JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Preliminary Analysis of Toxicity of Benzoxazinones and Their Metabolites for *Folsomia candida*

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The overall objective of this study was to explore the toxicity of benzoxazinone allelochemicals and their metabolites to *Folsomia candida* (Collembola: Isotomidae) (Willem, 1902). Experimental tests showed transformation products to have more pronounced toxicity than parent compounds. The underlying relationship between the chemical structure and toxicity was then studied using three-dimensional QSAR approaches, and results highlighted the role of the steric contribution.

KEYWORDS: ***Folsomia candida*; 3D QSAR; benzoxazinones; LC_{50}; CoMFA**

INTRODUCTION

Benzoxazinones are major allelochemicals (secondary metabolites self-produced during defense mechanisms) released by corn, wheat, and rye (*1*, *2*). They have been implicated as chemical factors of plant resistance to herbicides, insects, and diseases and have a wide variety of biological actions, including antifungal (*3*) and mutagenic activities (*4*). The predominant benzoxazinones in corn and wheat are 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its desmethoxy derivate (DIBOA), but these compounds exist prevalently as the corresponding stable inactive β-glucosides (2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one) in the living plants. Only upon plant cell disruption are they enzymatically converted to the active aglycones (DIMBOA and DIBOA) that are degraded spontaneously to the corresponding benzoxazolinones 6-methoxybenzoxazolin-2-one (MBOA) and benzoxazolin-2-one (BOA). Subsequently, the benzoxazolinones are transformed to phenoxazinone polycyclic products (APO, AAMPO, AAPO, AMPO) and to the acetamide (HPAA), which showed more pronounced biological activity and higher stability than the parent compounds (*5*−*12*). For this reason, in addition to the parent benzoxazinones, the following transformation products were also studied: 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 *N*-(2-hydroxyphenyl)acetamide (HPAA). However, the chemical mechanism of action of these compounds is not well understood, and we used three-dimensional (3D) QSAR approaches to study their activity and to extract as much information as possible from the experimental data obtained.

The aim of the present study was to predict LC_{50} values for the toxicological evaluation of the effects of benzoxazinones and their metabolites on beneficial nontarget soil organisms such as *Folsomia candida* and to extract relevant information for the understanding of their toxicological mechanism.

This study was conducted in two steps. First, toxicological standard tests with the benzoxazinones and their metabolites were performed on the selected test species *F. candida* (Willem, 1902) (Collembola: Isotomidae). Collembola are important, primarily wingless arthropods in the soil ecosystem, which mainly support the maintenance of soil fertility by their joint action with soil microorganisms as decomposers of different organic matters and in the mineralization process (*13*). Additionally, they serve as food source for other soil-dwelling arthropods, such as carabid beetles (*14*−*17*). *F. candida* was selected as a representative collembolan test species because of its widespread abundance, its cosmopolitan occurrence, its simplicity of mass rearing, and its short life cycle and high reproduction rate (*18*, *19*). Apart from that, this species has already been used as a standard test organism for about 30 years in pesticide side-effect testing (*20*) and was recommended as one of the standard soil test organisms that are used for the authorization of plant protection products (*21*−*23*) or for the notification of new chemicals in the European Union (EU Directive 67/548/EEC) (*24*).

The second step of the research was to extract relevant information for the understanding of the toxicological mechanism of these compounds to *F. candida*. This was possible by using computational approaches, such as quantitative structure−activity relationships (QSAR) methods, that are simplified mathematical representations of complex chemical−biological interactions (*25*−*27*). The expectation is that the factors governing the events in a biological test system are represented as a multitude of descriptors characterizing the compounds. The goal in any QSAR modeling is to obtain the mathematical

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10.1021/jf050916v CCC: $33.50
Published on Web 01/24/2006

expression that best describes the relationship between chemical structure and biological effect. QSAR predictions have the potential to save time, reduce costs, and minimize the use of biological testing. Moreover, theoretical approaches are being increasingly used by regulatory authorities, industries, and other institutions for assessing the risks of chemicals released to the environment (*28*−*30*).

In this study we explored LC_{50} (estimated concentration at which 50% of the test organisms are dead at the end of the test) toxicity data to obtain a QSAR that is able to give preliminary information of compounds not yet synthesized and which could serve as an aid in the study of new compounds.

