Chemical Basis for the Biological Activity of Imexon and Related Cyanoaziridines

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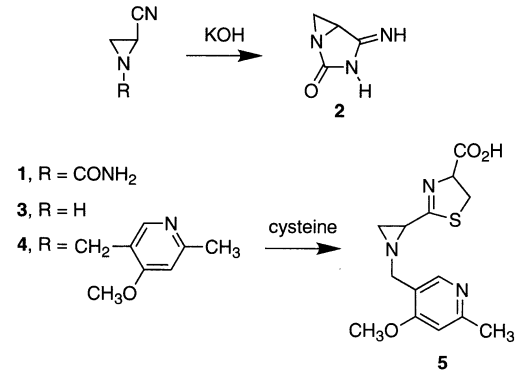
Chemical aspects of mode of action of imexon and related cyanoaziridines were studied. These compounds do not alkylate DNA nor react with the ϵ-amino groups of L-lysine, despite the presence of an aziridine ring. They do react readily with biologically important sulfhydryl compounds to give products derived from either aziridine ring opening, interaction with the cyano group of cyanoaziridines, or opening of the iminopyrrolidone ring of imexon. The products from reactions of imexon and related cyanoaziridines with thiols are not as potent as their parent compounds against tumor cells. These results are consistent with biological studies that show that the mechanism of cytotoxicity involves thiol depletion followed by oxidative stress leading to apoptosis.

Introduction

In 1975 Bicker reported a new type of carcinostatic aziridine, 2-cyanoaziridine-1-carboxamide (**1**).[1] Subsequent publications from his laboratory described the cytotoxicity of analogues in which the amide nitrogen was substitiuted with a variety of functional groups including methyl, phenyl, and 4-sulfamylphenyl.[2,3] Some of these compounds showed immunomodulatory properties, which prompted the suggestion that they act indirectly by an effect on immunological mechanisms.[1] More recently, a series of N-substituted 2-cyanoaziridine-1-carboxamides was shown to be cytotoxic in a large panel of cultured tumor cells including many species resistant to standard chemotherapeutic agents.[4] They gave a correlation between lipophilicity and potency.

When **1** was treated with KOH in methanol, it underwent cyclization to 4-imino-1,3-diazabicyclo[3.1.0]-hexan-2-one (**2**), which was named imexon.[5] Imexon is active against various transplanted syngeneic tumors in rodents[6] and transplanted human tumors in SCID mice.[7] Objective responses were found in a phase I clinical trial.[8] In fresh human tumors in clonogenic assay, imexon was selectively cytotoxic to multiple myeloma.[9] The activity in immunodeficient SCID mice, and in cell cultures lacking immune cell components, suggests that immunomodulation is not the mechanism of cytotoxic action.

One surprising property of 2-cyanoaziridine-1-carboxamide (**1**) was its failure to react with 4-(4-nitrobenzyl)pyridine in the standard assay for evaluating potential alkylating agents.[1] In contrast, cyanoaziridine (**3**) was reported to react readily with the stronger nucleophile cysteine.[1] Furthermore, ciamexon (**4**), a cyanoaziridine derivative which received brief clinical study in the late1980s, was found to deplete glutathione in liver microsomes, and to react readily with cysteine in vivo.[10] The product of this reaction was said to be a



thiazolidine, but the more reasonable thiazoline structure **5** would give the molecular ion of 307 found in its mass spectrum. It is noteworthy that the latter product would be formed by reaction of the sulfur atom with the cyano group in preference to the aziridine ring.

Biological studies by Dorr and colleagues support the idea that imexon does not alkylate DNA.[11] Instead, it is cytotoxic by a unique mechanistic pathway: induction of reactive oxygen species (ROS). They demonstrated that depletion of cellular thiols was dose- and time-dependent.[11] Oxidative stress was detected by confocal microscopy, which showed increased levels of 8-hydroxyguanosine in the cytoplasm, but not in the nucleus of imexon-treated 8226 myeloma cells. Interestingly, further studies have shown damage to mitochondrial but not nuclear DNA. These imexon-treated cells also showed the classic morphological features of apoptosis. Further depletion of glutathione by buthionine sulfoximide increased imexon cytotoxicity, and sulfhydryl replenishment with *N*-acetylcysteine blocked cytotoxicity. The cytotoxicity of imexon was shown to be time and concentration dependent with maximal effects noted after 48 h exposure at 37 °C.[11] Another study showed that 8226 myeloma cells which are sensitive to imexon undergo a significant loss of mitochondrial membrane potential (MMP).[12] This loss was shown to follow the generation of ROS. The release of cytochrome C from mitochondria was also documented to follow loss

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Table 1. Relative Alkylating Abilities of Imexon and Cyanoaziridines[a]

