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Structure–Toxicity Relationships for Methyl Esters of Cyanoacetic Acids to *Tetrahymena pyriformis*

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Toxicological properties of new chemicals are often inferred from structure/properties of similar compounds whose hazard is already quantified (McKinney et al. 2000). Cronin and Dearden (1995) reviewed the development of quantitative structure-activity relationships (QSARs) as related to aquatic toxicity. A fundamental tenant of such relationships is that the domain of similar chemicals shares common limiting steps and free energy requirements for the measured hazard. It is further presupposed that differences in rates give these requirements rise to the observed differences in quantitative potency. In relating structure to toxicity, the goal is to generalize from specific cases, develop an understanding of what constitutes the "domain" of like-acting molecules, and what molecular properties determine relative potency.

Recent work by Cronin et al. (2001) focused on modeling the ecotoxicity of aliphatic organic substances of the carbonyl chemical domain. These results describe with noted exceptions, small intrinsically reactive molecules, a quality fit of experimental toxicity data with that estimated by the QSAR based on hydrophobicity and electrophilic reactivity. The carbonyl chemical domain includes a variety of compounds (e.g., ketones, esters, formates, aldehydes, diones and lactones). All have in common the presence of a carbonyl [i.e., C(=O)] group.

Cyanoacetates are structurally characterized by the $N{\equiv}CCH_2C(=O)O$- moiety. The presence of a carbonyl group suggests that these compounds should be part of the carbonyl domain. However, the occurrence of multiple polarized π–bonds (i.e., both a cyano and carbonyl group) is a molecular structural indicator that such compounds may also be highly reactive.

Toxicity testing with the freshwater ciliate *Tetrahymena pyriformis* allows for the examination of a large number of organisms that possess characteristics of both single eucaryotic cells and whole organisms (Schultz 1997). Because of these traits, *Tetrahymena* have been used to generate toxicity data for the development of QSARs (Schultz et al. 1998).

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The purpose of this study was to examine the aquatic toxicity of selected methyl esters of cyanoacetic acids. The specific aims were to: (1) examine the *T. pyriformis* population growth kinetics of methyl cyanoacetate; (2) determine the toxic potency of each compound in the *T. pyriformis* population growth impairment assay, and (3) examine selected structure-toxicity relationships.

MATERIALS AND METHODS

Population growth kinetic assays were preformed with 2-methyl-cyanoacetate as described by Bearden et al. (1997). Briefly, 3 X 10^3 log growth cells/mL were inoculated at T_0. Five or 6 concentrations of each toxicant and a control containing no chemical were tested in duplicate. At T_0, after chemical and *T. pyriformis* were dispensed, samples were taken immediately. Samples were taken at 1, 2, 4, 6, 8, and 24 hours incubation. The experimental flasks were returned to the environmental chamber between samplings. Each sample consisted of diluting 2 mL of *T. pyriformis* culture into 50 mL of 10% Isoton. Five electronic counts using the Coulter Counter Model Zm were taken of each sample. Each experiment was conducted twice to verify observed trends. The mean count for duplicates were used for plots.

Eleven cyanoacetates, including the previously noted 2-methyl-cyanoacetate, were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) or Lancaster Synthesis Inc. (Windham, NH, USA). All had a purity of 95% or better. None were repurified prior to use. Stock solutions of each toxicant were prepared in dimethyl sulfoxide.

Tetrahymena pyriformis population growth impairment testing was executed following the protocol described by Schultz (1997). This static 40-hr assay used as its endpoint population density measured spectrophotometrically at 540 nm. Test conditions allow for 8-9 cell cycles in control cultures.

Each cyanoester was tested in a range finder prior to testing in duplicate for three additional replicates. Two controls, one with no test material but inoculated with *T. pyriformis*, and the other, a blank, which had neither toxicant nor ciliates were used to provide a measure of the acceptability of the test and a basis for interpreting treatment data. Each definitive test replicate consisted of six to eight different concentrations with duplicate flasks of each concentration. Only replicates with control-absorbency values > 0.6 but < 0.9 were used in the analyses. The effect levels were based on unmeasured toxicant concentrations. The 50% growth inhibitory concentrations, IGC_{50}, were determined by Probit Analysis of Statistical Analysis System (SAS) software (SAS Institute Inc. 1989).