MATERIALS AND METHODS

Experimental Evaluation. The particular benzoxazinones studied in this work were derived from wheat and were shown to degrade very rapidly in soil (*31*, *32*).

The *F. candida* trials were carried out according to ISO Standard 11267 (*33*), and the predefined validity criteria were applied. The objective of the test was to assess lethal (acute toxicity; mortality) effects of the test compounds on *F. candida*. In the range-finder test, 18 ± 4-day-old individuals were exposed to a range of test compound concentrations mixed with a defined artificial standard soil substrate. With regard to the age of the test organisms, a modification to ISO 11267 (1999) was made because several studies with juveniles had produced invalid results due to a control mortality exceeding the validity criterion of 20% (*44*−*47*). Treatments comprised the range-finder concentrations of the test compounds (five concentrations between 0.05 and 2.0 mg/kg of dry soil mass with three replicates, each with 10 individuals), a water control, and two toxic reference products, Betanal Plus (ai phenmedipham) and Perfekthion S (ai dimethoate). Lethal effects were evaluated once after 14 days of exposure to determine the range of test rates for the succeeding final test (five replicates per one to three test rates ranging between 1 and 10 mg/kg of dry soil mass including 10 test organisms per replicate) with a test duration of 4 weeks. Experimental conditions were as follows: temperature, 20 ± 1 °C; relative humidity, 70−90%; photoperiod, 16 h light/8 h dark (∼400−800 lx). Food (dried yeast) was provided ad libitum throughout the trial period. The LC_{50} values were assessed in milligrams per kilogram. The validity criteria applied were of a mean mortality of adults in the control samples of <20% at the end of the test, a minimum reproduction in each control container of ≥100 juveniles within 4 weeks, a coefficient of variation of <30% of reproduction in the control samples, and significant effects on reproduction of the reference substance Betanal Plus at 100 and 200 mg/kg of dry substrate (*34*, *35*).

3D QSAR Modeling. QSAR studies search for a relationship between the activity/toxicity of chemicals and the numeric representation of their structure and/or features. A 3D QSAR model reflects characteristics of chemicals derived from their three-dimensional organization in space, and therefore the structure of chemicals is a key factor. This kind of approach is made up of several steps (*36*): (1) evaluation of minimum energy conformation for each compound (geometry optimization); (2) calculation of descriptors related to each chemical structure [using comparative molecular field analysis (CoMFA) approaches, the structures have to be aligned to occupy the same position in space]; and (3) determination of the relationship between activity and descriptors by some statistical technique that gives a variable selection to ascertain the best predictive regression model.

The overall strategy of the molecular modeling followed in this work is shown in **Figure 1**.

Geometry Optimization. To correctly describe the 3D structural and electronic properties of a molecule under study in QSAR, one has to consider it in a stable (optimized) state, in which the internal strain of bonds, angles, and torsion angles is minimal. For this purpose the molecules were built from two-dimensional sketches, and full geometry optimization by ab initio quantum chemical methods was performed at Hatree−Fock level with a double-ζ basis set with polarization function 3-21G* as implemented in Gaussian 03W (*37*). The resulting geometries were checked for existence of imaginary frequencies by vibrational analysis (no imaginary frequency was found, i.e., structures represent gas-phase minima). Mulliken charges obtained in population analysis were used for the calculation of electrostatic properties.

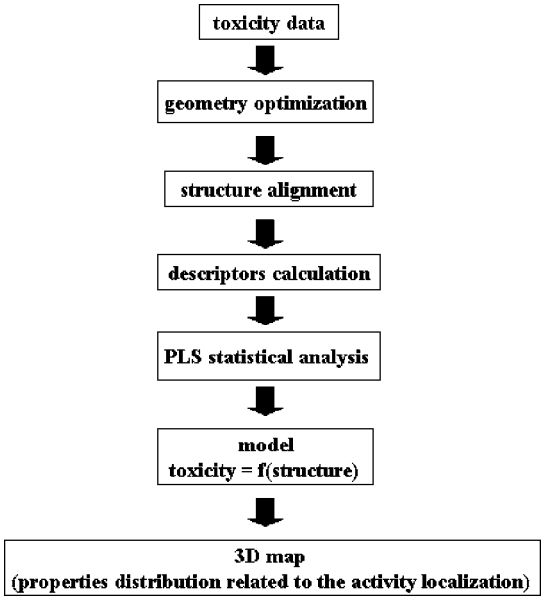


Figure 1. Flowchart of CoMFA model generation.