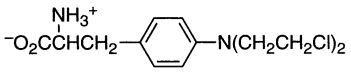
compd	relative absorbance at 560 nm
blank	0.03
2	0.05
3	0.04
6	0.04
7	0.36
9	0.47

[a] This assay was conducted according to a standard procedure (Preussmann, R.; Schneider, H.; Epple, F. Investigation on the Detection of Alkylating Agents. *Artzneimittelforsch.* **1969**, *19*, 1059−1073). Visible light absorbance was measured on a Perkin-Elmer Lambda 3A spectrophotometer.

of MMP. Overall, these studies suggest a sequential pathway for imexon-induced cytotoxicity, consisting of binding to sulfhydryls, accumulation of ROS, loss of MMP, release of mitochondrial cytochrome C, and induction of apoptosis.[12]

Chemistry

To gain a clearer picture of the alkylating ability of cyanoaziridines and imexon, we have undertaken a study on the comparative reactivity of certain of these compounds toward nucleophiles including 4-(4-nitrobenzyl)pyridine, 2′-deoxyguanosine, L-lysine, and cysteine. Furthermore, we have determined the structures of products from the reactions of these compounds with cysteine and other thiols of biological importance in vitro. Thus, a set of compounds including imexon (**2**), 2-cyanoaziridine (**3**), 2-cyanoaziridine-1-(*N*-phenylcarboxamide) (**6**), aziridine-1-(*N*-phenylcarboxamide) (**7**), and melphalan (**9**) were each treated with 4-(4-nitrobenzyl)pyridine at 100 °C under standard conditions.[2,13] In this assay, reactive compounds alkylate the pyridine nitrogen, and then treatment with piperidine removes a benzylic hydrogen to give a conjugated system with a purple color. The extent of reaction is determined by measuring light absorption at 560 nm. Compound **7**, which lacks a cyano group, was used to determine the importance of this substituent on alkylation. The bifunctional alkylating agent melphalan (**9**) was used as

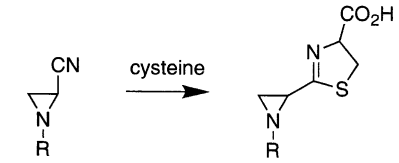


9

the positive control. The results of this assay are listed in Table 1. They show that imexon, **3**, and **6** were not alkylating agents; however, **7** was almost as reactive as melphalan. This observation suggests that the presence of a cyano group on the aziridine ring is sufficient to drastically reduce its alkylating ability. (Acylation of the aziridine nitrogen promotes reaction with nucleophiles because it participates in delocalization of the negative charge formed on the nitrogen in the transition state for nucleophilic attack on a carbon in the aziridine ring.)[14] These results contradict the previous report by Bicker and Fuhse that compound **6** was a more active alkylating agent than **7**.[2]

A qualitative comparison was made on the reactivity of some of the above compounds with 2′-deoxyguanosine in water at 100 °C. This assay involved visual observation of the disappearance of starting material and the appearance of a new spot or spots on TLC of the mixture. No attempt was made to isolate and identify the products. According to this procedure, imexon, **6**, and **7** remained unchanged, whereas melphalan reacted almost completely within 30 min. Another study was made using L-lysine as a model for the reactive groups in proteins. No reaction between imexon and L-lysine was observed under a variety of conditions including variation of pH from 7.0 to 9.4, and temperatures in the range from room temperature (21 °C) to 42 °C.

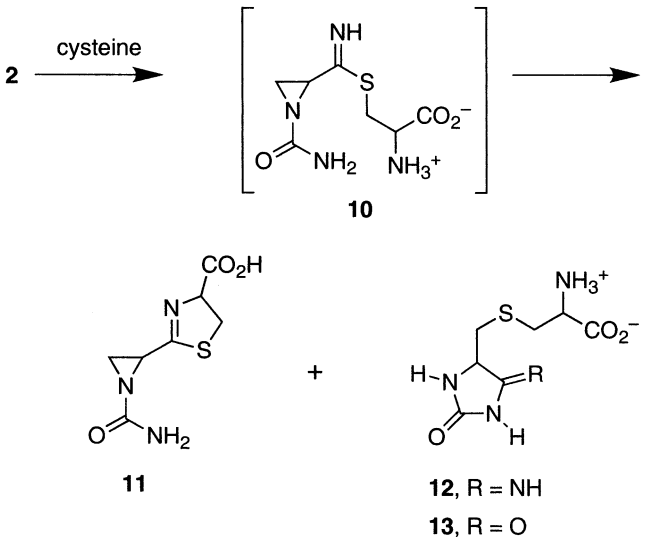
Imexon, **3**, and **8** each reacted readily with cysteine at room temperature. The products were isolated by preparative TLC (Experimental Section), and their



