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QSAR Models for *Daphnia magna* Toxicity Prediction of Benzoxazinone Allelochemicals and Their Transformation Products

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The overall objective of this study is the ecotoxicological characterization of the benzoxazinone 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the benzoxazolinones benzoxazolin-2-one (BOA) and 6-methoxybenzoxazolin-2-one (MBOA), and their transformation products: phenoxazinones 2-acetylamino-7-methoxy-3*H*-phenoxazin-3-one (AAMPO), 2-acetylamino-3*H*-phenoxazin-3-one (AAPO), 2-amino-7-methoxy-3*H*-phenoxazin-3-one (AMPO), and 2-amino-3*H*-phenoxazin-3-one (APO); aminophenol 2-aminophenol AP); acetamide *N*-(2-hydroxyphenyl)acetamide (HPAA); and malonamic acid amide *N*-(2-hydroxyphenyl)malonamic acid (HPMA). A comparison between empirical results and theoretical ones using rules-based prediction of toxicity was done, and it can be concluded that only the degradation metabolites exhibited significant ecotoxic effect. Using synthetic pesticides knowledge, several QSAR models were trained with various approaches and descriptors. The models generated exhibited good internal predictive ability ($R_{cv}^2 > 0.6$) and were used to predict the toxicity of the natural compounds studied.

KEYWORDS: QSAR; *Daphnia magna*; aquatic ecotoxicity; EC_{50}; benzoxazinones

INTRODUCTION

Because no land is free from attack by pests, plants have developed natural chemical defenses to survive. When plants are stressed or damaged, for example, during a pest attack, they may greatly increase their output of natural pesticides, such as allelochemicals, that is, benzoxazinones (*1*). Thus, natural compounds could be an alternative to synthetic pesticides. Because very little is known about the toxicity of these compounds, examination of their toxicity using both experimental and computational methods is of high interest.

In silico approaches are challenging methods to cover many knowledge gaps when experimental toxicity data are difficult to determine or not available at all. These approaches differ from laboratory experiments, in vivo and in vitro, because they do not involve the use of any biological system. They are based on the theoretical knowledge gained in different fields of the science and aided by the powerful computational capabilities of modern computers. Quantitative structure−activity relationship (QSAR) leads to finding a relationship (model) between the chemical structure of compounds and a given property (*2*−*4*). In this way QSARs are used for deriving models to predict property values for chemicals. The basic assumption of QSAR is that a quantitative relationship between the molecular structure of compounds and their biological, chemical, and physical properties does exist.

The characterization of chemicals from a toxicological point of view was done using both experimental animal tests and computational approaches. As the toxicological evaluation of synthetic pesticides is well documented, this information was used to train models and to assess the toxicity values of benzoxazinones and derivatives and in particular 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), benzoxazolin-2-one (BOA), and 6-methoxybenzoxazolin-2-one (MBOA). Moreover, as benzoxazinones from wheat were shown to degrade very rapidly in soil (*5*−*11*) and the transformation products showed more pronounced biological activity than the parent compounds from wheat (*12*−*19*), the toxicity of the following degradation compounds was also investigated: 2-amino-3*H*-phenoxazin-3-one (APO), 2-amino-7-methoxy-3*H*-phenoxazin-3-one (AMPO), 2-acetylamino-3*H*-phenoxazin-3-one (AAPO), 2-acetylamino-7-methoxy-3*H*-phenoxazin-3-one (AAMPO), 2-aminophenol (AP), *N*-(2-hydroxyphenyl)acetamide (HPAA), and *N*-(2-hydroxyphenyl)malonamic acid (HPMA).

Daphnia magna was used as a representative of the primary consumers in aquatic ecosystems (*20*). Those organisms are widely used for ecotoxicity and risk assessment in routine

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analysis, and the test procedures are known to be reproducible and to generate reliable results.

