

## Optimization of Benzoxazinones as Natural Herbicide Models by Lipophilicity Enhancement

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Benzoxazinones are plant allelochemicals well-known for their phytotoxic activity and for taking part in the defense strategies of Gramineae, Ranunculaceae, and Scrophulariaceae plants. These properties, in addition to the recently optimized methodologies for their large-scale isolation and synthesis, have made some derivatives of natural products, 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3-(4*H*)-one (DIBOA) and its 7-methoxy analogue (DIMBOA), successful templates in the search for natural herbicide models. These new chemicals should be part of integrated methodologies for weed control. In ongoing research about the structure–activity relationships of benzoxazinones and the structural requirements for their phytotoxicity enhancement and after characterization of the optimal structural features, a new generation of chemicals with enhanced lipophilicity was developed. They were tested on selected standard target species and weeds in the search for the optimal aqueous solubility–lipophilicity rate for phytotoxicity. This physical parameter is known to be crucial in modern drug and agrochemical design strategies. The new compounds obtained in this way had interesting phytotoxicity profiles, empowering the phytotoxic effect of the starting benzoxazinone template in some cases. Quantitative structure–activity relationships were obtained by bioactivity–molecular parameters correlations. Because optimal lipophilicity values for phytotoxicity vary with the tested plant, these new derivatives constitute a more selective way to take advantage of benzoxazinone phytotoxic capabilities.

**KEYWORDS:** Allelochemicals; DIBOA; DIMBOA; D-DIBOA; lipophilicity; herbicides; SAR

### INTRODUCTION

The intensive use of synthetic herbicides during the past 50 years has provoked different problems mainly connected to environmental impact (1, 2) and weed resistance to herbicides (3, 4). In the context of an increasing demand for high-quality feeding products and environmental concerns, it is urgent to change the philosophy applied to the research and development of strategies useful in crop protection. Chemical control of crop pests and diseases is one of the areas in which new methodologies mainly focused on the understanding and application of ecological phenomena should contribute to solve the problems created in the past (5).

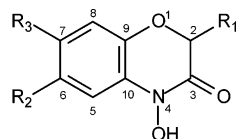
The employment of allelochemicals (natural plant toxins) as templates for the discovery of new phytotoxic agents useful in agriculture has developed into a convenient approach to chemical low-dose treatments with less environmental impact, higher selectivity, and alternative modes of action, which could decisively contribute to solve the problems associated with chemical weed control (6, 7). Commercial herbicides such as

cinmethylin (a derivative from natural monoterpene 1,4-cineole, which is widely distributed in plants) (8) and the herbicide family of triketones, which derive from leptospermane, a natural product from the plant genus *Callistemon* (9), exemplify the potential utility of plant allelochemicals for weed management.

Hydroxamic acids with a (2*H*)-1,4-benzoxazin-3(4*H*)-one skeleton have attracted the attention of phytochemistry and allelopathic researchers since the discovery of 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) (10) and 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) (11) (Figure 1). They were first isolated from rye and maize, respectively, being preserved as  $\beta$ -D-glucosides prior to their release (12–14). Soon after their discovery, they were found in a wide variety of plants mainly belonging to Gramineae, Ranunculaceae, and Scrophulariaceae families (15). Their anti-fungal (16), antimicrobial (17), and phytotoxic effects (18–22), in addition to their interesting ecological role (23), raised interest in their isolation and synthesis in the search for an accurate explanation of the defense mechanisms of benzoxazinone producer plants. Moreover, the discovery of microbial degradation phenomena (24, 25), which led to the detection of new chemicals with different structures, effects, ecological roles, and potential utilities, increased the interest in the development of analytical methods (26) and the design of experiments in

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$R_1=OH$ ;  $R_2=R_3=H$ : 2,4-Dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA)  
 $R_1=OH$ ;  $R_2=H$ ;  $R_3=OCH_3$ : 2,4-Dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA)  
 $R_1=R_2=R_3=H$ : 4-Hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA)  
 $R_1=H$ ;  $R_2=OCH_3$ ;  $R_3=H$ : 4-Hydroxy-6-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (6-MeO-D-DIBOA)

**Figure 1.** Molecular structures of benzoxazinones DIBOA, DIMBOA, D-DIBOA, and 6-MeO-D-DIBOA.

**Table 1.** Molecular Parameters Found To Be Relevant in Pharmaceutical and Herbicidal Activities, with their Optimal Values

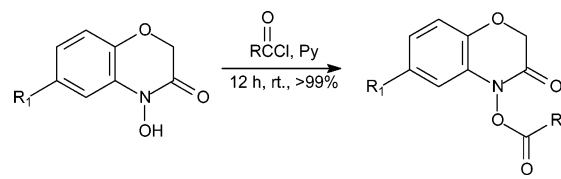
parameter	optimal value	
	Lipinski (pharmaceuticals)	Tice (postemergence herbicides)
H-bond donors	≤5	≤3
H-bond acceptors	≤10	≥2 and ≤12
molecular weight	≥500	≥150 and ≤500
cLog <i>P</i>	≤5	≤4
rotatable bonds		≤12

which all of the chemicals present in the plant–plant interaction involving benzoxazinones could be present (23). Finally, the availability of all those instruments makes benzoxazinones some of the most attractive groups of natural products to be employed in the design of new chemicals with potential use in weed management (27).

In the context of the new methodologies for bioactive compounds' design, quantitative structure–activity relationships studies (QSAR) constitute the key for a systematic analysis of structure and bioactivity properties. An adequate correlation of them to search for the most significant structural requirements is needed to achieve the desired physical, chemical, and biological properties (26) with the aid of combinatorial chemistry techniques (29).

QSAR methodology has been extensively employed for drug discovery, and its applications on new agrochemicals' design are starting to rise, as the potential applicability of the physical–chemical parameters employed in QSAR design of pharmaceuticals has been discussed (30). According to those studies, four parameters should be taken into consideration to generate leads for herbicide development: molecular mass, number of hydrogen-bond donors, number of hydrogen-bond acceptors, and log *P* (logarithm of the octanol/water partition coefficient), which constitutes a measurement of the aqueous solubility–lipophilicity ratio for a given chemical. On the basis of an empirical study on more than 2000 pharmaceuticals, Lipinski et al. proposed the optimal values for those parameters. Log *P*, previously employed to analyze the assimilation capacity of oral usage drugs (30), could also help to determine the bioavailability of a given organic chemical for a plant, as was stated by Tice (31), after the fitting of those parameters to the herbicidal and insecticidal activity requirements, and the addition of a new parameter (number of rotatable bonds). The optimal values of those parameters are shown in **Table 1**. These studies have been greatly influential as they provide useful clues for drug and agrochemical development on the basis of physicochemical parameters, which are easy to calculate.

