) * B (2,3)].

monitored by thin layer chromatography. The R_f values of products in different solvent systems showed the formation of two major creatinine derivatives. The fastest moving compound in each of the reactions was identified as the corresponding 5-n-alkoxy derivative. Several variations in the procedure were done for mechanistic interpretation. It was found that the presence of charcoal and oxygen is essential. There was no product formed in the presence of nitrogen or in the absence of charcoal. This clearly indicates catalytic oxidation involving reactive oxygen species that might be generating free radicals in adsorbed creatinine molecule at 5-position which then reacts with alkoxy radicals of alcohols (generated on carbon surface) to form the 5-substituted derivatives. This speculation is based on the report (14) that X-ray irradiated creatinine (guanidine derivative and the 'hydrated' form of creatinine) forms a radical at the α-methylene position by the elimination of a hydrogen atom.

Interestingly, these alkoxy derivatives were the predominant products in the reactions without ammonia (see reaction scheme). Reactions in both methanol and ethanol showed quantitative conversion of creatinine into 5-alkoxy compound over a period of 24 h and 48 h respectively, in the

TABLE 4

5- ETHOXYCREATININE

Final Fractional Positional and thermal Parameters with estimated standard deviations given in Parentheses

ATOM	X	Y	Z	B (Å^2)†
O4	0.3420 (6)	0.0332 (7)	0.7985 (6)	5.9 (1)
O5	0.1546 (7)	-0.1025 (8)	0.9190 (5)	6.5 (1)
N1	0.3090 (7)	0.0519 (8)	1.0511 (7)	5.0 (1)
N2	0.4286 (7)	0.3870 (8)	1.1149 (7)	5.2 (1)
N3	0.4064 (6)	0.2595 (12)	0.9448 (5)	4.6 (1)
C1	0.2921 (9)	-0.0362 (19)	1.1512 (8)	6.3 (2)
C2	0.3837 (8)	0.2388 (15)	1.0389 (6)	4.6 (1)
C4	0.3490 (8)	0.0773 (15)	0.8901 (8)	4.7 (1)
C5	0.2854 (8)	-0.0800 (15)	0.9582 (8)	4.9 (2)
C6	0.1186 (20)	-0.3400 (21)	0.8849 (19)	11.5 (4)
C7	-0. 0203 (20)	-0.3492 (23)	0.8594 (21)	12.6 (5)
H1N2	0. 410 (4)	0.382 (9)	1.177 (4)	3.9 (9)*
H2N2	0. 514 (3)	-0.009 (8)	1.394 (5)	4.3 (8)*
H1C1	0.299 (4)	0.075 (7)	1.201 (4)	4.5 (6)*
H2C1	0.250 (5)	0.104 (8)	1.182 (5)	4.7 (7)*
H3C1	0.207 (4)	-0.101 (7)	1.139 (4)	4.9 (6)*
H1C6	0.084 (8)	-0.272 (9)	0.941 (8)	6.1 (9)*
H2C6	0.193 (8)	-0.447 (8)	0.863 (9)	6.5 (9)*
H1C7	-0.031 (9)	-0.269 (10)	0.828 (9)	7.4 (9)*
H2C7	-0.027 (7)	-0.315 (7)	0.750 (9)	8.9 (9)*
H3C7	-0.055 (8)	-0.367 (9)	0.705 (10)	11.2 (9)*
HC5	0.318 (5)	-0.202 (5)	0.973 (9)	5.8 (7)*

†starred atoms were refined anisotropically. Anisotropically refined atoms are given in the form of the isotropic equivalent dispacement parameter defined as: (4/3) * [A2* B(1,1)+ B2* B(2,2)+ C2* B(3,3)+ AB (cos Gamma) * B (1,2)+ AC (cosBeta)* B(1,3)+ BC (cos Alpha) * B(2,3)].

presence of oxygen at 37°C. The one with lower R_f than creatinine gave the same value in all four reactions with different alcohols. Further, this same compound was formed in the reaction using 2 N ammonia solution. ^1H and ^{13}C NMR data showed changes in the chemical shifts involving the α-methylene group in the molecule. The mass spectrum of this compound gave ions at m/z 128 and m/z 256 suggesting that the parent molecule is a dimeric type of compound, probably an aldol type of compound formed between the methylene carbon of one creatinine molecule and the carbonyl carbon of another. The other possibility is the dimer involving a peroxy linkage between the methylene carbons of the two imidazole rings. Further refinement of data and additional information is needed to elucidate the structure of this common product. Interestingly, this derivative is not formed in the reactions using 50% alcohols instead of alcoholic ammonia.

Table 5
Creatine Monohydrate

Final Fractional Positional and anisotropic thermal Parameters with estimated standard deviations given in parentheses

