Antifeedants and Feeding Stimulants in Bark Extracts of Ten Woody Non-host Species of the Pine Weevil, *Hylobius abietis*

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Received: 25 November 2007 / Revised: 8 July 2008 / Accepted: 18 July 2008 / Published online: 22 August 2008
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Abstract Bark of ten woody species, known to be rejected as a food source by the pine weevil, *Hylobius abietis*, were sequentially extracted by a Soxhlet apparatus with pentane followed by methanol. Species were alder (*Alnus glutinosa*), aspen (*Populus tremula*), beech (*Fagus sylvatica*), guelder rose (*Viburnum opulus*), holly (*Ilex aquifolium*), horse chestnut (*Aesculus hippocastanum*), lilac (*Syringa vulgaris*), spindle tree (*Evonymus europaeus*), walnut (*Juglans regia*), and yew (*Taxus baccata*). Bark of each species was collected in southern Scandinavia during the summer. Resulting extracts were tested for antifeedant activity against the pine weevil by a micro-feeding choice assay. At a dose corresponding to that in the bark, methanol extracts from *Aesculus*, *Taxus*, *Ilex*, and *Populus* were antifeedant active, while pentane extracts of *Aesculus*, *Fagus*, *Syringa*, and *Viburnum* were stimulatory. Four known antifeedants against *H. abietis*, the straight-chained carboxylic acids, hexanoic and nonanoic acid (C6 and C9), carvone, and carvacrol were identified by gas chromatography (GC)–mass spectrometry (MS) in several extracts. The major constituents were identified and tested for feeding deterrence. The aromatic

compounds benzyl alcohol and 2-phenylethanol are new non-host plant-derived feeding deterrents for the pine weevil. Additionally, two feeding stimulants, β -sitosterol and 5-(hydroxymethyl)-2-furaldehyde, were identified. One active methanol extract of *Aesculus* bark was sequentially fractionated by liquid chromatography, and major compounds were tentatively identified as branched alcohols and esters of hexanoic acid. Five commercially available hexanoate esters and two commercially available branched alcohols were identified as new active antifeedants. Both stimulatory and inhibiting compounds were found in the same extracts and co-eluted in the same or adjacent fractions. The mix of semiochemicals of opposite activity in each extract or fraction could explain the stimulatory-, inhibitory-, or sometimes neutral activity. Generally, such co-occurrence confounds the isolation of antifeedants.

Keywords Phagostimulant · Carboxylic acid · Bark extract · Limonene · Carvone · Carvacrol · Eugenol · Benzyl alcohol · Benzoic acid · 2-Phenylethanol · β -Sitosterol · Hexanoate esters · Branched alcohols

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Introduction

Maturation feeding by juvenile and adult imagoes of the pine weevil, *Hylobius abietis* L., on small transplanted conifer plants causes severe problems in reforestation on felled areas in Northern Europe (Långström and Day 2004). Currently, insecticides, such as pyrethroids, are used to protect newly planted seedlings. Pyrethroids are toxic not only to insects but also to aquatic organisms. Indeed, cypermethrin, presently approved in Sweden, is even more toxic than the recently banned permethrin (McLeese et al. 1980). Thus, it is desirable that the use of such toxic

compounds be replaced by more environmentally friendly protection methods. One promising strategy is the application of plant-derived protective antifeedant compounds onto plants (Klepzig and Schlyter 1999; Bratt et al. 2001; Sibul et al. 2001; Thacker et al. 2003; Schlyter 2004).

The adult pine weevil accepts numerous non-host species for feeding but prefers Scots pine (*Pinus sylvestris* L.) over most woody species of the Scandinavian flora (Manlove et al. 1997; Månsson and Schlyter 2004). However, there are at least 11 woody species upon which the weevil will avoid feeding even in no-choice tests (Månsson and Schlyter 2004). These are alder (*Alnus glutinosa* (L.) Gaertner), aspen (*Populus tremula* L.), beech (*Fagus sylvatica* L.), guelder rose (*Viburnum opulus* L.), holly (*Ilex aquifolium* L.), horse chestnut (*Aesculus hippocastanum* L.), linden (*Tilia cordata* Mill.), lilac (*Syringa vulgaris* L.), spindle tree (*Evonymus europaeus* L.), walnut (*Juglans regia* L.), and yew (*Taxus baccata* L.). Our general hypothesis is that the avoidance of weevils feeding on these species is caused by antifeedants present in the bark. Thus, we investigated chemical compositions.

