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Functional Structure/Activity Relationships

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Phenylacetates as Antifeedants for the Pine Weevil, *Hylobius abietis,* Comparison with Benzoates and Phenylpropanoates

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Abstract –

This study concludes an extensive investigation of antifeedants for the pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae), an economically important pest of planted conifer seedlings. Building on previously reported antifeedant effects of benzoates and phenylpropanoids (aromatic compounds with one or three carbon atom substituents on the benzene ring) we here report the antifeedant effect of compounds with a two-carbon atom side chain (i.e. phenylacetates). We also present new results where the best antifeedants from the benzoate class were tested at tenfold lower concentrations in order to find the optimal antifeedants. Generally, for all three compound classes, efficient antifeedants were found to have one or two methyl, chloro or methoxy substituents on the aromatic ring. For monosubstituted phenylpropanoids the substituent preferably should be in the *para*-position. In search for synergistic antifeedant effects between the three compound classes, combinations of compounds from the three classes were tested in binary and ternary mixtures.

Key Words - Pine weevil, *Hylobius abietis,* synergism, conifer seedling protection, feeding deterrent, structure-activity relationships.

INTRODUCTION

Killing of planted conifer seedlings by feeding on the bark by the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) is a severe problem for the forestry industry in large parts of Europe.[1] If no countermeasures are taken, seedlings frequently suffer more than 80% mortality in areas with high population levels.[2-3] The likelihood of pine weevil attack can, however, be considerably reduced by various silvicultural practices,[4-6] such as soil scarification providing planting spots of mineral soil, which the weevils avoid.[7-8] Moreover, it is often necessary to protect the seedlings in the nursery, by insecticide applications or more recently, by a coating that physically protects the stem.[9-10] The use of insecticide treated seedlings poses health risks for forestry workers,[11] and to achieve the goal to completely abandon insecticides for seedling protection,[10] the need for new alternative methods remains high.[12] Furthermore, to ensure high seedling survival the protective effect needs to last for two seasons.[5]

An alternative to traditional insecticides is to apply antifeedant compounds. The strategy of utilizing compounds that deter feeding by specific pest insects without the intrinsic toxicity of pesticides has been applied for several decades across many systems. The early work on this approach has been reviewed by Jermy,[13] while the more recent advances in antifeedants and related repellents have been reviewed Deletre *et al*.[14] Lately, discoveries of antifeedant compounds derived from many plants, such as *Ginkgo biloba* against *Hyphantria cunea* (Lepidoptera: Arctiidae) larvae[15] and *Ajuga chamaepitys* extract against *Tuta absoluta* (Lepidoptera:

Gelechiidae),[16] to mention a few, have been reported. Individual compounds have been proven to be efficient antifeedants in several systems, for example cinnamaldehyde against the elm pest *Ambrostoma quadriimpressum* (Coleoptera: Chrysomelidae).[17] Extracts and individual compounds derived from various non-host plants have been shown to have antifeedant effects also for *H. abietis* or closely related species,[18-24] although these findings have not yet led to any practical use. Furthermore, several volatiles produced by bacteria and fungi associated with *H. abietis* have antifeedant properties or reduce the attraction of the weevils to host odors.[25-28]

In *H. abietis*, antifeedant compounds have specifically been found to be deposited during ovipositioning.[29] The eggs are laid at roots of recently dead conifer trees, where pine weevils also feed to a large extent (in addition to their feeding on seedlings).[30-31] In the oviposition process, feces are added to the eggs, and it is known that female feces possess antifeedant properties.[29] Bioassay guided fractionation of feces revealed aromatic compounds with low molecular weight as the main substituents of the most active fraction, and subsequently benzoate esters and phenylpropanoids with strong antifeedant activity were isolated.[29, 32] Chemically related antifeedants (ethyl cinnamate and ethyl 2,3-dibromo-3-phenylpropanoate) were also isolated from bark of lodgepole pine (*Pinus contorta* Douglas ex Loudon).[33]

Several synthetic analogues of the isolated active compounds from *H. abietis* feces and *P. contorta* bark have been synthesized and tested for antifeedant activity.[29, 32] Furthermore, active phenylpropanoids were derived from the lead compounds in

P. contorta[34-36] and benzoate ester analogues were prepared from leads in feces.[37] By making systematic variations in the structures, some correlations between structure and weevil response were revealed. Several structurally related compounds were found to be active in laboratory antifeedant bioassays.[34-35, 37-38] During these previous studies, a small number of phenylacetates were also tested.[37] The results from these preliminary trials showed great promise, which encouraged us to explore the antifeedant activities of this substance class more broadly in this current study, where the substituents on the aromatic ring were modified based on our results from our work with phenylpropanoids and benzoates. The overall aim was to identify new substances useful for practical conifer seedling protection. We synthesized seven new substituted phenylacetates and subsequently tested and evaluated these compounds as well as four acetate esters included in previous studies and two of the parent phenylacetic acids. The antifeedant activity of these compounds was compared with previously tested benzoates and phenylpropanoates. Based on our negative experiences with higher benzoates[37-38] and phenylpropanoids,[34-35] we limited our investigation to methyl esters. Additionally, for the first time in the series of investigations of *H. abietis* antifeedants, we include a test for synergistic effects of binary or ternary blends of compounds from all three substance classes of benzoate, acetate and propanoate esters: Three compounds with high antifeedant efficiency were selected and applied in a matrix on stems of coniferous seedlings.

