A Structure-Activity Relationship Study on a Natural Germination Inhibitor, 2-Methoxy-4-vinylphenol (MVP), in Wheat Seeds to Evaluate its Mode of Action

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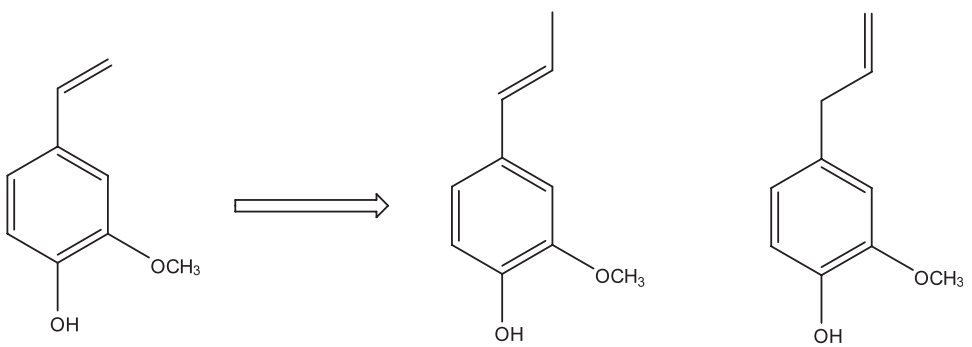
The first aim of the present study was to evaluate which structural elements of the 2-methoxy-4-vinylphenol (MVP) molecule (**1**) are responsible for its observed activity as germination inhibitor in wheat seeds. To find its mode of action, a series of compounds with varying functional moieties and substitution patterns were prepared and evaluated for their inhibitory activity. This systematic competitive inhibition study characterized two criteria for the effective increase of the inhibiting ability: (i) *ortho* substitution to each of the hydroxy and methoxy groups; (ii) alkene moiety on the ring. Understanding how the structure of natural compounds relates to their inhibition function is fundamentally important and may help to facilitate their application as novel inhibitors to restrain preharvest sprouting (PHS) in wheat fields. In this regard, in MVP and its natural analogues **8** and **9** as the most active inhibitors, the *ortho* substitution of hydroxy and methoxy groups plays a key role in their activity and, as well, the alkene moiety influences the activity significantly.

Key words: 2-Methoxy-4-vinylphenol (MVP), Germination Inhibitor, Structure-Activity Relationship

Introduction

Preharvest sprouting (PHS) of wheat can be a real agricultural disaster. It results from a weak dormancy of the grains and weather conditions favourable to germination at the time of seed maturation (Black, 1992; Busk *et al*., 1999; Mares, 1998; Walker-Simmons 1987; Weinder, 1987). Several natural compounds have been introduced which impose an exogenous dormancy to wheat seeds and, therefore, improve the resistance of seeds to PHS (Alizadeh *et al*., 2002; Gatford *et al*., 2002; Kato *et al*., 1973, 1977,1978, 2002, 2003; Lin *et al*., 1995; Mitchell and Tolbert, 1968; Stevens and Merril, 1980; Yanagawa *et al.*, 1972). One of them is 2-methoxy-4-vinylphenol (MVP, **1**); its mode of action for inhibiting binding with the wheat receptor has not been reported yet (Kato *et al*., 2002).

The objective of this study therefore was to evaluate which structural elements of the inhibitor **1** are important for its observed inhibition property. In a program of design and development of



1 **8** **9**

Fig. 1. Structures of the germination inhibitors MVP (**1**), iso-eugenol (**8**), and eugenol (**9**).

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potential inhibitors, as shown in Scheme 1, considering that the seed would be sensitive to the nature of the functional groups and their arrangement on the phenyl ring of **1**, we considered two factors: (a) the number and diversity of active structures from which critical structural elements are deduced, and (b) convincing evidence that the observed activity is due to a common mode of action. This situation provides an opportunity to build a discovery program focused on developing new inhibitors and on elucidating their mode of action.