The bark of one species, linden (*Tilia cordata*), contains nonanoic acid, which is a potent antifeedant against the pine weevil (Månsson et al. 2005). Several analogues of nonanoic acid are also active antifeedants (Månsson et al. 2006). While reports of the occurrence of similar carboxylic acids in bark tissue are sparse, several have been found in other tissues from some of the 11 woody species (Nahrstedt et al. 1981; Buttery et al. 2000).

To determine if the bark of the ten species rejected by the weevil, except for *Tilia*, also contains antifeedants against *H. abietis*, their barks were extracted with non-polar and polar solvents, and the extracts were tested for activity. Tests were made with doses corresponding to those occurring naturally in bark. The content of known antifeedants in the extracts was determined by gas chromatography (GC)–mass spectrometry (MS). The major compounds identified in the extracts were tested for activity. Additionally, an antifeedant active methanol extract of horse chestnut was fractionated. Stimulatory and inhibitory compounds in the resulting fractions were identified and bio-assayed.

Methods and Materials

Bark Extraction Branches and shoots (5- to 30-mm diam) from the ten plants, *Viburnum*, *Evonymus*, *Alnus*, *Juglans*, *Fagus*, *Aesculus*, *Taxus*, *Ilex*, *Populus*, and *Syringa*, were collected during the summer in southern Sweden. Only young plant material (second-year shoots and branches) was used. Preparation and extraction of the bark were performed as previously described (Månsson 2005; Månsson

et al. 2005). Prior to removal of bark from twigs, the total bark area was measured. Outer and inner bark were cut or scraped off the stem, immersed in liquid nitrogen, and ground in a mortar. Pulverized bark was extracted first in a Soxhlet apparatus (Furniss et al. 1989) with boiling pentane (300 ml). After 2 h, the solvent was changed to methanol (MeOH; 300 ml), and the extraction continued for another 2 h. The resulting extracts (one non-polar pentane and one polar MeOH for each species) were filtered through silica gel (~1 g), which was, in both cases, washed with MeOH (~5 ml) prior to being concentrated in a rotary evaporator.

Bioassays Adult *H. abietis* were collected and stored with a food source (pine twigs) and water in buckets kept in darkness at 8–10°C as described earlier (Klepzig and Schlyter 1999; Schlyter et al. 2004a). Before starting an assay, buckets were moved into a growth chamber in which the parameters were set to those found in the field at the time of the weevil collection [24°C, 75% RH, and a photo period of 20:4 h (L/D)]. Twigs were removed 6 days before the start of a micro-feeding assay (24 h before a twig test), and water was removed 1 day before (Schlyter et al. 2004a).

The micro-feeding assay is a choice test that uses two plates, cut from thin-layer cellulose chromatography Al plates (5×5 mm, Merck #1.0552), that were placed in Petri dishes (9-cm diam) and were allowed to run for 4 h. The method is described in detail previously (Schlyter et al. 2004a). Concentrated extracts were diluted with either pentane or methanol. The amount of solvent was determined on the basis of the total bark area extracted. Thus, if the bark area corresponded to, for example, 200 thin-layer chromatography (TLC) plates, each of 25 mm², solvent was added until a volume of 200×10 µl (2×5 µl) was reached. Then, 2×5 µl of the diluted extract were applied to the TLC test plate (Månsson et al. 2005). Hence, the dose (amount/area unit) of any compound on the test plate equaled that in the bark, assuming no loss during extraction and a bark thickness equal to the cellulose layer. These assumptions are not met exactly, but the deviations from the two assumptions will be of opposite directions and will partly cancel out.

