Ames Test of 1-(X-Phenyl)-3,3-dialkyltriazenes. A Quantitative Structure–Activity Study[1]

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The mutagenicity of 1-(X-phenyl)-3,3-dialkyltriazenes was tested in the Ames test using *Salmonella typhimurium* TA92. The following quantitative structure–activity relationship (QSAR) was formulated: $\log 1/C = 1.09 \log P - 1.63\sigma^{+} + 5.58$. In this expression, C is the molar concentration of triazene producing 30 mutations/10^8 bacteria above background. This equation is based on 17 congeners and has a correlation coefficient of 0.974. The QSAR for mutagenicity is compared with QSAR for antileukemia action and toxicity (LD_{50}) in mice. The mutagenicity of aflatoxin B ($\log 1/C = 9.5$) and DTIC ($\log 1/C = 3.0$) have also been determined.

The Ames test for the mutagenicity of organic compounds has almost become a household term. Accounts are now appearing in newspapers and magazines about the value of this test in estimating the carcinogenicity of hair dyes, grilled hamburgers, etc. Many of the large companies that produce our new drugs, pesticides, and other organic compounds destined for industrial production now routinely test prospective products in the Ames assay.

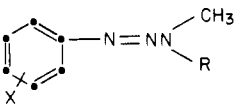
The Ames test[2-4] is based on strains of mutant bacteria which require histidine for growth. The strains have been selected for their sensitivity and specificity with which they revert back to the prototrophic wild type in the presence of mutagens. Preliminary evidence shows that there seems to be a close relationship between mutagenicity and carcinogenicity.[5]

There is now intense discussion about the ultimate value of the Ames test for predicting carcinogenicity.[6,7] Although the Ames test cannot be said to give a definitive answer about the carcinogenicity of a given compound in humans, it is becoming more or less accepted as the best estimate short of a direct and very expensive test for carcinogenicity in animals.

Because of the intense interest in and widespread use of the Ames assay, we believe it to be an excellent system for the study of quantitative structure–activity relationships (QSAR) which our laboratory has been developing.[8,9] There are a number of aspects of such a study which fascinate us. Of first importance is the determination of those structural features which increase the mutagenic potency of organic compounds. It has been pointed out[7] that there is a 1 000 000-fold range in the mutagenicity and carcinogenicity of organic compounds. Such sensitivity to changes in chemical structure is ideal for QSAR. The question then arises, would it be possible by substituent changes in a parent mutagenic compound to eliminate mutagenic (and probably carcinogenic) character while still maintaining some other desirable biological activity? This is an important question in the present search for better antitumor drugs. Almost all of the present drugs used in the treatment of cancer are carcinogenic. Finally, we believe that QSAR can offer valuable insight into the details of the mechanism of exactly how organic compounds produce mutations.

There has been concern that the Ames test cannot be placed on a quantitative basis so that numerical comparison can be made for the relative potency of various compounds.[6] This problem did not seem very serious to us, since in the past 15 years thousands of QSAR have been formulated for the action of organic compounds acting on hundreds of different biological systems, many of which are more complicated than the Ames system.

In initiating our study of mutagenicity, we have elected to investigate triazenes of type I in the *Salmonella typhimurium* strain TA92. QSAR for these triazenes acting as antitumor agents have already been formulated.[10,11] This allows us to compare the QSAR for mutagenicity (eq 1–4) with the QSAR for antitumor activity of the triazenes. It should be noted that these triazenes have definitely been established as carcinogenic in animal tests.[12]



I

One of Ames' important discoveries is that many compounds which are not mutagenic as such can be made to produce mutations in bacteria if rat liver microsomes are included in the test system. The triazenes are in this class; they do not appear to be carcinogenic or mutagenic until they have been activated by microsomes.

Experimental Section

Bacterial Strains. *Salmonella typhimurium* strain TA92 was obtained from Bruce N. Ames. Strain TA92 was originally derived from *Salmonella typhimurium* LT_2 and contains histidine mutation *hisG46* and R factor plasmid pKM101.

Media and Culturing. Manipulation of *S. typhimurium* TA92 was carried out essentially as recommended by Ames.[2] Frozen permanents were stored at −80 °C, while a working copy from which subcultures were obtained was kept at −20 °C. Portions of 5 mL of nutrient broth were subsequently inoculated with a small sample of bacteria from the working copy and incubated for 12–16 h at 37 °C, yielding approximately 10^9 cells/mL. Each subculture was tested for histidine deficiency by streaking on minimal nutrient plates both with and without histidine and for the presence of the R factor by observations of ampicillin resistance.

