Structure-Activity Relationships for Mitomycins. Application of the Distance and Charge Analysis Method

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Molecular orbital calculations based on coordinates from X-ray analysis have been performed for a set of 24 mitomycins, of which eight compounds have not been isolated so far. To prioritize the order of synthesis of these missing compounds, a new method named DISCA (*dis*tance and *c*harge *a*nalysis) has been developed. DISCA screens correlations between spatial distribution of charge in molecules and their biological activity. The spatial distribution of charge is represented by several indexes in DISCA. LD_{50} and ED_{50} were used as measures of biological activity. DISCA has successfully extracted indexes which have significantly high correlation coefficients. The indexes with the highest correlation coefficient were common to both LD_{50} and ED_{50}. By use of the correlation functions with high correlation coefficients DISCA has predicted that 9-epi-1a-*N*-demethylmitomycin D should have the best ED_{50} and a modest LD_{50} among the missing mitomycins.

Introduction

Mitomycin C is a prominent member of the antitumor agents which are applied clinically today. Inspite of its extraordinary potencies against tumors, severe side effects, i.e., bone marrow suppression or gastrointestinal damage, have limited its clinical application. Aiming to extract the maximum therapeutic advantage of the unique chemical skeleton, hundreds of analogues have been prepared and evaluated.[1] Minor constituents from the fermentation broth of mitomycins were also exhaustively screened and several noteworthy compounds were successfully discovered.[2] This research resulted in a considerable database of compounds with various structures and antitumor activity.

Many attempts have been made to correlate structures and physicochemical properties with biological activity for mitomycins. In previous QSAR studies, the correlations of the antitumor activity with partition coefficient,[3] quinone reduction potential,[4] and substituent size[5] have been investigated. The results were relatively unsatisfactory. Recently, however, Kunz et al.[6] tried to obtain reasonable correlations between the antitumor activity and the physicochemical properties for a set of 30 mitomycin C and mitomycin A analogues. By use of a more precise and reproducible in vitro assay system they found statistically significant correlations among antitumor activity, quinone reduction potential, and the logarithm of the partition coefficient, but there was no correlation between antitumor activity and calculated relative DNA binding strengths. Kunz et al.[6] concluded that tumor uptake and/or bioreductive activation are the limiting factors in antitumor potency of mitomycins.

The three-dimensional structure and the electron distribution in a molecule might be closely related to its physicochemical properties and biological activity. Partition coefficient and quinone reduction potential also largely depend upon the molecular structure. Most of the previous mitomycin QSAR studies, however, have not fully considered the three-dimensional structures of mitomycin molecules and the electron distribution in the molecules. We thought that it might be possible to obtain reasonable structure-activity relationships if we consider these factors by use of molecular orbital calculations together with X-ray analysis results. To disclose the inherent three-dimensional structures of the mitomycin skeleton and the structural variations due to different substituents, we have undertaken the X-ray analysis of various mitomycins.[7] On the basis of the precise geometrical information obtained by X-ray analysis, we have carried out molecular orbital calculations on a group of mitomycins.

The early studies on the structure-activity relationships implied that the aziridine ring, the carbamate substituent, and the mitosane skeleton are important structural features for good activity. The derivation of the essential structural features for activity is the central subject of the structure-activity relationships studies. The results of molecular orbital calculations usually bring an enormous number of figures. It is not necessarily easy to extract useful information from the results. To overcome this problem and to make the extraction process as routine as possible, we have developed a program named DISCA. DISCA stands for *dis*tance and *c*harge *a*nalysis.

As for mitomycins, hundreds of analogues have been synthesized and isolated from nature. If we focus on a family of mitomycins, however, all chemically possible analogues have not been obtained. In other words, there are some missing compounds in the family. Selection of candidates that should be synthesized next is a very important goal of drug-design programs. Therefore it would be most helpful if we could deduce the activity and toxicity of the missing compounds prior to their synthesis.