ATOM	X	Y	Z	B(1,1)	B(2,2)	B(3,3)	B(1,2)	B(1,3)	B(2,3)
N1	0.6719 (2)	0.1308 (4)	0.3807 (2)	0.0074 (1)	0.0395 (9)	0.0052 (1)	-0.0079 (6)	0.0063 (2)	-0.0019 (6)
C2	0.4956 (2)	-0.2429 (5)	0.3498 (2)	0.0055 (1)	0.0300 (9)	0.0061 (1)	-0.0009 (6)	0.0056 (2)	0.0033 (6)
N3	0.5645 (2)	-0.2159 (4)	0.2743 (2)	0.0054 (1)	0.0310 (8)	0.0057 (1)	-0.0010 (5)	0.0056 (2)	-0.0017 (5)
C3	0.3986 (1)	-0.0381 (5)	0.3295 (2)	0.0050 (1)	0.0326 (9)	0.0045 (1)	-0.0018 (6)	0.0039 (2)	-0.0014 (6)
O2	0.3482 (1)	-0.0286 (4)	0.4019 (1)	0.0070 (1)	0.0512 (9)	0.0056 (1)	0.0070 (5)	0.0070 (1)	0.0029 (5)
N2	0.6923 (2)	0.0282 (5)	0.2106 (2)	0.0069 (1)	0.0404 (9)	0.0055 (1)	-0.0061 (6)	0.0070 (2)	-0.0028 (6)
O1	0.3751 (1)	0.1047 (4)	0.2433 (1)	0.0085 (1)	0.0455 (9)	0.0059 (1)	0.0122 (6)	0.0074 (2)	0.0090 (5)
C1	0.6421 (2)	-0.0212 (5)	0.2889 (2)	0.0048 (1)	0.0311 (9)	0.0049 (1)	0.0017 (6)	0.0042 (2)	0.0012 (6)
C4	0.5284 (2)	-0.3673 (6)	0.1691 (2)	0.0089 (2)	0.0402 (11)	0.0073 (2)	-0.0076 (8)	0.0079 (2)	-0.0102 (8)
OW	0.3103 (2)	0.1403 (5)	0.0150 (1)	0.0130 (2)	0.0505 (9)	0.0054 (1)	0.0033 (7)	0.0063 (2)	-0.0050 (6)
H1OW	0.771 (3)	0.194 (8)	0.006 (3)	4.3 (8)*					
H2OW	0.339 (2)	0.116 (6)	0.089 (2)	2.6 (6)*					
H1C2	0.039 (2)	0.072 (5)	0.661 (2)	1.2 (5)*					
H2C2	0.452 (2)	0.227 (6)	0.570 (2)	1.6 (5)*					
H1C4	0.592 (3)	0.432 (6)	-0.150 (2)	3.2 (7)*					
H2C4	0.471 (3)	-0.505 (8)	0.171 (3)	4.3 (9)*					
H3C4	0.483 (3)	-0.252 (8)	0.105 (3)	4.5 (9)*					
H1N1	0.728 (2)	0.261 (6)	0.387 (2)	2.2 (6)*					
H2N1	0.650 (2)	0.100 (7)	0.443 (2)	2.3 (6)*					
H1N2	0.748 (2)	0.159 (6)	0.224 (2)	1.6 (6)*					
H2N2	0.171 (2)	0.550 (7)	0.141 (2)	3.1 (6)*					

starred B-values were refined isotropically. Anisotropically refined atoms are defined as: (4/3) * [A2* B(1,1)+ B2* B(2,2) + C2* B(3,3)+ AB cos (gamma)* B(1,2)+ AC cos (beta) * B (1,3) + BC cos (alpha) * B(2,3)].

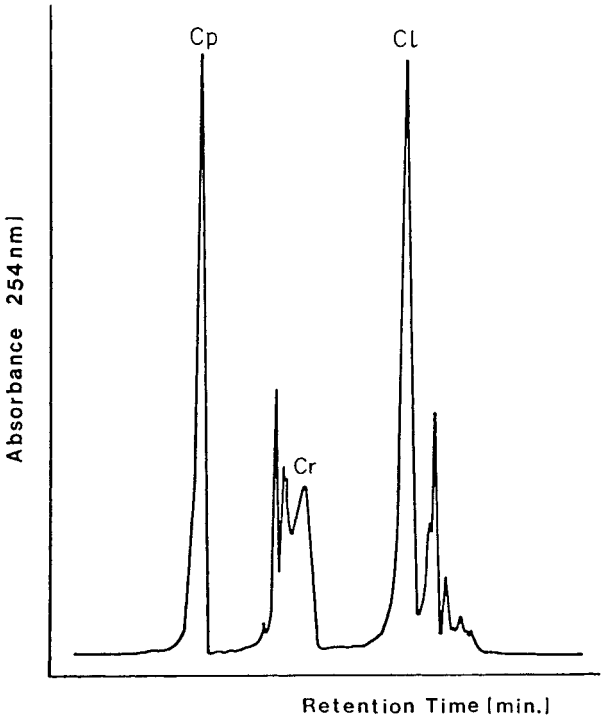


Fig.1. An HPLC elution profile for Creatinine- n-Propanol mixture.

The 5-ethoxycreatinine, co-chromatographed with the compound isolated from urine, had identical spectral properties. The structural identification of this compound was established from the study of the collision activation mass spectra which showed the presence of creatinine moiety (3).

The HPLC elution pattern shown in Fig. 1 is a typical profile for the oxidative reaction of creatinine with the aliphatic alcohols. This particular chromatogram shows 5-propoxycreatinine (Cp) eluting at 24 min in water-acetonitrile, 60 min gradient system while creatinine (Cr) and the dimer type derivative (Cl) elute early in the gradient. The propanol and butanol reaction mixtures had appreciable amounts of unreacted creatine as compared to the methanol and ethanol reactions. As shown by the HPLC elution profile, only the methanol and ethanol reactions showed a complete conversion of creatinine which showed the sluggishness of the reaction with alcohols containing longer alkyl chains. It is a well known fact that the adsorption capacity of charcoal decreases with increasing chain length of the alcohols and therefore, it is reasonable to assume this will also effect the rate of the reaction. The increase in R_f values and TLC and HPLC retention times as shown in Table 1 is in good agreement with the increasing order of nonpolarity and the size of the alkoxy substituent at the 5-position in the molecule.

The ultraviolet absorption spectra shown in Fig. 2 is clear evidence that the compound I isolated from urine and the chemically prepared 5-ethoxycreatinine are identical as both give same absorption curves at different pHs (see 2b and 2c). Compared to the parent molecule (Fig. 2a), 5-ethoxycreatinine does not exhibit any absorption maxima in acidic pH; only the end absorption is

