Biological Significance of the Electronic Structure of Alloxan and Its Tautomers

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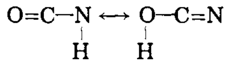
By means of a molecular orbital method which considers all valence electrons, INDO, we have calculated several electronic indices and determined the major tautomers in the lactam–lactim tautomerism of alloxan in the liquid and gaseous states. We have found that the principal tautomer, in both the gas phase and in solution, is the keto form; however, in solution, we have found a major contribution by the 2-hydroxy form. In addition, we have determined that the most reactive group, in all tautomers, to nucleophilic addition is the 5-CO group. We also made some observations concerning the effect of the electronic structure of alloxan on its diabetogenic activity.

The possibility of tautomerism in alloxan is quite important since it has been found to be very biologically active.[1-3] It has been detected in many biological fluids[4,5] and is also known to have an effect on the tissues of the endocrine glands.[6] It has also been found as a metabolite in urine.[7] Alloxan can inactivate encephalomyocarditis virus in vitro but not in vivo.[8] In addition, there have been responses to its presence by cell nuclei in the brain and nervous system.[9] Yet, its most important activity was shown by Dunn,[10] when he showed that alloxan can destroy β cells of the pancreatic islets. It apparently acts by attaching to the SH groups and lowering the blood glutathione, thereby producing hyperglycemia and diabetes. Mussgnug[3] noted that alloxan is an endogenously occurring natural substance. In addition, Dunn[10] felt that it might be a circulating hormone controlling insulin secretion and that its presence arises from the metabolism of pyrimidines and purines.

Although alloxan has been known for over a century since it was synthesized by Wohler[11] in 1838, very little is known either about its electronic structure or physiochemical properties with any degree of certainty. This is largely due to the fact that alloxan is a complex, unstable, and highly reactive substance. There have been several x-ray investigations[12,13] which have shown that there is no intermolecular hydrogen bonding in the solid state. In addition, there have been some studies by Raman spectroscopy[14] and NMR.[15] Whereas the earlier IR and UV studies gave spectral evidence to favor a lactim–lactam tautomerism, conclusions based on frequency shifts, the



$$O{=}C{-}\underset{H}{N} \longleftrightarrow \underset{H}{O}{-}C{=}N$$

most recent NMR study showed a lack of enolic signal in the alloxan solution studied. This indicates that the equilibrium lies very far toward the lactam form. In terms of molecular orbital studies, the only ones performed to date have been on alloxan itself and have been of the HMO variety,[16-18] which neglects σ valence electrons.

It is our intent to provide a much stronger foundation to the previous experimental and theoretical work by examining the electronic structure of alloxan, as well as its possible tautomers and rotamers, by utilizing the all-valence electron method, INDO.

Methods. We used the CNINDO[19] program obtained from QCPE, a version modified for use on the CDC 6400. Details about the method can be found in the literature.[20-22] We calculated several electronic indices, total energies, ionization potentials, and dipole moments for a total of 12 possible tautomers and rotamers of alloxan, by considering, in addition to the tautomers, the structures formed by rotation about the C–O bond. In addition, calculations were performed on the mono- and dianionic forms of alloxan. Since the results of these calculations depend to some extent on the geometries adopted, we resolved this problem by using the geometry of alloxan as determined by x-ray data for the keto form and by using standard bond lengths[22] for C–O and O–H bonds in all the forms. We maintained the ring structure as that of the parent and varied the COH bond angles. Limiting our calculations to this variable, we still examined approximately 120 configurations. The resulting geometries of the 12 tautomers and rotamers can be found in Figure 1.