MATERIALS AND METHODS

Biological Data. For this study, the toxic characterization of benzoxazinones and their degradation products was freshly investigated using the acute *D. magna* immobilization test DIN 38412 L30 (*21*), similar to OECD 202 (*22*). These data, which will be referred to in the remainder of the paper as the test set, contain benzoxazinones and benzoxazolinones (DIMBOA, BOA, and MBOA) and their transformation products (AAMPO, AAPO, AMPO, APO, AP, HPAA, and HPMA). The freshwater microcrustaceae *D. magna* STRAUSS was obtained in 1997 from the Higher School for Chemistry, Vienna, and kept in continuous culture in tap water at the IFA-Tulln. The biotests were performed in 100 mL beakers with 20 mL of tap water and five juvenile daphnia (ages between 4 and 24 h) in each vessel. The solvent solutions of the test substances were added in a fixed volume of 25 μL/100 mL of test medium. Positive references were done for each test series using potassium dichromate solutions to reach final concentrations of 0.9 and 1.9 mg/L (the recommended EC_{50} range for test validation). Each test was run in at least three replicates. The test run time was 48 h with an intermediate control after 24 h (which was not used for the final calculation). The conditions were 22 °C and a daylight/night cycle of 16/8 h. During this run time the animals were not fed, and any other disturbances were avoided. For the final evaluation the number of mobile (moving by themselves after a very gentle shake) individuals was counted in each beaker and treated as survivors. Only those test batches where not more than one immobile animal was found in the three control vessels were evaluated. The inhibition was calculated using the formula

$$\% \text{ inhibition} = N_{\text{sample}} \times 100/15 \quad (1)$$

where N_{sample} is the number of immobile daphnia in three sample beakers.

After the inhibition results had been fit to the Weibull equation (eq 2), the EC_{50} values were calculated from the function indices (eq 3).

$$y = 100 \times [a + (1 - a) \times (1 - \exp(-x/b)^c)] \quad (2)$$

$$EC_{50} = b \times \ln 2^{1/c} \quad (3)$$

where y is the inhibition in %, x is the concentration of the tested substance in mol/L, and a, b, and c are formular constants to be calculated by the curve-fitting algorithm.

On the other hand, toxicological evaluation of synthetic pesticides is well documented, and this information was used to train QSAR models. The training set, as the present data will be referred to, was built by selecting 86 synthetic pesticides with structures similar to those of the benzoxazinones (DIMBOA, BOA, and MBOA). In particular, all compounds contained an aromatic and/or heterocyclic ring with nitrogen and/or oxygen atoms. The toxicity values were collected from different sources: *The Pesticide Manual* (*21*), the U.S. EPA Ecotox database (*24*), and the CIRCA Website (*25*). The endpoint was EC_{50} (concentration causing immobilization to 50% of the test organisms) for *D. magna* exposed for 48 h.

Test and training data are expressed as follows: output = log(MW/EC_{50}) (MW = molecular weight) to refer to the moles of the chemical and not the weight, as good practice in QSAR studies (*26*).

Optimization. To correctly describe the three-dimensional (3D) structural and electronic properties of the molecule under study in QSAR, one has to consider it in a stable (optimized) conformation. The variations of torsion angles have the biggest impact on the energy of the molecule and determine the overall molecular shape. Theoretical−computational approaches in conformational searching are based on the variation of the degrees of freedom in the molecule. Afterward, the minimization of the internal strain of the molecule to find the global minimum has been done. The optimization procedure is driven by a gradient normal, which reflects the energy changes with respect to structural changes. When the gradient value becomes low, the structure optimization finishes in a minimum.

The Monte Carlo method (MCMM) implemented in MacroModel 8.1 was used for the conformational search of the studied compounds using Maestro 5.1 graphical user interfaces (*27*). For each compound the lowest energy conformation was used as the starting point for geometry optimization by a semiempirical AM1 method (*28*) as implemented in the Gaussian 03 package of computer codes (*29*). Vibrational analysis was performed at the optimized geometry to ensure that the found conformation is a minimum (no imaginary frequencies were found). Partial charges from Mulliken population analysis were used.

Chemical Descriptors. Once the structure is in the minimum energy state, it is possible to calculate the descriptors that mathematically characterize the molecule. The descriptors calculated can be divided according to type into two general classes.