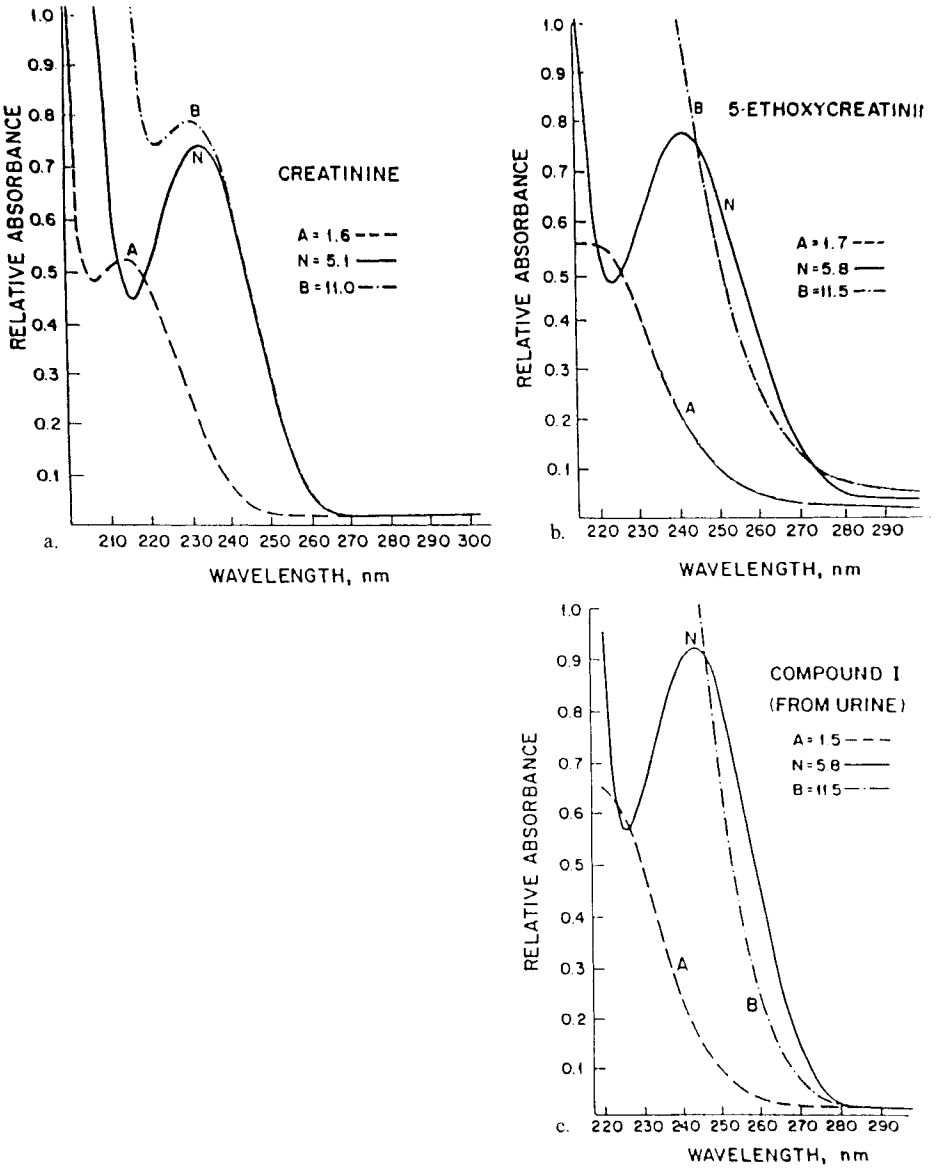


Fig.2. UV spectra showing the absorption curve shift in 5-ethoxycreratinine (b) as compared to creatinine (a). The absorption curve of compound isolated from human urine identical to that of (b) is shown in (c).

Table 6. UV Data: (Alkaline pH)

Compound		γ_{max} (nm)	ϵ
1.	Creatinine	234	4985
2.	5-Methoxycreatinine	242	5148
3.	5-Ethoxycreatinine	242	4898
4.	5-n-Propoxycreatinine	242	4446
5.	5-n-Butoxycreatinine	242	4588

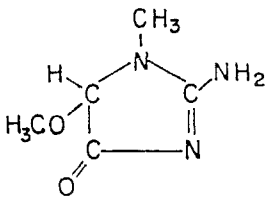
observed. This suggests ring degradation to form a creatine derivative. The absorption maxima values reported in Table 6 shows a characteristic shift for 5-alkoxy derivatives. All these different compounds showed absorption maxima in the range typical of glycoamidines of acylimino type (15). The EI mass spectra of all the alkoxy derivatives gave the corresponding molecular ion (see Table 7). The fragmentation pattern was similar in the four compounds. The common fragment ions at m/z 128 and 112 resulted from the loss of an alkyl group and an alkoxyl group respectively. Also, the fragment ion due to the loss of carbonyl group (M^+ -28) was observed. The collision activation mass spectra produced from m/z 112 and 113 was indistinguishable form the spectra generated in the same manner from creatinine. This demonstrated the presence of creatinine moiety in the derivative. As an example, the fragmentation pattern of 5-n-Butoxycreatinine is shown in Fig. 3. The mass spectral data for compound I from urine and 5-ethoxycreatinine were identical (3).

The PMR spectra of 5-ethoxycreatinine (Fig. 4a) exhibited characteristic resonance relative to TSP at γ3.03 (J, 3H) for the N-methyl group, γ3.58 (m, 2H) for the methylene protons of the ethyl group and γ5.10 (s, 1H) for the 5-methylene proton.

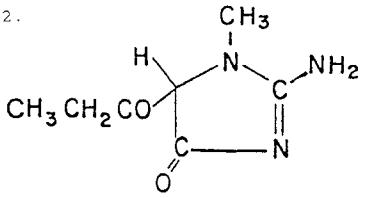
The resonance chemical shifts of compound I from urine (Fig. 4b) was identical to that of 5-ethoxycreatinine. Interestingly the splitting pattern of the methylene protons of ethyl group was a multiplet rather than a quartet. This multiplet collapsed into two adjacent doublets upon irradiation of the methyl resonance. Thus this distinct AB pattern suggests a nonequivalence of the methylene protons. Creatinine and the 5-alkoxy derivatives exhibited only one resonance for the amino protons

Table 7. Major Fragment Ions in 5-Alkoxy Creatinine.

1.

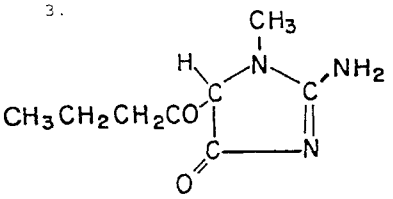


2.