MATERIALS AND METHODS

Collection and Maintenance of H. abietis. Both sexes of *H. abietis* were collected during spring migration at a sawmill in central Sweden (where they landed in large numbers in response to massive emissions of attractive conifer volatiles). The weevils were then stored in darkness at 10 °C and provided with fresh Scots pine (*Pinus sylvestris* L.) stems with tender bark as food. These storage conditions interrupted their reproductive development, so that females did not begin to oviposit until about a week after they had been transferred to the experimental conditions (a light regime of L18: D6 at 22 °C). This transfer was made at least 10 d before the weevils were used in bioassays.

Feeding Bioassays. All compounds in Figure 1and 2 were tested for their antifeedant effect on *H. abietis* by using a two-choice laboratory bioassay.[34] Fresh pieces of Scots pine twigs (50 mm long, 15 mm diam., and taken from one individual tree) were split, and each half (=test twig) was wrapped in aluminum foil. In each test twig, two sharp-edged metal rings (5 mm diam.) were punched 25 mm apart through the foil and into the bark. The rings and the pieces of aluminum foil inside them were then removed. The thin outer layers of corky bark inside the two circular areas on the surface of the twig were also carefully removed with a scalpel. Thereafter, new rings were fitted into the bark around the two exposed areas. Next, 100 μL of solvent (methanol or methyl acetate) with a concentration of 5 or 50 mM of the test compound was applied on the bark in one of the two rings. In the other ring, 100 μL of pure methanol or methyl acetate was added for control (so that solely the effect of the investigated compound was measured). When the solvent enclosed by the metal rings had evaporated and/or absorbed into the wood, the metal rings were removed (Figure 3). Each test twig was placed on moistened filter paper in a 142-mm-diam. Petri dish, with one weevil in each dish for 24 h. The assay was replicated 20 times for females and 20 times for males. Each weevil was used only once. The weevils were all in the reproductive phase of their life cycle and were starved for 24 h before the test period. The bioassays were conducted under a light regime of L18: D6 at 22 °C. After the 24 h test period, the amount of bark that had been removed by weevil feeding within the 20 mm^2 treatment and control area of each test twig were recorded by comparison with a square mm grid. There was generally no significant difference in response between the sexes, and the data presented were therefore pooled.

The effects of the various treatments are described by two variants of the antifeedant index, AFI:[39] 100x(C-T)/(C+T):

1) In AFIa, C represents the mean area of the control surfaces consumed and T represents the mean area of the treated surfaces consumed.

2) In AFIn, C represents the number of the control surfaces with any feeding and T represents the number of the treated surfaces without any feeding.

Hence, AFIa tends to be a measure that captures the reduction in feeding, whereas AFIn is a measure of complete inhibition of the initiation of feeding on the treated area. The two indices are fairly well correlated, but AFIa tend to be higher than AFIn because the antifeedant substances generally affect both the initiation of feeding and the amount of plant material consumed if feeding had started. For both indices, positive values (up to a maximum of 100) reflect an antifeedant effect, whereas negative values (down to a minimum of -100) indicate a stimulant effect on feeding.

Statistical differences in feeding/no feeding between treatment and control (i.e. the data used for calculating AFIn) were tested for each substance with Fisher´s exact test of a 2 x 2 table: *P<0.05, **P<0.01, ***P<0.001.

In the experiment to test synergistic effects the single compounds were tested at 15 mM concentration, in two-component lures at 7.5 mM of both components and in the test of the three-component lure at 5 mM of each component.

Test Compounds. The test compounds in Figure 1 and 2; **1, 2, 5, 6, 7, 9, 10, 13, 19, 20, 22, 23, 24, 30, 31, 32, 33**, **37**, **39**, 85, were purchased from Lancaster Synthesis, Lancaster, England and the test compounds **3, 8, 15, 17, 21**, **28**, **37** were purchased from (Sigma-Aldrich, Stockholm, Sweden). The compounds **11** and **35** were obtained from late prof. Holger Erdtman, KTH, Stockholm, Sweden.

The syntheses of the methyl hydroxy-methoxybenzoates **25**, **26**, and **29** were executed by regioselective synthetic sequences reported previously.[38] Some of the phenylpropanoids (**40** and **41**) were synthesized from the corresponding cinnamates. Methyl 3-(3,4-dimethoxyphenyl)propanoate (**42**) was obtained by reacting methyl 3-(3,4-dihydroxyphenyl)propanoate with sodium hydride and methyl iodide in THF according to the standard procedure.

