Chemical Basis for the Phytotoxicity of *N*-Aryl Hydroxamic Acids and Acetanilide Analogues

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Germination inhibition activity of *N*-aryl hydroxamic acids and acetanilide analogues was measured on lettuce seeds (*Lactuca sativa*). Lipophilicity of the compounds was determined by HPLC. A correlation between lipophilicity values and percentage of germination inhibition was established. A model mechanism of action for auxin was used for analyzing the effect of the substituent at the alpha carbon atom (Cα) on the polarization of hydroxamic and amide functions in relation to the germination inhibition activity observed. Results suggest that the lipophilic and acidic properties play an important role in the phytotoxicity of the compounds. A test with the microalga *Chlorella vulgaris* was used to evaluate the potential herbicide activity of the hydroxamic acids and acetanilides.

Key words: Hydroxamic Acids, Acetanilides, Phytoactivity

Introduction

Hydroxamic acids are excellent complexing agents with particularly interesting and relevant affinity for iron(III) (Crumbliss, 1990; Fernandes *et al.*, 1997), an essential element for life. This property plays an important role in iron uptake and metabolism in fungi, bacteria and plants. However, the hydroxamic acid function is a complex structure that displays other chemical characteristics such as acidity, nucleophilicity and electrophilicity. These features have also been considered to understand a wide range of biological activities of synthetic and naturally occurring hydroxamic acids. For example, cyclic hydroxamic acids (1,4-benzoxazin-3-ones, Hx) (Fig. 1) are present in several species of higher plants particularly in cereals of great agricultural importance, like maize, wheat and rye (Niemeyer, 1988; Sicker and Schuldz, 2002). Nucleophilicity and acidity of these molecules arise from the hydroxy group of the hydroxamic function. Consequently, nucleophilic substitution reactions can occur, and this mechanism has been suggested to explain the ability of Hx to detoxify herbicides (Tipton *et al.*, 1971; Raveton *et al.*, 1997). On the other hand, these molecules contain two electrophilic centres: (i) the nitrogen cation resulting from leaving of the hydroxy group; (ii) the hemiacetal function at C-2 which upon opening expresses an aldehyde group [Fig. 1, *N*-(2-hydroxy-phenyl)glyoxylohydroxamic acid (form A)]. For this reason bioactivity such as toxicity against insects, bacteria and fungi *in vitro* and resistance of cereal crops to pests and pathogens has been also related to electrophile-nucleophile interactions. These interactions arise from nucleophilic centres such as amine and thiol groups in amino acid residues present in the enzymes involved in fundamental processes (Queirolo *et al.*, 1983; Niemeyer *et al.*, 1982; Pérez and Niemeyer, 1989; Hashimoto *et al.*, 1991; Bravo *et al.*, 2001). Interesting allelophatic activities have also been reported for Hx (Sicker and Schuldz, 2002; Huang *et al.*, 2003; Burgos *et al.*, 2004; Friebe *et al.*, 1997). Phytotoxicity in plants could have some of the following origins: toxicity on the radicle growth of monocotyledonous plants, inhibitory effect on the energy metabolism of chloroplasts and mitochondria and/or modification of the binding affinity of the receptor sites of membranes. The role of the chemical properties of the hydroxamic function in phytotoxicity has not been reported. To gain a deeper understanding of the phytotoxicity of hydroxamic acids, we studied the potential herbicidal activity of a model series of *N*-(2-hydroxy-phenyl)glyoxylohydroxamic acid (form A), *N*-aryl hydroxamic acids and acetanilide analogues (Fig. 1). The role

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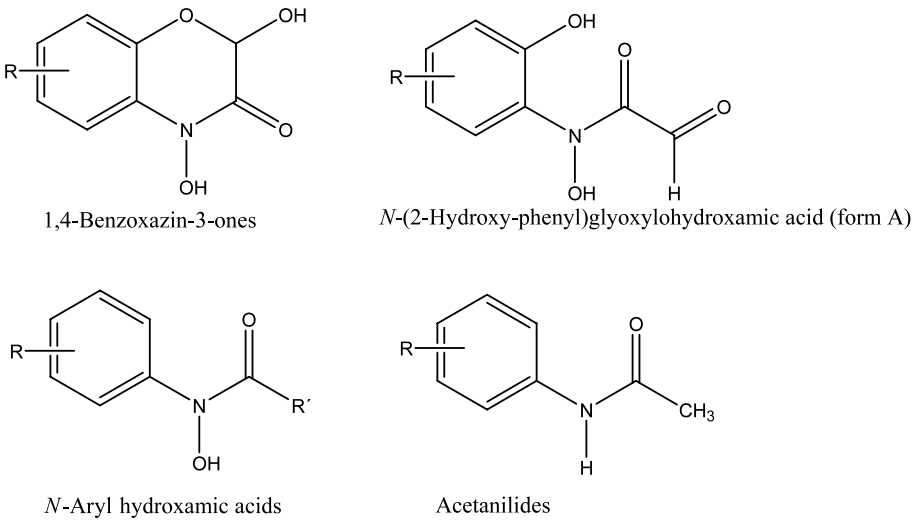