In this paper the family of compounds shown in Figure 1 is considered. The structure-activity relationships among the known compounds have been predicted by DISCA.

Experimental Section

Compounds. In Figure 1 two different substituents were considered for X, Y, and Z. As a substituent at 9 position, a

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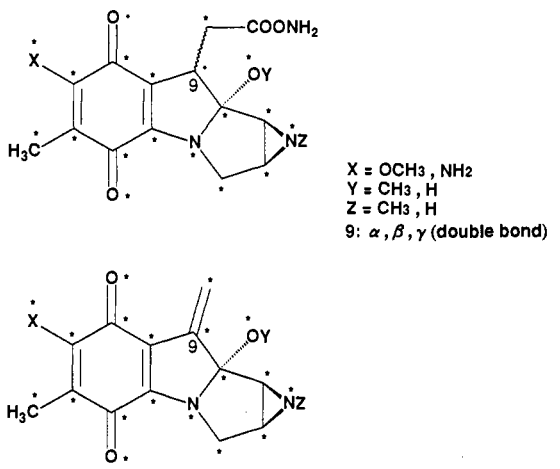

Figure 1. Chemical formula of the conventional mitomycins. The asterisked atoms were included in DISCA calculations.

Table I. Biological Activities of Mitomycins

					biological activities	
compd[a]	X	Y	Z	9	LD_{50} (mg/kg)	ED_{50} (mg/kg)
MB	OCH_3	H	CH_3	α	4.5	2.5
MJ	OCH_3	CH_3	CH_3	α	9.0	10.0
ME	NH_2	CH_3	CH_3	α	83.0	30.0
MD	NH_2	H	CH_3	α	430.0	150.0
DMJ	OCH_3	CH_3	H	α		
DMB	OCH_3	H	H	α		
DME	NH_2	CH_3	H	α		
DMD	NH_2	H	H	α		
MA	OCH_3	CH_3	H	β	2.1	1.1
MF	OCH_3	CH_3	CH_3	β	5.0	1.3
MC	NH_2	CH_3	H	β	8.4	4.4
9epiB	OCH_3	H	CH_3	β	7.5	4.9
POR	NH_2	CH_3	CH_3	β	57.0	22.0
9epiD	NH_2	H	CH_3	β	150.0	110.0
9epiDMD	NH_2	H	H	β		
9epiDMB	OCH_3	H	H	β		
MH	OCH_3	H	CH_3	γ	12.0	6.8
DMK	OCH_3	CH_3	H	γ	13.0	7.5
DMG	NH_2	CH_3	H	γ	75.0	18.0
MK	OCH_3	CH_3	CH_3	γ	22.0	35.0
MZ	NH_2	H	CH_3	γ	210.0	82.0
MG	NH_2	CH_3	CH_3	γ	130.0	100.0
DMH	OCH_3	H	H	γ		
DMZ	NH_2	H	H	γ		

[a] MA, mitomycin A; MB, mitomycin B; MC, mitomycin C; MD, mitomycin D; ME, mitomycin E; MG, mitomycin G; MH, mitomycin H; MJ, mitomycin J; MK, mitomycin K; MZ, mitomycin Z; POR, porfiromycin; D, demethyl.

(carbamoyloxy)methyl group with α and β configurations and a vinyl group were taken into account. All possible combinations make 24 compounds. Hereafter we will call these 24 compounds conventional mitomycins since most of the important members of the mitomycin family such as mitomycin C, mitomycin A, mitomycin B, and porfiromycin are included in this group. Sixteen compounds of the 24 combinations have been isolated and their biological activities have been evaluated as shown in Table I. The compounds with no data have not been isolated so far. The abbreviations of the 24 compounds are also given in Table I.