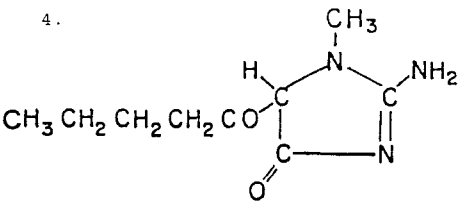


m/z	(Rel. Int.)	Ion	m/z	(Rel. Int.)	Ion
143	(12.5)	M^+	157	(27.39)	M^+
128	(8.62)	M^+-CH_3	128	(6.95)	M^+-CH_3
115	(62.93)	M^+-CO	129	(32.60)	M^+-CO
112	(14.22)	M^+-OCH_3	112	(16.08)	$M^+-C_2H_5$

3.



4.



m/z	(Rel. Int.)	Ion	m/z	(Rel. Int.)	Ion
171	(4.90)	M^+	185	(4.27)	M^+
143	(87.27)	M^+-CO	157	(92.72)	M^+-CO
128	(25.45)	$M^+-C_3H_7$	128	(41.81)	$M^+-C_4H_9$
112	(86.36)	$M^+-OC_3H_7$	112	(100)	$M^+-OC_4H_9$

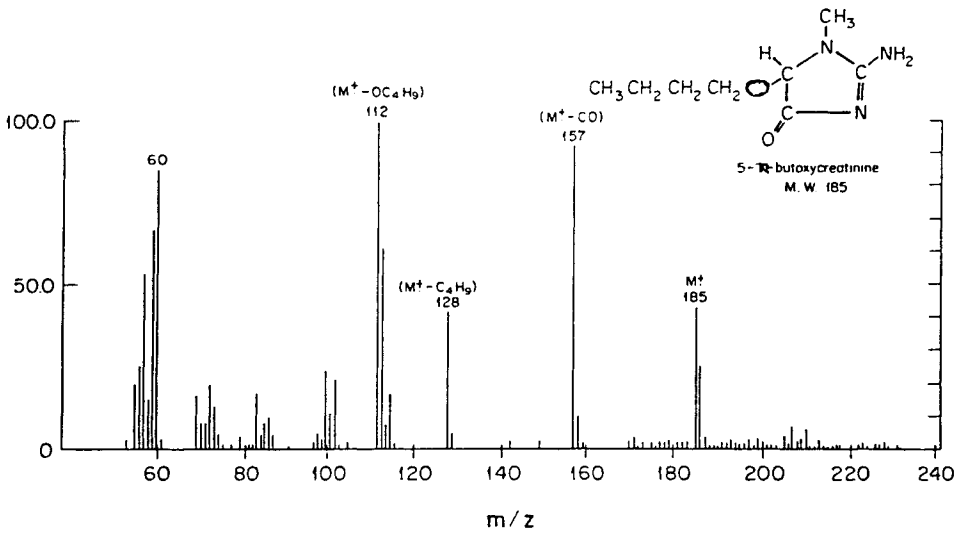


Fig.3. An EI- MS pattern of 5-n- butoxy creatinine obtained from Finnigan 4000 GC-MS Spectrometer.

in deuterated dimethyl sulfoxide which suggests the C=N bond is conjugated with the carbonyl group, that is, the creatinine moiety exists in the acylimino form. This data is in agreement with the finding of Kowalsky and Ratner (16) who observed only one type of nitrogen bound protons (amino) in creatinine. The methylene proton at C-5 did not exchange with deuterium. The proton chemical shift values for all the derivatives is given in Table 8. The methyl group on 5-methoxycreatinine (Fig. 4c) resonates at 3.12 ppm from TMS and shifts up field with the increasing alkyl chain as seen in the higher homologue.

Comparison of ^{13}C NMR spectral data of creatinine with that of the 5-alkoxy analogs (see Table 9) reveals that the substitution results in the down field shift of the α-carbon by about 32 ppm. An unusual trend was observed in the case of the carbonyl carbon which shifted by about 2.4 ppm up field. The guanidium carbon showed a slight up field shift. The spectrum in Fig. 5 depicts carbon resonances of 5-methyl creatinine.

These compounds were assayed for antiviral and antitumor activities and were found to be inactive. It appears that these alkoxycreatinine derivatives are non-toxic. Therefore, it is tempting