The barriers that a crop protection agent must cross to reach its molecular target site may vary: Postemergence herbicides must cross the leaf cuticle to reach the whole plant, having more potent effects when being phloem mobile (32). On the other hand, root and shoot uptake and xylem mobility are more



$R_2$	$R_1=H$	$R_1=OMe$
-CH <sub>3</sub>	ABOA	6-MeO-ABOA
-CH <sub>2</sub> -CH <sub>3</sub>	Pr-D-DIBOA	Pr-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>2</sub> -CH <sub>3</sub>	Bu-D-DIBOA	Bu-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>3</sub> -CH <sub>3</sub>	Val-D-DIBOA	Val-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>4</sub> -CH <sub>3</sub>	Hex-D-DIBOA	Hex-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>5</sub> -CH <sub>3</sub>	Hept-D-DIBOA	Hept-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>6</sub> -CH <sub>3</sub>	Oct-D-DIBOA	Oct-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>7</sub> -CH <sub>3</sub>	Non-D-DIBOA	Non-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>8</sub> -CH <sub>3</sub>	Dec-D-DIBOA	Dec-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>9</sub> -CH <sub>3</sub>	Undec-D-DIBOA	Undec-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>10</sub> -CH <sub>3</sub>	Lau-D-DIBOA	Lau-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>11</sub> -CH <sub>3</sub>	Tridec-D-DIBOA	Tridec-6-MeO-D-DIBOA
-(CH <sub>2</sub> ) <sub>12</sub> -CH <sub>3</sub>	Mir-D-DIBOA	Mir-6-MeO-D-DIBOA

**Figure 2.** Structures and acronyms of the tested benzoxazinone esters.

important for pre-emergence herbicides (33). Systemic transportation would be favored by a higher aqueous solubility, but some lipophilicity will be necessary to cross cell membranes and to reach the target site of action.

In this work, the algorithm included in OSIRIS Property Explorer (34) for cLog *P* calculation (35) was found to be the most adequate one, as it fits the previously reported values for the empirical rules mentioned above.

Herein we report the correlation of phytotoxic effects of two series of benzoxazinone ester derivatives with increasing lipophilicity in the search for the optimal value for log *P*. The lipophilicity was increased by preparing esters with increasing side chain length. This was found to be the most chemically convenient way, taking advantage of the hydroxyl group at N-4 present in the hydroxamic acids with a (2*H*)-1,4-benzoxazin-3(4*H*)-one skeleton selected for this study (**Figure 1**). In our previous papers on structure–activity relationship studies on benzoxazinones and related compounds (20–22), several requisites for phytotoxicity enhancement were displayed: the lack of functional groups at the aromatic ring, the lack of a hydroxyl group at C-2, and the presence of a hydroxamic acid moiety. 4-Hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA, **Figure 1**) was proposed as the optimal template for herbicide model development. Its derivative, 4-hydroxy-6-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (6-MeO-D-DIBOA, **Figure 1**), was included in the study to compare the effects on transport modulation in two structurally related chemicals, in order to assume or discard intrinsic phytotoxic effects from the ester side chains. Thus, 14 ester derivatives of D-DIBOA (**Figure 2**) were prepared and tested on the same species as in our previous studies. Their correlation to Tice's modification to Lipinski's rule of 5 (31), in the context of Hansch's transport theory (37), is discussed. These benzoxazinones constitute a second generation of chemicals from previously reported SAR studies of benzoxazinones and related compounds (20–22).

## MATERIALS AND METHODS

**General Experimental Procedures.** The purity of the compounds to test was determined by <sup>1</sup>H NMR and HPLC analyses and found to

be >98%.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded using  $\text{CDCl}_3$  as solvent in a Varian INOVA spectrometer at 399.99 and 100.577 MHz, respectively. The resonance of residual chloroform was set to  $\delta$  7.25. The solvent peak for  $^{13}\text{C}$  was set to  $\delta$  77.00 (chloroform) and used as internal reference. UV-vis spectra were acquired by means of a Varian Cary 50 BIO spectrophotometer, chloroform being used as solvent. Mass spectra (EIMS) were recorded by means of a Voyager Termoquest equipment. FTIR spectra were obtained by means of a Spectrum BX Perkin-Elmer FTIR system. Frequency values are given in  $\text{cm}^{-1}$ .

**Starting Material.** 4-Hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA, **Figure 1**) was synthetically obtained as previously reported (25). Acyl chlorides were purchased from Sigma-Aldrich Co. and used as received, except undecanoyl and tridecanoyl chlorides; these were synthesized as described below. 6-Methoxy-D-DIBOA was synthesized by using 4-methoxy-2-nitrophenol (purchased from Acros Organics, used as received) as starting material and following the same procedures as for D-DIBOA synthesis.

**Undecanoyl and Tridecanoyl Chloride Obtention.** These acyl chlorides were obtained by means of the typical thionyl chloride procedure. Five hundred milligrams of undecanoic or tridecanoic acid (purchased from Acros Organics Inc. and Sigma-Aldrich Co., respectively, used as received) were dissolved in thionyl chloride and refluxed for 4 h. After this, the excess thionyl chloride was distilled at reduced pressure, and the oily residue, containing the corresponding pure acyl chloride, was dissolved in dry THF (0.1 N assuming complete conversion) prior to their use.

**General Esterification Method.** One hundred milligrams of D-DIBOA or 6-methoxy-D-DIBOA was dissolved in 25 mL of dry pyridine, and 1.2 mol equiv of the suitable acyl chloride (commercial or readily synthesized and dissolved in THF for undecanoyl and tridecanoyl) was added dropwise at 0 °C (ice bath) and under a dry argon atmosphere. After the addition, the flask was allowed to warm to room temperature; 12 h later, 25 mL of EtOAc was added, and the mixture was transferred to an extraction funnel and washed with 50 mL of 0.1 N HCl (3 times) and 50 mL of 0.1 N  $\text{NaHSO}_4$  (three times). The organic phases were further combined and dried over anhydrous magnesium sulfate, and the solvent was removed (rotatory evaporator). The dry residue was chromatographed (CC,  $\text{SiO}_2$ , EtOAc/hexane 1:10) to obtain the benzoxazinone ester derivatives.

**Calculation of  $\text{IC}_{50}$  and Log *P*.** The phytotoxicity data were fitted to a sigmoidal dose-response model (constant slope) by employing the GraphPad Prism v. 4.00 software package (34).  $\text{cLog } P$  data were acquired by using the OSIRIS property explorer (35). This software uses the Chou and Jurs algorithm, based on computed atom contributions (36).

**Bioassays. Target Plants.** Selection of target plants is based on an optimization process made by us in the search for a standard phytotoxicity evaluation bioassay (38). After this process several standard target species (STS) were proposed, including monocots *Triticum aestivum* L. (wheat) and *Allium cepa* L. (onion) and dicots *Lycopersicon esculentum* Will. (tomato), *Lepidium sativum* L. (cress), and *Lactuca sativa* L. (lettuce), which were assayed for this study. The weeds *Lolium rigidum* Gaud. (rigid ryegrass) and *Avena fatua* L. (wild oat) were also tested by employing the same methodology.