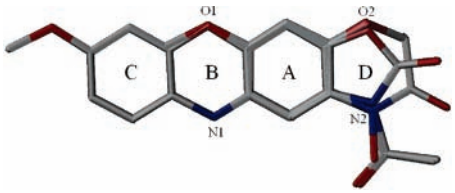


Figure 2. Alignment for CoMFA analysis. Hydrogen atoms are omitted for better visualization.

Structure Alignment. As CoMFA is a true 3D approach using grid point interaction energies as descriptors, prior to analysis, the studied structures have to be aligned to share common 3D spatial arrangement of major functional groups. Then the spatial regions (of points), which are strongly related to the activity, can be discovered. In this study the majority of the structures are rigid polycyclic systems, which were aligned with respect to the main structural motifs (**Figure 2**): aromatic ring A, oxygen atom O1, sp^2 nitrogen atom N1, amine (amide) nitrogen atom N2, and oxygen atom O2. The most toxic compound, APO, was used as a template.

Calculation of Descriptors (CoMFA Analysis). CoMFA describes the microenvironment surrounding the molecules looking at the molecules in three dimensions and describing the magnitude and directions of electronic and steric interaction (*38*). This technique measures the steric and electrostatic interaction energies between a small probe at a series of regular grid positions around the molecules.

The CoMFA was performed on a Linux-based PC workstation computer using the software package SYBYL version 6.91 (*39*). Grid dimensions were automatically set by the program, and 2 Å grid stepping was used. The steric and electrostatic field energies were calculated using an sp^3 carbon atom with a +1 charge as a probe.

Values of the logarithm of the octanol−water partition coefficient, log *P*, were calculated using the Pallas 3.0 (*40*) package. Because octanol can represent the cell membrane, log *P* indicates the ability of the chemical to permeate it and therefore to be available for interaction with the organism.

Statistical Analysis. Partial least-squares (PLS) regression (*41*) was performed to correlate the biological data and molecular fields. Leave-one-out (LOO) cross-validation (CV) was utilized to optimize the number of principal components and to evaluate the predictive capability of the model. The PLS procedures without cross-validation were performed to create predictive models.

PLS is based on a linear transformation of the descriptors' space, producing a new variable space based on a small number of orthogonal factors (latent variables) so that there is no correlation. In other words, factors are independent linear combinations of original descriptors. Latent variables are chosen in such a way as to provide maximum correlation with dependent variable; thus, the PLS model contains the smallest necessary number of factors (*42*). This method is particularly apt when the number of variables equals or exceeds the number of compounds (data points), because it leads to stable, correct, and highly predictive models even for correlated descriptors (*42*, *43*).

For the assessment of the goodness of the model the determination coefficient R^2 was used, which expresses the ability of the model to reproduce the training set and the standard error of estimate *S*. Coefficients are calculated as

$$R^2 = \frac{\mathrm{SD} - \mathrm{PRESS}}{\mathrm{SD}}; \quad \mathrm{SD} = \sum_i (y_i - \bar{y})^2; \quad \mathrm{PRESS} = \sum_i (y_i - \hat{y}_i)^2$$

$$S = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n}}$$

where y_i is the *i*th experimental value, $\bar{y}$ is the mean, $\hat{y}_i$ is the *i*th predicted value, and *n* is the number of data points.

Q^2 refers to the predictive power of the model and is computed using LOO CV. In a CV procedure, data are divided into *n* groups of equal size, and then *n* models are developed using all of the data but the *n*th group. The prediction of each model on the group left out contributes to the calculation of Q^2, using the previous formula. In the case of LOO only one object is left out every time from the model-building procedure and then predicted using the derived model.

RESULTS AND DISCUSSION

Table 1 lists the experimental LC_{50} values (milligrams per kilogram) expressed as log(MW/LC_{50}) (MW = molecular weight) together with the corresponding structure of the compounds studied. These results showed that APO was the compound most toxic to *F. candida* compared with the other test compounds of this study (LC_{50} up to 3×10^2 times lower). Similar effects were observed in refs *34* and *35*, where the LC_{50} of APO for *F. candida* was up to 6×10^4 below the ones of some pesticides commonly used.