Rat Liver Preparations. The liver homogenate fraction S-9 was prepared according to Ames.[13] Female albino rats (Sprague–Dawley/Bio-I strain) were maintained on Wayne Lab-Blox rat chow. Five days prior to sacrifice, the animals were injected intraperitoneally with Aroclor 1254 (diluted in corn oil to 200 mg/mL) at a dosage of 500 mg/kg. The rats were given water ad libitum and food until 12 h before sacrifice. They were killed by cranial fracture and cervical dislocation, their bodies were washed with alcohol within a sterile UV light transfer chamber, and the liver was removed and placed in a sterile ice-cold preweighed beaker. All steps were carried out at −0.4 °C with sterile solutions and glassware. The liver was washed with an equal volume of 0.15 M KCl solution and homogenized (3 mL/g of wet liver) in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged for 10 min at 9000*g* and the supernatant (S-9) fraction decanted. Aliquots of 1.5 mL were frozen in dry ice and stored at −80 °C. As they were required, the aliquots were thawed at room temperature and kept in ice. Unused portions were discarded. The "S-9 mix" was made to contain per milliliter: 0.2 mL of S-9 fraction, 8 mmol of $MgCl_2$, 33 mmol of KCl, 5 mmol of glucose 6-phosphate, 4 mmol of NADP, and 100 mmol of sodium phosphate buffer (pH 7.4).

Mutagenicity Test. Quantitative plate tests were performed as follows: plastic Agar plates (100 × 15 mm) were prepared in two steps in which approximately 10 mL of sterile minimal medium Vogel-Bonner E[14] plus 1.5% agar (Difico purified) and 2% glucose were used as the bottom layer and 2 mL of soft agar

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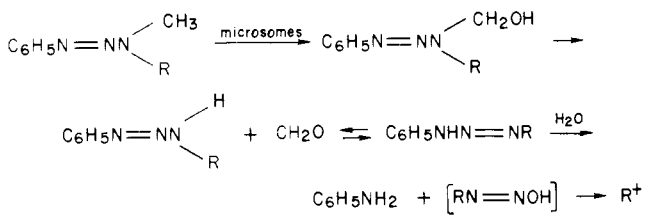
Table I. Parameters Used in the Formulation of Equation 1 for the Mutagenic Activity of X-$C_6H_4N{=}NN(CH_3)R$

no.	X	R	log 1/C obsd	log 1/C calcd	$\lvert\Delta$ log 1/C$\rvert$	log P	σ^+
1	4-$CONH_2$[a]	*t*-Bu	3.83	6.26	2.43	2.61	−0.30
2	3,5-CN	CH_3	3.46	3.50	0.04	2.18	1.12
3	4-SO_2NH_2	CH_3	3.49	3.15	0.34	0.98	0.57
4	3-$CONH_2$	CH_3	3.51	3.86	0.35	1.21	0.28
5	4-$CONH_2$	CH_3	4.04	3.72	0.32	1.20	0.36
6	4-$CONH_2$	allyl	4.16	4.65	0.49	2.09	0.36
7	3-$NHCONH_2$	CH_3	4.19	4.45	0.26	1.29	−0.03
8	4-CN	CH_3	4.43	4.47	0.04	2.39	0.66
9	4-$COCH_3$	CH_3	4.47	4.61	0.14	2.27	0.50
10	H	CH_3	5.32	5.75	0.43	2.59	0.00
11	4-$CONH_2$	*n*-Bu	5.41	5.03	0.38	2.46	0.36
12	4-$NHCONH_2$	CH_3	5.59	5.72	0.13	1.25	−0.84[b]
13	4-$NHCOCH_3$	CH_3	5.83	5.64	0.19	1.54	−0.60
14	4-CF_3	CH_3	5.99	5.91	0.08	3.70	0.61
15	3-CH_3	CH_3	6.44	6.14	0.30	2.85	−0.07
16	4-Cl	CH_3	6.48	6.34	0.14	3.33	0.11
17	4-CH_3	CH_3	7.00	6.61	0.39	2.93	−0.31
18	4-C_6H_5	CH_3	7.67	7.93	0.26	4.40	−0.18

[a] This data point not used in the derivation of eq 1. [b] Estimated value.

Scheme I



(0.6% agar, 0.6% NaCl) was used as the top layer. To each 100 mL of top agar, 10 mL of a sterile 0.5 mmol of L-histidine–0.5 mmol of *d*-biotin solution was added after autoclaving and cooling the agar to 45 °C. The top agar was used the day of preparation or stored overnight at 4 °C.