The rest of the non-commercial test compounds were synthesized from their corresponding carboxylic acids by acids by refluxing in the alcohol with H_2SO_4 as a catalyst. A typical procedure was as follows: The carboxylic acid (2,4-dimethoxyphenylacetic acid (275 mg, 1.40 mmol) was dissolved in 25 mL methanol and some drops of sulfuric acid were added. The reaction mixture was heated at reflux until completion (monitored by TLC, ca 3 h). The solvent was evaporated (10-20 mm Hg) and the crude product was dissolved in dichloromethane. The solution was washed twice with brine and once with water. Drying over magnesium sulfate and concentration gave the ester, in this case, 260 mg (1.24 mmol) of methyl 2,4-dimethoxyphenylacetate in 88% yield.

Final purities of all compounds ranged from 96 to 99%, and, if necessary, compounds were purified by preparative chromatography[40] or flash chromatography on silica gel 0.040-0.063 mm (Merck 60, Darmstadt, Germany).

Gas chromatography-mass spectrometry (GC-MS) and when appropriate, NMR, was used to confirm identity and purity of all compounds.

Analysis of Bioassay Results. The following factors were investigated for their importance for antifeedant activity:

(1) functional groups (carboxylic acid vs methyl ester) (Table 1);

(2) structure of substituents on the aromatic ring (Table 2);

(3) patterns of substituents on the aromatic ring (Table 3).

(4) effect of lowering the concentration of antifeedants (Table 3).

(5) synergy effects by blending compounds from three different substance classes.

Antifeedant activities (AFIa and AFIn) were compiled and compared for the structural features 1-3 above, for benzoates, phenylacetates and phenylpropanoids.

Tests for Synergistic Effect of Selected Antifeedants Three selected antifeedants were tested for synergy effects: (methyl 2,4-dimethoxybenzoate (**37**), methyl (4-chlorophenyl)acetate (**45**), and methyl 3-(4-methylphenyl)propanoate (**55**). The selection was based on the antifeedant activity for each substance class, with the additional criterion of selecting compounds with different substituent types on the aromatic ring. The 4-chloro-analogue was selected from the phenylacetates, the 2,4-dimethoxy-analogue from the benzoate group and the 4-methyl-analogue from the phenylpropanoate group. Although a promising antifeedant, the latter was not the most active phenylpropanoate, but it was found appealing to use a third type of substituent. If antifeedant activity is a result of multiple interactions with several receptors, the selection of substances with multiple substituents would increase the chances of beneficial synergy effects. The experimental design is presented in Table 4 and 5 together with the results.

RESULTS AND DISCUSSION

Laboratory Bioassays The selected 13 phenylacetates and two phenylacetic acids were tested for antifeedant activity and our new results were compared with the results of our previous studies of benzoate[37-38] and phenylpropanoid[34-35] antifeedants in Tables 1-3.

Effect of Functional Groups. From the studies of benzoic and phenylpropanoic antifeedants we concluded that the carboxylic acids tested were inactive as antifeedants. This result was confirmed for the two tested phenylacetic acids, which both showed low antifeedant activity (Table 1, **1-18**).

Effect of Structure of the Substituents on the Aromatic Ring. One or two hydroxy groups on the aromatic ring seem to reduce the antifeedant activity (Table 2, **19-24**). The negative effect of the hydroxy group on the aromatic ring seems to be eliminated by an additional methoxy groups for many test compounds (Table 2, **12 + 25-30**). Apparently a methoxy group results in a favorable interaction with the antifeedant receptors for many substrates, as compounds with exclusively methoxy substituents, **31–41**, are generally strong antifeedants for all substance classes (Table 2). An exception is the relatively long 3,4-dimethoxyphenylpropanoid **42**, for which the antifeedant receptors do not seem to be able to accommodate both methoxy groups. Methyl and halogen substituents seem to yield relatively strong antifeedants for all three substance classes, although very few methylated benzoates were tested (as their relatively high volatility would make them unsuitable to use as antifeedants in practical applications) Table 2, **43-61**.

Effect of Patterns of Substituents. In order to find general trends, the effect of aromatic ring substituent pattern on antifeedant activity was studied. It was difficult to reveal any clear trends. For instance, of the compounds tested, all dimethoxy derivatives except 2,6-dimethoxybenzoate[37] and 3,4-dimethoxyphenylpropanoate derivatives, i.e. **42**, exhibited strong antifeedant activity, Table 2. For five of the 3-chloro and 4-chloro derivatives, AFIa reached ≈100, thus indicating very strong AF activity, Table 2, **44-46** and **52-53**.