Fig. 1. Structures of 1,4-benzoxazin-3-ones, *N*-aryl hydroxamic acids and acetanilides.

of molecular lipophilicity, of the hydroxy group at the nitrogen atom and the effect of the substituent at the alpha carbon atom were analyzed from the standpoint of a structure-phytotoxicity relationship against lettuce seed (*Lactuca sativa)* and the alga *Chlorella vulgaris,* one of the most commonly used species in microalga toxicity testing (Rioboo *et al.*, 2002)

Experimental

Syntheses

The *N*-aryl hydroxamic acids and acetanilides were synthesized as previously described (Brink and Crumbliss, 1982; Hibbert *et al.*, 1998). Products were purified by preparative LC (benzene/diethyl ether, 1:1 v/v) to achieve sufficiently high purity levels for biological assays.

Log P_{HPLC} values

Lipophilicities (log P_{HPLC}) for the *N*-aryl hydroxamic acids and acetanilides were obtained from the relationship: log P_{HPLC} = 0.7914 log K′ + 0.1612 log K′ with K′ = $(t_R - t_M)/t_M$, where t_R is the retention time of the analyse compounds and t_M is the retention time of the unretarded compound (thiourea), determined by RP-HPLC on a C_{18} column (Hollosy *et al.*, 2002; Minick *et al.*, 1988). The mobile phase was methanol/water (pH 3.0), 30:70 v/v.

Germination assays

45 lettuce seeds (*Lactuca sativa)* were uniformly placed on Petri dishes covered with cotton. In order to maintain individual compound concentrations, each plate was watered with 8 mL of an aqueous solution of 500, 250 or 100 μg/mL of each compound. Then, the plates were sealed and incubated at (25 ± 2) °C in an 8 h:16 h light:dark cycle for 7 d. Controls were incubated only with water. Each assay was replicated three times. Germination inhibition (I%) was expressed as percentage of the control.

Antialgal test

Test compounds were dissolved in nutrient growth media (Gibco) with the aid of either ultrasound or gentle heating. *Chlorella vulgaris* (Laboratory of Microbiology, Faculty of Science, University of Chile, Santiago, Chile) was ground in nutrient growth medium. Samples were incubated at 25 °C for 10 d in test tubes containing $4.0 \cdot 10^4$ colony forming units (CFU) under continuous cold white fluorescent light with an intensity of 200 ft-c. The growth of *C. vulgaris* was assessed by turbidity measured spectrophotometrically at 600 nm.

Percentage inhibition was obtained according to: $I\% = \frac{100\ (Ts - Tc)}{100 - Tc}$, where Ts is the sample transmittance and Tc the control transmittance.

Results and Discussion

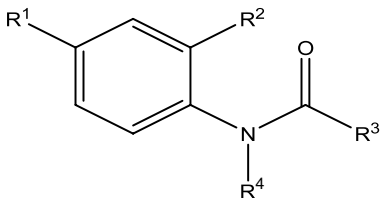
Table I shows a structure-germination inhibition activity relationship for *N*-aryl hydroxamic acids and acetanilide analogues. In the concentration range studied (100–500 μg/mL), germination inhibition (I%) of lettuce seeds of 90–100% was found at the highest concentrations of both groups of compounds, with the exception of **10**.

These results suggest that the hydroxy group at the nitrogen atom does not determine germination inhibition. In the lower dose range the percentages are more diverse and allow the evaluation of structural effects. At 100 μg mL^{-1}, compounds substituted by chlorine at the alpha carbon atom displayed germination inhibition between 65–96% (except for compound **8**) and the non-chlorinated compounds between 3.4–40%. Compounds substituted in the *p*-position of the aromatic ring showed the lowest activities (compounds **5**, **10**, **11**). Therefore, the halogen substituent at the alpha carbon atom could play an important role in the germination inhibition activity.