Molecular Orbital Calculations. All calculations were carried out using the MNDO-PM3 semiempirical molecular orbital method[8] in the MOPAC program suite version 6.01.[9] Starting coordinates of MB,[7a] MD,[7b] MA,[7c] MC,[7d] 9epiB,[10] Por,[7e] MH,[7f] and MG[7g] were from crystal structures. Other structures were built on the basis of the most similar crystal structures and standard bond lengths and angles. All structures were energy minimized using the Broyden–Fletcher–Goldfarb–Shanno method.[11] Minimization was performed until the energy gradient norm was less than 0.5 kcal/mol·A. The atomic charges were calculated

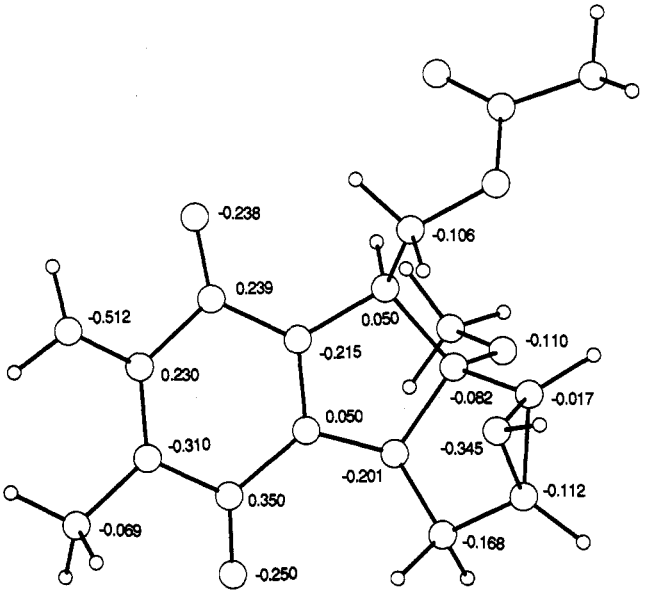

Figure 2. An optimized geometry of mitomycin C. The formal atomic charge, except the terminal moiety of the carbamoyl group, is also shown.

Table II. The Indexes with High Correlation Coefficients (>0.80)[a]

index	R	σ	a	b
	$\log LD_{50} = a \times \text{index} + b$; $n = 16$			
CHRS(X,C9a)	0.91	0.28	2.79	0.02
PDS(X,C9a)	0.91	0.28	0.46	0.02
CHRM(X,O9a)	0.91	0.29	11.9	0.66
PPM(X,O9a)	0.90	0.29	85.6	0.66
CHRM(X,C10)	0.90	0.29	9.34	0.67
PPM(X,C10)	0.90	0.29	51.5	0.66
CHRM(C6,O9a)	0.87	0.33	21.1	0.56
PPM(C6,O9a)	0.87	0.33	124	0.33
PDS(N4,C6′)	0.85	0.36	−1.45	0.45
CHRS(N4,C6′)	0.85	−0.36	−7.36	0.45
PDS(C7,O9a)	0.84	0.37	0.74	0.07
CHRS(C7,O9a)	0.84	0.37	4.36	0.07
	$\log ED_{50} = a \times \text{index} + b$; $n = 16$			
CHRS(X,C9a)	0.85	0.35	2.58	−0.15
PDS(X,C9a)	0.85	0.35	0.42	−0.15
CHRS(C6′,C10)	0.82	0.38	−4.56	0.67
PDS(C6′,C10)	0.82	0.38	−0.68	0.67
PDS(C7,C10)	0.81	0.39	0.79	−0.14
CHRS(C7,C10)	0.81	0.39	3.67	−0.14
PPM(X,C9a)	0.81	0.39	−59.3	0.62
CHRM(X,C9a)	0.81	0.39	−9.67	0.62

[a] R designates correlation coefficient. X denotes oxygen and nitrogen atoms in the methoxy and amino groups, respectively.

with the electrostatic potential method.[12] The optimized geometry of mitomycin C together with the atomic charge is shown in Figure 2.