The Effect of Lowering the Concentration of Antifeedants. It was observed that at 50 mM concentration, maximum or close to maximum antifeedant index was obtained for several compounds. To differentiate between some of the most promising antifeedant compounds and understand the relation of antifeedant activity with concentration, the tests for a subset of compounds were repeated at 5 mM. Monosubstituted benzoates were not tested due to their relatively high volatility, giving them less potential to be long-lasting antifeedants. All compounds showed lower antifeedant activity when the concentration was lowered. In the comparison of *ortho- meta-* or *para*-substituted monosubstituted phenylacetates and phenylpropanoates, no general correlation between antifeedant activity and substituent position on the aromatic rings could be revealed (Table 3). Both monomethoxylated phenylacetate **34** and monochlorinated phenylacetate **45** had much higher AFI than the corresponding monomethoxylated phenylpropanoate **34** and monochlorinated phenylpropanoate **46** (Table 3). Dimethoxy-substituted phenylacetates **14**, **39** and benzoates **2**, **37** showed good to excellent antifeedant activity. Some methylsubstituted phenylacetates **54**, **59** and phenylpropanoates **55-57**, **60-61** were top performing antifeedants (Table 3). Phenylpropanoates **46, 49** and **50** halogenated in position 4 were all good antifeedants while chlorination in position 2 or 3 lead to a decrease in activity (**47-48** and **53**). Interestingly, all three chlorinated phenylacetates tested **43-45** had high antifeedant activities although the *para*-isomer was the most active (Table 3). The 3,4-dichlorophenylacetate **52** also had relatively high AFI at 5 mM.

To summarize, efficient antifeedants for the pine weevil *H. abietis* are found among benzoates, phenylacetates and phenylpropanoates with one or two methyl, chloro or methoxy substituents on the aromatic rings.

Tests for Synergistic Effect of Selected Antifeedants. Three selected antifeedants were tested for synergistic effects (Table 4 and 5).

No synergy effects were found in the tests employing various combinations of three antifeedants from the different substance classes. All mixtures and single compounds tested resulted in AFIa values of 60-70, with the exception of the binary mixture of methyl (4-chlorophenyl)acetate (**45**) and methyl 3-(4-methylphenyl)propanoate (**55**), showing an AFIa as low as 37.

DISCUSSION

Several compounds covered in this study, or closely structurally related analogues thereof, have been reported previously as biologically active in various systems. Potentially relevant for this study is the report that methyl 4-methoxyphenylacetate (**34**) is emitted by sporulating tree-decaying fungi.[41-42] This compound may function as a signal that the tree stump is infested with fungi and in a state of decay, thus making it unsuitable for oviposition to *H. abietis* females.[43] Compounds with remotely similar structures have been reported as antifeedants and oviposition deterrents for other insects. Ethyl 3-(4-nitrophenyl)acrylate was reported as an oviposition deterrent for the onion fly *Delia antiqua* (Diptera: Anthomyiidae),[44] although showed no antifeedant activity in *H. abietis*,[35] while cinnamaldehyde acted as an antifeedant for *Tribolium* and *Sitophilus* store product beetles (Coleoptera: Tenebrionidae and Curculionidae)[45] as well as the elm pest *Ambrostoma quadriimpressum* (Coleoptera: Chrysomelidae).[17] Esters of phenylacetic acids have been found to act as inhibitors of soybean lipoxygenase[46] and were evaluated as anti-allergenic agents after showing degranulation inhibitory effects.[47] Benzoic- and cinnamic acid esters were recently reported to have strong antifungal activity against *Candida albicans*, a relevant fungus for human infections.[48]

For *H. abietis*, over a hundred derivatives of benzoic-, acetic-, phenylpropanoic- and cinnamic acid have been prepared and tested for antifeedant activity. Despite the discovery of many active compounds, it has always been difficult to identify the mode of action of these compounds. Several attempts to correlate physical properties of compounds with antifeedant activity using both traditional structure-activity relationship correlations such as the Topliss approach[35] and computational structure activity relationship studies[34] have been made on various subclasses of these small aromatic compounds. None of these investigations has given any clear indication of which properties are the key to a potent antifeedant. Substituents with very different electronic and steric properties have shown strong activity and the optimal substitution pattern has varied between substituent types as well as type of carboxylic acid.

We believe that our current study has merit not only in reporting the activity of methyl esters of phenylacetic acids as antifeedants, but also serves as a concluding study comparing the best antifeedants from similar types of compounds in literature. The results from this study are very well aligned with the results from previous studies [35, 37-38] and it is interesting that even if no “magic bullet” was discovered, many active compounds were revealed. From practical considerations, our results provide many alternatives for forestry protection applications. For such work, there are several other factors such as volatility, stability and toxicology that are highly important, and from the array of strongly active antifeedants presented not only from the phenylacetic acid derivatives, but also from related classes of compounds, there is a good chance that suitable compounds or combinations of compounds could be utilized. In our previous studies we did not test for possible synergistic effects between compounds from the three different substance classes.