Each test compound was weighed in a sterile screw-cap vial. All of the following steps were performed inside a sterile transfer chamber. The compounds were serially diluted in Me_2SO to the appropriate test concentration, and 0.1 mL of the mutagen solution was added to capped sterile tubes. To this was added 0.1 mL of the bacterial strain and 0.5 mL of the chilled S-9 mix. To increase the sensitivity of the assay, the tubes were removed from the chamber and preincubated for 20 min in a 30 °C water bath. Immediately after preincubation, 2 mL of the top agar solution was added to each tube and the entire mixture poured onto the Petri plates containing the bottom agar. The top agar was allowed to harden for 30 min to 1 h, and the plates were then placed in a dark incubator at 37 °C for 2 days.

The number of bacteria contained in 0.1 mL of the nutrient broth was determined by a 10^6 dilution in 0.85% saline solution and plating on a high-nutrient agar (1% tryptone, 0.5% NaCl, 0.5% yeast extract, and 1.5% agar Difco). The number of bacteria contained per milliliter of the undiluted overnight broth was determined from the number of revertants produced on these plates.

In order to measure the number of spontaneous revertants per plate, controls of 0.1 mL of Me_2SO, 0.1 mL of bacteria, and 0.5 mL of S-9 mix without mutagens present were run. The number of revertants produced on the control plates (about 50) were subtracted from the number of revertants on the test plates. All data points were determined in triplicate, and the average log 1/*C* was used. Each compound was tested at least twice. We elected to use 30 revertants/10^8 bacteria as our standard end point, since this allowed us to avoid extrapolations into the toxicity range of some of the compounds. In all cases, a plot of activity vs. concentration of mutagen yielded a "parabolic curve". At the higher concentrations, the toxicity of the compounds killed many of the bacteria. In most cases, only small linear interpolations were needed to get the desired log 1/*C*.

Scoring of the Results. After 2 days of incubation at 37 °C, all revertants per plate were hand counted with the aid of an electronic bacterial colony counter.

Physicochemical Constants. The σ^+ and log *P* values were taken from our recent study.[10] Some values were calculated using the correlation equation developed in this study.

Results and Discussion

We have derived eq 1 and 2 from the data in Table I.

$$\log 1/C = 1.01\ (\pm 0.48)\ \log P + 2.84\ (\pm 1.2) \quad (1)$$

$$n = 17;\ r = 0.759;\ s = 0.875$$

$$\log 1/C = 1.09\ (\pm 0.17)\ \log P - 1.63\ (\pm 0.35)\ \sigma^+ + 5.58\ (\pm 0.95) \quad (2)$$

$$n = 17;\ r = 0.974;\ s = 0.315$$

C in these equations is the molar concentration of triazene producing 30 mutations/100 million bacteria. Using σ in place of σ^+ in eq 2 gives a much poorer correlation (r = 0.946; s = 0.452). Equation 2 is a significant improvement over eq 1 ($F_{1,14}$ = 102; $F_{1,14;\alpha=0.001}$ = 17.1). Adding a term in $(\log P)^2$ to eq 2 does not result in a reduction of the variance.

Equation 2 shows that increasing lipophilicity and increased electron release via through resonance increase mutagenicity. Years ago we found[15] in our first QSAR for the carcinogenicity of polycyclic aromatic hydrocarbons and acridines that increased electron density, and, up to a point, increased lipophilicity, produced more carcinogenic congeners. It is not yet evident from eq 2 what the ideal value of log *P* in the Ames test with the triazenes is.

There is a 16 000-fold range in the mutagenicity of the triazenes, and eq 2 predicts potency within a factor of ±2. These results provide preliminary evidence that quantitative comparisons of mutagenicity can be made via the Ames assay.

Since the first step in the mutagenic process is the activation of the triazenes by the microsomes, one might expect the same dependence on log *P* that one finds in the microsomal oxidation of organic compounds.[16] To a certain degree, eq 3 supports this idea. In this equation K_M is the

$$\log 1/K_M = 0.69 \log P + 2.90 \quad (3)$$

$$n = 14;\ r = 0.920;\ s = 0.330$$

Michaelis constant for the microsomal oxidation of a variety of organic compounds (barbiturates, dimethylanilines, etc.). The parameter V_{max} for this series of compounds was almost constant so that oxidation depends mostly on binding of the drugs to the microsomes.

Equation 3 shows this to be a highly log P dependent process with a dependence similar to that of eq 2. The first step in the microsomal oxidation of the triazenes is removal of a hydrogen from an N-alkyl group. Electron release as characterized by σ^+ would appear to aid this process.