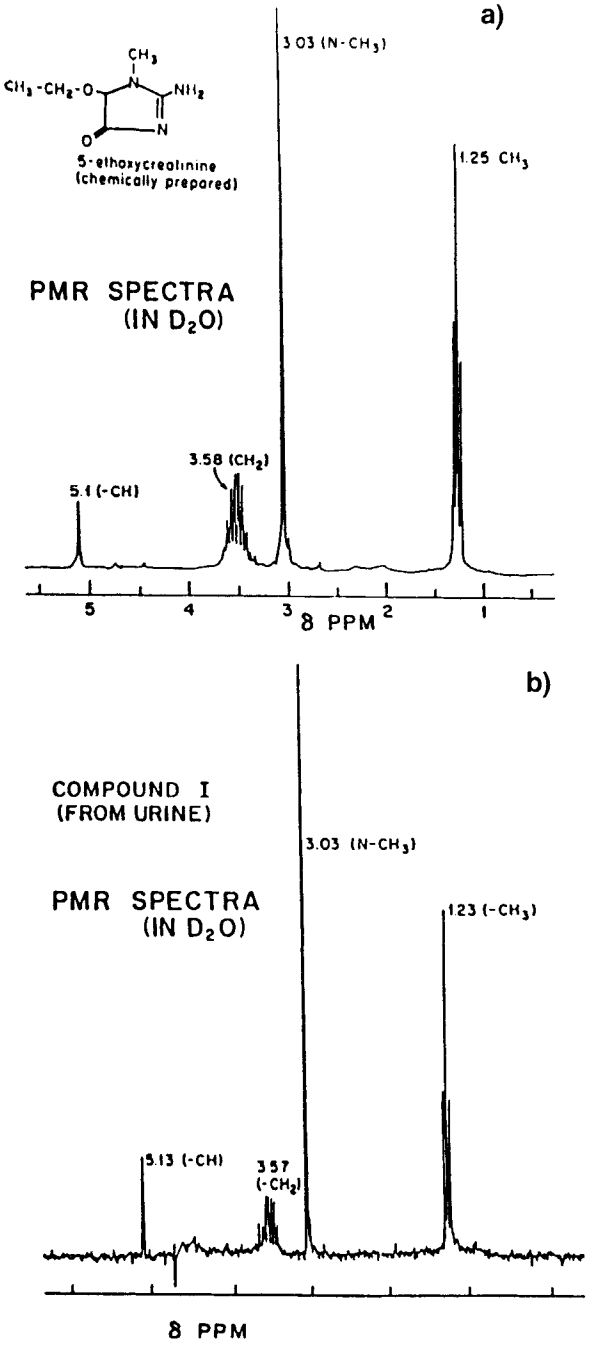


Fig. 4 (a) ^{1}H- NMR spectra of 5-ethoxycreatinine (synthesized) and (b) isolated from human urine.

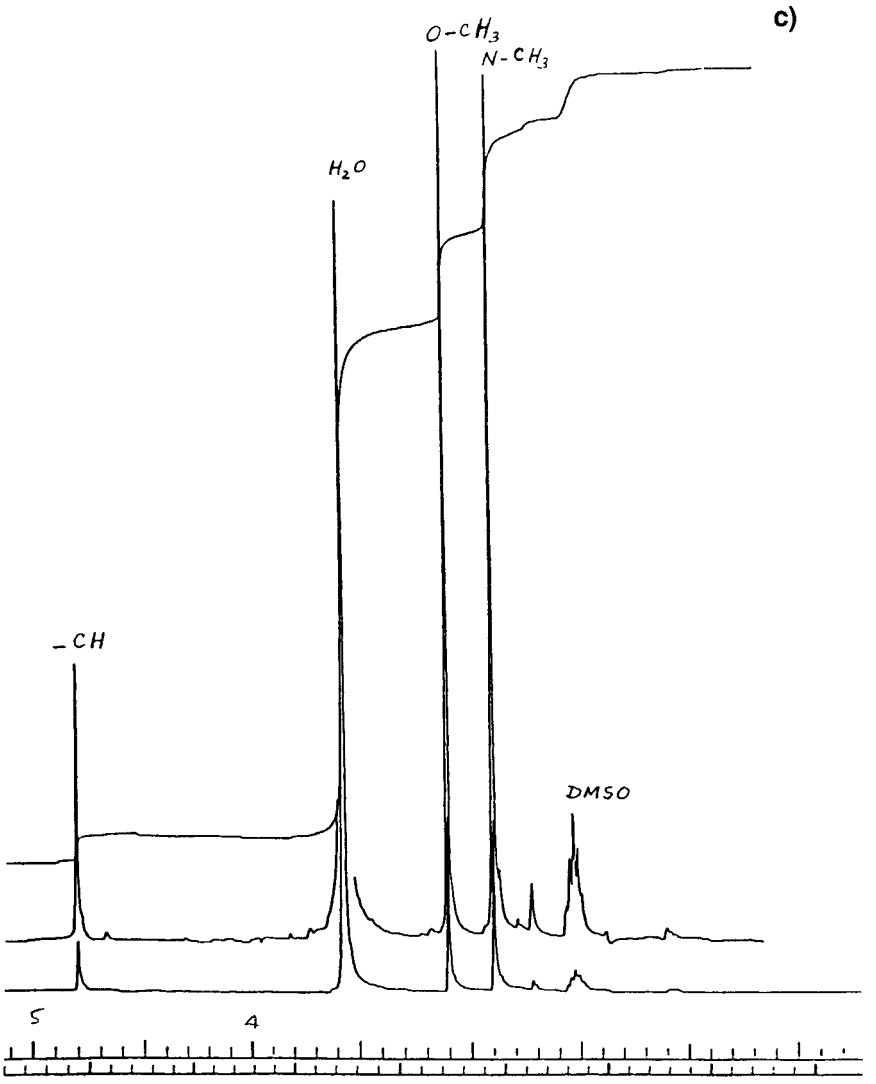
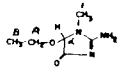
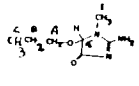
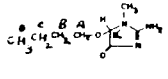


Fig. 4 (c) ^{1}H- NMR spectrum of 5- methoxycreatinine.

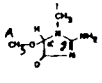
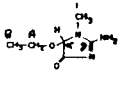
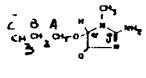
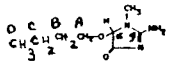
to speculate on the use of creatinine for alcohol toxicity, that would lower the (methanol or ethanol) alcohol content of the blood by forming non-toxic metabolites. Further studies are necessary to understand the role of the alkoxy derivatives, especially methoxy- and the ethoxy-derivatives in metabolism. There is a good possibility that the 5-substituted creatinine may be generated in vivo under suitable oxidizing conditions. The formation of 5-alkoxy derivatives through a novel reaction of creatinine with various

TABLE 8:PROTON NMR DATA FOR CREATININE AND ITS 5-ALKOXY DERIVATIVES *

COMPOUND	H_α	H_1	H_A	H_B	H_C	H_D
1. 	3.72 (S,2H)	2.94 (S,3H)				
2. 	4.80 (S,1H)	2.90 (S,3H)	3.12 (S,3H)			
3. 	4.78 (S,1H) **5.10 (S,1H)	2.90 (S,3H) 3.03 (S,3H)	3.30 (M,2H) 3.58 (M,2H)	1.10 (T,3H) 1.25 (T,3H)		
4. 	4.80 (S,1H)	2.90 (S,3H)	3.30 (M,2H)	1.50 (M,2H)	0.90 (T,3H)	
5. 	4.78 (S,1H)	2.90 (S,3H)	3.30 (T,2H)	1.40 (M,2H)	1.40 (M,2H)	0.90 (T,3H)

* PPM VALUES IN DMSO RELATIVE TO TMS.