The lipophilicity of bioactive molecules is important to establish quantitative structure-biological activity relationships. The lipophilic-hydrophilic balance which is expressed by the partition coefficient (log P) is a measure of the distribution behaviour of a chemical in a biphasic system, and is critical for the absorption and transport processes of the whole molecule to the receptor compartment. Thus, we have determined log P values using a HPLC method (Hollosy *et al.*, 2002; Minick *et al.*, 1988). Table I also displays log P_{HPLC} values for the hydroxamic acids and acetanilides. All of them show low log P values (≤ 1.0). In general, the lowest ones correspond to the compounds not substituted at the alpha carbon atom (**1**, **6**, **10**). Other insights into the role of lipophilicity on the germination inhibition activity were obtained from relationships between log P_{HPLC} values and I% for the *p*-unsubstituted compounds. As shown in Fig. 2, the activity increased linearly with increasing lipophilic character of the molecules. The monohalogen derivatives displayed an activity higher than predicted (**2** and **7**).

Hansch *et al.* (1963) proposed a theory to rationalize the relations between chemical structure and biological activity of auxins. The hypothesis assumes that auxins with an aromatic ring and a side chain react with a plant substrate *via* two points,

Table I. Percentage of germination inhibition (I%) of lettuce seeds after seven days and log P_{HPLC} values.



Compound	R^1	R^2	R^3	R^4	I% [mM]			log P_{HPLC}
					100 μg/mL	250 μg/mL	500 μg/mL	
1	H	H	CH_3	OH	40.0 (0.7)	97.3 (1.6)	100 (3.3)	0.48
2	H	H	CH_2Cl	OH	96.2 (0.5)	100 (1.3)	100 (2.7)	0.70
3	H	H	$CHCl_2$	OH	65.0 (0.4)	97.0 (1.1)	100 (2.3)	0.88
4	H	H	CCl_3	OH	87.5 (0.4)	100 (1.0)	100 (2.0)	0.86
5	CN	H	CH_3	OH	9.10 (0.6)	85.2 (1.4)	100 (2.8)	Nd
6	H	OH	CH_3	H	26.5 (0.7)	37.9 (1.6)	100 (3.3)	0.38
7	H	OH	CH_2Cl	H	91.7 (0.5)	100 (1.3)	100 (2.7)	0.68
8	H	OH	$CHCl_2$	H	19.3 (0.5)	84.1 (1.1)	98.9 (2.3)	0.95
9	H	OH	CCl_3	H	84.0 (0.4)	100 (1.0)	100 (1.9)	1.01
10	OH	H	CH_3	H	13.1 (0.7)	22.6 (1.6)	38.1 (3.3)	−0.05
11	CH_3	OH	CH_3	H	3.40 (0.6)	44.8 (1.5)	91.9 (3.2)	0.73

Nd, not detected.
Values in parenthesis show mM concentration. Each value corresponds to the mean of three samples; replicate values show errors below 5% in all cases.

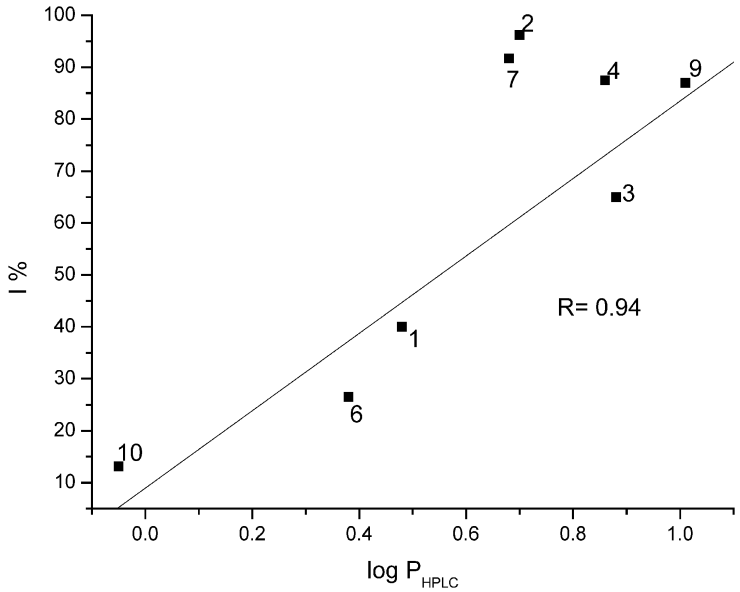


Fig. 2. Relationship between log P_{HPLC} and percentage of germination inhibition of lettuce seeds (I%) for *N*-aryl hydroxamic acids and acetanilide analogues. The number at each point (1, 2, 3, 4, 6, 7, 9, 10) corresponds to the compounds indicated in Table I.