**Methodology.** Bioassays used Petri dishes (90 mm diameter) with one sheet of Whatman no. 1 filter paper as substrate. Germination and growth were conducted in aqueous solutions at controlled pH by using  $10^{-2}$  M 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1 M NaOH (pH 6.0). Compounds to be assayed were dissolved in DMSO (0.2, 0.1, 0.02, 0.01, and 0.002 M), and these solutions were diluted with buffer (5  $\mu\text{L}$  of DMSO solution/mL of buffer) so that test concentrations for each compound ( $10^{-3}$ ,  $5 \times 10^{-4}$ ,  $10^{-4}$ ,  $5 \times 10^{-5}$ , and  $10^{-5}$  M) were reached. This procedure facilitated the solubility of the assayed compounds. The number of seeds in each Petri dish depended on the seed size; 25 seeds were used for tomato, lettuce, cress, and onion, 15 seeds were used for rigid ryegrass, and 10 seeds were used for wheat and wild oat. Five milliliters of treatment, control, or internal reference solution was added to each Petri dish. Four replicates were used for tomato, cress, onion, lettuce, and rigid ryegrass; 10 replicates were used for wheat and wild oat.

After the addition of seeds and aqueous solutions, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were further incubated at 25 °C in a Memmert ICE 700 controlled-environment growth chamber, in the absence of light. Bioassays took 4 days for cress, 5 days for lettuce, tomato, rigid ryegrass, wild oat, and wheat, and 7 days for onion. After growth, plants were frozen at  $-10$  °C for 24 h to avoid subsequent growth during the measurement process. This helped in the handling of the plants and allowed a more accurate measurement of root and shoot lengths.

The commercial herbicide Logran, a combination of *N*-(1,1-dimethylethyl)-*N'*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (Terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-[[[4-methoxy-6-methyl-1,3,5-triazin-2-yl]amino]carbonyl]benzenesulfonamide (Triasulfuron, 0.6%), was used as internal reference, according to a comparison study previously reported (38). It was used at the same concentrations ( $10^{-3}$ ,  $5 \times 10^{-4}$ ,  $10^{-4}$ ,  $5 \times 10^{-5}$ , and  $10^{-5}$  M) and in the same conditions as the compounds in study. Negative control samples (buffered aqueous solutions with DMSO and without any tested compound) were used for all of the plant species assayed.

**Bioassay Data Acquisition.** Evaluated parameters (germination rate, root length, and shoot length) were recorded by using a Fitomed system (39), which allowed automatic data acquisition and statistical analysis by its associated software.

**Statistical Analysis.** Data were statistically analyzed using Welch's test, with significance fixed at 0.01 and 0.05. Results are expressed in bar charts in which the null value represents control, negative values represent inhibition, and positive values represent stimulation of the studied parameter (39). Statistical significance is expressed by means of letters, "a" meaning significantly different from control with 0.01 confidence and "b" meaning significantly different from control with a confidence from 0.01 to 0.05. The absence of a letter indicates no significant difference from control values. Phytotoxic activities expressed in this way can be found in the Supporting Information for all chemicals and species.

Once the germination and growth data had been acquired, cluster analysis was used to group compounds with similar phytotoxicity behaviors and associate them with their molecular structure. Complete linkage was used as an amalgamation rule, and the distance measurement was based on squared Euclidean distances (41), given by the equation

$$d(x,y) = \sum_i (x_i - y_i)^2$$

where  $d(x,y)$  is the squared Euclidean distance ( $i$ -dimensional),  $i$  represents the number of variables, and  $x$  and  $y$  are the observed values.

The cluster was obtained by using Statistica v. 5.0 software (41). Germination rate, shoot length, and root length effects, for all tested species, were included in the analysis to acquire an overall view of the phytotoxicity and its relationship with chemical structure.

$\text{EC}_{50}$  values were obtained after the phytotoxicity data had been adjusted to concentration (logarithmic scale), to a constant slope sigmoidal dose-response curve, defined by the equation

$$Y = Y_{\min} + \frac{Y_{\max} - Y_{\min}}{1 + 10^{\log \text{EC}_{50} - X}}$$

where  $X$  indicates the logarithm of concentration,  $Y$  indicates the response (phytotoxicity), and  $Y_{\max}$  and  $Y_{\min}$  are the maximum and minimum values of the response, respectively. Goodness of fit is described by the determination coefficient ( $r^2$ ). The adjustment and the  $r^2$  were obtained by using GraphPad Prism software v. 4.00 (39).

## RESULTS AND DISCUSSION

**Synthesis of D-DIBOA, 6-Methoxy-D-DIBOA, and Their Esters.** D-DIBOA was obtained according to the previously reported methodology. 6-Methoxy-D-DIBOA was obtained following the same methodology with 4-methoxy-2-nitrophenol as starting material, with a 65% yield after two reaction steps. Quantitative yield was achieved in all esterification reactions.



**Table 2.** Molecular Parameter Values Found for D-DIBOA and Its Esters<sup>a</sup>

	molecular weight	cLog <i>P</i>	rotatable bonds	H-bond donors	H-bond acceptors
D-DIBOA	165.15	0.42	0	1 <sup>b</sup>	4
ABOA	207.19	1.18	2	0	5
Pr-D-DIBOA	221.21	1.65	3	0	5
Bu-D-DIBOA	235.23	2.11	4	0	5
Val-D-DIBOA	249.25	2.58	5	0	5
Hex-D-DIBOA	263.27	3.04	6	0	5
Hept-D-DIBOA	277.29	3.5	7	0	5
Oct-D-DIBOA	291.31	3.97	8	0	5
Non-D-DIBOA	305.33	<b>4.43</b>	9	0	5
Dec-D-DIBOA	319.35	<b>4.89</b>	10	0	5
Undec-D-DIBOA	333.37	<b>5.36</b>	11	0	5
Lau-D-DIBOA	347.39	<b>5.82</b>	12	0	5
Tridec-D-DIBOA	361.41	<b>6.29</b>	13	0	5
Mir-D-DIBOA	375.43	<b>6.75</b>	14	0	5

<sup>a</sup> Values outside the optimal ranges for activity are shown in boldface type.

<sup>b</sup> Number of donors was found to be pH-dependent, value at pH 6.0 being displayed.

**Table 3.** Comparison of Phytotoxic Effects of D-DIBOA and Its Esters<sup>a</sup>

	germination rate	root length	shoot length
<i>L. sativum</i>	+	+	+
<i>A. cepa</i>	0	–	–
<i>L. esculentum</i>	–	0	0
<i>L. sativa</i>	0	–	–
<i>T. aestivum</i>	0	0	0
<i>L. rigidum</i>	0	+	0
<i>A. fatua</i>	++	+	0

<sup>a</sup> 0, D-DIBOA phytotoxicity not significantly affected by esterification; –, effects decreased by esterification; +, effects increased by esterification; ++, effects highly increased by esterification.

The structures of all synthesized chemicals were confirmed by spectroscopy methods (see Supporting Information).

**Fitting to Lipinski's Rule of 5 (Tice Approach for Agrochemical Design).** The parameters included in Lipinski's rule of 5, as they were reinvestigated by Tice as a useful approach for agrochemicals design, are summarized in **Table 2** for all of the obtained chemicals. All tested compounds after Dec-D-DIBOA did not fit the rule, as they have cLog *P* values >5, which agrees with Lipinski's requirements. In addition to this, Tridec- and Mir-D-DIBOA exceeded the number of rotatable bonds. Compounds from D-DIBOA to Oct-D-DIBOA have molecular properties adequate to reach good phytotoxicity values, according to Tice's optimal cLog *P* range.