Table I. Dipole Moments of Alloxan and Its Tautomers and Rotamers

	Dipole moment[a]	θ[b]
Alloxan (I)	2.25	0
4-Hydroxy (II)	1.30	89
4-Hydroxy (III)	3.00	185.8
2-Hydroxy (IV)	6.04	24.9
2-Hydroxy (V)	8.06	48.3
4,6-Dihydroxy (VI)	1.61	180
4,6-Dihydroxy (VII)	7.50	180
4,6-Dihydroxy (VIII)	4.83	160.8
2,4-Dihydroxy (IX)	4.41	53.6
2,4-Dihydroxy (X)	5.48	94.9
2,4-Dihydroxy (XI)	7.49	70.1
2,4-Dihydroxy (XII)	1.99	102.1

[a] Dipole moment (μ) is in Debye units. [b] θ is in degrees and is defined as the angle with respect to the C(2)–C(5) vertical axis, counterclockwise.

Results and Discussion

We can group these structures according to their dipole moments. Structures I, II, VI, and XII have small dipole moments, approximately 1–2 D, whereas structures IV, V, VII, and XI have large dipole moments, between 6 and 8 D. Because of these large differences in values, experimental measurements may represent a useful way of identifying members of the two groups, frequently difficult to identify by other physicochemical techniques. In the case of alloxan I, we calculated a dipole moment of 2.25 D and the experimental value[16] is 2.10 D, giving good agreement. Table I gives the results for the dipole moments and the angle of the dipole with the C(2)–C(5) axis. In the cases where the molecular geometry can be described by the C_{2v} point group, such as structures I, VI, and VII, the dipole vector is parallel to the C(2)–C(5) axis, with the largest dipole associated with structure VII. Shifting the protons from the nitrogens to the oxygens, in these cases, changes the dipole vector angle by 180°. Rotation about the CO bond causes the largest change in dipole moment for the 2,4-dihydroxy and 4,6-dihydroxy cases.

In examining Figures 2–4, which show the σ, π, and total net charges for alloxan and its respective tautomers and rotamers, one finds that the largest concentration of positive charge is at C(2). This remains true even for the possible tautomers, as shown by the structures II and IX. Although position C(2) has a larger net positive charge than C(5), the free valence is greater for C(5). Therefore, in terms of reactivity, it would be expected that C(5) is still the most reactive center to nucleophilic attack. This is

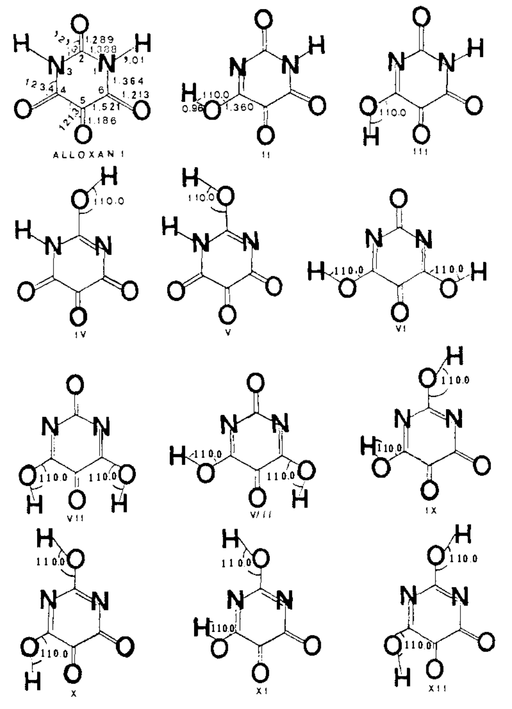


Figure 1. Geometry of alloxan and its tautomers and rotamers.