Herein, we wish to report our discovery program in order to explore molecular information on the possible site and mode of action of **1** and, as well, to develop very potent analogue(s) by a proper structure optimization (Fig. 1). This principle has emerged from a comparative study including 15 different compounds with varying function moieties and substitution patterns.

Materials and Methods

General experimental procedures

The structure and purity of all compounds were supported by gas chromatography-mass spectrometry (GC-MS) analyses. GC-MS analyses were carried out on a Fisons 8000 gas chromatograph fitted with a fused CP-sil 5 CB column (30 m × 0.25 mm I. D., 0.4 μm film thickness), coupled to a Trio 1000 mass detector. Column temperature was programmed from 80 to 280 °C at 10 °C/min. Injection was performed at 220 °C. Helium was used as carrier gas (20 ml/min). Mass spectra were recorded in the scan mode at 70 eV; 1 μl of sample in CH_2Cl_2 was injected in the split injection and the pressure was 10 Psi.

The germination assay was performed in an incubator at 20 °C under continuous lighting with a 15 W fluorescent lamp. Water for the assay was prepared by distillation and deionization. All chemicals used for the preparation of MVP (**1**) were commercially available. Compounds **4**–**6**, **8**, **9**, **14** and **15** are commercially available.

2-Methoxy-4-vinylphenol (**1**) was prepared in three steps starting from vanillin according to the original report with a few modifications (Kato *et al.*, 2002). Compounds **3** and **10** (Ault, 1998), **7** and **13** (Siegel, 1991), **11** (Moher *et al.*, 1992) and **12** (Corey *et al.*, 1978) were prepared based on the literature.

Plant material

One variety of non-dormant sprouting-susceptible wheat cultivar, *Shiroodi,* was obtained from Golestan County in Iran. It was harvested in Golestan fields in June 2005.

Germination and growth assay

Standard germination assays were conducted in 3 replicates with the average deviations being less than ±10% in each experiment. For each assay, 20 hand-threshed seeds (grains) collected by random sampling were placed with the embryo side up in a glass Petri dish (Ø 9 cm, 1.2 cm depth) and in each Petri dish 7 ml of test solution were added. The test solution contained the sample in distilled water and a control experiment was conducted by inserting seeds in distilled water. Then the dishes were transmitted to a transparent chamber, the bottom of which was covered with a sheet of wet filter paper. Incubation was at 20 °C for 4 d. Results were evaluated in comparison with the control experiment. The germinated seeds (2–3 mm root and hypocotyls lengths) were counted daily for 4 d and germination assays were stopped when no new seed germinated for consecutive 5 d. Furthermore, the germination percentage as germination index (GI) and growth ratio (%) were calculated according to

$$\textit{germination percentage}\ (\%) = (N/S) \times 100, \quad (1)$$

where N is the total number of germinated seeds and S the total number of seeds in each Petri dish (Liu *et al.*, 2005), and

$$\textit{growth ratio}\ (\%) = E_i/E_{\text{control}} \times 100, \quad (2)$$

where E_i is the mean elongation of roots and sprouts of culture for compound i on the 4$^{\text{th}}$ day and E_{control} is the mean elongation of roots and sprouts of the control experiment on the 4$^{\text{th}}$ day.

Tetrazolium chloride (TZ) test

All unsprouted seeds in solutions were tested for viability using a tetrazolium chloride stain. Seeds were incubated in 1% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) which reduces the colourless tetrazolium salt into tri-phenyl formazon. Formazon stains living cells (respiring) with a red colour while dead cells (not respiring) remain colourless. Highly viable seeds were uniformly stained red (Liu *et al.*, 2005). We applied such a high concentration to make the seeds completely