Synthetic compounds tested (Tables 1, 2, and 3) were applied in 1.5-µl aliquots in a 10% MeOH solution. After solvent evaporation from both the treatment and solvent blank plates, 5 µl of 1 M sucrose in water were added as a feeding stimulant to both (Schlyter et al. 2004a).

Twig no-choice tests were performed on 12-mm long *P. sylvestris* twig sections of approximately equal diameter (6- to 10-mm diam), which were dipped in solutions of the compound to be tested. The method is described in detail (Klepzig and Schlyter 1999; Schlyter et al. 2004a).

The Anti-Feedant Index, AFI, was the variable used to quantify results from the assays (Schlyter et al. 2004a). The

Table 1 Content of known antifeedants identified in pentane and methanol extracts of ten woody non-host species and their activity against *Hylobius abietis*

Compound	AFI ±SE (n)	<i>Viburnum opulus</i>	<i>Evonymus europaeus</i>	<i>Alnus glutinosa</i>	<i>Juglans regia</i>	<i>Fagus sylvatica</i>	<i>Aesculus hippocastanum</i>	<i>Taxus baccata</i>	<i>Ilex aquifolium</i>	<i>Populus tremula</i>	<i>Syringa vulgaris</i>	Reference
Hexanoic acid	0.98* ±0.01 (26)			X					X			Månsson et al., 2006
Nonanoic acid	0.94* ±0.05 (41)								X			Månsson et al., 2006, Månsson et al., 2005
Carvone**	0.80* ±0.06 (49) R-enantiomer 0.92* ±0.14 (19) S-enantiomer					X			X			Schlyter et al., 2004b
Carvacrol	1.00* ±0.00 (24)						X					Schlyter et al., 2004b

*) AFI significantly different from 0 (zero) by 95% confidence interval for the mean of *n* observations (negative values indicate feeding stimulants)

**) Stereoisomeric composition in the extract not determined

area eaten on the control plate (*C*) is related to the area eaten on the test plate (*T*): $AFI = (C - T)/(C + T)$. Negative values (<0) indicate a feeding stimulation, while a value of 1.00 corresponds to total feeding inhibition. A value of 0.50 indicates a half effect and 0 no effect. AFI values are presented as their arithmetic means ±SE (standard error of the mean), and activity is considered significant if the 95% confidence interval for the mean (95% CI) does not overlap zero.

The AFI obtained from the dose-response test with 10%, 1%, and 0.1% solutions in the micro-assays provides an ED₅₀ value. The ED₅₀ is the effective dose (expressed as concentration in percent of the treatment solution) that is needed for achieving a 50% feeding deterrent effect (i.e., the dose required to get an AFI=0.5).

Fractionation of *Aesculus* Methanol Extract All solvents were distilled prior to use. The MeOH extract from *Aesculus* bark (M1) was fractionated by reverse phase LC by using octadecyl functionalized silica gel (12 g, Sigma-Aldrich Sweden AB) in a flash column (10 mm i.d.). The gradient used was one-column volume (10 ml) of MeOH followed by 10 ml of 50% CH₂Cl₂ in MeOH, 10 ml CH₂Cl₂, 10 ml 50% pentane in CH₂Cl₂, and finally, 20 ml of pentane. In total, 14 fractions, each of ≈4 ml, were collected. The fractions containing similar compounds (as judged by TLC) were pooled. Each of the resulting eight

fractions (M1:1–8) were concentrated to 1 ml by flushing a gentle stream of argon over the solution, and they were subsequently tested in the micro-feeding assay.

One fraction (M1:3) had antifeedant activity and was further fractionated on silica gel 60 (6.5 g, Fluka) on a flash column (10 mm i.d.). The elution gradient was 20 ml of CH₂Cl₂ followed by 10 ml of 10% MeOH in CH₂Cl₂, 10 ml 20% MeOH in CH₂Cl₂, 10 ml 50% MeOH in CH₂Cl₂, 10 ml 75% MeOH in CH₂Cl₂, and finally, 50 ml MeOH. In total, 30 fractions, each of ≈4 ml, were collected. By merging fractions of similar chemical content as determined by TLC, the number of fractions was reduced to nine (M1:3:1–9), concentrated, and tested in the micro-feeding assay.