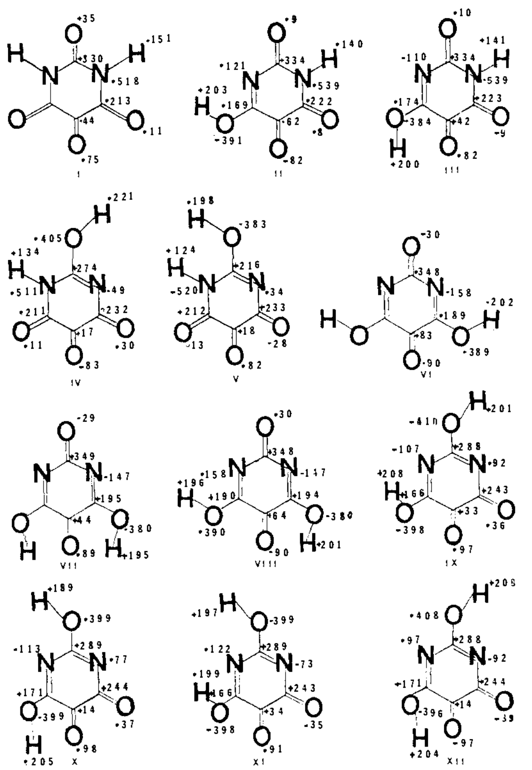


Figure 2. σ net charges (10^{-3} e).

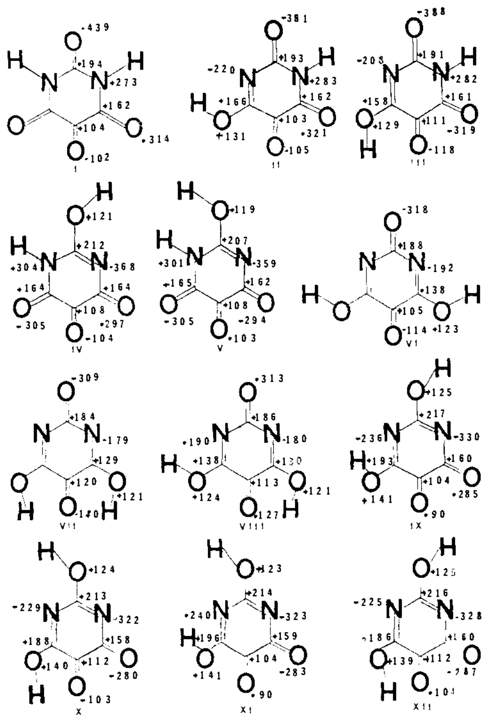


Figure 3. π net charges (10^{-3} e).

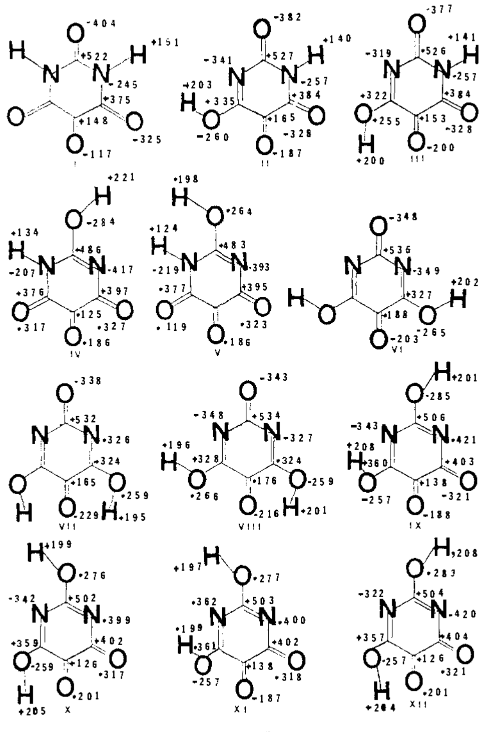


Figure 4. Total net charges (10^{-3} e).

experimentally verified by the reactions of α-amino acids in a bimolecular mechanism at the 5-keto group to produce alloxantin.[24]

Because of the strong dipolar nature of the carbonyl group, the intermolecular interaction in the solid state for alloxan I is not hydrogen bonding, but C=O···C.[12,13] Pullman explained this on the basis of π net charges; however, as can be seen from Figure 2, there is also a positive contribution by the σ net charge. For all structures, the largest positive σ net charge is at C(2), followed by C(4) and C(6). In alloxan I, there is a positive net π charge on the nitrogens and a negative net σ charge, which gives an overall negative charge to the nitrogen centers. The largest net negative charge is at O(2) in structures I and IV and at O(6) in structure IX. Rotation about the C–O bond does not significantly change these observations. Because of the large negative charges at O(2) and N(1), as well as N(3), the possibility for chelation is increased at these positions by metal cations, such as Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}. In fact, Lange and Foye[25] show chelation with alloxan takes place with a prevention of a conversion to alloxanate.