3, R = H **14**, R = H

8, R = $CONHCH_3$ **17**, R = $CONHCH_3$

structures were determined by proton NMR and by mass spectrometry. The structures of the products varied with the natures of the compounds. Thus, imexon reacted with cysteine at room temperature to furnish the thiazoline conjugate **11** in which the five-membered ring has opened to provide a carboxamido group. The

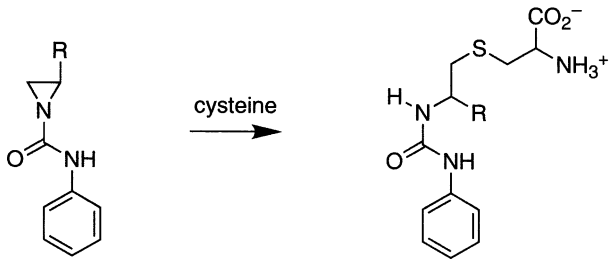


mechanism of this process is unknown, but it can be visualized as an attack of the cysteine sulfur atom on the cyclic amidine functionality of imexon, with ring opening facilitated by relief of ring strain. Cyclization of the hypothetical intermediate **10** would then give the thiazoline ring of **11** with loss of ammonia. An alternative mechanism might be opening of the five-membered ring of **2** to give **1**, followed by reaction with cysteine to give **11**. We have observed that **1** is slowly formed when **2** is kept in aqueous solution, although it takes days to form appreciable quantities of **1**.

When imexon and cysteine were reacted at 50 °C, thiazoline **11** was still obtained, but it was accompanied by **12**, the product of aziridine ring opening. Compound **12** was identified by its ^1H NMR spectrum. When the NMR solution was concentrated after two weeks and used to determine the mass spectrum, it was found that

the imine group had hydrolyzed to give imidazolidinedione derivative **13**. The composition of **13** was determined by HRMS.

2-Cyanoaziridine (**3**) reacted with cysteine by way of its cyano group to give the thiazoline **14**. Bicker suggested previously that **3** reacts at its cyano group, but proposed no structure for the product.[1] In contrast, we have found that 2-cyanoaziridine-1-(*N*-phenylcarboxamide) (**6**) reacted with cysteine through its aziridine ring to furnish compound **15**. This difference is attributed

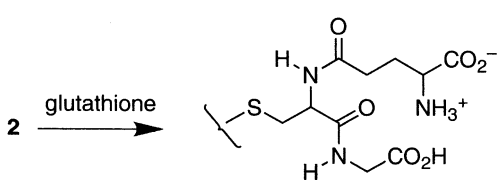


6, R = CN
7, R = H

15, R = CN
16, R = H

to the fact that **6** had to be heated to 100 °C because of its very low water solubility. Even then it reacted as a suspension. Apparently the high temperature promotes aziridine ring opening. This reaction occurs to some extent when imexon is heated to 50 °C, as described above. Although the ^{1}H NMR spectrum of **15** is consistent with the depicted structure, it does not rule out one in which the aziridine ring has been opened by nucleophilic attack of sulfur on the carbon bearing the cyano group. There is, however, literature precedent for opening of the cyanoaziridine rings by nucleophilic attack on the less hindered methylene group of the aziridine.[15] The corresponding compound **7**, without a cyano group, also has very low water solubility and had to be reacted at 100 °C. It can only react by opening of its aziridine ring. Although the crude product of this reaction was so insoluble that it could not be characterized completely, its mass spectrum showed a molecular ion and daughter peaks which were consistent with the expected product **16**. The 2-cyanoaziridine-1-(*N*-methylcarboxamide) (**8**) has good water solubility and it reacted readily with cysteine at room temperature to provide **17**.

Previous biological experiments indicated that imexon reacts readily with glutathione to deplete this important thiol.[11] We found that equimolar amounts of imexon and glutathione react completely within 1 h at room temperature. A completely pure sample of the product **18**

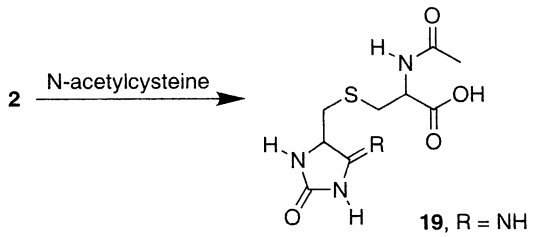


18

was not obtained; however, the ^{1}H NMR spectrum indicated that the aziridine ring of imexon had been opened with incorporation of glutathione. In particular, the peaks for protons on the aziridine ring at 2.0, 2.14, and 2.83 ppm were absent, and new peaks at 2.95−3.2 and 3.85−3.95 ppm, corresponding to the open-chain structure **18**, were present. The HRMS analysis indicated that a NH had been replaced by O, suggesting that the imine group had been hydrolyzed to a carbonyl group, as occurred in compound **13**. Aziridine ring opening when imexon reacts with glutathione is reasonable because the γ-glutamyl substituent on the nitrogen of the cysteine residue of glutathione prevents imidazoline formation.