If there were in fact several taste receptors involved in the interaction with the antifeedants, this possibility is most likely reality. Therefore, we here compared three selected representative strong antifeedants. The results were unequivocal: no relevant synergistic effects were observed. Although arguably possible to predict, this result is important for improving our understanding of the molecular interaction between this type of antifeedants and the insect taste receptor. The antifeedants of these types (esters of benzoic-, phenylacetic- and phenylpropanoic acid) may act on the same receptor. Based on this prediction, we believe that after evaluating over a hundred related compounds, the probability to find significantly more active antifeedants based on the tentative hypothesis of an oviposition deterrent is slim. In our opinion, further work on practical applications would be most effective by utilizing best fit candidates from the set of compounds already tested. Other types of antifeedants, that may signal food quality may however prove to be important. For example, 2-phenylethanol, an ubiquitous bacterial metabolite, is a strong antifeedant for *H. abietis* and has been suggested as a candidate for use to protect conifer seedlings.[25]

Since the techniques for application of protective stem coatings for conifer seedlings have evolved rapidly during the last decade,[10, 49] it is a tempting strategy to combine the flexible coating concept with an effective, non-toxic antifeedant. Currently used coatings often contain hard particles providing the physical protection but also making the application process more cumbersome and costlier. A coating containing an effective antifeedant may therefore offer a less complicated application process and maybe even an enhanced protective effect. An urgent task for future research is therefore to find compatible combinations of coating material and antifeedant that can provide protection against pine weevil feeding for two seasons without any detrimental effects on the seedling.

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Figure Captions

Figure 1. Structures of compounds tested for antifeedant activity.

Figure 2. Structures of compounds tested for antifeedant activity.

Figure 3. Scots pine twig with treatment and control area used in the two-choice feeding bioassay with *H. abietis*.

Table Legends

Table 1. Effect of Functional Group and the Substituents on the Aromatic Ring on the Activity of Antifeedant for the Pine Weevil, *Hylobius abietis*

Table 2. Effect of Aromatic Ring Substituents on Antifeedant Activity for the Pine Weevil, *Hylobius abietis*.[a]

Table 3. Antifeedant Activity at Low Concentration, 5 mM, for the Pine Weevil, *Hylobius abietis* sorted after AFIa Rank.

Table 4. Test Setup for Synergetic Effects between Three Selected Antifeedants from the Three Substance Classes.

Table 5. Antifeedant Activity of the Treatments in the Synergy Experiment for the Pine Weevil, *Hylobius abietis*.

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[a] Ranking between all 61 compounds tested at 50 mM concentration.

Table 1. Effect of Functional Group and the Substituents on the Aromatic Ring on the Activity of Antifeedant for the Pine Weevil, *Hylobius abietis*.[a]

Compound No.	Compound	AFIa	Rank AFIa	AFIn	Rank AFIn	Fisher test [b]
1	3,5-Dimethoxybenzoic acid	-4	60	2	57	ns
2	Methyl 3,5-dimethoxybenzoate	95	17	84	17	***
3	3,4-Methylenedioxybenzoic acid	14	55	11	48	ns
4	Methyl 3,4-methylenedioxybenzoate	57	38	25	43	**
5	2-Hydroxy-5-methoxybenzoic acid	17	54	2	57	ns
6	Methyl 2-hydroxy-5-methoxybenzoate	74	34	56	32	***
7	2-Hydroxy-3-methoxybenzoic acid	22	50	3	56	ns
8	Methyl 2-hydroxy-3-methoxybenzoate	95	17	85	16	***
9	3,4-Dimethoxybenzoic acid	7	58	2	57	ns
10	Methyl 3,4-dimethoxybenzoate	81	31	66	28	***
11	(4-Hydroxy-3-methoxyphenyl)acetic acid	10	57	5	52	ns
12	Methyl (4-hydroxy-3-methoxyphenyl)acetate	21	51	9	49	ns
13	3,5-Dimethoxyphenylacetic acid	1	59	-4	61	ns
14	Methyl (3,5-dimethoxyphenyl)acetate	98	10	93	12	***

---

[a] Ranking between all 61 compounds tested at 50 mM concentration.

[b] * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

15	3-(2-Methylphenyl)propanoic acid	47	44	12	47	*
16	Methyl 3-(2-methylphenyl)propanoate	91	21	75	24	***
17	3-(2-Methoxyphenyl)propanoic acid	12	56	4	53	ns
18	Methyl 3-(2-methoxyphenyl)propanoate	97	13	95	7	***

Table 2. Effect of Aromatic Ring Substituents on Antifeedant Activity for the Pine Weevil, *Hylobius abietis*.[a]

Compound No.	Compound	AFIa	Rank AFIa	AFIn	Rank AFIn	Fisher test [b]
19	Methyl 2-hydroxybenzoate	21	51	13	45	*
20	Methyl 4-hydroxybenzoate	34	48	26	41	**
21	Methyl 3-(4-hydroxyphenyl)propanoate	21	51	8	50	ns
22	Methyl 2,4-dihydroxybenzoate	46	45	8	50	ns
23	Methyl 3,4-dihydroxybenzoate	-7	61	2	57	ns
24	Methyl 3,5-dihydroxybenzoate	23	49	13	45	ns
25	Methyl 4-hydroxy-2-methoxybenzoate	35	46	4	53	ns
26	Methyl 4-hydroxy-3-methoxybenzoate	53	42	22	44	*
12	Methyl (4-hydroxy-3-methoxyphenyl)acetate	21	51	9	49	ns
27	Methyl 3-(4-hydroxy-3-methoxyphenyl)propanoate	54	39	32	38	***
28	Methyl 3-hydroxy-4-methoxybenzoate	65	35	32	38	***
29	Methyl 3-hydroxy-5-methoxybenzoate	54	39	26	41	***
30	Methyl 2-hydroxy-4-methoxybenzoate	60	37	52	33	***
31	Methyl 2-methoxybenzoate	80	32	51	34	***

---

[a] Ranking between all 61 compounds tested at 50 mM concentration.