TABLE 9: CARBON-13 CHEMICAL SHIFTS FOR CREATININE AND ITS ALKOXY DERIVATIVES*

COMPOUND	C_O	C_G	C_α	C_1	C_A	C_B	C_C	C_D
1. 	184.34	171.14	55.96	30.63				
2. 	182.80	170.00	88.44	28.32	51.24			
3. 	182.64	170.04	87.92	28.31	59.71	14.99		
4. 	182.76	170.34	87.92	28.28	65.48	22.32	10.45	
5. 	182.73	170.29	87.92	28.28	63.48	31.07	18.66	13.57

*PPM VALUES IN DMSO RELATIVE TO TMS.

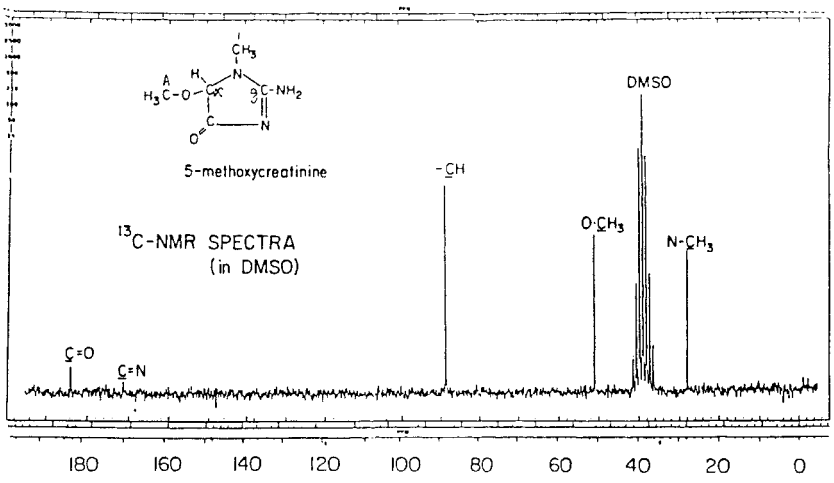


Fig. 5. [13] C- NMR spectrum of 5-methoxycreatinine.

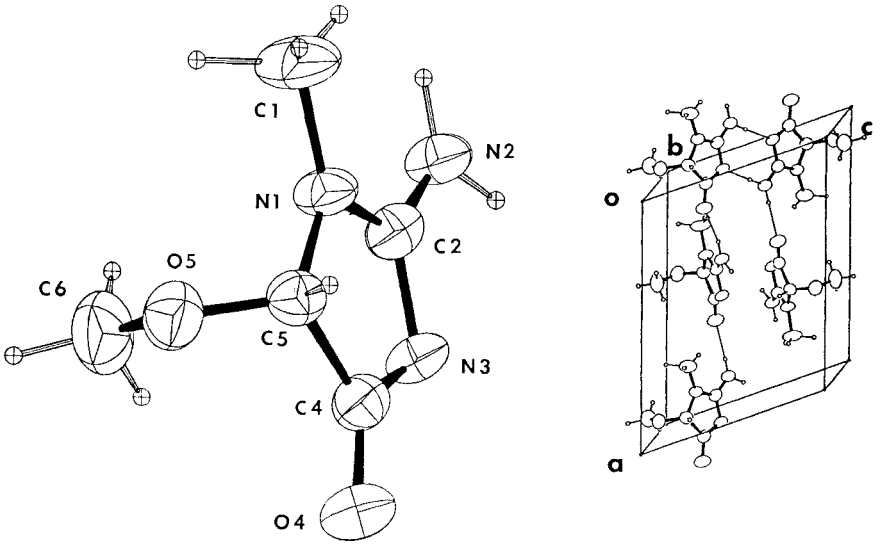


Fig. 6 (a) An ortep diagram of 5-methoxycreatinine showing the conformation of the molecule and the atomic numbering scheme and Fig. 6 (b) An unit cell packing diagram projected down the b-axis.

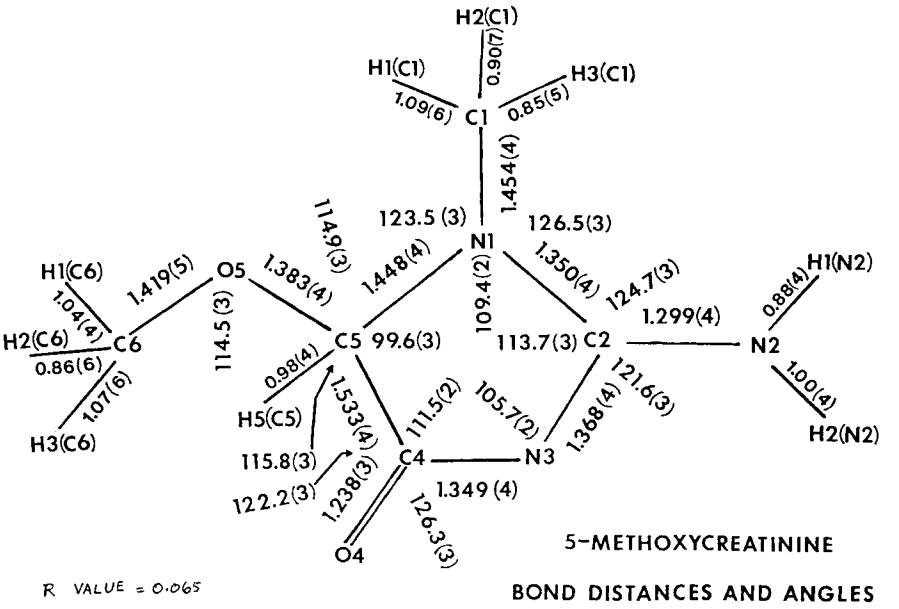


Fig. 7. Intramolecular bond distances (Å) and bond angles (°) in 5-methoxycreatinine.