Chemical Analysis Bark extracts were analyzed by GC–MS on a Varian 3800 GC instrument with a CP-sil 5CB low-bleed column (Varian, 30 m×0.25 mm i.d., 0.25 μm film thickness), in series with a Saturn 2000 MS, ion trap detector, EI mode. All compounds in Tables 1 and 2 were identified by comparing their retention times and mass spectra with those of the reference compounds co-injected on the same instrument under the same analytical conditions.

Chemical Compounds (*Z*)-3-Hexenyl hexanoate was purchased from Bedoukian Research, β-sitosterol was purchased from Fluka. Tetradecyl hexanoate was synthesized

Table 2 Identified and tested compounds in pentane and methanol extracts of the ten woody non-host species and their activity against *Hylobius abietis*

Compound	AFI ±SE (<i>n</i>)	<i>Viburnum opulus</i>	<i>Evonymus europaeus</i>	<i>Alnus glutinosa</i>	<i>Juglans regia</i>	<i>Fagus sylvatica</i>	<i>Aesculus hippocastanum</i>	<i>Taxus baccata</i>	<i>Ilex aquifolium</i>	<i>Populus tremula</i>	<i>Syringa vulgaris</i>
Hexadecanoic acid	0.16 ±0.09 (28)	X	X	X	X	X	X	X	X	X	X
Linoleic acid	-0.15 ±0.10 (29)	X	X	X	X	X	X	X	X	X	X
Linolenic acid	-0.09 ±0.08 (36)									X	
Limonene**	0.05 ±0.13 (19)		X				X		X		
Eugenol	1.00* ±0.00 (14)	X				X	X	X			
Benzyl alcohol	0.59* ±0.17 (17)				X				X	X	
Vanillin	0.01 ±0.25 (14)		X	X		X		X	X		
Benzoic acid	-0.18 ±0.13 (19)		X		X	X	X	X	X	X	X
Cinnamic acid	-0.02 ±0.11 (28)							X		X	
2-Phenylethanol	1.00* ±0.01 (19)								X	X	
Benzaldehyde	-0.16 ±0.12 (20)		X				X	X	X	X	X
2-Furaldehyde	-0.16 ±0.13 (24)	X	X	X	X	X	X			X	X
5-(Hydroxymethyl)-2-furaldehyde	-0.32* ±0.13 (19)				X		X			X	X
β-Sitosterol**	-0.27* ±0.09 (38)	X	X	X	X	X	X	X		X	X

*) AFI significantly different from 0 (zero) by 95% confidence interval for the mean of *n* observations
(negative values indicate feeding stimulants)

**) Stereoisomeric composition in the extract not determined

Table 3 The antifeedant activity in *Hylobius abietis* of hexanoate esters and branched aliphatic alcohols, lead compounds tentatively identified from horse chestnut extracts

Compound	AFI $\pm$ SE (n)	ED ₅₀ (%) $\pm$ SE (n)	AFI twig $\pm$ SE (n)
(Z)-3-Hexenyl hexanoate	0.99 ^a $\pm$ 0.01 (10)		
Hexyl hexanoate	0.87 ^a $\pm$ 0.10 (19)		
2-Methylbutyl hexanoate	0.86 ^a $\pm$ 0.07 (7)		
3-Methylbutyl hexanoate	0.88 ^a $\pm$ 0.06 (10)		
iso-Butyl hexanoate	0.83 ^a $\pm$ 0.10 (10)		
Tetradecyl hexanoate	0.32 $\pm$ 0.16 (19)		
2-Butyl-1-octanol	0.86 ^a $\pm$ 0.10 (27)	0.76 $\pm$ 0.59 (2)	0.21 ^a $\pm$ 0.07 (20)
2-Ethyl-1-hexanol	0.93 ^a $\pm$ 0.08 (27)		0.11 $\pm$ 0.16 (10)
2-Hexyl-1-decanol	0.17 $\pm$ 0.09 (29)		0.24 ^a $\pm$ 0.10 (10)

^a AFI significantly different from 0 (zero) by 95% confidence interval for the mean of *n* observations

from hexanoic acid and tetradecanol; spectral data (¹H, ¹³C NMR, and MS) were satisfactory. All other chemical compounds in Tables 1, 2, and 3 were purchased from Sigma-Aldrich Sweden AB.