The development of imexon resistance in 8226 myeloma cells is correlated in part with an increase in the enzyme thioredoxin.[16] This result suggested that it might be valuable to investigate the reaction of imexon and related cyanoaziridines with thiols more like cysteine incorporated into enzymes than to free cysteine. *N*-Acetylcysteine was chosen for this purpose because its amino group is substituted and thus not available for thiazoline formation. Glutathione is another example of this type of compound.

Imexon reacted with *N*-acetylcycteine at 37 °C in water to afford a product that appeared to be a mixture of iminoimidazolidolinone **19** and the corresponding imidazolidinedione **20** according to mass spectrometry,



19, R = NH
20, R = O

which showed appropriate MH^+ ions at 284 and 285 *m*/*z*, respectively. The proton NMR spectrum was consistent with these structures. When this solution was kept for 7 days, the peak at 284 disappeared, suggesting complete hydrolysis to dione **20**. A new peak appeared at *m*/*z* 347, but the mixture was not investigated further.

In contrast to imexon, 2-cyanoaziridine-1-(*N*-methylcarboxamide) (**8**) failed to react with *N*-acetylcysteine in water at 37 °C. Decomposition resulted at 55 °C.

The foregoing results suggest that imexon is more reactive than related cyanoaziridines toward thiols. A direct competition experiment was conducted to gain more information on this possibility. In this experiment, a solution containing equivalent amounts (molar basis) of imexon, 2-cyanoaziridine-1-carboxamide (**1**), and cysteine in D_2O was examined by proton NMR spectrometry. The amounts of unreacted imexon and **1** were estimated by comparing the integrals of the doublets for their CH protons at 3.8 and 3.38 ppm, respectively. Solutions containing only imexon, **1**, or cysteine in D_2O were run as standards. This experiment showed that imexon was consumed approximately two times faster than **1**.

To gain insight into the relative reactivity between imexon and standard thiol trapping agents, a competition between imexon and *N*-methylmaleimide was conducted. *N*-Methylmaleimide was chosen because the two protons on a double bond are clearly separated from all imexon protons, and the methyl group can serve as a standard for the relative amounts of other protons.

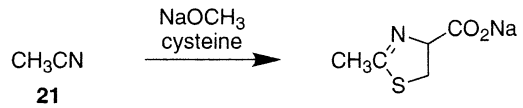
Table 2. Cytotoxicity of Cyanoaziridines and Related Compounds

	IC_{50} (μM)[a]		
compd	L1210/S	L1210/MDR	myeloma 8226/S
6	1.2, 38.4	11, 41.2	5.3
7	2.1, 27.1	5.8, 28.4	5.5
1	>500	>500	>500
11	>500	>500	>500
3	>500	>500	>500
14	>500	>500	>500
8	25.1	36.7	15.5
17	>500	>500	>500
15	73.9	>500	44.9
20	73.7	116.4	56.3

[a] Cytotoxicity against the L1210 leukemia cells, both sensitive and multidrug resistant (MDR), and the human myeloma 8226 cells was determined by the MTT assay (Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Schoemaker, R. H.; Boyd, M. R. Feasibility of Drug Screening with Panels of Human Tumor Cell Lines Using a Microculture Tetrazolium Assay. *Cancer Res.* **1998**, *48*, 589−601.

Furthermore, it is widely used in biological studies involving thiols. In this experiment, 1 equiv of cysteine in water was treated with 1 equiv of imexon and 1 equiv of *N*-methylmaleimide. The ^{1}H NMR spectrum was determined after 6 h. It showed almost complete loss of the two protons on the double bond of *N*-methylmaleimide and the generation of three protons on aliphatic carbons formed by saturation of the double bond. The protons attributable to imexon were unchanged in location and intensity. Standards consisting of imexon, *N*-methylmaleimide, and cysteine singly in D_2O were used in this comparison.

The current results show that the aziridine ring activates the cyano group toward reaction with cysteine. Condensation of cysteine with the simpler cyano compound, acetonitrile (**21**), to afford thiazoline **22** is described in the literature;[17] however, it requires the



presence of a base (sodium ethoxide) and reflux temperature, and the product is the sodium salt of the carboxylic acid group. We confirmed this reaction, but found that there was no reaction when the base was omitted. This finding suggests that small amounts of the thiolate anion, formed in equilibrium, are required for addition to the cyano group of **21**. Enhanced reactivity of 2-cyanoaziridine over acetonitrile toward cysteine presumably results from the electron-withdrawing property of the α-nitrogen atom. The ^{1}H NMR spectrum of the acidic form of **22** was useful for assigning the protons in the spectra of the thiazolines described above.