**Phytotoxic Activity.** All tested compounds, when active, showed inhibitory profiles. The esters were more or less active than D-DIBOA depending on the tested plant and the evaluated parameter (germination rate, root length, and shoot length). The persistence, the increase or decrease of the phytotoxic effect with cLog *P*, was also plant and parameter dependent. In general terms, root length was the most affected parameter in all species. A qualitative general comparison of the esters with D-DIBOA, with regard to plant species and parameters, is given in **Table 3**. The addition of an ester function to D-DIBOA provoked a significant phytotoxicity enhancement on cress (with increasing germination rate, root length, and shoot length inhibition), as the most affected STS, and the weeds rigid ryegrass (in which root length inhibition increases) and especially wild oat (with a drastic difference on germination inhibition rate and highly significant effects on root length).

Cress was more affected by chemicals of medium polarity of the series: Valeryl and heptanoyl D-DIBOA derivatives

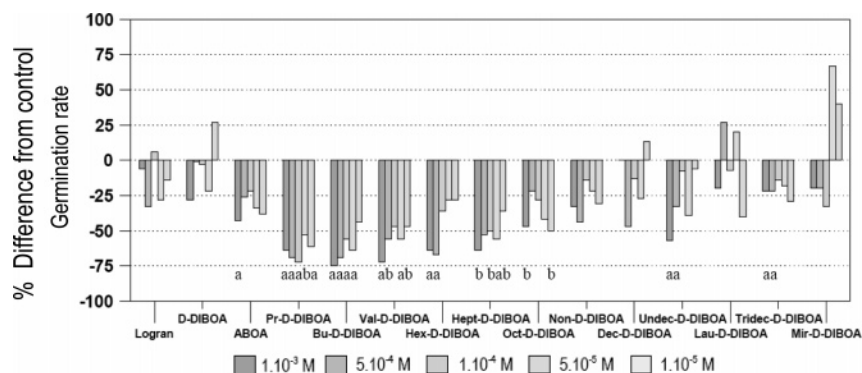
strongly inhibited root and shoot length. Low values of IC<sub>50</sub> were reached, especially on shoot length inhibition [IC<sub>50</sub> = 82.4 and 87.0 μM for valeryl and heptanoyl derivatives, respectively, with excellent fitting to the dose–response model ( $R^2 > 0.99$  in both cases)]. Valeryl D-DIBOA also provoked a significant effect on germination rate (IC<sub>50</sub> = 580 μM,  $R^2 = 0.9374$ ), maintaining significant activity values at all concentrations when D-DIBOA was found to be active only at the most concentrated treatment.

With regard to the phytotoxicity on weeds, D-DIBOA and ABOA were the most active chemicals on *L. rigidum* if joint effects on roots and shoots were considered. Nevertheless, heptanoyl and octanoyl derivatives were also active on roots, with IC<sub>50</sub> values around 100 μM (123.4 and 107.4 μM, respectively, with  $R^2 > 0.95$  in both cases). No significant effects were observed for germination rate.

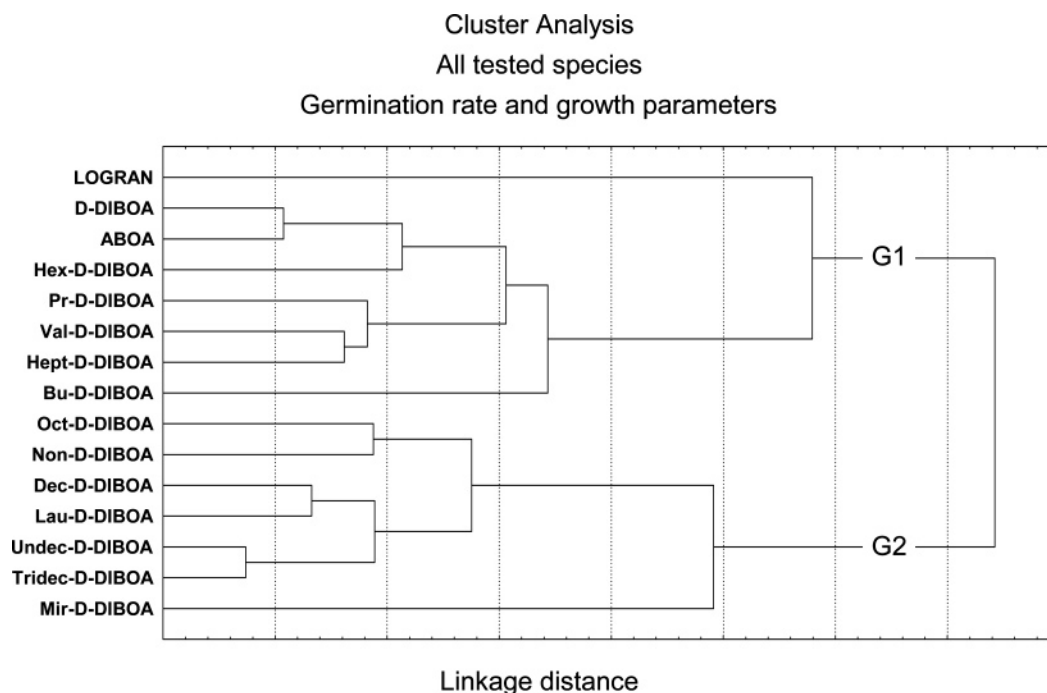
Among the different weed species tested to date, *Avena fatua* (wild oat) has been the one most affected by benzoxazinone treatments, according also to our previous research (21, 23). Wild oat is the only plant of all that were studied that showed a significant effect on its germination rate, this effect being closely related to lipophilicity (**Figure 3**). The effects drastically increase from Pr-D-DIBOA (cLog *P* = 1.65) to Hept-D-DIBOA (cLog *P* = 3.50). A loss of the effect is observed from the octanoyl derivative to the end of the series, this behavior being in good accordance with the empiric rules of Tice (29). Optimal values of cLog *P* are in the propanoyl–valeryl interval (1.65–2.58). Compounds outside the cLog *P* values that fit the rule (**Table 2**) did not provoke any significant effect on germination rate. Pr-D-DIBOA affected root length like no other chemical of the series, preserving an almost complete inhibition effect in the first three concentrations, which provides a very low IC<sub>50</sub> (27.8 μM,  $R^2 = 0.9772$ ). Without presentation of those so potent values, Val-D-DIBOA is the only chemical to provoke consistent effects on the three parameters for this plant, the effects on shoot being of medium potency (IC<sub>50</sub> = 509.6 μM,  $R^2 = 0.9800$ ). The consistency of the effects of the tested compounds on wild oat makes this plant the optimal one to attempt a mathematical correlation of lipophilicity, expressed as cLog *P*, with phytotoxicity values (see next section).

It is interesting to point out the effects of these chemicals on wheat. The IC<sub>50</sub> (root length) for the commercial herbicide Logran was 7268 μM ( $R^2 = 0.9410$ ) and 7026 μM for D-DIBOA ( $R^2 = 0.9789$ ). They are much higher than the highest tested dose, so they were obtained by extrapolation. All IC<sub>50</sub> values for D-DIBOA esters are >1 mM, the maximum tested dose. A detailed analysis of the dose–response curves for Pr-D-DIBOA on *A. fatua* and wheat (root length) revealed a highly relevant result from the point of view of the selectivity (see below).