Logarithm of the 1-octanol/water partition coefficient (log K_{ow}) value for each compound was secured as an estimated or measured value from CLOGP for Windows software (BIOBYTE Corp., Claremont, CA, USA).

The energy of the lowest unoccupied molecular orbital (E_{LUMO}) is reported as the mean of three replicates. Each value was determined using Alchem 2000 software (Tripos Inc., St Louis, MO, USA). Initially, each ester was built as a 2-dimensional structure and converted to a 3-dimensional structure prior to energy minimization. Subsequently, each molecule was geometrically optimized and molecular orbital quantum chemical calculations performed using the PM3 Hamiltonian in the MOPAC6 program.

Excess toxicity (T_e) evaluations were conducted for each chemical by calculating the molar ratio of predicted toxicity to observed toxicity (Lipnick et al. 1987). Experimental toxicity was measured as the IGC_{50} in mM. Predicted baseline (i.e., neutral or non-polar narcosis) toxicity was determined from the relationship,

$$\log (IGC_{50}^{-1}) = 0.74 (\log K_{ow}) - 1.86;$$

$$n = 148, r^2 = 0.96, s = 0.21, F = 3341, Pr > F = 0.0001 \qquad \text{Eq. [1]}.$$

developed by Schultz et al. (1998) with data for neutral organic chemicals.

QSARs were determined using the log of the inverse of the IGC_{50} ($\log (IGC_{50}^{-1})$) in mM as the dependent variable, and $\log K_{ow}$ and E_{LUMO} as the independent variables. Data were modeled using least-squares regression (general linear model procedure of SAS). Fit of the data to the model was quantified with the coefficient of determination (r^2 value) and the root of the mean square for error (s value). [In addition, the Fisher statistic (F value), and the probability greater than the F value (Pr > F) were noted.]

RESULTS AND DISCUSSION

Typical population growth kinetics for *T. pyriformis* exposed to 2-methyl cyanoacetate are shown in Figure 1. They reveal a direct relationship between the concentration of the cyanoacetate and the generation time of the ciliates. This relationship is consistent with the results reported by Bearden et al. (1997) for ciliates exposed to ethanol or acetone. These growth kinetics suggest that methyl ester of cyanoacetic acids act via a non-covalent mode of toxic action. In addition, there is no observable lag phase in growth (Bearden et al. 1997).

In contrast, the population growth kinetics for *T. pyriformis* when exposed to electrophiles reveal a threshold concentration where death of the initial inoculum occurred. At concentrations below this threshold, populations exhibit a short lag phase without any cell death followed by growth at rates (i.e., slope) similar to control populations, while at concentrations larger than the threshold, a concentration dependent death of the initial inoculum occurred (Bearden et al. 1999). Bearden et al. (1999) further noted that *Tetrahymena,* which survived, grew at rates similar to control populations. All these observations were confirmed by Seward et al. (2000).