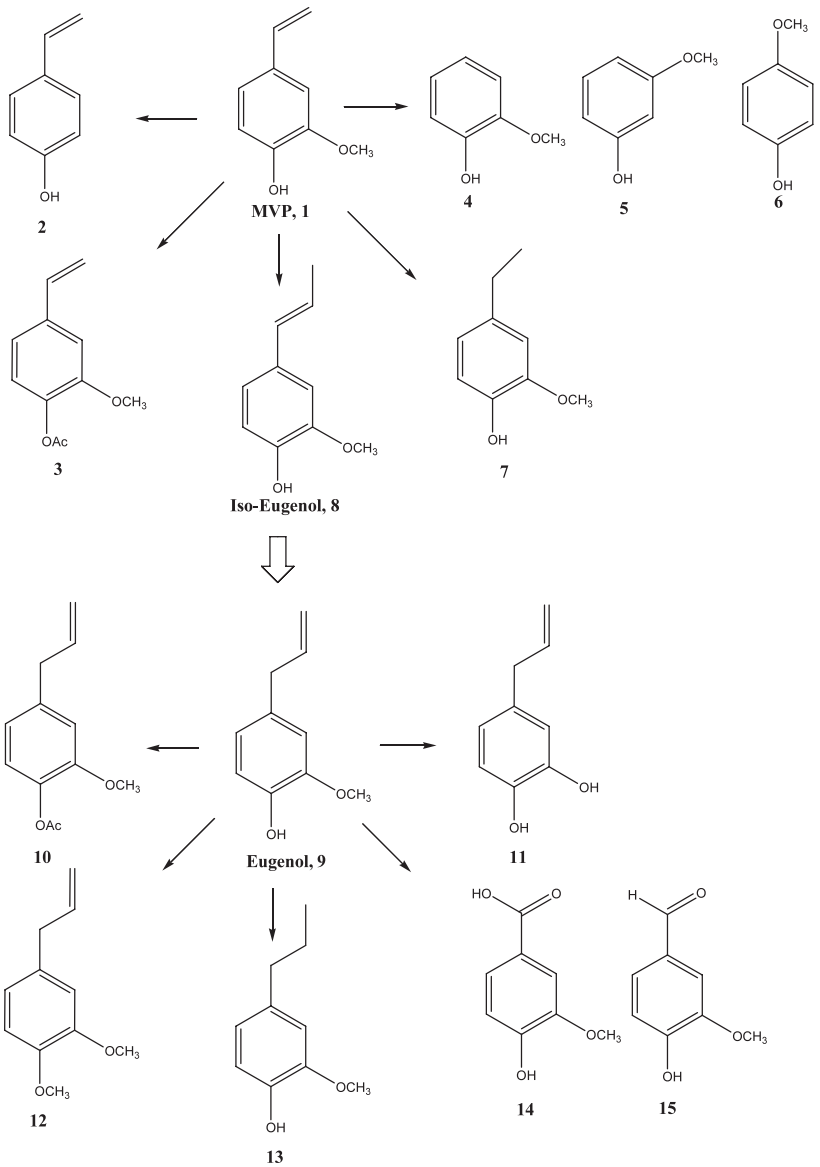
dead so that the sprouting inhibition was caused by death of the seeds.

Results and Discussion

The mode of action of MVP as germination inhibitor in wheat seeds has not been elucidated. A library of 15 compounds containing a wide range of functional groups with different electronic properties and patterns have been tested. In order to develop a quantitative structure-activity relationship, the compounds were arranged and studied within two steps of evaluation, as shown in Scheme 1.

Germination inhibition

Although the inhibitory activity of **1** on non-dormant grains of *Shiroodi* at 1000 ppm is 100%, it was found that its activity at 400 ppm is *ca.* 60% after 4 days. Therefore, to gain insight into the inhibitory action of all studied chemicals, the germi-



Scheme 1. Quantitative structure-activity relationship of **1**.