DISCA. In addition to the three-dimensional disposition of functional groups, the spatial distribution of the electric charge in a molecule must be closely correlated to its physicochemical properties and also could be decisive for intermolecular interactions with other molecules. We thought that the relative disposition of charge in a molecule is more important. To represent the three-dimensional disposition of functional groups and charge in molecules we adopted the following five indexes by combination of formal charge and interatomic distance: $d(i,j)$, DIST(i,j); $c(i) - c(j)$, CHRS(i,j); $c(i)c(j)$, CHRM(i,j); $(c(i) - c(j))d(i,j)$, PDS(i,j); $(c(i)c(j))/d(i,j)$, PPM(i,j). $c(i)$ stands for the atomic formal charge of atom i and $d(i,j)$ the interatomic distance between atoms i and j. DIST, CHRS, CHRM, PDS, and PPM are abbreviations for those indexes and are defined below. The distance between atoms are calculated on the basis of the geometry optimized by molecular orbital calculations. The indexes are calculated for all pairs of non-hydrogen atoms in each molecule

Table III. Predicted log LD_{50} and log ED_{50} (in Parentheses) for the Missing Mitomycins[a]

R	index	mitomycins (predicted biological activity)							
					log LD_{50}				
0.91	PDS(X,C9a)	DMJ (0.46)	9epiDMD (0.55)	DMB (0.64)	DMH (0.89)	9epiDMB (1.50)	DME (1.58)	DMD (1.74)	DMZ (1.94)
0.90	PPM(X,O9a)	DMJ (0.85)	DMB (0.99)	9epiDMD (1.01)	DMH (1.01)	DME (1.74)	DMD (2.37)	9epiDMB (2.39)	DMZ (2.50)
0.90	PPM(X,C10)	9epiDMD (0.72).	DMJ (0.93)	DMB (0.93)	DMH (0.98)	9epiDMB (1.28)	DME (2.10)	DMD (2.26)	DMZ (2.58)
0.87	PPM(C6,O9a)	DMJ (0.69)	DMB (0.72)	DMH (1.04)	9epiDMD (1.19)	DME (1.65)	DMD (2.20)	DMZ (2.37)	9epiDMB (2.40)
0.85	PDS(N4,C6′)	DMJ (0.43)	DMB (0.92)	9epiDMD (1.12)	DMH (1.18)	DME (1.65)	9epiDMB (1.95)	DMZ (2.16)	DMD (2.16)
0.84	PDS(C7,O9a)	DMJ (0.67)	DMB (1.07)	DMH (1.30)	9epiDMD (1.32)	DME (1.82)	DMD (2.18)	DMZ (2.23)	9epiDMB (2.31)
					log ED_{50}				
0.85	PDS(X,C9a)	DMJ (0.26)	9epiDMD (0.34)	DMB (0.42)	DMH (0.66)	9epiDMB (1.21)	DME (1.29)	DMD (1.44)	DMZ (1.62)
0.82	PDS(C6′,C10)	9epiDMD (−0.02)	DMJ (0.84)	DMB (0.85)	9epiDMB (0.93)	DMH (1.22)	DME (1.73)	DMD (1.94)	DMZ (2.18)
0.81	PDS(C7,C10)	9epiDMD (0.04)	DMB (0.81)	DMJ (0.89)	DMH (1.11)	9epiDMB (1.12)	DME (1.83)	DMD (1.90)	DMZ (2.09)
0.81	PPM(X,C9a)	DMJ (0.68)	9epiDMD (0.71)	9epiDMB (0.71)	DMB (0.73)	DME (0.79)	DMH (0.82)	DMD (1.08)	DMZ (1.52)

[a] See Table I for compound abbreviations.

