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Chemical Basis for the Antifeedant Activity of Natural Hydroxamic Acids and Related Compounds

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Natural hydroxamic acids and related compounds derived from the 1,4-benzoxazin-3-one structure show antifeedant activity against the aphid *Rhopalosiphum padi*. This antifeeding activity is based on the electrophilic character of the hydroxamic acid function, the opening of the hemiacetal function and the lipophilic character of the molecule. In addition, the antifeedant activity of the aqueous extracts of different tissues of *Acanthus mollis* (Acanthaceae) was determined. The activity observed is attributed to the presence of 2,4-dihydroxy-1,4-benzoxazin-3-one in the extracts.

KEYWORDS: Antifeedant activity; hydroxamic acids*; A. mollis*; *Rhopalosiphum padi*

INTRODUCTION

Secondary metabolites with toxic properties toward pests and pathogens have become part of modern pest control strategies (*1*−*3*). Cyclic hydroxamic acids are secondary metabolites that occur naturally in several species of higher plants as 2-*β*-*0*-D-glucopyranosides (*4*−*8*) particularly in cereals of great agricultural importance like maize, wheat, and rye. Cyclic hydroxamic acids have been associated with resistance to insects, fungi, and bacteria (*4*). The chemical compound 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) is the aglycone of the main hydroxamic acid present in maize and wheat, and its demethoxylated analogue (DIBOA) is found in rye. These cyclic hydroxamic acids are hydrolyzed by glucosidases released after plant tissue disruption (*9*). The concentration increases upon germination and decreases thereafter (*10*−*12*). This fact could imply that the cyclic hydroxamic acids might be responsible for the chemical resistance of young plants against the attack of pathogens. Insecticidal activity of cyclic hydroxamic acids has been studied mainly against aphids (*4*) commonly found on cereals. These insects obtain their food from phloem by penetrating the plant tissue through epidermal and mesophyll cell layers using their stylet-like mouthparts to feed on photoassimilates translocated in the phloem sieve elements (*13*).

The ability of these molecules to repel insects is related to their concentration and chemical characteristics. Electronically monitored feeding assays have shown that DIMBOA has antifeedant effects at concentrations as low as 1 mM, but when the concentration of DIMBOA reaches 12 mM, feeding is completely inhibited (*14*). On the other hand, the biological activity of cyclic hydroxamic acids has been associated with their electrophilic behavior. Electrophilicity allows the molecule to react with nucleophilic centers such as amine or thiol groups of amino acid residues present in enzymes involved in fundamental processes (*15*−*17*). The aglycones DIMBOA, DIBOA, and analogues have been found in species of the Acanthaceae (*8*). Recently, the content of DIBOA in different morphological parts of *Acanthus mollis* has been documented. The high content of DIBOA in younger leaves has been considered as a basis for natural resistance against phytophagous insects found on cereals (*18*). Other properties of the 1,4-benzoxazin-3-ones related to plant defense are allelopathic effects (*19*−*21*) and herbicide detoxification (*22*−*24*). All this information supports the inclusion of cyclic hydroxamic acids in strategies for the integrated control of plant pests. The antifeedant activity of 1,4-benzoxazin-3-ones can be rationalized on the basis of structure−activity relationships. In this work, we studied the effect of the electrophilicity and lipophilicity of DIMBOA and DIBOA isolated from cereals and of a series of synthetic cyclic derivatives against the aphid *Rhopalosiphum padi*. In addition, the antifeedant activity of aqueous extracts of *A. mollis* was related to the concentration of DIBOA.