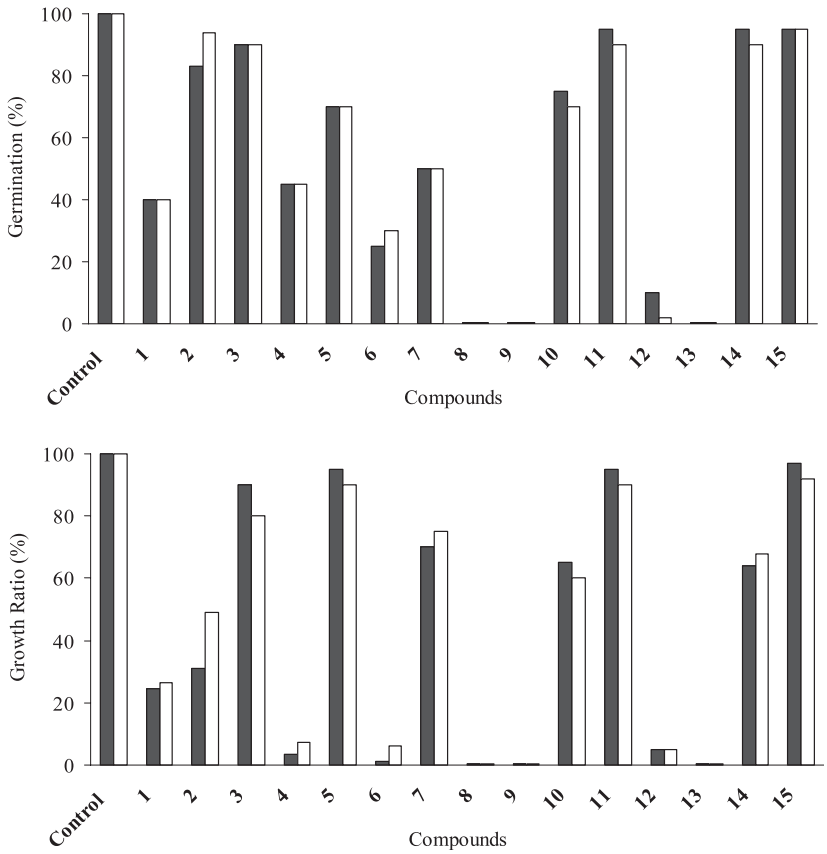


Fig. 2. Structure-activity relationship of compounds **1–15** in the viewpoint of germination index (up) and growth ratio (below) on *Shiroodi* cultivar at 400 ppm after 4 days (sprout □, root ■).

nation inhibition experiments were carried out in 400 ppm aqueous solution.

In the first step of inhibition studies, the relative activity of MVP (**1**) and its seven direct structural analogues **2**–**8** was determined. The structural analogues had the MVP side groups, hydroxy (OH), methoxy (OCH_3), and alkene (C=C), bound to the basic benzene ring either singularly or in combinations.

A schematic summary of the structure-activity relationships for the germination and growth inhibition of compounds **1**–**8** is shown in Fig. 2. In the first comparison, a big difference in the inhibition activity of compounds **1** and **3** was observed. The free OH group establishes more favourable interaction with the active site of the receptor in the wheat seed. Analysis of isomers of the methoxyphenols **4**–**6** suggested that the methoxy group can influence the inhibitory activity. The activity is in decreasing order as follows: **6** > **4** >> **5**. The results of **5** and **6** are not characteristic because in the assay the seed and solution were deeply coloured, may be due to another type of interaction with seeds. As a further support, the removal of the methoxy group from MVP (**1**) gave 4-hydroxystyrene (**2**), resulting in a remarkable reduction in activity (Kato *et al.*, 2002).

The effect of the vinyl group on the activity of MVP is proved when compared to **7**. In addition, the natural compound iso-eugenol (**8**) is a very good candidate to support this effect because it differs only in the alkene group, ($CH{=}CH_2$) in **1** or ($CH{=}CHCH_3$) in **8**. As shown in Fig. 2, **8** has a remarkable higher activity effect than **1**. This big difference is possibly due to the instability of **1** which is apt to polymerize. In contrast, **8** is quite stable in aqueous solution and its higher activity compared to compounds **1**–**7** reflects that the structure relates to the inhibition function.