[b] * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

32	Methyl 3-methoxybenzoate	89	24	65	30	***
33	Methyl 4-methoxybenzoate	54	39	44	36	***
34	Methyl (4-methoxyphenyl)acetate	100	1	100	1	***
35	Methyl 3-(4-methoxyphenyl)propanoate	96	15	89	14	***
18	Methyl 3-(2-methoxyphenyl)propanoate	97	13	95	7	***
36	Methyl 3-(3-methoxyphenyl)propanoate	95	17	80	18	***
10	Methyl 3,4-dimethoxybenzoate	81	31	66	28	***
37	Methyl 2,4-dimethoxybenzoate	99	7	95	7	***
2	Methyl 3,5-dimethoxybenzoate	95	17	84	17	***
14	Methyl (3,5-dimethoxyphenyl)acetate	98	10	93	12	***
38	Methyl (2,5-dimethoxyphenyl)acetate	96	15	77	22	***
39	Methyl (2,4-dimethoxyphenyl)acetate	88	26	65	30	***
40	Methyl 3-(2,3-dimethoxyphenyl)propanoate	86	28	77	22	***
41	Methyl 3-(3,5-dimethoxyphenyl)propanoate	89	24	70	27	***
42	Methyl 3-(3,4-dimethoxyphenyl)propanoate	35	46	4	53	ns
43	Methyl (2-chlorophenyl)acetate	85	30	78	20	***
44	Methyl (3-chlorophenyl)acetate	100	1	100	1	***
45	Methyl (4-chlorophenyl)acetate	100	1	100	1	***

46	Methyl 3-(4-chlorophenyl)propanoate	100	1	100	1	***
47	Methyl 3-(2-chlorophenyl)propanoate	78	33	51	34	***
48	Methyl 3-(3-chlorophenyl)propanoate	86	28	78	20	***
49	Methyl 3-(4-bromophenyl)propanoate	97	13	89	14	***
50	Methyl 3-(4-fluorophenyl)propanoate	98	10	90	13	***
51	Methyl 3,5-dibromobenzoate	50	43	36	37	***
52	Methyl (3,4-dichlorophenyl)acetate	100	1	100	1	***
53	Methyl 3-(3,4-dichlorophenyl)propanoate	98	10	94	11	***
54	Methyl (4-methylyphenyl)acetate	87	27	74	26	***
55	Methyl 3-(4-methylphenyl)propanoate	99	7	95	7	***
56	Methyl 3-(2-methylphenyl)propanoate	91	21	75	24	***
57	Methyl 3-(3-methylphenyl)propanoate	90	23	66	28	***
58	Methyl 3,5-dimethylbenzoate	61	36	32	38	**
59	Methyl (3,5-dimethylphenyl)acetate	94	20	80	18	***
60	Methyl 3-(2,4-dimethylphenyl)propanoate	100	1	100	1	***
61	Methyl 3-(3,4-dimethylphenyl)propanoate	99	7	95	7	***

Table 3. Antifeedant Activity at Low Concentration, 5 mM, for the Pine Weevil, *Hylobius abietis* sorted after AFIa Rank.

Compound No.	Compound	AFIa	Rank AFIa	AFIn	Rank AFIn	Fisher test [a]
45	Methyl (4-chlorophenyl)acetate	76	1	58	2	***
37	Methyl 2,4-dimethoxybenzoate	74	2	61	1	***
34	Methyl (4-methoxyphenyl)acetate	70	3	38	3	***
49	Methyl 3-(4-bromophenyl)propanoate	42	9	20	16	**
56	Methyl 3-(2-methylphenyl)propanoate	52	4	30	7	***
54	Methyl (4-methylyphenyl)acetate	52	4	33	6	***
55	Methyl 3-(4-methylphenyl)propanoate	46	6	24	10	**
50	Methyl 3-(4-fluorophenyl)propanoate	44	7	35	5	***
57	Methyl 3-(3-methylphenyl)propanoate	43	8	23	11	***
59	Methyl (3,5-dimethylphenyl)acetate	42	9	23	12	**
52	Methyl (3,4-dichlorophenyl)acetate	42	9	21	15	*
43	Methyl (2-chlorophenyl)acetate	41	12	27	8	**
44	Methyl (3-chlorophenyl)acetate	41	12	20	16	**