**Lipophilicity–Activity Correlations.** Taking into consideration effects on all parameters and species, a cluster analysis was made to classify the tested chemicals according to their phytotoxicity and properties. The results are shown in **Figure 4**. Two main groups of compounds can be discerned according to their phytotoxicity profiles: G1, in which the commercial herbicide Logran, D-DIBOA, and their esters from acetyl to heptanoyl are included, and G2, containing D-DIBOA esters from octanoyl to miristoyl. G1 is formed by the most phytotoxic chemicals on most of the tested species. This result clearly agrees with the proposed dependence of phytotoxicity with cLog *P*, as the chemicals fitting the proposed optimal cLog *P* interval are grouped as the most active ones, except the octanoyl derivative, which is still active in some cases (IC<sub>50</sub> = 316.8

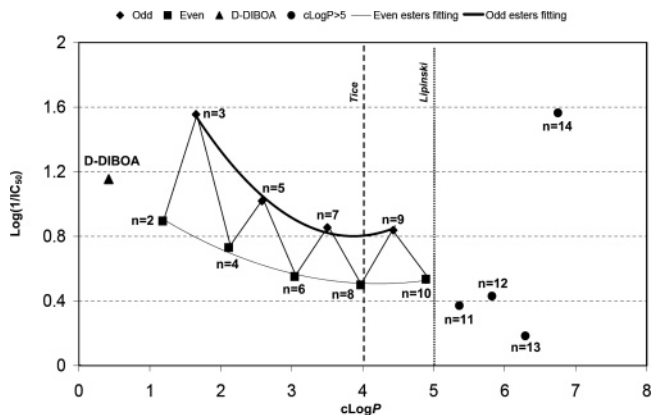


**Figure 3.** Phytotoxic effects of D-DIBOA esters on *A. fatua* L. (germination rate). If it is not indicated,  $P > 0.05$  for Welch's test: (a) values significantly different at  $P < 0.01$ ; (b) values significantly different at  $0.01 < P < 0.05$ .



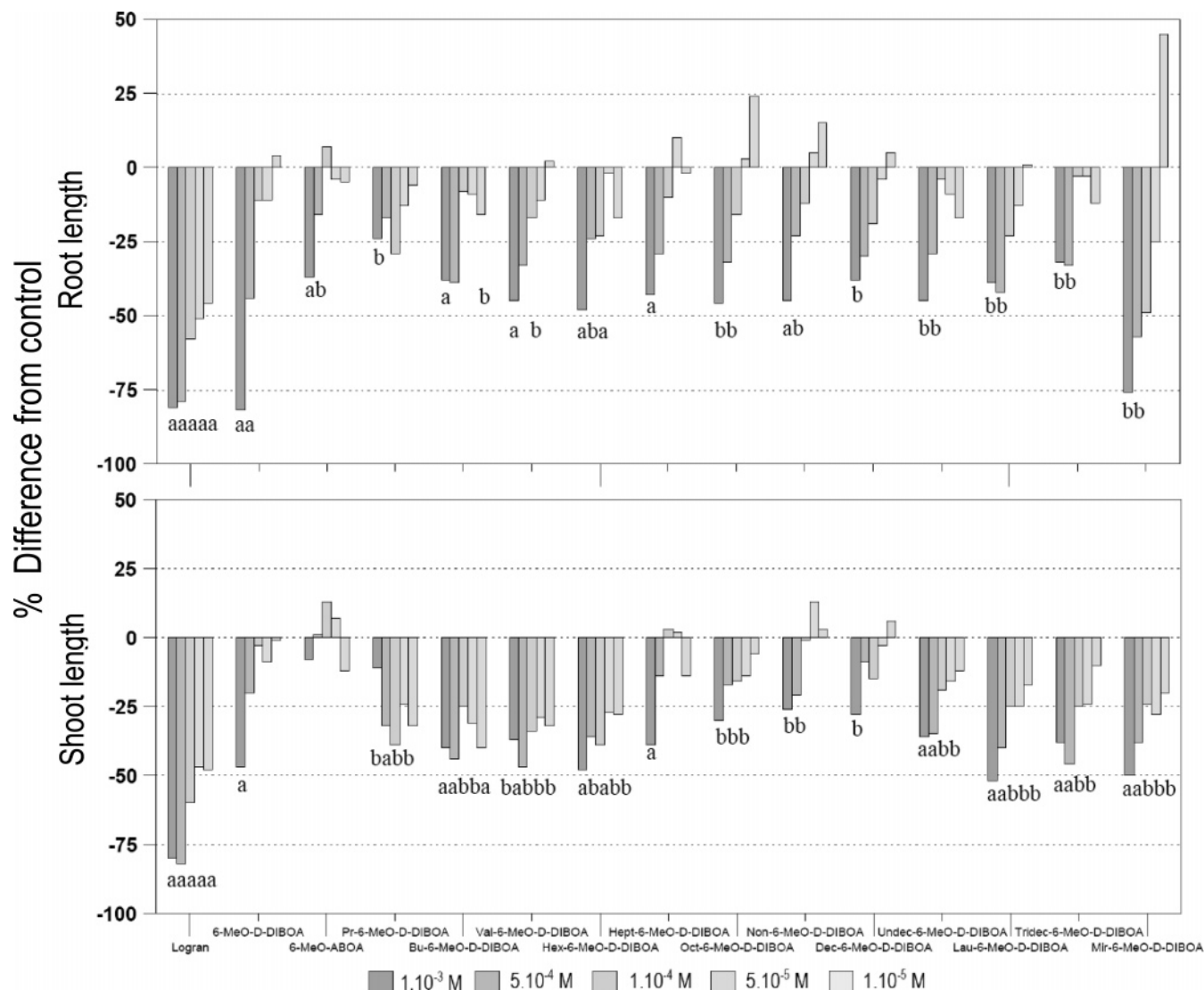
**Figure 4.** Cluster analysis for D-DIBOA esters, made on the basis of germination and growth effects for all tested plants.

$\mu\text{M}$ ,  $R^2 = 0.9570$ , *A. fatua*, root length), but with a phytotoxicity profile much closer to the lipophilic side of the series. Those differences are very clearly displayed in several cases: germination rate on *A. fatua* and germination and root and shoot lengths on *L. sativa* and *L. esculentum* (all parameters, but on root length especially). This loss of phytotoxicity with lipophilicity could agree with the Lipinski and Tice models, as compounds with lipophilicities higher than that of octanoyl-D-DIBOA ( $c\text{Log } P = 3.97$ ) do not fit the optimal requirements for phytotoxicity (Table 2). This fact makes difficult a mathematical correlation of  $c\text{Log } P$  values with phytotoxicity results, at least if the most lipophilic chemicals are included. These kinds of correlations have been made recently though, yielding a lipophilicity–activity relationship and an effect on activity related to the even or odd character of side chains as the most interesting results (42). In that paper, the authors employ the wheat coleoptile bioassay as a phytotoxicity prediction, finding good phytotoxicity– $\text{Log } P$  correlations. They also detect some influence on the activity, depending on the odd or even nature of the ester. Herein we present a similar correlation made by means of the results found for a whole plant, *A. fatua*, which was the tested weed in which higher phytotoxicity (21, 22) for benzoxazinones was found, seemed to be adequate for this study. The phyto-