The overlap populations were calculated by means of Kaufman's method.[16] These values were included in Table

Table II. Total Overlap Populations for Alloxan and Its Dianion

	Alloxan			Alloxan (2^-)		
Bond	σ	π	Total	σ	π	Total
C(2)-O(2)	1.7940	0.8139	2.6079	1.7298	0.7129	2.4427
C(2)-N(1)	1.7304	0.3780	2.1084	1.7073	0.4366	2.1439
C(2)-N(3)	1.7304	0.3780	2.1084	1.1070	0.4366	2.1439
N(1)-C(6)	1.5851	0.4051	1.9902	1.5802	0.5962	2.1764
C(6)-O(6)	1.9402	0.8620	2.8022	1.8758	0.7225	2.5983
C(5)-C(6)	1.7571	0.2396	1.9967	1.7891	0.2340	2.0231
C(5)-O(5)	1.6476	0.9338	2.5814	1.5626	0.9166	2.4792
C(4)-C(5)	1.7571	0.2396	1.9967	1.7891	0.2340	2.0231
C(4)-O(4)	1.9402	0.8620	2.8022	1.8758	0.7225	2.5983
C(4)-N(3)	1.5851	0.4051	1.9902	1.5802	0.5962	2.1764
N(1)-H(1)	1.6432		1.6432			
N(3)-H(3)	1.6432		1.6432			

II, for the σ, π, and total overlap populations. We can see from Table II that our calculated values for the total overlap populations of the C(6)–N(1) bonds and C(5)–C(6) bonds in alloxan are very close in value, with the C(5)–C(6) total overlap population being slightly stronger. Yet, there is even a larger difference in the σ overlap population, favoring the strength of the C(5)–C(6) bond. As a consequence of the stronger C(5)–C(6) bond, an internal benzilic acid rearrangement takes place, with the breaking of the C(6)–N(1) bond and not the C(5)–C(6) bond to form alloxanate. The breaking of the C–N bond, as opposed to the C–C bond, was suggested by Spayd[26] as the result of his C-14 labeling experiments. As alloxan is converted from its neutral form to the dianion, there is a slight change in the total overlap population but the C–C σ bond still remains the strongest; therefore, a similar rearrangement should take place in the dianion. Work by Kwart and his co-workers[27,28] has shown this to be the case.

The explanation of many of the biological and chemical properties of alloxan is based on the supposition that lactam–lactim tautomerism may take place. It, therefore, seemed of interest to investigate the stabilities of our 12 structures. In the gas phase, the keto form (I) is favored by as much as 19 kcal mol^{-1} over the nearest energetic structure, a 4-hydroxy form (II). This is in accordance with the crystallographic data and the recent NMR study in Me_2SO.[15] However, this does not explain the strong pK_a of 7.20 obtained by Richardson and Cannan.[29] They felt that this acidity is due to the labile hydrogen resulting from enolization. Therefore, in Table III, the results for ΔE, difference in energy of structures with respect to the keto form, are given in both vapor phase and in solution. The results for water were determined by using the reaction field equations which were discussed by Rein.[30] He applied these equations to the tautomerism of adenine. The energy of each tautomer is determined in solution as follows

$$E_{\text{soln}} = E_{\text{gas}} - 0.5R\mu^3 \tag{1}$$

where

$$R = (2\epsilon - 2)/(\epsilon + 1)a^3 \tag{2}$$

and

$$\mu^2 = \mu^2_{\text{gas}}(1 - \alpha R) \tag{3}$$

where the symbols are defined as follows: ϵ = dielectric constant of the solvent, a = cavity size, μ = dipole moment, and α = polarizability. Rein[30] noted that changing the dielectric constant of the solvent has a pronounced effect on the tautomeric equilibria, particularly where there exists a large difference in dipole moment between the tautomers. This has proven to be the case for alloxan. When each of the structures is examined in solution, there is a reduction in the energy difference such that although alloxan I is still the dominant form, structure V, which differed by as much as 30 kcal mol^{-1} in the gas phase, now differs by only 3 kcal mol^{-1}. Even though the other forms are reduced in energy difference, none become as important as this 2-hydroxy form, tautomer V. This would appear to be in accordance with the fact that the second proton does not dissociate readily[29] but, instead, has a pK_a of 10 or above. This would imply that instead of the picture which is usually displayed of a quinoid as the enol intermediate, such as tautomers VI and VII, the structure that is the actual intermediate in the conversion from the keto form to the anion is tautomer V, as shown in Figure 1.