---

[a] * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

39	Methyl (2,4-dimethoxyphenyl)acetate	40	14	22	13	**
60	Methyl 3-(2,4-dimethylphenyl)propanoate	40	14	20	16	ns
14	Methyl (3,5-dimethoxyphenyl)acetate	38	16	19	20	*
46	Methyl 3-(4-chlorophenyl)propanoate	37	17	36	4	***
2	Methyl 3,5-dimethoxybenzoate	37	18	19	19	**
61	Methyl 3-(3,4-dimethylphenyl)propanoate	33	19	26	9	**
58	Methyl 3,5-dimethylbenzoate	31	20	22	13	ns
62	Methyl 3-phenylpropanoate	28	21	15	21	*
36	Methyl 3-(3-methoxyphenyl)propanoate	27	22	15	21	ns
48	Methyl 3-(3-chlorophenyl)propanoate	25	23	13	24	ns
53	Methyl 3-(3,4-dichlorophenyl)propanoate	24	24	15	21	*
47	Methyl 3-(2-chlorophenyl)propanoate	21	25	5	26	ns
35	Methyl 3-(4-methoxyphenyl)propanoate	13	26	6	25	ns
18	Methyl 3-(2-methoxyphenyl)propanoate	0	27	2	27	ns

Table 4. Test Setup for Synergetic Effects between Three Selected Antifeedants from the Three Substance Classes.

Treatments	1	2	3	4	5	6	7
Test compounds	mM						
Methyl 2,4-dimethoxybenzoate (**37**)	15			7.5	7.5		5.0
Methyl (4-chlorophenyl)acetate (**45**)		15		7.5		7.5	5.0
Methyl 3-(4-methylphenyl)propanoate (**55**)			15		7.5	7.5	5.0

Table 5. Antifeedant Activity of the Treatments in the Synergy Experiment for the Pine Weevil, *Hylobius abietis*.

	AFIa	Rank AFIa	AFIn	Rank AFIn	Fisher test [a]
Tr 1	61	5	51	3	***
Tr 2	72	1	53	1	***
Tr 3	58	6	41	6	***
Tr 4	62	4	44	5	***
Tr 5	71	2	48	4	***
Tr 6	37	7	15	7	ns
Tr 7	69	3	52	2	***

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[a] * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

Figure 1.

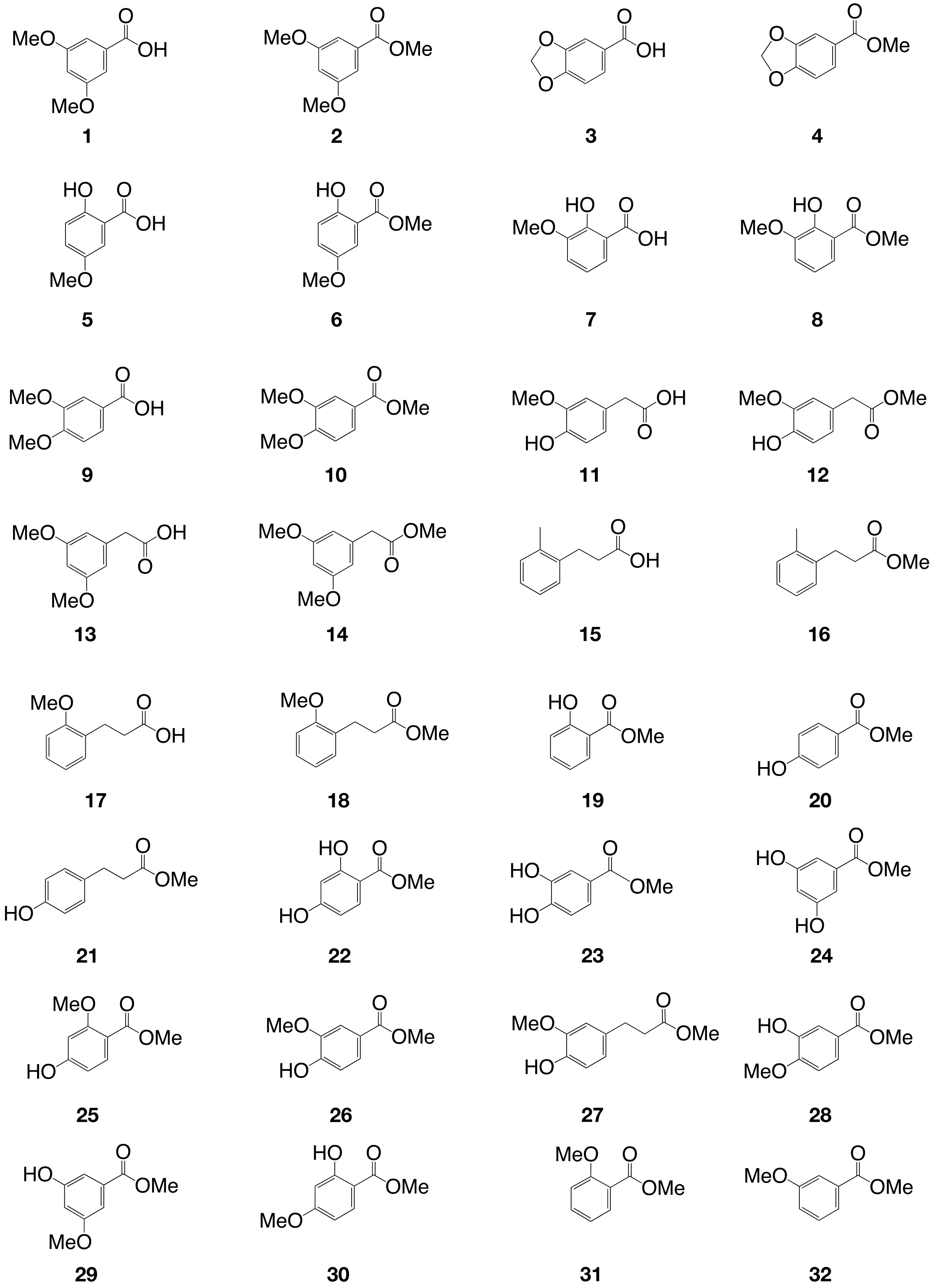
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Figure 2.