We used the **Table 1** values to construct a 3D QSAR trying to extract more information about the properties of these compounds.

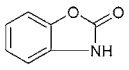
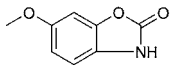
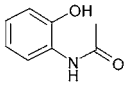
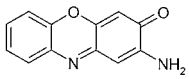
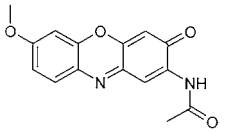
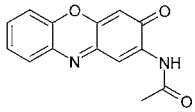
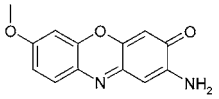
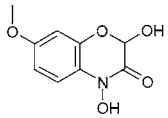
Looking for a good CoMFA relationship for the benzoxazinones and their derivatives, we found that excluding compound DIMBOA increased the goodness of the relationship. DIMBOA is an unstable reactive compound because it decomposes to 6-methoxybenzoxazolin-2-one (MBOA) spontaneously in an aqueous solution (*5*). Moreover, from the structural point of view DIMBOA is different from the other compounds studied, because it contains a nonplanar saturated heterocyclic ring, which could not be properly aligned onto a common template and caused problems in the CoMFA analysis due to its well-known sensitivity of this technique to minor superposition variations in combination with the low number of studied structures.

The final relationship was obtained using only three components, log *P* and steric contribution. Column filtering of 2.0 kcal/mol was performed by omitting from the analysis those columns (lattice points) with an energy variance of <2.0 kcal/mol, reducing the variables used from 577 to 48.

The statistical parameters and field contributions of the relationship found (excluding DIMBOA from the data set) are summarized in **Table 2**. The determination coefficient, R^2 value, was 0.97, the Q^2 leave-one-out cross-validated was 0.79, and the standard error of estimate was 0.24.

Electrostatic descriptors were not selected for PLS analysis of the CoMFA model, because they did not provide any additional contribution to the model predictivity. This is caused by the fact that prior to calculation of molecular interaction fields, a pharmacophore-like alignment (overlapping common structural motifs present in all compounds) was used to orient the molecules in the 3D grid space. Additionally, most of the studied compounds are rigid polycyclic systems, and there is

Table 1. Schematic Illustration of the Compounds Used in the Development of 3D QSAR Together with the Experimental LC_{50}, Expressed Also as log(MW/LC_{50}),[a] and log *P* Values

Compound	LC50 (shortterm toxicity) in mg/kg dry soil	log (MW/LC50)	logP
BOA 	3.4	1.60	1.56
MBOA 	2.9	1.76	1.35
HPAA 	0.1	3.18	0.78
APO 	0.01	4.33	1.62
AAMPO 	32.1	0.95	2.26
AAPO 	0.3	2.93	2.15
AMPO 	0.5	2.69	2.13
DIMBOA 	2.2	1.98	0.28

[a] MW, molecular weight.

Table 2. Summary of Statistics for the Obtained Relationship

no. of compounds	7
optimal no. of components	3
Q^2	0.79
standard error of estimate	0.24
R^2	0.97
contributions	
log *P*	0.37
steric	0.63

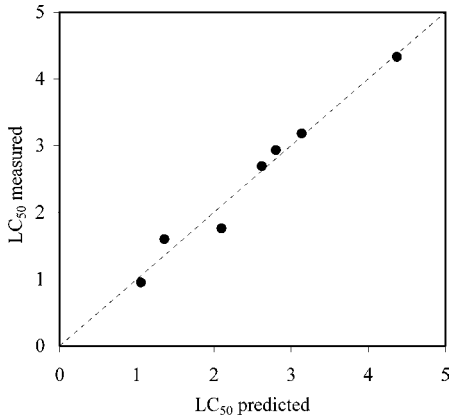


Figure 3. Correlation plot between the predicted and experimentally measured toxicity values.

very little variability among them from the point of view of electronic distribution. Thus, the resulting electrostatic interaction maps are very similar for all of the compounds. To identify significant electrostatic interactions, a larger set containing compounds lacking one or more pharmacophoric groups would be needed.