**Figure 5.** QSAR correlations of phytotoxic effects with lipophilicity.  $n$  indicates number of carbons for the esters. Vertical lines indicate maximum lipophilicity according to Tice and Lipinski models.

toxicity [expressed as  $\log(1/\text{IC}_{50})$ ] was correlated with  $c\text{Log } P$  as shown in Figure 5. Two zones can be clearly separated: the lower  $c\text{Log } P$  zone (fitting the Lipinski  $c\text{Log } P$  requirements for optimal bioactivity), in which some correlations can be made, and the zone of higher  $c\text{Log } P$  values, in which the phytotoxic



**Figure 6.** Phytotoxic effects of 6-MeO-D-DIBOA esters on *A. fatua* L. (root and shoot lengths). If it is not indicated,  $P > 0.05$  for Welch's test: (a) values significantly different at  $P < 0.01$ ; (b) values significantly different at  $0.01 < P < 0.05$ .

effect of the chemicals could not be adjusted to a parabolic equation, as Hansch's transport model proposes (37).

The phytotoxicity data for the lower  $c\text{Log } P$  zone did not fit the quadratic model correctly when treated all together, but the separation of even and odd side chains provided a couple of excellent fittings. This effect was previously found and discussed (42), so that a relationship with the capacity of the esters' side chains to interact with the cell membrane lipidic bilayer was proposed to be the cause for this phenomenon. Even and odd side chains reasonably could behave differently, on the basis of the side-chain lipidic bilayer van der Waals interactions that occur. In this case, as in the previous paper, odd esters are more active than the even ones. This could be a result of a stronger van der Waals interaction with the lipidic bilayer of the even ones, resulting in a low access to the corresponding target site(s) of action. Thus, a good permeability through the lipidic bilayer (measured in terms of  $\text{Log } P$ ), but weak interactions with it, favor the phytotoxic effect. Logically, the closer the side chains are to the cell membrane fatty acid length (and lipophilicity), the better the interaction, resulting in a retention of the phytotoxins and the subsequent lower effects. The tendency of both even and odd series helps to explain the disposition of butyric ester in the cluster analysis (Figure 4), which seems not to be ordered. As can be seen,  $\text{IC}_{50}$  values of

Bu-D-DIBOA are very close to the ones for hexanoyl, heptanoyl, and also octanoyl D-DIBOA, located in the  $c\text{Log } P$  region in which the distance from one curve to another is narrower. This puts Bu-D-DIBOA in the middle of both clusters. In addition to this, the even-odd effect is also observed in the cluster analysis, if compounds other than the butyric ester are grouped following this behavior in the G1 group. The most lipophilic side of the series, in which the transport model has no application (G2), does not follow this pattern. The QSAR correlations of even and odd esters separately resulted in the following equations:

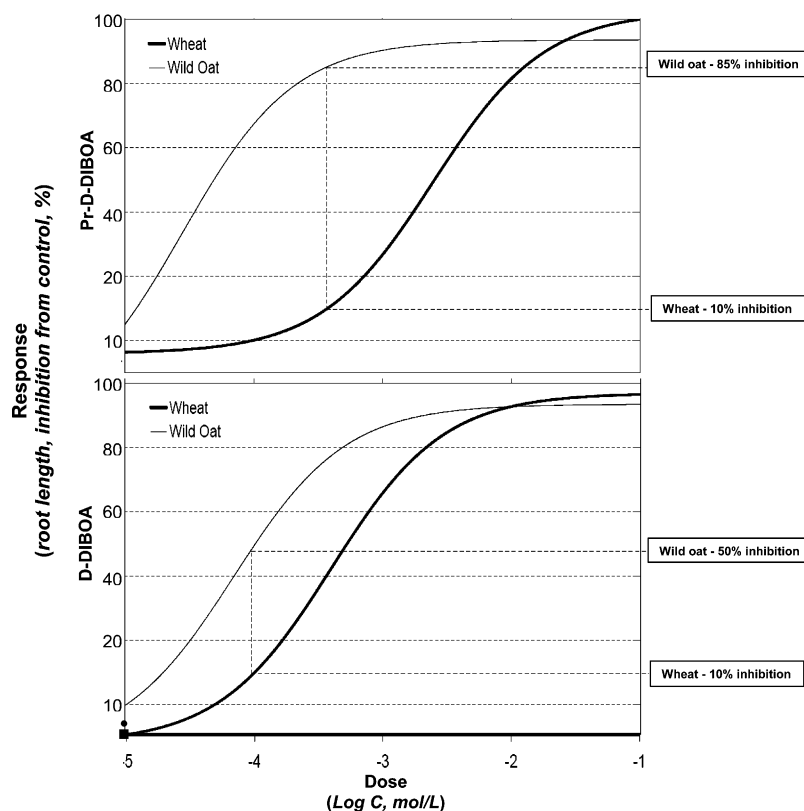
odd esters

$$\log(1/\text{IC}_{50}) = 0.151(c\text{Log } P)^2 - 1.1685(c\text{Log } P) + 3.0612, \\ R^2 = 0.9929$$

even esters

$$\log(1/\text{IC}_{50}) = 0.138(c\text{Log } P)^2 - 1.0626(c\text{Log } P) + 2.473, \\ R^2 = 0.9640$$

Lipophilicity is not the only molecular parameter modified by the systematic esterification of D-DIBOA. The increasing



**Figure 7.** Dose–response curves for D-DIBOA and its propanoyl ester (Pr-D-DIBOA) on wheat and wild oat (root length). Inhibitions provoked on wild oat growth at wheat IC<sub>10</sub> are indicated.

ester length provides a larger number of rotatable bonds, whereas a new H-bond acceptor (carboxyl group) is added and a H-bond donor (hydroxylic proton of the hydroxamic acid moiety) is lost. The numbers of H-bond donors and acceptors, which are relevant in terms of H-bond interaction of the phytotoxin with the target site of action, remains within the model limits throughout the series. The increasing number of rotatable bonds lowers the conformational stability of the molecule, making it difficult to fit the target site of action. Nevertheless, some conformational flexibility is needed for this purpose, as compounds with 12 or fewer rotatable bonds fit the rule. Thus, the ester derivatives of D-DIBOA provide conformational flexibility to the molecule (which lacks rotatable bonds) in addition to lipophilicity. All of the tested chemicals fit the model in terms of molecular weight.