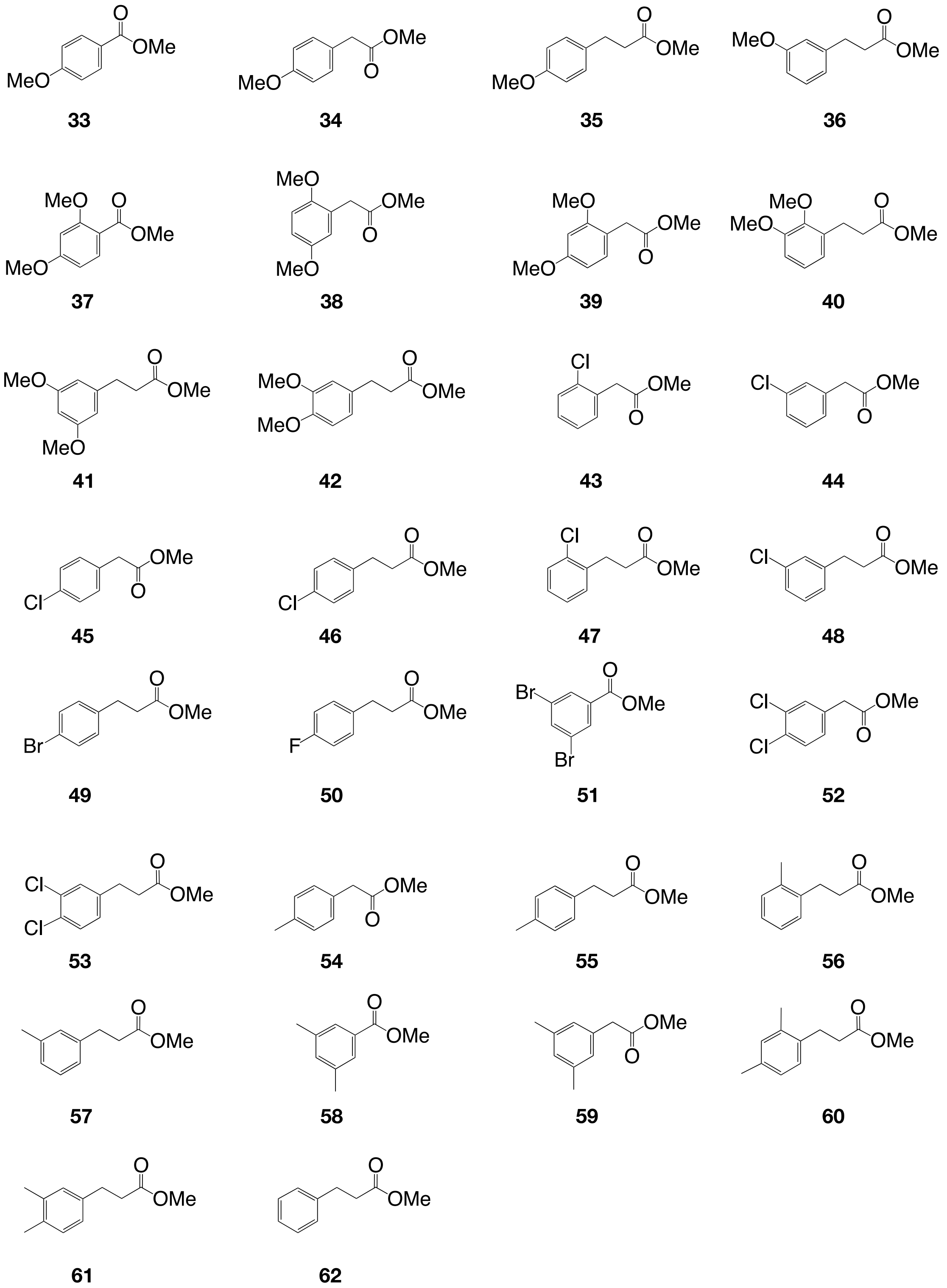

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Figure 3.

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