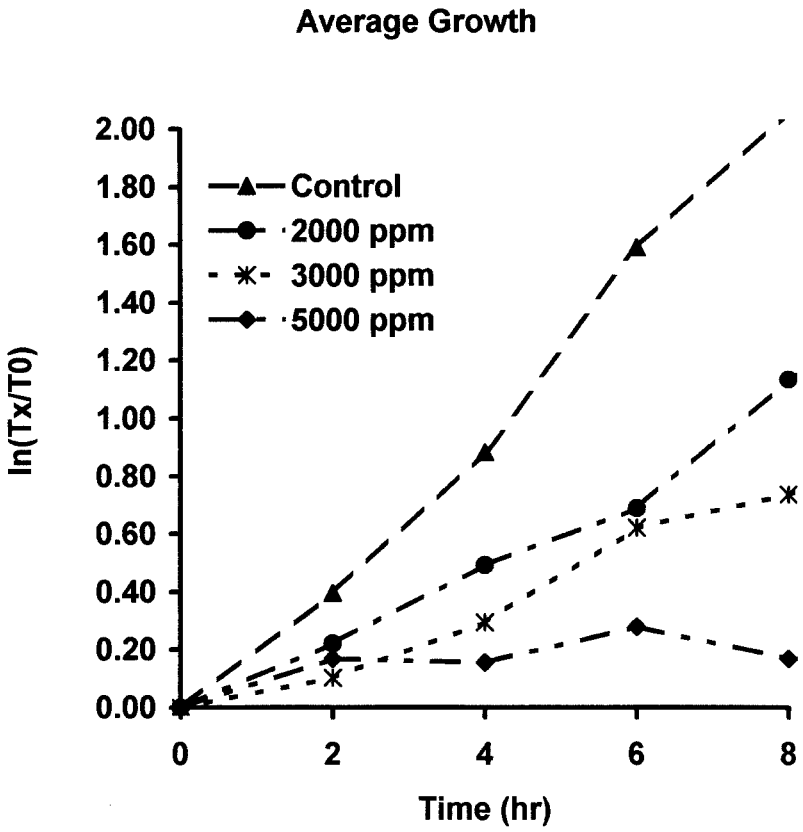

Figure 1. Population growth kinetics for *T. pyriformis* exposed to 2-methyl cyanoacetate.

A summation of the Chemical Abstract Service registry numbers, toxicity, hydrophobicity, and electrophilic reactivity are given in Table 1. Toxicity varies over about 3 orders of magnitude, while hydrophobicity varies over about 5 orders. However, electrophilicity varies over a very narrow range.

The excess toxicity (T_e) exhibited by the cyanoacetates indicates they are not baseline toxicants (Table 1). While the growth kinetics for 2-methyl cyanoacetate indicates a noncovalent mode of toxic action, the T_e values in relationship to baseline narcosis suggest the specific mechanism is different than baseline or neutral narcosis.

Simple regression analysis of the cyanoester data yielded the hydrophobic-dependent toxicity model:

$$\log (IGC_{50}^{-1}) = 0.64 (\log K_{ow}) - 1.08;$$

$$n = 11, r^2adj. = 0.973, s = 0.16, F = 361, Pr > F = 0.0001 \quad \text{Eq. [2].}$$

Evaluation of the standardized residuals based on Eq. [2] showed one derivative, the allyl cyanoester, to be an outlier and more toxic than predicted. However, due to the high quality of Eq. [2] no effort was made to improve the model.

Table 1. Toxicity and molecular descriptor values for cyanoacetates.

Cyanoacetic Acid	CAS Number[a]	Log $1/IGC_{50}$	Log K_{ow}[b]	E_{LUMO}[c]	T_e[d]
Methyl ester	105-34-0	-1.34	-0.47m	0.497	7.38
Allyl ester	13361-32-5	-0.79	-0.08e	0.469	13.46
Ethyl ester	105-56-6	-1.23	-0.04e	0.379	4.57
Isopropyl ester	13361-30-3	-0.95	0.25e	0.553	5.31
n-butyl ester	5459-58-5	-0.61	1.02e	0.387	3.13
Cyclohexyl ester	52688-11-6	-0.09	1.44e	0.533	5.06
n-amyl ester	17686-39-4	-0.31	1.52e	0.389	2.66
Ethylphenyl ester	4553-07-5	-0.02	1.63e	0.120	4.30
2-ethylhexyl ester	13361-34-7	0.89	2.98e	0.379	3.51
n-octyl ester	15666-97-4	0.90	3.14e	0.383	2.73
n-decyl ester	17686-43-0	1.70	4.17e	0.378	2.98