Biology

The potencies of imexon and related cyanoaziridines in cultures of various tumor cells have been reported previously.[4] In Table 2 the relative potencies of the noncyano compound aziridine-1-(*N*-phenylcarboxamide) (**7**) and its 2-cyano analogue **6** are compared. Both compounds were active in the micromolar range against sensitive L1210 murine leukemia cells. This observation is interesting in view of the finding described above that **7** appears to alkylate DNA, whereas **6** does not. 2-Cyanoaziridine (**3**) and 2-cyanoaziridine-1-carboxamide (**1**) were not active against either L1210 leukemia or 8226 myeloma cells in culture; however, the *N*-methyl derivative **8** and the *N*-phenyl derivative **6** of **1** were cytotoxic in these cell lines. This difference may be caused by the greater lipophilicity of **6** and **8**. A correlation between lipophilicity and cytotoxicity was previously found in a larger set of 2-cyanoaziridine-1-carboxamides.[4] Table 2 shows that none of the products containing both an aziridine ring and a thiazoline ring (**11**, **14**, and **17**) was cytotoxic. Somewhat surprisingly, compounds **15** and **20**, in which the aziridine ring has been opened by reaction with cysteine, retain some cytotoxicity. They are less potent than cyanoaziridines **6** and **8**, but clearly more potent than compounds **11**, **14**, and **17**, which retain the aziridine ring. It is possible that they act with cells by a mechanism different from that of imexon and cyanoaziridines.

Conclusions

The results of chemical studies on the mode of action of imexon and related cyanoaziridines with nucleophiles were consistent with the published results of biological and biochemical studies. These compounds did not appear to be DNA alkylators, according to both the 4-(4-nitrobenzyl)pyridine color test and direct treatment with 2′-deoxyguanosine. They also did not react with the ϵ-amino group of L-lysine. In contrast, they reacted readily with cysteine. Cysteine reacts preferentially with the cyano group of cyanoaziridines or the amidine system of imexon at room or physiological temperatures. At higher temperatures, some aziridine ring opening occurs. Glutathione, which is incapable of forming a thiazoline, reacts by opening the aziridine rings of imexon or cyanoaziridines. Imexon reacts more rapidly than the cyanoaziridines. Imexon also reacts with *N*-acetylcysteine, a model compound for cysteine residues in proteins, but cyanoaziridine **8** fails to react with *N*-acetylcysteine even at 42 °C. The aziridine ring of cyanoaziridines enhances reactivity of the cyano group toward thiols, as indicated by the unreactivity of acetonitrile (**21**). Imexon reacts readily with thiols, but more slowly than *N*-methylmaleimide.

The products of reaction of imexon or related cyanoaziridine-1-carboxamides lose cytotoxic potency; however, this decrease is greater for compounds that retain their aziridine rings and form thiazolines than for those that lose their aziridine rings and retain their cyano groups.

It appears that the aziridine ring is required for significant potency in imexon and its analogues, but it might not be the actual target for nucleophilic attack by biological thiols in some analogues containing cyano groups.

Experimental Section

Melting points were recorded on a Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Unity-300 spectrometer using the indicated solvents and internal standards. NMR shift values are expressed in ppm.

Comparison of the Alkylating Abilities of Imexon, Cyanoaziridines, and Melphalan. A 1 mL quantity of 0.005 mM solutions of each compound, including imexon (**2**), cy-

anoaziridine (**3**), 2-cyanoaziridine-1-(*N*-phenylcarboxamide) (**6**), aziridine-1-(*N*-phenylcarboxamide) (**7**), and melphalan (**9**) in 2-methoxyethanol was treated with 1 mL of a 5% solution of 4-(4-nitrobenzyl)pyridine in 2-methoxyethanol, and each resulting solution was heated at 100 °C for 1 h. A control solution involving 5% 4-(4-nitrobenzyl)pyridine in 2-methoxyethanol at the same concentration was used. The solutions were then cooled and treated with 0.5 mL of piperidine and 2.5 mL of 2-methoxyethanol. The solutions from **2**, **3**, and **6** and the control remained colorless, whereas those from **7** and **9** turned purple. The absorbance of each solution was measured at 560 nM. The results are given in Table 1.