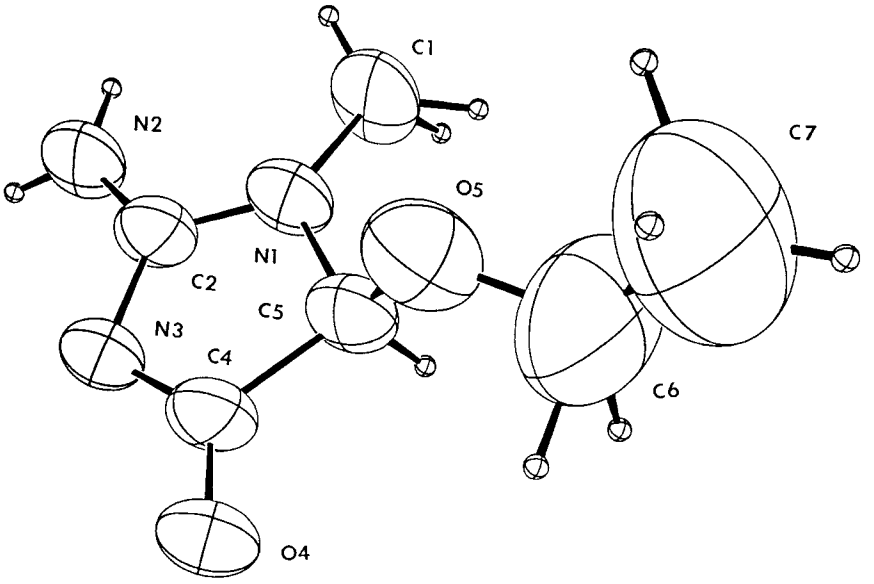


Fig. 8. An ORTEP diagram showing the conformation of 5-ethoxycreatinine. The atoms C6 and C7 of the terminal ethoxy group have higher temperature factors than the rest of the molecule.

Table 10

Bond distances (Å) and Bond Angles (°) for 5- EthoxyCreatinine

Bond Distances

N1- C1	1.453 (1)	C1- H1C1	0.94 (2)
N1- C2	1.346 (1)	C1- H2C1	0.91 (2)
C2- N3	1.359 (2)	C1- H3C1	1.19 (2)
N3- C4	1.352 (2)	N2- H1N2	0.87 (2)
C4- C5	1.535 (1)	N2- H2N2	0.81 (2)
C5- O5	1.399 (1)	C5- HC5	0.99 (2)
O5- C6	1.458 (2)	C6- H1C6	1.19 (2)
C6- C7	1.415 (3)	C6- H2C6	1.18 (2)
C2- N2	1.309 (2)	C7- H1C7	0.78 (3)
C4- O4	1.224 (1)	C7- H2C7	0.99 (3)
		C7- H3C7	1.17 (3)

Bond Angles

C5-N1-C1	123.0 (2)	O4-C4-C5	122.9 (1)
C5-N1-C2	108.8 (2)	C4-C5-N1	100.5 (2)
C2-N1-C1	125.2 (1)	C4-C5-O5	110.6 (1)
N1-C2-N3	114.1 (1)	C5-O5-C6	113.3 (2)
C2-N3-C4	106.1 (1)	O5-C6-C7	111.0 (3)
N3-C4-O4	126.9 (1)	N1-C2-N2	124.2 (1)
N3-C4-C5	110.2 (1)	N3-C2-N2	121.7 (1)

Table 11

HYDROGEN BOND DISTANCES (IN Å) AND ANGLES (°) IN ETHOXYCREATININE

SERIAL NO.	DONOR D	HYDROGEN H	ACCEPTOR A	DISTANCES IN Å D-H	H...A	D...A	ANGLE D-H...A	DATA SET
1	N2	H1N2	O4	0.87	2.02	2.841	157°	$(\bar{2}\ 0\ 0\ \bar{1})$
2	N2	H2N2	N3	0.81	2.15	2.945	167°	$(\bar{2}\ 0\ 0\ \bar{1})$
3	C1	H1C1	O4	0.94	2.59	3.475	157°	$(\bar{2}\ 0\ 0\ \bar{1})$

HYDROGEN BOND DISTANCES (IN Å) AND ANGLES (°) IN METHOXYCREATININE

SERIAL NO.	DONOR D	HYDROGEN H	ACCEPTOR A	DISTANCES IN Å D-H	H...A	D...A	ANGLE D-H...A	DATA SET
1	N2	H1N2	O4	0.88	1.97	2.830	164°	$(\bar{2}\ \bar{1}\ 0\ 0)$
2	N2	H2N2	N3	0.99	1.96	2.944	173°	$(\bar{1}\ 0\ 1\ 1)$
3	C1	H2C1	O4	0.95	2.74	3.381	150°	$(\bar{2}\ \bar{1}\ \bar{1}\ 0)$

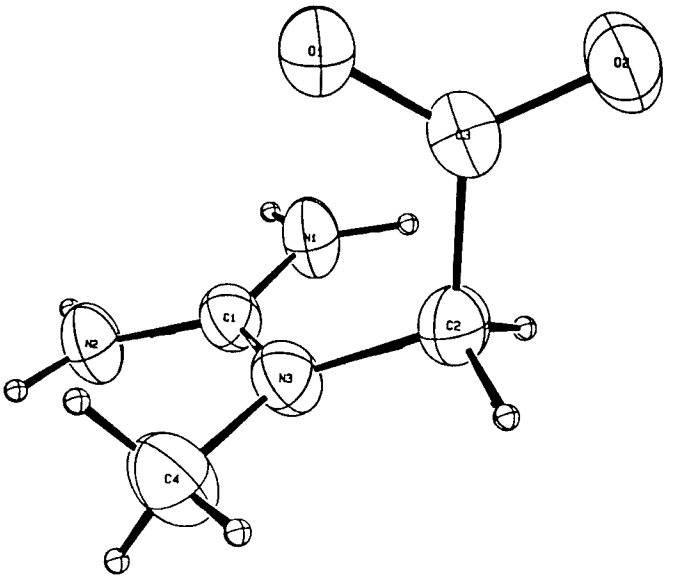


Fig. 9 (a) An Ortep diagram showing the conformation of the Creatine molecule.