**6-Methoxy-D-DIBOA Esters.** Once the lipophilicity–phytotoxicity relationships have been determined, and in the context of Hansch’s transport theory (37), in which bioactivity is described as a function of transport phenomena and “intrinsic” bioactivity, it would be interesting to discover if the phytotoxic effects observed for the D-DIBOA esters are provoked for a higher bioavailability of the active part of the molecule, or the ‘intrinsic’ phytotoxicity of the fatty acid residues attached to D-DIBOA could have. If side chains were responsible for those phytotoxicity values, a change in the “active” part of the molecule would not significantly affect the phytotoxicity. 6-Methoxy-D-DIBOA (**Figure 1**), a benzoxazinone derivative with phytotoxicity values very similar to those found for D-DIBOA (see Supporting Information), is examined through the same methodology as D-DIBOA on *A. fatua*. Phytotoxicity results are shown on **Figure 6** (growth parameters). The zone of the series in which the maximum root length effects are

obtained is slightly displaced to the lipophilic side in comparison to the D-DIBOA series (which could be caused by the higher aqueous solubility of the 6-methoxy derivative, with  $c\text{Log } P = 0.32$ ). Neither acetyl nor propanoyl derivatives affected root length significantly. Moreover, shoot length is the most affected parameter instead of root length. The phytotoxic effect of the more lipophilic compounds could be due to their accumulation in the seed surface during the experiment, avoiding the seed water uptake (as could happen with Mir-D-DIBOA), rather than a “true” phytotoxic effect in which the active chemical reaches the corresponding molecular target site. Thus, 6-methoxy-D-DIBOA derivatives modulate the transport phenomena for this compound, enhancing the phytotoxic effect in the case of *A. fatua* shoot length, but in the context of the intrinsic activity of this chemical, in which the methylation of the aromatic ring makes it less active, as in the case of the previously reported 7-methoxy derivative (20, 22).

**Selective Action of D-DIBOA Esters: Pr-D-DIBOA.** Pr-D-DIBOA was the most active tested chemical on *A. fatua* root length ( $\text{IC}_{50} = 27.8 \mu\text{M}$ ,  $R^2 = 0.9772$ ). The introduction of an ester function in D-DIBOA provokes a modulation of the transport phenomena, resulting in an increase or decrease of phytotoxicity. This effect could be different, depending on the tested plant, as their morphological structures, then the barriers the chemical needs to cross to reach the target site of action, could be different.

One of the determinant parameters in the optimization of herbicides is the selectivity: A herbicidal treatment, when made to a crop, involves necessarily a loss in the crop production due to the phytotoxicity of the treatment. Thus, it is necessary to compare the effects that a herbicide candidate can produce on crops and their common weeds. As *A. fatua* is a common



wheat weed, the influence of D-DIBOA and its propanoyl derivative was studied for both plants, by means of the comparison of their dose–response curves. The main intention of this correlation was to discover if Pr-D-DIBOA, in addition to a phytotoxicity increase, affects selectivity of action on wheat and its common weed, *A. fatua*. **Figure 7** include two sets of curves, one for each chemical, in which the comparative effects on wheat and wild oat can be examined. D-DIBOA was more active on wild oat than on wheat. The D-DIBOA dose provoking a 10% inhibition of wheat root length causes a 50% inhibition on wild oat. D-DIBOA propanoyl ester, at the same low-activity dose on wheat, causes an almost complete inhibition of root length on wild oat (–85%). In other words, propanoyl ester effects on wheat and wild oat (two Gramineae species) are much more from one another than the ones provoked by D-DIBOA.

The systematic esterification of D-DIBOA was effective for the purpose of studying the influence of transport phenomena in the phytotoxic effect of this chemical, a useful lead for natural herbicide model development (20–22). These derivatives displayed a modulated phytotoxicity, which could be related to their interactions with cell membrane lipidic bilayers, and the subsequent capability of reaching the D-DIBOA target site of action. Propanoyl and valeryl derivatives resulted as the most interesting ones from the point of view of their effects, establishing the optimal cLog *P* in the range from 1.65 to 2.58. These lipophilic D-DIBOA derivatives could constitute a second generation of leads for natural herbicide development (43), in which lipophilicity, a determinant parameter in drug and agrochemical design (30–31), is optimized. Pr-D-DIBOA also possesses a much higher selectivity of action than D-DIBOA, being then potentially able to control a common wheat weed (*A. fatua*) population without significantly affecting the crop. This is the first time in which this kind of analysis is reported with allelochemical derivatives, by using whole plants as target sites. This work also sheds light on the usefulness of QSAR analysis, more likely applied in drug design, to the development of phytotoxins useful in the search for new herbicide models.

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**Supporting Information Available:** Physical data for all tested chemicals (FTIR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, EIMS); phytotoxicity data for D-DIBOA series chemicals on all species; and phytotoxicity data for 6-MeO-D-DIBOA series on *A. fatua*. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## LITERATURE CITED

- Sullivan, T. P.; Sullivan, D. S. Vegetation management and ecosystem disturbance: impact of glyphosate herbicide on plant and animal diversity in terrestrial systems. *Environ. Rev.* **2003**, *11*, 37–59.
- Wang, Y. S. Environmental impact of herbicide use in the subtropics. *Food Sci. Agric. Chem.* **1999**, *1*, 165–179.
- Preston, C. Herbicide detoxification: herbicide selectivity in crops and herbicide resistance in weeds. *ACS Symp. Ser.* **2005**, No. 899, 195–204.
- Owen, M. D. K.; Zelaya, I. A. Herbicide-resistant crops and weed resistance to herbicides. *Pest Manage. Sci.* **2005**, *61*, 301–311.
- Macías, F. A.; Molinillo, J. M. G.; Oliveros-Bastidas, A.; Marín, D.; Chinchilla, D. Allelopathy. A natural strategy for weed control. *Commun. Agric. Appl. Biol. Sci.* **2004**, *69*, 13–23.
- Dayan, F.; Romagni, J.; Tellez, M.; Rimando, A.; Duke, S. Managing weeds with natural products. *Pestic. Outlook* **1999**, *10*, 185–188.
- Macías, F. A.; Molinillo, J. M. G.; Galindo, J. C. G.; Varela, R. M.; Simonet, A. M.; Castellano, D. The use of allelopathic studies in the search for natural herbicides. *J. Crop Prod.* **2001**, *4*, 237–255.
- Grayson, B. T.; Williams, K. S.; Freehauf, P. A.; Pease, R. R.; Ziesel, W. T.; Sereno, R. L.; Reinsfelder, R. E. The physical and chemical properties of herbicide cynmethylin. *Pestic. Sci.* **1987**, *21*, 143–153.
- Lee, D. L.; Prisybilla, M. P.; Cromarthe, T. H. The discovery and structural requirements of inhibitors of *p*-hydroxyphe-nylpyruvate dioxygenase. *Weed Sci.* **1997**, *45*, 601–609.
- Virtanen, A. I.; Hietala, P. K. The structures of the precursors of benzoxazolinone in rye plants. II. *Suom. Kemistil. B* **1959**, *32*, 252.
- Hamilton, R. H.; Bandurski, R. S.; Reusch, W. H. Isolation and characterization of a cyclic hydroxamate from *Zea mays*. *Cereal Chem.* **1962**, *39*, 107–113.
- Wahlroos, Ö.; Virtanen, I. The precursors of 6-methoxy-benzoxazolinone in maize and wheat plants, their isolation and some of their properties. *Acta Chem. Scand.* **1959**, *13*, 1906–1908.
- Hofman, J.; Hofmanova, O. 1,4-Benzoxazine derivatives in plants. *Eur. J. Biochem.* **1969**, *8*, 109–112.
- Hofman, J.; Hofmanova, O. 1,4-Benzoxazine derivatives in plants: absence of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one from uninjured *Zea mays* plants. *Phytochemistry* **1971**, *10*, 1441–1444.
- Sicker, D.; Schulz, M. Benzoxazinones in plants: occurrence, synthetic access, and biological activity. *Stud. Nat. Prod. Chem.* **2002**, *27*, 185–232.
- Honkanen, E.; Virtanen, A. I. Synthesis of some 1,4-benzoxazine derivatives and their antimicrobial activity. *Acta Chem. Scand.* **1960**, *14*, 1214–1217.
- Escobar, C. A.; Sicker, D.; Niemeyer, H. M. Evaluation of DIMBOA analogs as antifeedants and antibiotics towards the aphid *Sitobion avenae* in artificial diets. *J. Chem. Ecol.* **1999**, *25*, 1543–1554.
- Schulz, M.; Friebe, A.; Kueck, P.; Seipel, M.; Schnabl, H. Allelopathic effects of living quack grass (*Agropyron repens* L.). Identification of inhibitory allelochemicals exuded from rhizome borne roots. *Angew. Bot.* **1994**, *68*, 195–200.
- Wolf, R. B.; Spencer, G. F.; Plattner, R. D. Benzoxazolinone, 2,4-dihydroxy-1,4-benzoxazin-3-one, and its glucoside from *Acanthus mollis* seeds inhibit velvetleaf germination and growth. *J. Nat. Prod.* **1985**, *48*, 59–63.
- Macías, F. A.; Marín, D.; Oliveros-Bastidas, A.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Structure–activity relationships (SAR) studies of benzoxazinoids, their degradation products and analogues. Phytotoxicity on standard target species (STS). *J. Agric. Food Chem.* **2005**, *53*, 538–548.
- Macías, F. A.; Chinchilla, N.; Varela, R. M.; Oliveros-Bastidas, A.; Marín, D.; Molinillo, J. M. G. Structure–activity relationship studies of benzoxazinones and related compounds. Phytotoxicity on *Echinochloa crus-galli* (L.) P. Beauv. *J. Agric. Food Chem.* **2005**, *53*, 4373–4380.
- Macías, F. A.; Marín, D.; Oliveros-Bastidas, A.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Structure–activity relationship (SAR) studies of benzoxazinones, their degradation products, and analogues. Phytotoxicity on problematic weeds *Avena fatua* L. and *Lolium rigidum* Gaud. *J. Agric. Food Chem.* **2006**, *54*, 1040–1048.
- Macías, F. A.; Oliveros-Bastidas, A.; Marín, D. The allelopathic phenomenon, a dynamic process. In *Proceedings of the 4th World Congress on Allelopathy*; Harper, J. D. I., An, M., Wu, H., Kent, J. H., Eds.; The Regional Institute Limited: Gosford, Australia, 2005; pp 77–85.