Atom-based descriptors describe only the magnitude of particular physical properties but no directional preferences that these properties may have. One hundred and seventy-three descriptors were calculated including the atoms themselves, molecular fragments, or substructures (functional groups), molecular indices derived by topological methods (molecular connectivity indices, related to the degree of branching in the compounds), atomic properties (electrotopological indices or atomic polarizability), geometrical properties (molecular surface area and volume, moment of inertia, shadow area, projections, and gravitational indices), electrostatic properties (partial atomic charges and others depending on the possibility to form hydrogen bonds), using CODESSA (comprehensive descriptors for structural and statistical analysis) software (*30*), and physicochemical properties (log *P*) using Pallas 3.0 (*31*). Using this approach, 174 chemical descriptors were calculated and assigned the name data set A.

Field-based descriptors describe the microenvironment surrounding the molecules (molecular electrostatic and steric potential and van der Waals volume). This approach, which is called comparative molecular field analysis (CoMFA), looks at the molecules in three dimensions and describes the magnitude and directions of electronic and steric interaction (*32*). This technique measures the steric and electrostatic interaction energies between a small probe at a series of regular grid positions around the molecules. It is important to emphasize that the structures have to be aligned (superimposed) to occupy the same position in space. The CoMFA calculations were done with Chem-X software (*33*). A sufficiently large box was positioned around the molecules, and grid spacing of 1 Å was defined. For steric and electrostatic fields we used the distribution of van der Waals volume around the compounds and positive dot charge unit, respectively. Energy cutoff was 20 kcal/mol for the electrostatic field. The standard deviations of the energy columns were in accordance with the generally accepted threshold of 1−2 kcal/mol. A common phenyl ring was used as a template to align the compounds, and for compounds with two or more phenyl rings, the phenyl rings attached to the heterocycle ones were selected. The ligands were superimposed by the flexible fitting option in Chem-X. This approach was used only on aromatic compounds (76 chemicals) because the phenyl ring was used to align them. Therefore, 585 chemical descriptors were calculated, and we called this matrix data set B.

Statistical Analysis. Once biological data have been collected and chemicals have been associated with a proper set of descriptors, mathematics takes care to extract the information hidden in the numbers. We used different approaches for the different data sets called A and B, applying different statistical analyses to select the variables or to construct the model.

On data set A (86 compounds and 173 *atom-based* descriptors calculated) we used multiple linear regression (MLR) and the group method of date handling (GMDH).

MLR is the first and most diffuse technique in chemometrics and, especially, in QSAR studies. It was coupled with a genetic algorithm (GA) variable selection to reduce the noise caused by irrelevant variables. The GA procedure is described in detail in ref *34*; in this case the objective function is the MLR regression model derived on the training set.

Table 1. R_{cv}^2 and Number of Variables Used for Different Approaches: Models Built Using Atom-Based Descriptors (MLR/GA and Linear, Nonlinear, and Combined GMDH Neural Network Models), Followed by the Values Obtained Using Field-Based Descriptors (PLS Model) and Only the Aromatic Compounds (76 Molecules)

	atom-based descriptors							field-based descriptors
		GMDH neural networks						
	MLR/ GA	M1 (linear)	M2 (linear)	M3 (linear)	M4 (nonlinear)	M5 (nonlinear)	M6 (combined)	PLS
R_{cv}^2	0.76	0.68	0.59	0.72	0.65	0.64	0.79	0.97
no. of variables	10	9	6	8	4	5	5	6

Table 2. External Test Set with Experimental Toxicity Values Expressed as − log EC_{50} Together with the Theoretical Values Obtained Using Different Approaches: Values Predicted Using Atom-Based Descriptors (MLR/GA Model and Linear, Nonlinear, and Combined GMDH Neural Network Models), Followed by the Values Obtained Using Field-Based Descriptors (PLS Analysis)

		atom-based descriptors							field-based descriptors
			GMDH neural networks						
molecule	exptl value	GA/MLR	M1 (linear)	M2 (linear)	M3 (linear)	M4 (nonlinear)	M5 (nonlinear)	M6 (combined)	PLS
AAMPO	<2.15	0.8	0.02	0.79	0.79	1.18	0.56	0.41	1.56
AAPO	1.99	0.7	–0.08	0.64	0.84	1.41	0.88	0.64	1.05
AMPO	<1.39	0.7	0.06	0.79	0.46	0.83	0.93	0.26	1.78
AP	2.45	–1.1	1.09	0.55	0.29	0.11	1	0.45	0.92
APO	2.82	0.3	–0.16	0.5	0.43	0.90	1.31	0.42	1.36
BOA	<1.82	0.3	0.84	0.74	1.40	0.64	1.39	1.16	1.17
DIMBOA	<2.02	0.8	0.92	0.6	0.42	0.20	1.51	0.62	2.25
HPAA	<1.89	0.3	0.78	0.61	0.43	0.30	1.18	0.49	0.75
HPMA	<2.00	0.8	1.04	0.3	0.41	0.70	1.16	0.84	1.04
MBOA	<1.92	1	0.80	0.8	1.07	0.31	1.57	0.9	1.8