Contributions are parameters that depict the relevance of descriptors to the model. For the optimized model, contributions from steric fields and log *P* are 0.63 and 0.37, respectively, emphasizing the greater and most important steric properties.

The octanol−water partition coefficient log *P* is often used in QSAR studies as a measure of molecular hydrophobic properties, and it is important information of the membrane penetration. The steric fields used are more general descriptors related not only to the bulkiness of the molecules but also to the orientation of the molecules and the interaction in the binding site.

The correlation plot between the experimentally measured and predicted toxicity values is graphically illustrated in **Figure 3**. The good correlation is indicative of the closeness of the predicted values of the dependent variable (biological activity) with respect to the experimentally measured values.

The CoMFA steric contour map indicates that there is a small sterically favorable area (green) in which the presence of bulky functional groups increases the toxicity. This area is located near the C ring (see **Figures 2** and **4**) of the phenoxazinone polycyclic system. The CoMFA relationship also identifies two sterically unfavorable (yellow) areas: one smaller on the same side of the sterically favorable (green) area and parallel to it, corresponding to the position of the methoxy substituent of the phenoxazinone polycycle, and another, bigger, one located on the opposite side.

The existence of a smaller yellow area is consistent with the decreased toxicity of 7-methoxy-substituted phenoxazinones and is related to the lower toxicity of AAMPO and AMPO with respect to APO. This means that the bulkiness of chemical

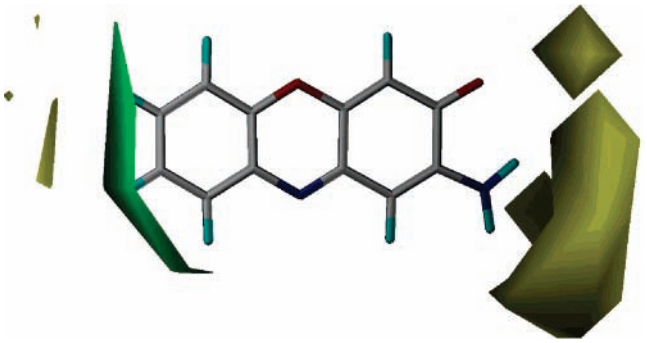


Figure 4. Steric CoMFA contour map using APO, the most toxic compound, for visualization: greater toxicity is correlated with more bulky groups near green areas and less bulky groups near yellow ones.

groups in this area determines the decrease of toxicity, suggesting a weaker receptor interaction due to a bad fit in the binding site.

On the other hand, the presence of the large sterically unfavorable (yellow) areas close to the 2-amino group on the phenoxazinone polycycle is related to the fact that 2-aminophenoxazinone polycyclic compounds are more toxic than 2-acetylaminophenoxazinone analogues. This finding is consistent with the lower toxicity of AAMPO and AAPO, which both have the acetylamino group in this position, because the introduction of any bulky substituent in this area is expected to decrease the activity of the compound. This suggests that this amino group is important for the favorable interaction with the receptor site as it may serve as a donor for hydrogen bonding with the receptor's counterparts.

We can conclude that a successful strategy was used to have a preliminary analysis of toxicity of benzoxazinones and their metabolites, constructing a statistically significant CoMFA relationship.

Preliminary results obtained using CoMFA on a small set of benzoxazinones and their metabolites show that this kind of 3D QSAR approach could be useful for finding valid relationships between their toxicity and structure. Mechanistic interpretation of the statistically significant CoMFA model based on steric interactions and log *P* highlighted the importance of the free amino group at position 2 on the phenoxazinone polycycle for the overall toxicity. The CoMFA model and the information reported in this study could be used for rough toxicity estimation and could enlarge our insight into the world of the allelochemical compounds, a family of secondary metabolites self-produced during defense mechanisms, which are still not well-known.

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Received for review April 20, 2005. Revised manuscript received July 29, 2005. Accepted December 6, 2005. The research described in this paper was performed as part of the project "FATEALLCHEM", "Fate and Toxicity of Allelochemicals (natural plant toxins) in Relation to Environment and Consumer". The project was carried out with financial support from the Commission of the European Communities under the Work Program Quality of Life, Contract QLK5-CT-2001-01967.

JF050916V