Table III. Total Energy of Tautomers and Rotamers and Their Energy Difference in Gas and Aqueous Solution

	Total energy,[a] au	ΔE_{gas}, kcal mol^{-1}	ΔE_{soln}, kcal mol^{-1}
Alloxan (I)	−121.3203		
4-Hydroxy (II)	−121.2902	+18.9	+19.4
4-Hydroxy (III)	−121.2867	+21.1	+20.3
2-Hydroxy (IV)	−121.2775	+26.9	+16.1
2-Hydroxy (V)	−121.2737	+29.2	+3.0
4,6-Dihydroxy (VI)	−121.2509	+43.5	+44.0
4,6-Dihydroxy (VII)	−121.2443	+47.7	+27.1
4,6-Dihydroxy (VIII)	−121.2477	+45.6	+40.6
2,4-Dihydroxy (IX)	−121.2470	+46.0	+42.5
2,4-Dihydroxy (X)	−121.2414	+49.5	+41.9
2,4-Dihydroxy (XI)	−121.2463	+46.4	+26.0
2,4-Dihydroxy (XII)	−121.2435	+48.2	+48.4

[a] 1 au = 27.210 eV = 627.49 kcal/mol.

Even though the quinoid structure is not the favored one in the enol, it does not mean it might not be the favored one in the anionic forms. In the case of the monoanion, there may be a tautomeric shift to a quinoid structure. The fact that the keto form is dominant in both the gas phase and in solution is in agreement with observations concerning other hydroxypyrimidines. It has been determined that 2-, 4-, and 6-hydroxypyrimidines should all be represented mainly in the keto form.[31]

From an examination of the chemical and electronic properties of alloxan, we can see that the hydration at 5-CO is more likely than hydration at the other CO bond, as suggested by Lagercrantz and Yhland.[32] Since the possibility of enolization at both the 4 and 6 positions is remote, the reactivity of the 5-CO is not reduced. On the basis of our calculations in both the gaseous state and in solution, we found that the 4,6-dihydroxy forms are not important tautomers, as shown by their large difference in energy. Because of this, it is no longer difficult to understand why alloxan is reduced to dialuric acid and not to isodialuric acid. In the case of isodialuric acid, these

Table IV. Energies and Nature of the Two Highest Occupied and Lowest Unoccupied Molecular Orbitals and Ionization Potentials

	HOMO1 (n), au	HOMO2 (π), au	LUMO (π^*), au	I_1 (n), eV
Alloxan (I)	−0.4364	−0.4751	0.0304	11.87
4-Hydroxy (II)	−0.4371	−0.4576	0.0037	11.89
4-Hydroxy (III)	−0.4326	−0.4582	0.0009	11.77
2-Hydroxy (IV)	−0.3888	−0.4232	0.0332	10.58
2-Hydroxy (V)	−0.3898	−0.4241	0.0316	10.61
4,6-Dihydroxy (VI)	−0.4015	−0.4385	−0.0107	10.92
4,6-Dihydroxy (VII)	−0.3977	−0.4413	−0.0164	10.82
4,6-Dihydroxy (VIII)	−0.3996	−0.4399	−0.0136	10.87
2,4-Dihydroxy (IX)	−0.3806	−0.4073	−0.0058	10.36
2,4-Dihydroxy (X)	−0.3786	−0.4075	−0.0086	10.30
2,4-Dihydroxy (XI)	−0.3801	−0.4070	−0.0063	10.34
2,4-Dihydroxy (XII)	−0.3790	−0.4079	−0.0082	10.31

quinoid enol forms are required as intermediates.[29]