Table IV. Weighted Average of log LD_{50} and log ED_{50}

			$S(LD_{50})$[a]				
DMJ	DMB	9epi-DMD	DMH	DME	9epi-DMB	DMD	DMZ
0.67	0.87	0.98	1.06	1.76	1.96	2.15	2.30
			$S(ED_{50})$[a]				
9epi-DMD	DMJ	DMB	DMH	9epi-DMB	DME	DMD	DMZ
0.27	0.66	0.70	0.95	0.99	1.41	1.59	1.85

[a] $S(LD_{50}) = \sum_{i=1}^{6} R_i(\log LD_{50})_i / \sum_{i=1}^{6} R_i$ $S(ED_{50}) = \sum_{i=1}^{4} R_i(\log ED_{50})_i / \sum_{i=1}^{4} R_i$ where R_i is the correlation coefficient of the ith index in Table III.

under consideration. Correlation functions between biological activity and all the computed indexes are further examined to extract the indexes which have intimate correlations with observed biological activity. All the possible combinations are examined completely. Therefore the process is quite routine, and no prior knowledge about substantial pharmacophores is required. Only the linear regression function is considered in the current version of DISCA.

The meanings of DIST, CHRS, and CHRM are obvious. PDS corresponds to a moment around the midpoint between atoms i and j and could play a role in steering mitomycins to interact with other molecules. Locating and orienting a drug effectively at the receptor site are very important steps for drug action. Therefore PDS could have high correlation with biological activity. PPM is related to the electrostatic potential between atoms i and j in a molecule and could contribute to determining the stiffness or softness of the conformation. PPM might also contribute to the strength of the intermolecular interactions between mitomycins and relevant receptors.

All 19 non-hydrogen atoms with an asterisk in Figure 1 were included in the DISCA calculations. The (carbamoyloxy)methyl side chain adopts various conformations in some mitomycin crystal structures.[13] The torsion angle of C9–C10–O10–C10a is in particular full of variety. This indicates that the side chain is quite flexible. We have no good reasons to restrict the conformation of the side chain. The binding sites on mitomycin C have been identified to be C1 and C10.[13] This implies that the terminal part of the side chain may not be so important for the binding process itself. In the recent model of a monovalent adduct between a mitomycin and DNA,[14] the terminal part protrudes from the minor groove. In addition we take the γ series compounds without the side chains into consideration. Under these circumstances, we felt it was better to leave the flexible part of the side chain out of consideration. Therefore only the C10 atom was included in the calculations.

All ${}_{19}C_2$ combinations for each index were calculated for each compound. Correlation functions between biological activity and 855 different indexes were examined exhaustively for the 16 mitomycins which have already been isolated and for which biological activities have been measured.

LD_{50} and ED_{50} were used as the measure of biological activity.[15] LD_{50} values of ip administration were calculated in male ddY mice by probit analysis. ED_{50} doses that gave 50% inhibition of tumor growth were calculated from the dose–response curve. Sarcoma 180 cells (5×10^6/mouse) were inoculated sc into ddY mice on day 0, and drugs were injected ip on day 1. Tumor volume was measured on day 7.

Results and Discussion

The indexes with the highest correlation coefficients (>0.80) are tabulated in Table II. Hereafter we will designate the correlations between the indexes and LD_{50} and ED_{50} as LD_{50} and ED_{50} correlations, respectively.

The indexes PDS (X, C9a) and CHRS (X, C9a) with the highest correlation coefficients are common to both biological activities. That the index DIST (X, C9a) does not have any high correlations with both LD_{50} and ED_{50} is noteworthy. It clearly shows that the relative disposition of X and C9a atoms are fixed but that the charge distribution is significantly different. The information about the three-dimensional arrangements of pharmacophores is most essential for effective drug design. Therefore the information which was obtained automatically from DISCA is very valuable.