Equation 2 can be compared with the antitumor activity[10] of the triazenes against L1210 leukemia, as correlated by eq 4. Equation 4, which is based on 61

$$\log 1/C = 0.10 \log P - 0.042(\log P)^2 - 0.31\sigma^+ - 0.18\mathrm{MR}_{2,6} + 0.39E_s\text{-R} + 4.12 \quad (4)$$

$$n = 61;\ r = 0.836;\ s = 0.191;\ \log P_0 = 1.18$$

congeners, contains a number of analogues having substituents in the ortho position. Their deleterious effect is parameterized by $\mathrm{MR}_{2,6}$. The E_s-R term shows that bulky groups on the nitrogen side chain also reduce activity. The most potent antitumor triazenes have log P values of 1.18. The antitumor activity correlated by eq 4 is also promoted by electron-releasing substituents via through resonance (σ^+); however, the coefficient with the σ^+ term in eq 4 is much smaller than in eq 2. Why mutagenicity is so much more sensitive than antitumor activity to the electronic effect of substituents is, as of the present, not clear.

The general toxicity of the triazenes is correlated[11] by eq 5 for the LD_{50} with mice. The σ^+ coefficient in eq 5 is

$$\log 1/C = -0.024(\log P)^2 - 0.26\sigma^+ + 3.49 \quad (5)$$

$$n = 11;\ r = 0.913;\ s = 0.110$$

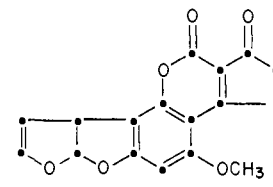
essentially the same as in eq 4. Both gross toxicity and antitumor activity in mice show the same dependence on the electronic effects of substituents. It is possible that the different electronic effects in both eq 2 and 4 may be related to the bacterial DNA which is presumably being attacked. We plan to test the triazenes in the sister chromatid exchange assay so that mutagenicity in bacterial cells can be compared with that in mammalian cells.

One congener is of particular interest. The compound with the *tert*-butyl group in the side chain is poorly fit by eq 2 and, in fact, was not used in deriving eq 1 and 2. This congener is completely inactive as an antitumor agent, which suggests a steric problem of the triazenes in their reaction with DNA.

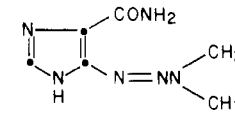
It has been postulated that the triazenes undergo the reactions shown in Scheme I after microsomal oxidation. Thus, the active species attacking DNA in mutagenisis or antileukemic action is a carbonium ion. The very low activity of 1 in Table I suggests steric hindrance in the attack of $(CH_3)_3C^+$ on DNA. It may be possible to take advantage of this steric effect to design more selective mutagens.

Using eq 1 and 4 we can illustrate how mutagenicity (and presumably carcinogenicity) can be minimized with relatively little loss in antitumor potency. For the congener of I where 4-X = H and R = CH_3, eq 4 gives a calculated value of log $1/C$ of 3.58; where X = 4-SO_2NH_2 and R = CH_3, the calculated value is 3.48. Antitumor potency has been reduced by 0.10 or a factor of 1.2. Equation 1 predicts log $1/C$ of 5.75 and 3.15 for the same two congeners with a 2.6-unit or 400-fold decrease in mutagenic potency. By manipulating the electronic and lipophilic character of substituents, much can be done to decrease mutagenicity without destroying the pharmaceutical properties of a drug. This possibility has also been discussed by Bueding and Batzinger.[17]

For benchmarks for comparative mutagenic potency, aflatoxin B_1 (II) and DTIC (III) were assayed to find log



II



III

$1/C$ for 30 revertants/10^8 bacteria. Log $1/C$ for aflatoxin was found to be 9.48, and the value for DTIC was 3.00.

Aflatoxin B_1 is one of the most carcinogenic compounds known. It is about 10^7 (on our scale) more mutagenic than DTIC. This supports Ames' view[7] that there is a wide range in mutagenicity and carcinogenicity.

DTIC continues to be widely used in clinical antitumor studies. We cannot include it in eq 1 because we lack the appropriate σ constant. The log P for DTIC is −0.24; this alone accounts for most of its relative lack of mutagenic activity.