Table IV contains the energies and nature of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). In all cases, the HOMO is an n occupied molecular orbital, whereas the lowest unoccupied molecular orbital is a π antibonding molecular orbital. This differs from most of the pyrimidines in that they have a π molecular orbital for their HOMO. In the case of alloxan and its tautomers and rotamers, the highest occupied π molecular orbital differs by only 1 eV from the n occupied molecular orbital. The electron-donor properties, which are determined by the ionization potential, tend to increase as we go from alloxan I to its 2,4-dihydroxy tautomer, XII. These ionization potentials were achieved by utilizing an application of Koopmans' theorem,[33] which says

$$I_1 = E^+ - E_g = -\epsilon_{26} \tag{4}$$

where ϵ_{26} represents the energy of the highest molecular orbital. The fact that all forms have a high ionization potential for both the π and n molecular orbitals as compared with other pyrimidines[18] indicates that alloxan and its tautomers are particularly bad electron donors. The results for alloxan I agree with the conclusions based on the HMO analysis by Pullman.[17] In addition to the nature of the electron donor capabilities, we can see that the small value of the LUMO indicates the electron affinity or electron-acceptor capabilities. The fact that such a small value exists for alloxan shows that it is a very good electron acceptor. This is in accord with ESR studies in a water–methanol solution by Orr.[34]

It has been noted that there are very few derivatives of alloxan or related compounds which are diabetogenic. In fact, Hidy[35] observed that substitution at both N atoms resulted in a loss of diabetogenic activity. This may be due to the lack of enolization, as he suggested since the possibility of the 2-hydroxy tautomer V is now blocked. We have shown, from our calculations, that this is a major enol contributor in solution. In fact, this appears to be the intermediate before ionization.

Brückman and Wertheimer[36,37] studied the diabetogenic activity of several substances, including the following: barbituric acid, violuric acid, uramil, parabanic acid, and ninhydrin. They found that all of these substances were nondiabetogenic, even though each contained a significant portion of the alloxan structure. What they all lack is the highly reactive 5-CO portion of the alloxan molecule. The fact that this is such a highly reactive portion is in agreement with our findings.

From our calculations, we therefore feel that two things are important in the diabetogenic activity of alloxan. First, that alloxan must be able to tautomerize to its 2-hydroxy form, which increases the lability of the hydrogen atom and enables ionization with a low pK_a. Second, we feel that the –CO–CO–CO group is necessary. In fact, it appears that the most important part of this group is the highly reactive 5-CO moiety. After ionization, we have found that the binding capacity of the C(5) atom increases, which indicates that the reactivity of this group increases. The importance of the –CO–CO–CO group is in accord with the observations of Brückmann and Wertheimer.[36,37]

Conclusions

The calculations indicate that alloxan does display lactam–lactim tautomerism in solution but not to any extent in the vapor state. The lactam–lactim equilibrium is affected by the nature of the solvent, cavity size, and dipole moments of the tautomers in solution. Nevertheless, the major tautomer is the keto form, in both the gas phase and in the high dielectric solvent, water; however, a significant contribution is made by the 2-hydroxy form in water. From our calculations, we can rule out any significant contribution by the 4,6-dihydroxy forms. This suggests that the quinoid structure is not directly realized by enolization and explains why alloxan is reduced to dialuric acid and not to isodialuric acid. The fact that the 2-hydroxy form makes a significant contribution in solution explains the lability of the hydrogen atom and concomitant low pK_a.

An analysis of the charge distributions and total overlap populations has shown that, in an internal benzilic acid rearrangement, the C(6)–N(1) bond is broken, instead of the C(5)–C(6) bond being broken. In addition, the most reactive group to nucleophilic attack is the 5-CO moiety. It is at this group that the reactions involving amino acids, water, and thiols take place. This group is shown to be reactive in all tautomers studied.