Comparative Reactivities of Imexon, Cyanoaziridines, and Melphalan with 2-Deoxyguanosine. A mixture of each of the following compounds: imexon, **6**, **7**, and melphalan, with 1 equiv of 2′-deoxyguanosine in water was heated at reflux temperature. The reaction was monitored by thin-layer chromatography on silica gel with methanol/chloroform (2:8 v/v) as solvent. Melphalan reacted within 30 min, with two new spots formed and only a trace of starting material remaining. Only starting materials were observed with imexon and **6** even after 48 h. Compound **7** decomposed partially, but there was no change in the 2′-deoxyguanosine present.

Reaction of Imexon with Lysine. Solutions containing imexon (10 mg, 0.09 mmol) and L-lysine (15 mg, 0.09 mmol) in deionized water were treated under the following sets of conditions: (A) pH 9.4 and room temperature for 48 h; (B) pH 7.0 (tris buffer) and room temperature for 48 h; (C) pH 7.0 at 37−42 °C for two h. These solutions were monitored by TLC on silica gel plates with MeOH−$CHCl_3$ (2:8 v/v) as solvent. No new spots were detected in any of the chromatograms.

Reactions of Imexon with Cysteine. A. At Room Temperature. A solution of imexon (**2**, 10 mg, 0.09 mmol) and DL-cysteine (12 mg, 0.1 mmol) in deionized water was kept at room temperature for 2 h. TLC analysis (2:8 v/v MeOH−$CHCl_3$ on silica gel) then indicated complete conversion of starting material. The solution was concentrated under reduced pressure, and the white solid residue was was stirred 30 min with ethanol and then filtered. The residue **11** was dried under high vacuum. It did not melt below 250 °C; ^{1}H NMR (D_2O + DSS) δ 2.86 (d, 2), 2.91 (d,1), 3.09 (br s, 1), 4.8 (under DOH, 1); MS (elctrospray) indicated $MH^+ = 216$; HRMS (FAB^+) calcd for $C_7H_{10}N_3O_3S$ 216.04426, found MH^+ 216.04420.

B. At Elevated Temperatures. A mixture of 30 mg (0.27 mmol) of imexon (**2**), 36 mg (0.297 mmol) of DL-cysteine, and 1.0 mL of deionized water was warmed at about 50 °C to maintain a clear solution and then allowed to cool to room temperature during 1 h. TLC (silica gel with methanol−chloroform 1:4 v/v) then showed no starting material. The mixture was concentrated by rotary evaporation, and the residual solid was divided into two approximately equal portions. An HPLC separation of this mixture using an analytical C18 column and solvent consisting of 10% acetonitrile and 90% water containing 0.1% trifluoroacetic acid under isocratic conditions gave two main peaks. The solid residue obtained from concentration of the larger and faster-moving peak (**12**) decomposed without melting when heated; ^{1}H NMR (D_2O + DSS) δ 3.06 (d, 1, J = 9 Hz), 3.13 (d, 1, J = 4.2 Hz), 3.20 (d, 1, J = 7.2 Hz), 3.25 (d, 1, J = 4.5 Hz), 4.22 (dd, 1, J = 7.2 Hz, 4.5 Hz), 4.57 (dd, 1, J = 9 hz, 4.2 Hz).

The material from the smaller, slower-moving peak was identical in *Rf* value on HPLC with the sample of **11** obtained in method A.

The second portion of the crude product was subjected to HPLC−mass spectrometry using an identical instrument, column, and conditions as above for the HPLC separation. The ionization mode was electrospray. The spectrum obtained from the larger, faster-moving peak showed a molecular ion (MH^+) at *m*/*z* 233 and fragment ions at 217, 173, 161, 145, 122, 112, 100, 88, and 69, all appropriate for **12.** HRMS (FAB) on the 233 peak gave a MW of 233.0684. Calcd for $C_7H_{13}N_4O_3$: 233.0708.

After two weeks, the ^{1}H NMR sample of **12** was concentrated to dryness and treated repeatedly with H_2O followed by concentration to ensure that all of the deuterium was exchanged for hydrogen. When the resulting solid was examined by mass spectrometry using the conditions described above, there was no peak at *m*/*z* 233, but there was one at *m*/*z* 234. HRMS on this peak showed a MW of 234.0544, which is appropriate for the chemical formula (MH^+) $C_7H_{12}N_3O_4$ (calcd 234.0548). This change is probably produced by hydrolysis of the C═NH group to a C═O group to afford imidazolidinedione **13**.

When imexon was reacted with dl-cysteine at 37 °C and the same concentrations as used in method A, the products were the same as those found in method A except for a trace amount of product possibly produced by hydrolysis of the imine group to a carbonyl group.