MATERIAL AND METHODS

Chemicals. DIBOA and DIMBOA were isolated from Et_2O extracts of maize (*Zea mays* L. cv. T-128) and rye (*Secale cereale* L. cv. Tetra), respectively (*15*, *25*). DIBOA derivatives substituted at position 7 of the aromatic ring and C_2 of the heterocyclic ring and the respective lactams (1,4-benzoxazin-3(4H)-ones) have been reported elsewhere (*26*).

log P_{HPLC} Values. These values were obtained from the relationship $\log P_{HPLC} = 0.7914 \log K' + 0.1612$. $\log K'$ ($K' = t_R - t_0/t_o$) was determined by an RP-HPLC method (*27*, *28*). The mobile phase was methanol−water (pH = 3.0), 30:70 v/v with a C18 column. Thiourea was used as nonretarded compound (t_0)

Analysis of Diffusion of Hydroxamic Acids into the Leaves. A solution of hydroxamic acid 10% w/v in acetone was sprayed on the leaf surface. After 24 h the leaves were washed with acetone to eliminate the hydroxamic acid residues from the surface and macerated with water, and the hydroxamic acids present in the extract were analyzed by a previously described HPLC method (*18*).

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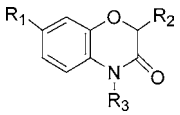
Table 1. Repellency Index (RI) of Natural Benzoxazinones and Derivatives against *Rhopalosiphum padi*



compd	R_1	R_2	R_3	RI (%)[a]
1	H	OH	OH	92.3 ± 6.0[b]
2	CH_3O	OH	OH	74.0 ± 5.0[c]
3	CH_3O	H	OH	52.0 ± 3.0[d]
4	CH_3O	CH_3O	OH	33.3 ± 3.0[e]
5	CH_3O_2C	H	OH	32.0 ± 3.0[e]
6	COOH	H	OH	2.5 ± 0.2[f]
7	H	CH_3O	OH	0.0
8	H	OH	H	38.0 ± 4.0[b]
9	CH_3O	H	H	18.0 ± 2.0[g]
10	H	H	H	17.0 ± 2.0[g]
11	H	H	OH	26.0 ± 3.0[e]
12	CH_3O	OH	H	35.0 ± 4.0[e]

[a] Each value is the mean of 20 samples ± one SE. [b–g] ANOVA showed significant differences among treatments. Same letters denote nonsignificant difference.

Feeding Choice Assay. This bioassay was used to determine the antifeedant properties of the compounds against *R. padi*. Aphids were collected from naturally infested barley and allowed to reproduce on barley plants kept under a light/dark photoperiod of 12/12 h at 20 ± 5 °C.

Each assay was done on 10 plates, with 10 individuals in each plate. Third-instar individuals were used to facilitate manipulation. Aphid feeding deterrence assays were conducted using two pieces of young barley leaf (1 cm^2) over a thin layer of agar for to diminish dehydration (hydroxamic acids are not present naturally in barley). One piece of leaf was sprayed with 10 μL of a 10% solution of the compound in acetone (w/v), and the other was sprayed only with the solvent (control). Once the solvent had evaporated, 10 individuals were distributed at random on each plate. After 24 h, the number of insects on each piece of leaf was counted. Individuals not located on the leaf pieces were not considered. A total of 20 trials were done under the same conditions. The repellency index was calculated as $RI = [1 - (T/C)] \times 100$, where T = number of individuals on the piece of leaf with compound and C = number of individuals on the piece of leaf without compound.

***A. mollis* Extracts.** Plants were cultivated for a period of 32 weeks from the end of April until November of the year 2002. Plant age was recorded from the moment of planting the shoots for rooting. Solutions used for the test were prepared by weighing 0.1 g of *A. mollis* tissues (leaves, sepals, petals, pistils, and stamens) and macerated with 2 mL of water. The aqueous extracts were left at room temperature for 1 h. This procedure has been used in the chemical quantification of DIBOA (*18*).