Kolar and his co-workers have made extensive studies on the mechanism of the biological action of triazenes.[18] They have also shown that triazenes produce mutations[18a] using *Salmonella typhimurium hisG46*. However, they did not attempt to formulate a QSAR with their results. It is noteworthy that Kolar et al.[18e] were able to obtain mutations by triazenes with yeast cells (*Saccharomyces cerevisiae*) without the use of microsomes. Three triazenes of type I (4-OMe, 4-OH, and 4-NHCOMe) showed high activity. Analogues with electron-attracting groups showed low activity. The authors conclude that mutagenicity may be due to the triazene hydrolyzing and the resulting diazonium compound decomposing to yield a carbonim ion. However, we were unable to produce mutations above background in TA92 using congeners with the most electron-releasing groups (e.g., 4-Me) or any of the other congeners. The triazenes are very toxic to TA92 and it may be that a fragment such as $C_6H_5^+$ is not as mutagenic as CH_3^+; if so, the higher concentration of triazene required might inhibit cell growth. Kolar et al.[18e] and others have pointed out that triazenes may produce mutations by two different mechanisms. Our finding shows there may be considerable variation among different organisms.

In closing, it should be pointed out that we have selected as a standard response the molar concentration of mutagen which produces a standard number of mutations. This conforms with our general model for correlating chemical structure with biological activity. The problem of defining biological response in numerical terms for correlation analysis has been discussed by Martin[19] and Cammarata and Rogers.[20]

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References and Notes

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Computer-Assisted Structure–Activity Studies of Chemical Carcinogens. A Heterogeneous Data Set

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A structure–activity relations study has been performed on a heterogeneous set of organic compounds to develop predictive ability for carcinogenic potential. The compounds employed came from more than 12 structural classes and numbered 130 carcinogens and 79 noncarcinogens. A set of 28 calculated molecular structure descriptors was identified that supported a linear discriminant function able to completely separate 192 compounds into the carcinogenic and noncarcinogenic classes. A predictive ability of 90% for carcinogens and 78% for noncarcinogens was obtained in randomized testing. The results demonstrate that pattern-recognition methods can be used to analyze a diverse set of compounds each represented by calculated molecular structure descriptors for a common biological activity.

The attempt to rationalize the connection between the molecular structures of organic compounds and their biological activities comprises the field of structure–activity relations (SAR) studies. Correlations between structure and activity are important for the development of pharmacological agents, herbicides, pesticides, and chemical communicants (olfactory and gustatory stimulants) and the investigation of chemical toxicity and mutagenic and carcinogenic potential. Practical importance attaches to these studies because the results can be used to predict the activity of untested compounds. In addition, SAR studies can direct the researcher's attention to molecular features that correlate highly with biological activity, thus suggesting mechanisms or further experiments. SAR studies have been used to some extent in the pharmaceutical and agricultural industries. The methods are beginning to be applied to the important problems of chemical toxicity and chemical mutagenesis and carcinogenesis.

Evidently, chemical carcinogenesis poses a public health problem of enormous magnitude. A dilemma confronts regulatory agencies and chemically related industry, namely, how to test the enormous numbers of compounds that are produced or could be produced in order to avoid exposure to toxic materials which could lead to adverse effects among the population. While the most satisfactory approach is to use rodent testing, this is uneconomical for the large number of compounds involved. Thus, a new set of short-term tests have been applied to the problem.[1,2] Recently, a set of techniques drawn from SAR studies in pharmacology have been applied to sets of chemical carcinogens in an attempt (a) to develop predictive capability for unknown compounds and (b) to further fundamental understanding of the structural features of molecules that can lead to carcinogenic potential.

The superior way to develop predictive capability is to understand, at the molecular level, the mechanisms that lead to the undesired carcinogenic initiation reaction. Unfortunately, this knowledge is not yet available for most classes of chemical carcinogens. Furthermore, the progress made through a living system by a carcinogen or its precursors is not usually known. Thus, two choices are presented: study the mechanisms for a very few compounds to develop fundamental information for those few compounds, or use empirical methods to study larger sets of compounds with correlative methods. The latter method comprises an SAR approach to the study of chemical carcinogenesis. Thus, one has available a set of compounds that has been tested in rodent tests and the observations that resulted from the tests. One can then search for correlations between the structures of the compounds tested and the biological observations reported. One is actually modeling the entire process of uptake, transport, metabolism, cell penetration, binding, etc.

The discovery and design of biologically active compounds (drug design) is a field that has been subject to widespread and well-documented[3-9] changes in the past decade. A host of new techniques and perspectives have evolved. While these techniques have been used largely for the development of pharmaceuticals, they can also be applied to the rationalization of structure–activity relations among sets of toxic, mutagenic, or carcinogenic compounds.

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