By use of the correlation functions with high correlation coefficients and the calculated indexes for the missing compounds we can predict the biological activities for them. The predicted LD_{50} and ED_{50} for the missing compounds are tabulated in Table III. In Table III CHRS and CHRM are not included because the correlations for them are almost equivalent to the corresponding PDS and PPM, as shown in Table II. This also indicates that the distance between two atoms which contribute to PDS or PPM with high correlations is almost constant. The indexes with high correlation coefficients are not totally independent of each other. At present, however, it is not easy to extract the independent indexes only. Therefore multiple indexes are used here to predict the activity. All indexes are not necessarily in harmony with each other.

By combination of multiple correlations we can determine the order and approximate magnitude of the activity. In drug-design strategy it is usually important to determine the derivatives which should be synthesized next. Therefore the aforementioned information is sufficient enough for the decision making. In Table IV weighted averages of log LD_{50} and log ED_{50} are shown. The correlation coefficients are used as weights and weighted logarithms are averaged for the indexes listed in Table III. $S(LD_{50})$ and $S(ED_{50})$ could be good standards to determine the candidates to be synthesized. Among the missing compounds, 9epiDMD is the best compound to be prepared next. DMZ, DMD, and DME may be trivial compounds. The predicted ED_{50} and LD_{50} for 9epiDMD are calculated to be 1.8 and 9.6, respectively. The ED_{50} value is lesser and the LD_{50} is marginally larger than the corresponding values of mitomycin C. Contrary to our expectations, the prediction has obviously shown that the probability of finding decidedly better drugs than mitomycin C among the missing compounds is low.

Conclusions

DISCA has been applied to a set of conventional mitomycins and successfully found several indexes which have significant correlations with biological activity. On the basis of the correlation functions a prediction has been made to select candidates for synthesis. Although the candidates are expected to possess reasonably high potency against tumors and modest toxicity, the prediction should be confirmed by experiments in the future. Unfortunately the predicted biological activity is not definitely superior to that of mitomycin C, which has been the best drug in the mitomycin family so far. Therefore the validity of the method presented in this paper has not been verified yet as far as the structure–activity relationships of mitomycins are concerned.

The method, however, is a general one and can be applicable to other structure–activity relationships programs. In drug-design programs there is usually a large set of compounds. Due to practical reasons, however, all chemically possible combinations are not necessarily in hand. In some cases it is not easy to synthesize these missing compounds and it is most desirable to estimate their toxicity and activity before making the decision to synthesize them. A routine application of DISCA to these problems could give some ideas about the priority of synthesis and the expectations of the results. If we apply the conventional QSAR methods to problems of this kind, we should have some information about pharmacophores and some properties which are related to the biological activity. Application of DISCA, however, does not require any prior knowledge at all and is quite routine. Therefore we believe that DISCA has a very wide application.