This data revealed that, in addition to the *ortho* arrangement of hydroxy and methoxy substitutions on the ring, an alkene group in conjugation with the benzene ring plays a critical role on the activity of the inhibitor. However, the question is posed whether the alkene effect is due to its electronic effect on the ring or not. In this regard, the structure of the natural compound eugenol (**9**) is very similar to **8** and differs only in the alkene group, ($CH{=}CHCH_3$) in **8** or ($CH_2CH{=}CH_2$) in **9**.

Therefore the second step of our studies was based on the structure of **9** and the related compounds **10**–**15** (Fig. 1). Such a comparative study on **8** is impossible because it is a mixture of *E*- and *Z*-isomers (in this study *E*:*Z* = 87:13). The similar activities of **8** and **9** suggest that this kind of alkene group is not definite towards inhibition activity, at least at 400 ppm. Furthermore, a comparison among compounds **9**, **11** and **12** revealed that their inhibitory activities are in decreasing order as follows: **9** > **12** >> **11**.

It is of importance to clarify whether the complete inhibition of germination of the tested seeds was caused by fatal damage due to the supposed phytotoxic nature of eugenol (**9**) or not. For studying the viability of seeds, seeds of the eugenol solution with various concentrations were treated with TZ solution. The results indicated clearly that the tested grains are fully alive but sleeping under the examined condition which is deeply related to the concentration of each test solution.

Structure-activity relationship

An analysis closely related to structure-activity among the tested compounds was adapted to evidence feasible effects because of functional groups and substitution patterns. Since the benzene ring has a low flexibility and a high lipophilicity, it is beneficial for affinity because the delocalized electron cloud of the unsaturated benzene ring allows the hydrogen of the hydroxy group to act in an acidic manner.

This systematic inhibition study characterized the critical role of a hydroxy and a methoxy substituent on the ring in *ortho* position. Together this is a selective group and the potent inhibitor provides structural rigidity in the sense of a locked orientation. To find the best type of *ortho* substituents as a binding pocket with receptor in seeds, a comparison among compounds **9**, **11** and **12** was performed. It was found that replacement of the methoxy group in **9** by a hydroxy group in **11** or of the hydroxy group in **9** with a methoxy group in **12** resulted in decreased activity.

An obvious extension of this study is that the affinity-enhancing effects of each substituent are additive. For example, the addition of any type of alkene moiety to a compound (*e. g.*, **4** to **1** or **8** or **9** in Fig. 2) resulted in a significant improvement in activity or the removal of the one group from the inhibitor (*e. g.*, **1** to **2**) resulted in a remarkable reduction in activity.

To prove the structurally important role of the alkene moiety, compounds **7** and **13** having an alkyl moiety were prepared. Generally, as shown in Figs. 2 and 3, replacement of an alkene group in **1**, **8** and **9** by an alkyl group in **7** and **13** resulted in decreased activity. To complete the best structural requirements, the most powerful inhibitors **8**, **9** and **13** were assayed in lower content (200 ppm)

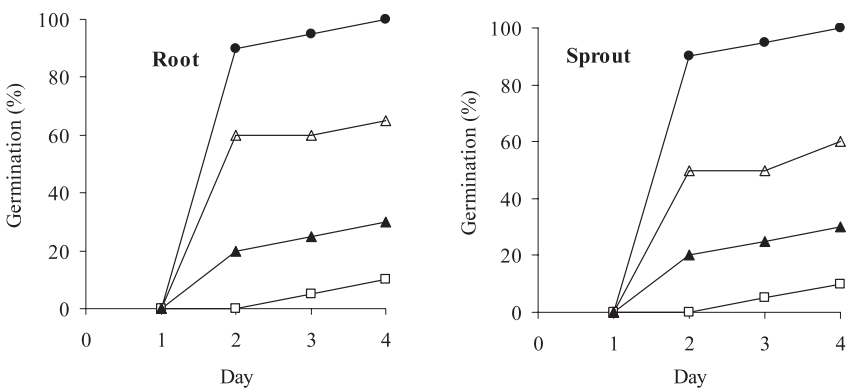


Fig. 3. Inhibitory effects of iso-eugenol (**8**) (□), eugenol (**9**) (▲), and **13** (Δ) at 200 ppm, and control (●) toward germination of wheat seed.