[a]Chemical Abstract Services registry number
[b]1-octanol/water partition coefficient
[c]energy of the lowest unoccupied molecular orbital
[d]excess toxicity as compared to baseline neutral narcosis
e = estimated value; m = measured value

Quantum chemical descriptors, especially molecular orbital energies (Seward et al. 2001), often quantify ecotoxic reactivity. The E_{LUMO} value is related to electron affinity and characterizes the susceptibility of a chemical towards nucleophilic attack; as such, it is a gross quantifier of "soft" electrophilicity (Veith and Mekenyan 1993). E_{LUMO} values did not model the toxicity of all 11 cyanoesters simultaneously ($r^2adj.$ = 0.10). When the two variables log K_{ow} and E_{LUMO} (which are orthogonal: $r^2adj.$ = 0.025) are combined, the fit of the model ($r^2adj.$ = 0.971) was less than that observed for log K_{ow} alone (see Eq. [2]). Moreover, E_{LUMO} only was significant at the 0.594 level. These relationships would be expected since the test set is a congeneric series with a single functional group and thus a limited range of reactivity.

A transparent approach to modeling toxicity across mechanisms of action is the response-surface. This approach typically models toxic potency as a plane determined by hydrophobicity and electrophilic reactivity (Schultz 1999). Cronin

et al. (2001) model a variety of carbonyl-containing aliphatic compounds with the QSAR;

$$\log (IGC_{50}^{-1}) = 0.62 (\log K_{ow}) - 1.04 (E_{LUMO}) - 0.55;$$

$$n = 135, r^2 = 0.853, s = 0.39, F = 382, Pr > F = \text{(not given)} \qquad \text{Eq. [3].}$$

Using Eq. [3] predicted toxic potencies were estimated. Simple regression analysis;

$$\text{Obs. } \log (IGC_{50}^{-1}) = 0.97 (\text{Pred. } \log (IGC_{50}^{-1})) + 0.08;$$

$$n = 11, r^2\text{adj.} = 0.951, s = 0.23, F = 195, Pr > F = 0.0001 \qquad \text{Eq. [4].}$$

The slope of near one and the intercept of near zero reveal that Eq. [3] does an excellent job of predicting the ecotoxicity of methyl esters of cyanoacetic acids.

The response-surface model of Cronin et al. (2001) is a multiple linear regression approach utilizing quantitative descriptors for passive transport (i.e., uptake by the biophase) as well as electrophilic reactivity (i.e., interaction) with the molecular site of action. This approach is mechanistic-based in that the descriptors are selected *a priori* and limited to global parameters estimating toxicokinetic and toxicodynamic effects.

The model of Cronin et al. (2001) demonstrated that the ecotoxicity data to the freshwater ciliate of chemicals with a carbonyl moiety could be fitted as a plane in a 3-dimensional surface. The model described toxic potency by using the hydrophobicity (log K_{ow}), to model the biouptake, and the (E_{LUMO}) to model electrophilic interaction. The resulting toxic space was a continuum, with narcotic toxicants as ketones and monoesters being distributed along the potency-log K_{ow} aspect of the surface. At the same time, toxicants with strong electrophilic reactivity polarized alpha-beta unsaturates such as alkenals, were distributed nearer the potency-E_{LUMO} aspect of the space. The methyl esters of cyanoacetic acids exhibit toxic potency that resides as an isoelectrophilic window (Bradbury 1995) in the middle of the plane described by Eq. [3]. It was emphasized that the plane described by Eq. [3] combined chemicals with different modes and mechanisms of toxic action into a single QSAR (Cronin et al. 2001).

In conclusion, the results of this investigation indicated that the methyl esters of cyanoacetic acids exhibited toxic potency in excess of baseline narcosis. Based on

population growth kinetics this activity is considered to be non-covalent in character. Within the congeneric cyanoester series, toxicity is hydrophobic-dependent. While the specific mechanism of toxic action is unclear, the hydrophobic and electrophilic response-surface domain of aliphatic carbonyl-containing toxicants can be extended to include cyanoesters.

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