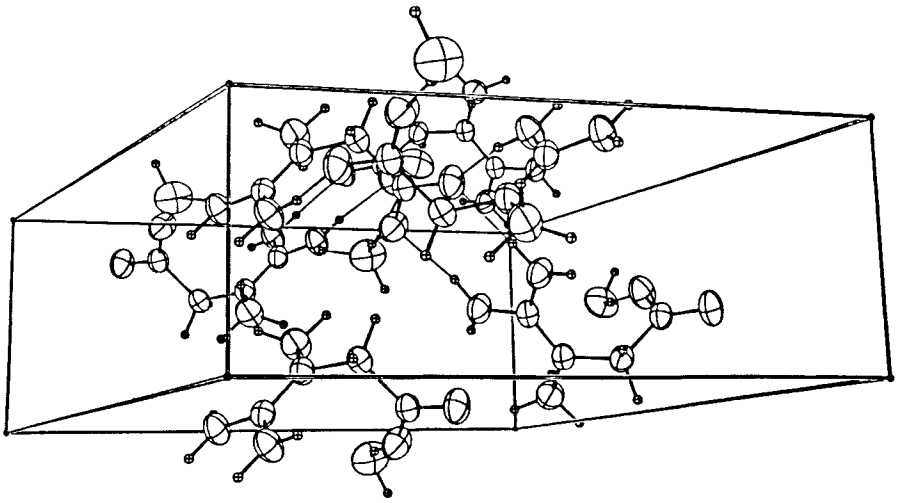


Fig. 9 (b) An ORTEP diagram showing the packing of the molecules in the unit cell. Some of the intermolecular hydrogen bonds are shown by solid lines.

TABLE 12

Intramolecular bond distances (Å) and Bond angles (°) for Creatine monohydrate

Bond Distances

N1- C1	1.328 (1)	N1- H1N1	0.91 (2)
C1- N3	1.332 (1)	N1- H2N1	0.93 (2)
C1- N2	1.333 (1)	N2- H1N2	0.92 (2)
N3- C4	1.459 (1)	N2- H2N2	0.91 (2)
N3- C2	1.457 (1)	C4- H1C4	0.93 (3)
C2- C3	1.526 (2)	C4- H2C4	0.99 (2)
C3- O1	1.246 (1)	C4- H3C4	0.99 (3)
C3- O2	1.249 (1)	C2- H1C2	1.00 (2)
		C2- H2C2	1.02 (2)
		OW- H1OW	1.15 (2)
		OW- H2OW	0.88 (2)

Bond Angles

N1- C1- N2	117.7 (2)	N3- C2- C3	113.9 (2)
N1- C1- N3	121.8 (2)	C2- C3- O1	118.8 (1)
N2- C1- N3	120.6 (2)	C2- C3- O2	116.2 (1)
C1- N3- C4	120.2 (2)	O1- C3- O2	125.1 (2)
C1- N3- C2	120.7 (2)	H1OW- OW- H2OW	101.9 (5)
C4- N3- C2	117.7 (2)		

Table 13

HYDROGEN BOND DISTANCES (IN Å) AND ANGLES (°) IN CREATINE MONOHYDRATE

SERIAL	DONOR	HYDROGEN	ACCEPTOR	DISTANCES IN Å			ANGLE(°)	
NO.	D	H	A	D-H	H...A	D...A	D-H...A	DATA SET
1	N1	H1N1	O2	0.93	1.97	2.852	178°	($\bar{1}$ 1 0 1)
2	N1	H2N1	O2	0.92	1.96	2.844	164°	(2 0 0 0)
3	N2	H1N2	O1	0.92	1.91	2.818	172°	(2 0 0 0)
4	N2	H2N2	OW	0.91	2.09	2.934	155°	($\bar{1}$ 1 0 0)
5	OW	H1OW	OW	0.95	1.93	2.877	179°	($\bar{2}$ 0 0 0)
6	OW	H2OW	O1	0.88	1.83	2.708	169°	(1 0 0 0)
7	C4	H2C4	O1	0.99	2.59	3.333	159	(1 0 1 0)

aliphatic alcohols in presence of charcoal and air via a free radical pathway has a synthetic value as it extends the scope for the facile synthesis of alkoxy adducts involving active methylene substrates.

X-RAY CRYSTALLOGRAPHY:

An ORTEP drawing of the 5- methoxycreatinine is given in Fig. 6 (a). Fig. 6 (b) gives the packing of the molecules in the unit cell. The molecules are connected by N-H...O and N-H... N hydrogen bonds which are shown by solid lines in the packing diagram. The intramolecular bond distances and angles are shown in Fig.7. Fig. 8 is an ORTEP diagram of 5- ethoxy creatinine. The terminal alkyl side chains in both these crystals have higher thermal vibrations than the rest of the molecule. The vibrations of the ethoxy carbon atoms are much higher than the methoxy side chains. Table 10 gives the bond distance and angles for 5-ethoxycreatinine.Table 11 gives the hydrogen distances and angles for both the ethoxy and the methoxycreatinine derivatives. Fig. 9 (a) shows the conformation of the creatine molecule as observed in the monohydrate form. Fig. 9 (b) shows the packing of the molecules in the unit cell . The intramolecular distances and angles in creatine monohydrate are given in Table 12. Table 13 gives the hydrogen bond distances and angles in creatine monohydrate. The molecules are stabilized by a dimeric type of hydrogen bonds involving the amino nitrogen atom as donors and the carbonyl oxygen as acceptors. The water molecules stabilize the creatine molecules by a net work of strong N-H...O and O-H...O hydrogen bonds.

In conclusion, the 5-alkoxy derivatives of creatinine were synthesized and purified by reverse phase HPLC and their structure determined by spectral (MS, NMR and UV) and elemental analysis. Further proof for the structures for 5-methoxy and 5-ethoxy compounds came from x-ray diffraction studies. The assay for antiviral and antitumor activity were carried out on all the analogs.

ACKNOWLEDGMENTS

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