Results

Twenty extracts (one pentane and one methanol of each species) of *Alnus*, *Populus*, *Fagus*, *Ilex*, *Aesculus*, *Viburnum*, *Syringa*, *Evonymus*, *Juglans*, and *Taxus* were tested twice in the micro-assay. In the first run, the highest antifeedant activity was found for the methanol extract of *Aesculus* (*N*=7). Combining the results from both test runs, the methanol extracts of *Populus*, *Ilex*, *Aesculus*, and *Taxus* were all active in inhibiting feeding (AFI 95% CI were all >0, Fig. 1). None of the pentane extracts displayed any antifeedant activity in these species. In contrast, the pentane extracts of *Fagus*, *Viburnum*, *Aesculus*, and *Syringa* significantly stimulated feeding (AFI 95% CI were all <0, Fig. 1).

The content of known antifeedants, such as straight-chained aliphatic carboxylic acids in the range C6–C9 (Månsson et al. 2006), carvone, and carvacrol (Schlyter et al. 2004b) in the 20 extracts was determined by GC–MS (Table 1). Among known active carboxylic acids, the feeding deterrents hexanoic acid (in the pentane extract of *Ilex* and *Alnus*) and nonanoic acid (in the pentane extract of *Ilex*) were detected. The known pine weevil antifeedants carvone and carvacrol were found in the pentane extracts of *Fagus* and *Ilex*, and in the methanol extract of *Aesculus*, respectively (stereoisomeric composition of carvone in the extracts was not determined; however, both enantiomers are active antifeedants, Table 1).

Additionally, major compounds in the extracts were identified, and those available were tested in the micro-assay (Table 2). The antifeedant active methanol extracts of *Aesculus*, *Taxus*, and *Populus* contained one or more of the deterrent, aromatic compounds carvacrol, eugenol, or benzyl alcohol (Tables 1 and 2). To our knowledge, the latter, together with 2-phenylethanol that was found in the

pentane extracts of *Ilex* and *Populus*, have not been reported previously as feeding deterrents for the pine weevil. Two aldehydes, 2-furaldehyde (furfural) and 5-(hydroxymethyl)-2-furaldehyde (5-hydroxymethylfurfural), were found in some extracts with antifeedant activity. While no activity was found for 2-furaldehyde, 5-(hydroxymethyl)-2-furaldehyde stimulated feeding (AFI significantly <0). Another feeding stimulant, β -sitosterol, was detected in the majority of the extracts (Table 2). Hexadecanoic acid, which was identified in almost all extracts (both pentane and methanol), did not exhibit any antifeedant activity (Table 2). Two other long-chain carboxylic acids, linoleic acid that was found in several pentane and methanol extracts and linolenic acid present in the pentane extract of *Populus*, seemed to

Fig. 1 Antifeedant index (AFI) of pentane (lines with open circles) and methanol (lines with filled squares) extracts in micro-feeding assay choice test of ten woody non-hosts. Plants extracted included spindle tree (*Evonymus europaeus*), alder (*Alnus glutinosa*), horse chestnut (*Aesculus hippocastanum*), walnut (*Juglans regia*), beech (*Fagus sylvatica*), yew (*Taxus baccata*), holly (*Ilex aquifolium*), aspen (*Populus tremula*), lilac (*Syringa vulgaris*), and guelder rose (*Viburnum opulus*). *N* on the x-axis is the number of data points (individual beetles in Petri dishes) where an AFI could be calculated, i.e., when feeding occurred on at least one of the paired plates in the dish. AFI relates the area eaten on the control plate (*C*) to that on the test plate (*T*): $AFI = (C - T)/(C + T)$ and ranges from -1 to +1

stimulate feeding but not significantly so (both AFI <0). Five common plant constituents, limonene, vanillin, benzoic acid, cinnamic acid, and benzaldehyde, were identified in some extracts but showed no effect on the weevil (AFI ~0, Table 2).