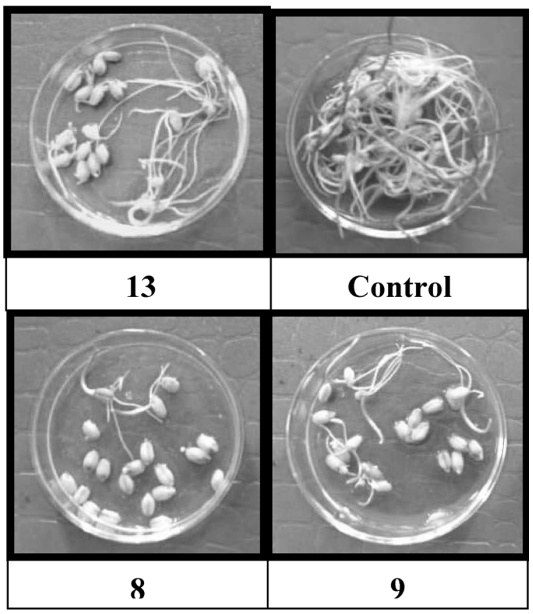


Fig. 4. Inhibitory assay of MVP analogues after 4 days.

because the inhibition activity is related to both power and number of binding sites of the inhibitor on the receptor. As shown in Figs. 3 and 4, this assay proved a more critical role of the conjugated alkene over an allyl moiety in the structure of the inhibitor.

Fashion of binding

This systematic competitive inhibition study characterized the key bifunctional inhibitors possessing two active sites: (i) *ortho* substitution of hydroxy and methoxy groups on the ring; (ii) alkene moiety on the ring. The physiological mechanism of MVP (**1**) and its analogues **8** and **9** on the inhibition is not still understood. It is known that enzyme α-amylase is essential for seed germination by making starch degradation in the endosperm of seeds (Perata *et al.*, 1992, 1997). It might be possible that MVP (**1**) and its analogues **8** and **9** inhibit the germination owing to the inhibition of α-amylase activity in the seeds. Possibly the receptor has a complementary carboxlic residue able to accept this hydrogen bond at a favourable distance to the hydroxy group. This fashion of binding to the receptor is supported by assaying of vanilic acid (**14**) and vanillin (**15**), because different binding modes may account for the different fashion of inhibition of the compounds. Therefore, as shown in Fig. 2, MVP (**1**) and its analogues **8** and **9** exhibited a greater activity compared to **14** and **15** showing that their fashion of binding to the receptor is different. It is noteworthy that **9** has exhibited greater antimicrobial activity compared to **15** (Boonchird and Flegel, 1982). These two compounds are structurally similar and differ only in one functional group, aldehyde (–CHO) in **15** or allyl ($-CH_2CH=CH_2$) in **9**.

In general, the increased activity of **9** over **15** is either due to the greater hydrophobicity of the former or due to the preferential association of the carbonyl groups, instead of the hydroxy moiety, to the hydrophilic site of the receptor, not resulting in formation of the association suitable to inhibit germination.

In conclusion, this study was designed to provide a better understanding of the structure-function relationships of the different substituent groups within the MVP molecule (**1**). In this regard, compounds **8** and **9** were discovered as the most active inhibitors, even better than of MVP. In general, the *ortho* substitution of hydroxy and methoxy groups plays a key role in their activity and the alkene moiety influences the activity significantly.

These relationships may be used to optimize the substituents further and constitute a base for future designed libraries in which all positions will be varied at the same time in order to reveal interaction effects between the different substituents.

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