GMDH neural networks (*35*) are able to self-organize an optimal complex model composed of a set of self-selected relevant descriptor variables starting from a completely unknown and not predefined model structure. In this way it is possible to systematically self-organize not overfitted linear or nonlinear models, making this knowledge extraction technology well suited for QSAR modeling. On data set A, variable selection was done using this kind of approach, and the algorithm comes up with a final model composed of a few self-selected and relevant descriptors only. Such a model can be linear or nonlinear, which in our case means automatically obtaining a linear or nonlinear multivariate polynomial regression model. When one uses the predicted values of the generated individual linear or nonlinear models as inputs instead of the intitial descriptor values, GMDH neural networks self-organize an optimal combination of the individual models, ending in a combined model the performance of which usually increases compared with individual ones.

On data set B containing 76 chemicals (only the aromatic compounds of data set A) and 585 *field-based* descriptors we used partial least-squares (PLS) analysis.

PLS (*36*, *37*) is especially useful when the number of independent variables is comparable to or greater than the number of compounds (data points) and/or there exist other factors responsible for correlations between variables, because it leads to stable, correct, and highly predictive models even for correlated descriptors (*38*, *39*).

In all of these different approaches a model validation step has been done to ensure the model's predictivity. Therefore, internal and external validations were included in the process. Each model was first validated using leave-one-out cross-validation (LooCV) (*33*) (internal validation). In this method each chemical in the training set is systematically excluded once from the data set, after which its activity is predicted by a model derived from the remaining chemicals. During this process the cross-validated R^2 will be derived. A model with $R_{cv}^2 > 0.5$ is normally considered to have a significant predictive ability (*32*). This internal validation assesses the model's extrapolation within the training set, but the only way to truly test any QSAR model is to use it to predict the activities of compounds that have not been included in designing the model. Therefore, the models obtained here are then tested on the external test set.

RESULTS AND DISCUSSION

The results summarized in **Table 1** represent the training set compounds (synthetic pesticide) with experimental toxicity values together with the values predicted using different computational approaches. Additionally, R_{cv}^2 and the number of selected variables for each model are shown. Using *atom-based* descriptors (data set A containing 86 compounds) we built one GA/MLR model and six different GMDH neural network models: three linear, two nonlinear, and one, called combined, obtained from a combination of the previous five models. Moreover, using only aromatic compounds (76 molecules), we calculated *field-based* descriptors (data set B) and built a PLS model. The results showed for all models good regression coefficients ($R_{cv}^2 > 0.6$) except for the M2 linear model that we reported because it was used, together with all other GMDH neural network models, to build the M6 combined one.

Then we used these models to predict the toxicity of the natural compounds studied (benzoxazinoids and their transformation products), using them like an external test set.

In **Table 2**, the experimental toxicity values obtained for benzoxazinones and their transformation products, expressed as −log EC_{50}, are summarized. For the three substances AP, APO, and AAPO, reliable EC_{50} values were obtained from the Weibull curve fit. For all other tested substances no significant inhibitions were measured and no curve-fitting was possible. The maximum test concentration was reached by the individual solubility of each substance; no suspensions were used for the tests. Therefore, the results are given as EC_{50} > maximum tested concentration.