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References

(1) Remers, W. A.; Dorr, R. T. Chemistry, Biology, and Therapeutics of the Mitomycins. In Alkaloids: Chemical and Biological Perspectives: Pelletier, S. W., Ed.; Wiley-Interscience: New York, 1988; Vol. 6, pp 1–74.
(2) Shirahata, K.; Kono, M.; Matsubara, I.; Kasai, M. New Mitomycins, Structure Determination, Derivation and Their Activity. Abstracts of 23rd Symposium on the Chemistry of Natural Products, 1980, p. 608–615.
(3) Sami, S. M.; Iyengar, B. S.; Tarnow, S. E.; Remers, W. A.; Bradner, W. T.; Schurig, J. E. Mitomycin C Analogues with Aryl Substituents on the 7-Amino Group. *J. Med. Chem.* **1984**, *27*, 701–708.
(4) Matsui, M.; Yamada, Y.; Uzu, K.; Hirata, T. Studies on Mitomycins. III. The Synthesis and Properties of Mitomycin Derivatives. *J. Antibiot.* **1968**, *21*, 189–198.
(5) Moriguchi, I.; Komatsu, K. Adaptive Least-Squares Classification Applied to Structure-Activity Correlation of Antitumor Mitomycin Derivatives. *Chem. Pharm. Bull.* **1977**, *25*, 2800–2802.
(6) Kunz, K. R.; Iyengar, B. S.; Dorr, R. T.; Alberts, D. S.; Remers, W. A. Structure-Activity Relationships for Mitomycin C and Mitomycin Analogues. *J. Med. Chem.* **1991**, *34*, 2281–2286.
(7) (a) Hirayama, N.; Shirahata, K. Structural Studies of Mitomycins. I. Absolute Configurations of Mitomycins A and B. *Acta Crystallogr.* **1987**, *B43*, 555–559. (b) Hirayama, N.; Arai, Y.; Kasai, M. Structural Studies of Mitomycins. VII. Structure of Mitomycin D. *Acta Crystallogr.* Submitted. (c) Hirayama, N.; Shirahata, K. Structural Studies of Mitomycins. II. Structure of Mitomycin A Hemihydrate. *Acta Crystallogr.* **1989**, *C45*, 1780–1783. (d) Shirahata, K.; Hirayama, N. Revised Absolute Configuration of Mitomycin C. X-ray Analysis of 1-N-(p-Bromobenzyl)mitomycin C. *J. Am. Chem. Soc.* **1983**, *105*, 7199–7200. (e) Hirayama, N. Unpublished work. (f) Hirayama, N. Structural Studies of Mitomycins. V. Structure of Mitomycin H. *Acta Crystallogr.* **1991**, *C47*, 604–606. (g) Hirayama, N.; Arai, Y.; Kasai, M. Structural Studies of Mitomycins. VII. Structure of Mitomycin D. *Acta Crystallogr.* Submitted.
(8) (a) Stewart, J. J. P. Optimization of Parameters for Semiempirical Methods. I. Method. *J. Comput. Chem.* **1989**, *10*, 209–220. (b) Stewart, J. J. P. Optimization of Parameters for Semiempirical Methods. II. Applications. *J. Comput. Chem.* **1989**, *10*, 221–264. (c) Stewart, J. J. P. MOPAC: A General Molecular Orbital Package (Version 6.0). *QCPE Bull.* **1990**, *10*, 86–87.
(9) Hirano, T. MOPAC Version 6: Revised as Version 6.01 by T. Hirano for UNIX Machines. *JCPE Newslett.* **1991**, *3*, 28–29.
(10) Egbertson, M.; Danishefsky, S. J.; Schulte, G. On the Remarkable Stability of Derivatives of Leucomitomycin F. Novel Mitomycin Analogues. *J. Org. Chem.* **1987**, *52*, 4424–4426.
(11) Shanno, D. F. An Example of Numerical Nonconvergence of a Variable-Metric Method. *J. Optimization Theory Appl.* **1985**, *46*, 87–94.
(12) Besler, B. H.; Merz, K. M.; Kollman, P. A. Atomic Charges from Semiempirical Methods. *J. Comput. Chem.* **1990**, *11*, 431–439.
(13) Moore, H. W. Bioactivation as a Model for Drug Design Bioreductive Alkylation. *Science (Washington, D.C.)* **1977**, *197*, 527–532.
(14) Arora, S. K.; Cox, M. B.; Arjunan, P. Structural, Conformational, and Theoretical Binding Studies of Antitumor Antibiotic Porfiromycin (N-Methylmitomycin C), a Covalent Binder of DNA, by X-ray, NMR, and Molecular Mechanics. *J. Med. Chem.* **1990**, *33*, 3000–3008.
(15) Morimoto, M.; Ahizawa, T.; Ohno, H.; Azuma, M.; Kobayashi, E.; Okabe, M.; Gomi, K.; Kono, M.; Saitoh, Y.; Kanda, Y.; Arai, H.; Sato, A.; Kasai, M.; Tsuruo, T. Antitumor Activity of 7-N-{{2-{-[2-(γ-L-Glutamylamino)ethyl]dithio}ethyl]}-mitomycin C. *Cancer Res.* **1991**, *51*, 110–115.