Statistical Analysis. We performed ANOVA and Tukey tests to evaluate the significance of differences between treatments

RESULTS AND DISCUSSION

The DIBOA and DIMBOA aglycones present two electrophilic centers: (i) a hemiacetal function at C_2, which upon opening exposes an aldehyde group; and (ii) a nitrogen atom at the hydroxamic acid function. The effect of each one on the feeding deterrency activity was evaluated by comparing the repellency index (RI) of DIBOA, DIMBOA and a series of cyclic hydroxamic acids substituted in the aromatic ring at C_2 and at the nitrogen atom. RI values after 24 h of treatment are shown in **Table 1**. DIBOA and DIMBOA were the most active compounds. DIBOA showed higher activity than did DIMBOA. Part of the activity of these aglycones could be explained by the presence of the aldehyde group, which reacts with nucleophiles such as thiols and amines that are present in biomolecules involved in essential biochemical processes (*16*, *17*). These chemical reactions could explain the weaker deterrent effects found when the opening of DIMBOA (**2**) and DIBOA (**1**) is blocked as in compounds **7** and **11**, and **3** and **4**, respectively. Similar effects were obtained against the aphid *Sitobion avenae* (*29*)

The electrophilicity of the nitrogen atom has been explained by the leaving group ability of the substituent on this atom (*30*). This suggests that derivatives with a poor negative leaving group such as the hydrogen atom should present lower activity. Probably a previous metabolic *N*-acylation step may be required to produce the electrophilic nitrogen atom in these molecules. On the basis of this assumption, we can say that when the *N-hydroxyl* group in DIBOA and DIMBOA is substituted by a hydrogen atom as in lactams 2-Hydroxy-1,4-benzoxazin-3(4H)-one (**8**) and 7-methoxy,2-hydroxy-1,4 benzoxazin-3(4H)-one (**12**), it should decrease the deterrent effect. This effect was more pronounced in the derivatives in which opening of the hemiacetal function is blocked as for lactams 7-methoxy-2H-1,4 benzoxazin-3(4H)-one (**9**) and 2H-1,4-benxozazin-3(4H)-one (**10**).

This evidence suggests that the antifeedant activity can also be controlled by the same chemical properties that are responsible for the antimicrobial activity (*26*, *31*).

An important role in this mechanism as well as in the quantitative structure−biological activity relationships is played by the lipophilicity of the bioactive molecules. The lipophilic−hydrophilic balance, which is expressed by a partition coefficient (log P) a measure of distribution behavior of a chemical in a

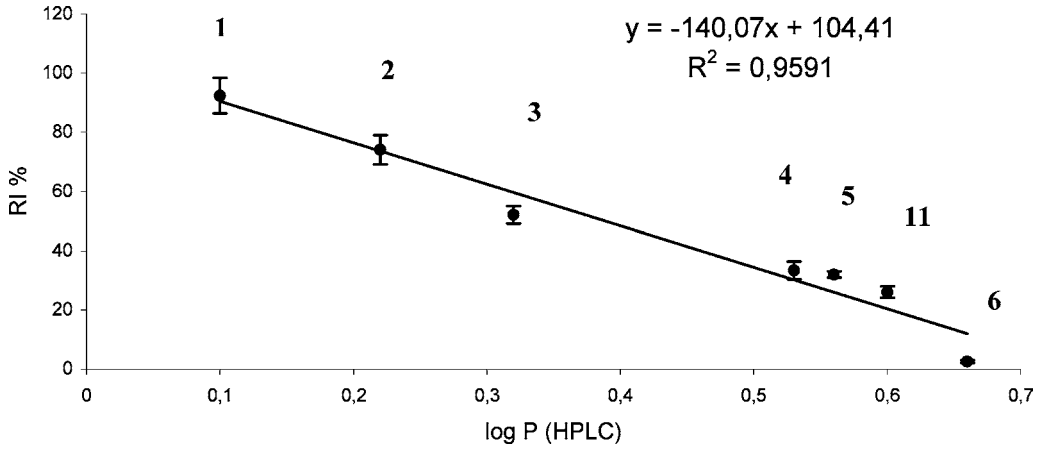


Figure 1. Structure activity relationship between log $P_{(HPLC)}$ and repellency index of hydroxamic acids. Each point represents the mean of 20 samples (±SE). The number over each point (1,2,3,4,5,6,11) corresponds to the compound indicated in **Table 1**