From an evaluation of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), it can be seen that alloxan is a poor electron donor and a good electron acceptor. These properties are exhibited by all the tautomers and rotamers considered. This result indicates that alloxan is a good oxidizing agent but a poor reducing agent.

In terms of diabetogenic activity, the structure of alloxan has a highly reactive 5-CO group which is absent from substances found to be nondiabetogenic. In addition, by tautomerizing to the 2-hydroxy structure, and after ionization, the binding power of the C(5) atom increases; therefore, the reactivity of the 5-CO group increases. Besides these facts, the negative charge on the nitrogen increases; therefore, there is an increased possibility of chelation by Zn^{2+} ions.

In conclusion, these results seem to provide a stronger theoretical foundation for the many diverse observations found over the last century for alloxan, in the fields of biology and chemistry.

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Synthesis of Analogues of *N*-(2-Chloroethyl)-*N′*-(*trans*-4-methylcyclohexyl)-*N*-nitrosourea for Evaluation as Anticancer Agents

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The superior activity of *N*-(2-chloroethyl)-*N′*-(*trans*-4-methylcyclohexyl)-*N*-nitrosourea (MeCCNU) against advanced murine Lewis lung carcinoma in comparisons with the cis form and other nitrosoureas prompted the synthesis of a number of MeCCNU analogues, including several cis–trans pairs. The methyl group was replaced by a variety of substituents (CO_2H, CH_2CO_2H, CO_2Me, CH_2OAc, CH_2Cl, OMe); the *trans*-3-methylcyclohexyl, *cis*-2-methyl-1,3-dithian-5-yl, *cis*- and *trans*-2-methyl-1,3-dithian-5-yl tetraoxide, and 1-methylhexyl (open-chain) analogues were also prepared. Preliminary tests against murine leukemia L1210 revealed therapeutic indices (ED_{50}/LD_{10}) ranging from 0.26 to 0.79; all but three analogues effected 50% cure rates at nontoxic doses, the open-chain analogue being one of the least active. In terms of therapeutic index, diequatorial (trans-4) isomers were, with one exception, as active as or, in four of the eight examples, somewhat more active than the corresponding axial–equatorial (cis-4) isomers. In this series, four of the five 2-fluoroethyl analogues prepared were clearly inferior to the corresponding 2-chloroethyl analogues.

The synthesis and anticancer evaluation of numerous nitrosoureas[2-4] established a structure–activity pattern that led to clinical interest in *N*,*N′*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU, carmustine), *N*-(2-chloroethyl)-*N′*-cyclohexyl-*N*-nitrosourea (CCNU, lomustine), and *N*-(2-chloroethyl)-*N′*-(*trans*-4-methylcyclohexyl)-*N*-nitrosourea (1, MeCCNU, semustine).[5] Although most nitrosoureas of this type are highly active against murine leukemia L1210 implanted intraperitoneally and many are active against the same disease implanted intracerebrally, the superior activity of MeCCNU against advanced murine Lewis lung carcinoma[6] in comparisons with BCNU, CCNU, and the cis form of MeCCNU (**2**) suggested a conformational dependence that warranted further investigation. To this end, a number of MeCCNU analogues, including several cis–trans pairs of isomers, were prepared for evaluation. This paper concerns these syntheses (Tables I and II) and consequent evaluations against leukemia L1210 (Table III), but the results of evaluations against solid tumors, more discriminating in their response to nitrosoureas, are reserved for later, expanded treatment (see the following paper in this issue).

Chemistry. In a resynthesis of *cis*-MeCCNU (**2**), a two-step conversion of *trans*-4-methylcyclohexyl tosylate involving inversion with sodium azide and catalytic reduction was found more amenable to scale-up than the previously described[4] one-step ammonolysis. In a resynthesis of the fluoro analogue[4] **3** of MeCCNU, the intermediate urea was prepared by a different (not necessarily better) method involving the treatment of MeCCNU with 2-fluoroethanamine. The previously reported[3] *N*,-*N″*-(*trans*-1,4-cyclohexanediyl)bis[*N′*-(2-chloroethyl)-*N′*-