Reaction of 2-Cyanoaziridine with DL-Cysteine. A mixture of 2-cyanoaziridine (**3**, 136 mg, 2 mmol) and DL-cysteine in 2.5 mL of deionized water was warmed to approximately 40 °C to produce a solution and then immediately cooled to room temperature. After 2 h, TLC analysis (3:7 MeOH−$CHCL_3$ v/v on silica gel) showed at least four new spots, but no starting material remained. This mixture was concentrated under reduced pressure and separated by preparative TLC on a silica gel plate (20 cm × 20 cm × 0.2 cm) using the solvent system described above. The major band (slow moving) was extracted with methanol, and the extract was filtered and evaporated under reduced pressure to afford 11 mg of **14** as pale yellow solid which decomposed above 220 °C; ^{1}H NMR (D_2O, DSS) δ 1.93 (split doublet, 1), 2.21 (d,1), 3.07 (m,1) 3.35−3.64 (m, 2), 4.95 (bs s, partially under DOH); MS (LR, ES) *m*/*z* 173 (MH^+ and base peak), 128, 101, 69, 59; HRMS (FAB^+) calcd for $C_6H_8N_2O_2S$ (MH^+) 174.0418; found 174.0420.

Reaction of 2-Cyano-1-(*N*-phenylcarboxamide) with DL-Cysteine. A mixture of **6** (57 mg, 0.3 mmol) and DL-cysteine (40 mg, 0.33 mmol) in 1 mL of deionized water was heated to about 100 °C for 15 min and then stirred at room temperature for 3 h. TLC analysis (2:8 MeOH−$CHCl_3$ on silica gel) then showed no remaining starting material. The mixture was concentrated under reduced pressure, and the residue was dissolved in 1 mL of MeOH and purified by preparative TLC on a silica gel plate (20 × 20 × 0.2 cm) with 3:7 MeOH−$CHCl_3$ (v/v) as solvent. The slow moving band was extracted with MeOH, and this extract was filtered and concentrated. This procedure afforded 10 mg of **15** as off-white solid which decomposed above 200 °C; ^{1}H NMR (DMSO-d_6, TMS) δ 3−4 (m, 6), 7−7.5 (br s, 5); MS (LR, ES) *m*/*z* 309 (MH^+); HRMS (FAB^+) calcd For $C_{13}H_{16}N_4O_3S$ 309.1021, found 309.1029.

Reaction of 2-Cyanoaziridine-1-(*N*-methylcarboxamide) with DL-Cysteine. A solution of 25 mg of **8** and 25 mg of DL-cysteine in 1 mL of deionized water was stirred at room temperature for 2 h and then evaporated under reduced pressure. The white solid residue was stirred with 2 mL of ethanol, the mixture was filtered, and the solids were dried under high vacuum. Product **17** did not melt below 200 °C; ^{1}H NMR (D_2O, DSS) δ 2.7 (s, 3), 2.9−3.0 (br s, 2), 3.4−3.7 (m, 3), 5.0 (t, 1); MS (ESI) *m*/*z* 230 (MH^+, base peak) and 173 (MH^+ − CH_3NCO); HRMS (FAB^+) calcd for $C_8H_{11}N_3O_3S$ (MH^+) 230.0599; found 230.0590.

Reaction of Imexon with Glutathione. A mixture of imexon (13 mg, 0.12 mol), glutathione (36 mg, 0.12 mmol), and 1 mL of deionized water was stirred for 1 h at room temperature. The resulting solution then showed complete consumption of the imexon according to TLC on silica gel with 2:8 MeOH−$CHCl_3$ (v/v) as solvent. It was concentrated under reduced pressure, and the white solid residue was stirred 30 min with ethanol (2 mL) and filtered. The resulting solid **18** was dried under high vacuum. It did not melt below 250 °C; ^{1}H NMR (D_2O, DSS) δ 2.1 (d, 2, CH_2CO), 2.5 (m, 2, CH_2 CH_2), 2.9 (m, 2, CH_2SH), 2.95−3.2 (m, 2, CH_2 derived from imexon), 3.75 (t, 1, C*H*(NH_2)CO_2H), 3.80 (s, 2, CH_2), 3.85−3.95 (m, 1, CH derived from imexon), 4.55 (t, 1, C*H*(NH_2)CO_2H); HRMS (FAB^+) calcd for $C_{14}H_{21}N_5O_8S$ (MH^+) 420.11885; found 420.1195.

Synthesis of Aziridine-1-(*N*-phenylcarboxamide), 7. Ethyleneimine was prepared from 2-aminoethyl hydrogen

sulfate (11.3 g) and 25 mL of 40% aqueous sodium hydroxide as described in the literature.[18]

A 250 mg (6 mmol) portion of the ethyleneimine was treated with 0.65 mL (6 mmol) of phenyl isocyanate in 2 mL of toluene at 0−5 °C for 12 h. Removal of the toluene under reduced pressure afforded the product **7** in 55% yield. It had mp 78−80 °C; ^{1}H NMR ($CDCl_3$, TMS) δ 2.2 (s, 4, aziridine), 7.1 (t,1), 7.3 (t,2), 7.5 (br s, 2), 7.9 (br s, 1, NH).