Table 2 contains also the values predicted by the different models for the external test set. The boldfaced compounds (DIMBOA, BOA, MBOA) are the ones containing the structures (**Figure 1**) more similar to the training set (pesticides). Unfortunately, for these compounds numerical values are not available; we have only an indication of the relevant range (<). Anyway, for these three compounds the experimental toxicity

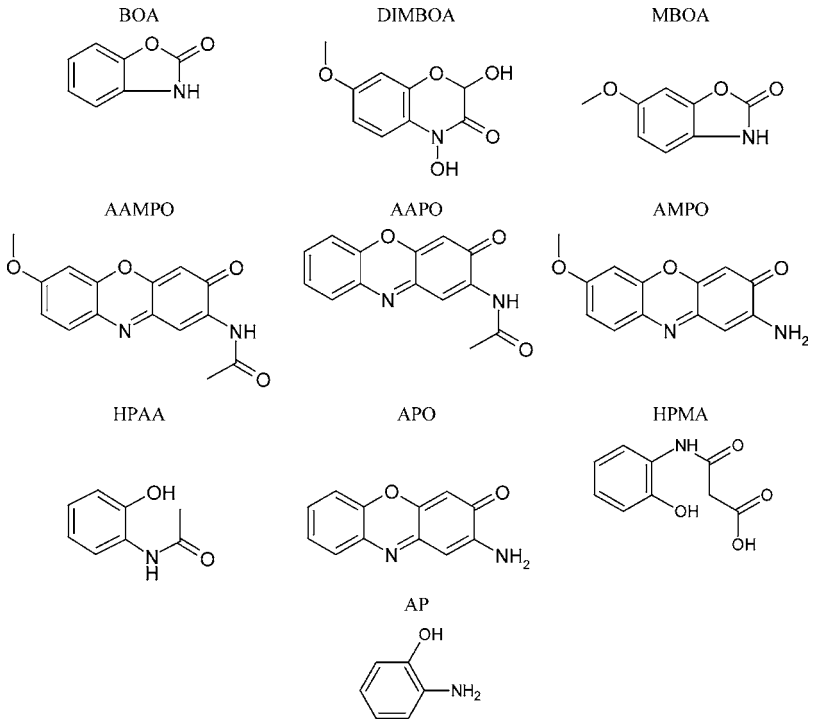

Figure 1. Schematic illustration of the compounds studied.

values are in agreement with the ones predicted using the different approaches, except for one compound (DIMBOA), for which the model that was built using PLS analysis and *field-based* descriptors predicted a toxicity value ($-\log EC_{50} = 2.25$) slightly outside the range of experimental toxicity found ($-\log EC_{50} < 2.02$). However, for BOA and MBOA all of the predicted values are in agreement with the experimental values obtained (<1.82 and <1.92, respectively).

Moreover, because benzoxazinones were shown to degrade very rapidly in soil and the transformation products exhibit more pronounced biological activity than the parent compounds, the model was used to predict the toxicity of the degradation compounds: APO, AMPO, AAPO, AAMPO, AP, HPAA, and HPMA. From these results it is evident that when the experimental values obtained are categorical (as for AAPO, AP, and APO), in general the CoMFA analysis is able to give a better prediction compared to the other approaches used.

We can conclude from the biotest results that none of the natural wheat benzoxazinones or benzoxazolinone derivatives, namely, BOA, MBOA, and DIMBOA, exhibited significant ecotoxic effects to *D. magna*. Only the degradation metabolites AP, APO, and AAAPO affected the freshwater animals (*14*). Currently, it is unclear what the ecological effect of the bioactivation due to microbial metabolization of those benzoxazinones in soil could be. Further research would be necessary to investigate degradation pathways for soil and for the aquatic environment and to identify all of the degradation metabolites that may cause toxic effects to human, animals, and plants. Nevertheless, it could be demonstrated that biotests with a nontarget organism could be a base for a preliminary risk assessment and fit well to theoretical data. A detailed risk assessment can be done only if allelochemicals would be used as natural pesticides and application amounts in the field known.

We also developed models to predict toxicity of these compounds using different approaches. Three-dimensional models, using *field-based* descriptors, gave good results, which also indicate important probable common toxic processes. Vice versa, using *atom-based* descriptors and statistical methods based on automatic extraction of knowledge, we extracted information more independent of mechanism, and due to their lower performances, we can conclude that benzoxazinones and benzoxazolinone derivatives have very similar toxicity modes of action to each other and also compared to the synthetic pesticides used to build our models. Therefore, in silico approaches using data of synthetic pesticides with structures similar to that of benzoxazinones can be usefully applied to predict the toxicity of benzoxazinones and derivatives toward *D. magna*.

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