- (24) Macías, F. A.; Oliveros-Bastidas, A.; Marín, D.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Degradation studies on benzoxazinoids. Soil degradation dynamics of 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and its degradation products, phytotoxic allelochemicals from Gramineae. *J. Agric. Food Chem.* **2004**, *52*, 6402–6413.
- (25) Macías, F. A.; Oliveros-Bastidas, A.; Marín, D.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Degradation studies on benzoxazinoids. Soil degradation dynamics of (2*R*)-2-*O*- $\beta$ -D-glucopyranosyl-2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-Glc) and its degradation products, phytotoxic allelochemicals from Gramineae. *J. Agric. Food Chem.* **2005**, *53*, 554–561.
- (26) Eljarrat, E.; Guillamón, M.; Seuma, J.; Mogensen, B. B.; Fomsgaard, I. S.; Oliveros-Bastidas, A.; Macías, F. A.; Stochmal, A.; Oleszek, W.; Shakaliene, O.; Barceló, D. First European interlaboratory study of the analysis of benzoxazinone derivatives in plants by liquid chromatography. *J. Chromatogr. A* **2004**, *1047*, 69–76.
- (27) Macías, F. A.; Marín, D.; Oliveros-Bastidas, A.; Chinchilla, D.; Simonet, A. M.; Molinillo, J. M. G. Isolation and synthesis of allelochemicals from Gramineae: benzoxazinones and related compounds. *J. Agric. Food Chem.* **2006**, *54*, 991–1000.
- (28) Kubinyi, H. Strategies and recent technologies in drug discovery. *Pharmazie* **1995**, *50*, 647–662.
- (29) Baum, R. M. Combinatorial approaches provide fresh leads for medicinal chemistry. *Chem. Eng. News* **1994**, *Feb 7*, 20–26.
- (30) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **2001**, *46*, 3–26.
- (31) Tice, C. M. Selecting the right compounds for screening: does Lipinski's rule of 5 for pharmaceuticals apply to agrochemicals? *Pest Manag. Sci.* **2001**, *57*, 3–16.
- (32) Kleier, D. A.; Grayson, B. T.; Hsu, F. C. The phloem mobility of pesticides. *Pestic. Outlook* **1998**, *9*, 26–30.
- (33) Sicbaldi, F.; Sacchi, G. A.; Trevisan, M.; Del, Re, A. A. M. Root uptake and xylem translocation of pesticides from different chemical classes. *Pestic. Sci.* **1997**, *50*, 111–119.
- (34) *PRISMA 4.0*; GraphPad Software, Inc.: San Diego, CA, 2003.
- (35) Sander, T. OSIRIS Property Explorer. Actelion Property Explorer, 2001. <http://www.chemexper.com/tools/propertyExplorer/main.html>.
- (36) Chou, J.; Jurs, P. *J. Chem. Inf. Comput. Sci.* **1979**, *19*, 172.
- (37) Hansh, C.; Leo, A. J. *Substituent Constants for Correlation Analysis in Chemistry*; Wiley: New York, 1979.
- (38) Macías, F. A.; Castellano, D.; Molinillo, J. M. G. Search for a standard phytotoxic bioassay for allelochemicals. Selection of standard target species. *J. Agric. Food Chem.* **2000**, *48*, 2512–2521.
- (39) Castellano, D.; Macías, F. A.; Castellano, M.; Cambronero, R. M. FITOMED (automated system for measurement of variable lengths). Sp. Patent P9901565.
- (40) Macías, F. A.; Velasco, R. F.; Viñolo, V. M. I.; Castellano, D.; Galindo, J. C. G. Cluster analysis. A valuable tool for allelopathic SAR studies? In *Allelopathy: from Molecules to Ecosystems*; Reigosa, M. J., Pedrol, N., Eds.; Science Publishers: Enfield, NH, 2002; pp 305–316.
- (41) *Statistica for Windows 5.0*; Statsoft Inc.: Tulsa, OK, 1984–1995.
- (42) Macías, F. A.; Velasco, R. F.; Castellano, D.; Galindo, J. C. G. Application of Hansch's model to guaianolide ester derivatives: a quantitative structure–activity relationship study. *J. Agric. Food Chem.* **2005**, *53*, 3530–3539.
- (43) Macías, F. A.; Molinillo, J. M. G.; Marín, D.; Carrera, C.; Chinchilla, N.; Oliveros-Bastidas, A. 4-Aciloxyloxy-2*H*-1,4-benzoxazin-3(4*H*)-one derivatives with phytotoxic activity. Sp. Patent P200601039.

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