Table 2. DIBOA Contents in Different Extracts of *A. mollis* and Repellency Index (RI) against *Rhopalosiphum padi*

tissue	RI (%)	DIBOA[d] μmol g^{-1} f.w.
younger leaves (less than 4 weeks)	50.4 ± 4.0[a]	18.0 ± 2.10
older leaves (more than 13 weeks)	33.0 ± 3.0[b]	3.5 ± 1.10
stamens	65.4 ± 3.0[c]	56.5 ± 9.15
petals	37.8 ± 2.0[b]	13.3 ± 1.89
sepals	34.0 ± 0.9[b]	9.82 ± 0.98
pistils	82.4 ± 5.0[d]	10.3 ± 1.42

[a–c] Each value of RI is the mean of 20 samples ± one SE. Same letters denote nonsignificant difference. [d] Each value of DIBOA concentration is the mean of five samples ± one SE.

biphasic systems, is critical for the absorption and transport processes of the whole molecule to the receptor compartment. Little information about the effect of this property on the bioactivity of the 1,4-benzoxazin-3-ones has been found in the literature. Antialgal activity of 1,4-benzoxazin-3-ones with a blocked hemiacetal function increases with the lipophilic character of the aromatic ring substituent (π parameter) (26). We determined log *P* values of some cyclic hydroxamic acids using an HPLC method. All of them show low values for log *P* (<1.0). The lowest ones belong to the derivatives with a $C_{(2)}$-OH group. **Figure 1** shows the structure−activity relationships between log P_{HPLC} and the repellency index. We can conclude that feeding deterrency decreases with the lipophilic character of the hydroxamic acid.

As reported previously, the antifeedant activity of the chemicals might be mediated by the sensitive hairs present on the proboscis, antennae and legs of the aphids (*32*). This activity depends on the concentration of the compounds deposited on the contact surface and the diffusion into the leaf tissues, which are determined by lipophilicity. This might explain the decrease in repellent activity with increasing log *P* values. Another result supporting this hypothesis was obtained from the barley leaves used in the choice feeding assay with **1**, the most active compound (RI = 92.3%; log *P* = 0.10), and with the least active derivative, **7** (RI = 0%; log *P* = 0.66). The chromatographic analysis showed that only **7** was present in detectable amounts in the macerated tissues. However, further experiments will be necessary to fully clarify the role of this property in the deterrency of the hydroxamic acids

According to previous results, DIBOA is the most active natural cyclic hydroxamic acid. On the other hand, the antifeedant activity of *A. mollis* against herbivorous insects is attributed to the DIBOA content in leaves (*18*). Contents of DIBOA vary in the different parts of this plant; therefore, to clarify the relationship between the antifeedant activity of *A. mollis* and the contents of DIBOA, we used aqueous extracts of different morphological parts of *A. mollis* to evaluate the deterrency against *R. padi*. **Table 2** shows the RI of aqueous extracts of different parts of *A. mollis*. All the extracts showed deterrent effects against *R. padi*; however, the pistil extract proved to be the most active. The DIBOA content in the aqueous extracts of the different tissues was verified as described (*18*) and included in **Table 2**. A correlation between these contents and the RI values was obtained. In accordance with the results, the deterrency of the extracts increases linearly ($r^2 = 0.94$) with the content of DIBOA, with the exception of the pistil extract, which showed higher activity than predicted, probably due to the presence of other bioactive compounds. This correlation suggest that the antifeedant activity of aqueous extracts of *A. mollis* increases linearly with the content of DIBOA. In conclusion, the antifeedant activity of DIBOA in aqueous extracts continues to be shown, and this supports the hypothesis that DIBOA could also be the main antifeedant factor of *A. mollis* against phytophagous insects.

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