Reaction of Aziridine 1-(*N*-phenylcarboxamide) with DL-Cysteine. A mixture of aziridine-1-(*N*-phenylcarboxamide) (16 mg, 0.1 mmol) and DL-cysteine (12 mg, 0.1 mmol) in 1 mL of deionized water was heated to 95−100 °C for 6 h in a tightly capped glass vial. The resulting mixture was cooled to room temperature and filtered. The solids were washed with 10 mL of ethyl acetate to remove unreacted starting material. They were then dried under vacuum. This crude product was not soluble in water or organic solvents. The only data obtained was a mass spectrum determined in the ABCI mode; *m*/*z* 283.9 (M^+), 196.9 ($M^+ - H_2C{=}C(NH_2)CO_2H$), 164.9 ($M^+ - C_6H_5NCO$), 162.9 ($M^+$ − cysteine), 136.9 ($C_6H_5NHCONH_2$). It suggests the possible presence of compound **16**.

Reaction of Imexon with *N*-Acetylcysteine. A solution of imexon (**2**,110 mg, 1 mmol) and *N*-acetylcysteine (165 mg, 1 mmol) in 2 mL of Tris buffer (pH 7.0) was warmed at 37 °C for 24 h. TLC on silica gel with MeOH−$CHCl_3$ (2:8 v/v) then indicated that all starting material was consumed. The solution was concentrated under reduced pressure, and the residual solid was extracted with 5 mL of MeOH. This extract was filtered and concentrated under reduced pressure, affording 67 mg (24.3% yield) of a mixture of **19** and **20** as white crystals, which had mp 102−105 °C; ^{1}H NMR (D_2O, DSS) δ 2.06 (d, 3, N−CH_3), 2.9−3.2 (m, 4, CH_2), 4.365 (1, CH), 4.51 (1, CH); MS (APCI) *m*/*z* 275 (MH^+) and 233 ($MH^+ - CH_2CO$) for **19** ($C_9H_{13}N_3O_5S$), 276 (MH^+) and 234 ($MH^+ - CH_2CO$) for **20** ($C_9H_{13}N_4O_5S$); HRMS (FAB) 275.0818 (calcd for **19** MH^+, 275.08137), 276.06630 (calcd for **20** MH^+, 276.06538).

The sample from NMR was concentrated under reduced pressure, and the residue was dissolved in water, kept at room temperature for one week, and evaporated under reduced pressure. The mass spectrum (APCI) then showed only the peak at *m*/*z* 276 and no peak at *m*/*z* 275, indicating completion of the hydrolysis of **19** to **20**.

Relative Reactivities of Imexon and 2-Cyanoaziridine-1-carboxamide with Cysteine. A solution of 11 mg (0.1 mmol) of imexon (**2**), 11 mg (0.1 mmol) of 2-cyanoaziridine-1-carboxamide (**1**), and 12 mg (0.1 mmol) of DL-cysteine in 0.6 mL of D_2O, containing DSS as internal standard, was analyzed by ^{1}H NMR spectrometry after 6 h. The integrals of the dd at 3.8 ppm for the CH of imexon and the dd at 3.38 ppm for the CH in **1** were compared with the corresponding peaks in separate solutions of each of these compounds in the same solvent. These spectra showed that the ratio of imexon to **1** consumed was 4:1.

Relative Reactivities of Imexon and *N*-Methylmaleimide. A solution of imexon (11 mg, 0.1 mmol), *N*-methylmaleimide (11 mg, 0.1 mmol), and cysteine (12 mg, 0.1 mmol) in 0.6 mL of D_2O containing DSS as an internal standard was examined by ^{1}H NMR spectrometry after 6 h at room temperature. The resulting spectrum was compared with reference spectra of imexon and of *N*-methyleimide without cysteine in the same solvent. These spectra showed that the imexon in the mixture was unchanged, whereas the *N*-methyleimide was nearly completely reacted, as indicated by the almost total loss of the vinyl proton singlet at 6.83 ppm.

Treatment of Acetonitrile with Cysteine. A mixture of acetonitrile (**21,** 0.5 mL) and DL-cysteine (40.9 mg) in 2 mL of deionized water was stirred at room temperature and monitored by TLC on a silica gel plate with 10% methanol in chloroform as solvent. There was no reaction indicated at 6 h. Increasing the time another 6 h at 40 °C did not produce any reaction. In contrast, when the reaction was run in the presence of sodium ethoxide as described in the literature,[17] the sodium salt of 2-methylthiazoline-4-carboxylic acid (**22**) was formed.

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