one on the side chain and the other one on the aromatic ring. When the auxins act to initiate the growth process, the mechanism of phytotoxicity has three critical steps:

$$\text{auxin in solution} \xrightarrow[\text{step 1}]{k_1} \text{site of action} \xrightarrow[\text{step 2}]{k_2} \text{one point attachment}$$

$$\xrightarrow[\text{step 3}]{k_3} \text{two point attachment.}$$

A critical step should be the movement of the compounds from solution to the action sites (step 1). The partition coefficient is essential in the penetration rate. The correlations observed agree with this model. Thus, the effect of the lipophilic character has an important role in the penetration of the compounds into seeds.

To understand the mechanism of action at the side chain attachment point (steps 2 and 3) it is necessary to consider the polarization of hydroxamic and amide functions owing to the delocalization of the unshared electrons of the nitrogen atom towards the oxygen atom of the carbonyl group. This effect is favoured by electron-accepting substituents at the alpha carbon atom.

The dissociation constant values for the ionization of N–H bond in acetanilide and the O–H bond in the hydroxamic acids can be useful to understand the effect described above. pK_a values for acetanilide were previously reported (Pollack and Bender, 1979; Struffer, 1972; Bravo *et al.*, 2003). The α-trifluoro derivatives were 10^4–10^5 times more acidic than the methyl derivatives. We have determined spectrophotometrically the pK_a values for the hydroxamic acids **1**–**5**: **1**, 8.3; **2**, 7.7; **3**, 5.8; **4**, 5.6; **5**, 8. 2. It can be seen that chloro derivatives were 10^2–10^3 times more acidic than the unsubstituted compound **1**. Our series does not allow to evaluate the effect of the aromatic substituent on the acidic properties from the structure-reactivity relationship.

However, the results strongly suggest that the electron-accepting effect of the substituent at the alpha carbon atom also plays an important role in the polarization and acidic properties of the *N*-aryl hydroxamic acids. On the other hand, halogenated derivatives also showed the highest percentage of germination inhibition of lettuce seeds and the methylated derivatives displayed the lowest (Table I). These results suggest that the ionization of the N–H and O–H bond might be an important feature in the molecular mode of action of the side chain.

Table II. Percentage of *in vitro* growth inhibition (I%) of the microalga *Chlorella vulgaris* by *N*-aryl hydroxamic acids and acetanilide analogues. For the structure of the compounds see Table I.

Compound	R^1	R^2	R^3	R^4	I% [mM]		
					100 μg/mL	250 μg/mL	500 μg/mL
1	H	H	CH_3	OH	0.0 (0.7)	55.0 (1.6)	85.5 (3.3)
12	Cl	H	CH_3	OH	2.3 (0.5)	95.0 (1.3)	95.5 (2.7)
13	Br	H	CH_3	OH	1.3 (0.4)	96.0 (1.1)	96.7 (2.2)
14	CH_3	H	CH_3	OH	2.9 (0.60	29.4 (1.5)	100 (3.0)
3	H	H	$CHCl_2$	OH	28.1 (0.5)	86.4 (1.1)	100 (2.3)
7	H	OH	CH_2Cl	H	49.1 (0.5)	100 (1.3)	100 (2.7)
15	H	OH	CH_2Br	H	14.1 (0.4)	75.1 (1.1)	96.0 (2.2)
9	H	OH	CCl_3	H	2.7 (0.4)	97.2 (1.0)	93.3 (1.9)

Values in parenthesis show mM concentration. Each value corresponds to the mean of three samples; replicate values show errors below 5% in all cases.

Microalgae respond rapidly to environmental changes owing to their short generation time. Green microalgae such as *Chlorella* are taxonomically classified as plants bearing some similarity to higher plants. For this reason, microalgae tests may be used to evaluate the herbicidal activity against higher plants. Phytotoxicity of some *N*-aryl hydroxamic acids and acetanilide analogues were measured against the fresh water green alga *Chlorella vulgaris*. Percentages of growth inhibition are shown in Table II. All compounds tested displayed toxic effects against *C. vulgaris* closely related to their effects on the inhibition of germination of lettuce seeds. Growth inhibition of about of 90% at the highest concentration (500 μg mL^{-1}) was observed. In general, halogenated derivatives displayed the highest activity at all concentrations and the methylated derivatives showed the lowest one. Therefore, the results suggest that the *C. vulgaris* test should be as useful laboratory assay to predict the potential herbicidal properties of *N*-aryl hydroxamic acids and acetanilides.

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