Since *Aesculus* was the only species that yielded both inhibitory (methanol) and stimulatory (pentane) extracts and was active in the first micro-feeding test, the active methanol extract (M1) was chosen for fractionation. Reverse-phase liquid chromatography resulted in eight fractions, of which several (M1:4 to M1:8) were phagostimulatory. Only fraction M1:3 displayed significant anti-feedant activity (Fig. 2). However, when the eight fractions were combined, the activity was lost (M1 sum, Fig. 2). Fractions M1:2 (slightly antifeedant) and M1:3 (active antifeedant) contained similar patterns of compounds with one exception; fraction M1:2 contained a large amount of the feeding stimulant 5-(hydroxymethyl)-2-furaldehyde, while M1:3 did not.

A second chromatographic separation of the antifeedant active fraction M1:3 on straight phase silica gel resulted in nine fractions of which the last (M1:3:9) exhibited the highest activity (Fig. 3). In contrast, several fractions had AFI <0, and fraction M1:3:5 strongly (AFI <<0) and significantly stimulated weevil feeding on treated plates (Fig. 3). Two compounds were common in fractions M1:3:8 and M1:3:9, and their mass spectra showed they were aliphatic esters of hexanoic acid. The mass spectra and retention times of these compounds were compared with

Fig. 2 Feeding activity of fractions of the first *Aesculus* methanol extract in micro-feeding assay no-choice tests. *N* on the x-axis is the number of data points (individual beetles in Petri dishes) where an AFI could be calculated, i.e., when feeding occurred on at least one of the paired plates in the dish. M1:sum joined extract from pooling all fractions

Fig. 3 Feeding activity of the sub-fractions of fraction M1:3 from the first *Aesculus* methanol extract in micro-feeding assay no-choice tests. *N* on the x-axis is the number of data points (individual beetles in Petri dishes) where an AFI could be calculated, i.e., when feeding occurred on at least one of the paired plates in the dish

those of reference samples of some esters of that type (tetradecyl-, tridecyl-, dodecyl-, and hexyl hexanoate), but neither the mass spectra nor the retention times were identical with any of the reference esters. Interestingly, when six available esters of this type were tested in the micro-feeding assay, five out of six were active antifeedants with AFIs >0.80 (Table 3). The most active was (*Z*)-3-hexenyl hexanoate, followed by hexyl hexanoate, 2-methylbutyl hexanoate, 3-methylbutyl hexanoate, and *iso*-butyl hexanoate. The heavier tetradecyl hexanoate was not active.

The mass spectra of other major components in the slightly antifeedant active fraction M1:3:8 indicated they were branched aliphatic alcohols. Three alcohols of that type were available, namely 2-butyl-1-octanol, 2-ethyl-1-hexanol, and 2-hexyl-1-decanol, but their retention times were not identical with those in the extracts. In the micro-feeding assay at a 10% dose, they displayed varying antifeedant activities (Table 3): high activity for 2-butyl-1-octanol and 2-ethyl-1-hexanol but low activity for 2-hexyl-1-decanol. The ED₅₀ for 2-butyl-1-octanol in the choice micro-assay was as low as 0.8% (±0.6 *N*=2), indicating high activity. When tested on twigs, less activity than in the micro-feeding assay was obtained for 2-butyl-1-octanol and 2-ethyl-1-hexanol, while 2-hexyl-1-decanol showed the same low activity as in the micro-feeding assay.

Discussion

Eleven new active compounds were found during this study. Two of these, β-sitosterol and 5-(hydroxymethyl)-2-

furaldehyde (5-hydroxymethyl furfural), are the first reported feeding stimulants from non-host plants of the pine weevil. Another two, benzyl alcohol and 2-phenylethanol, were new antifeedants that were identified in several non-host plants. Aliphatic esters of hexanoic acid and branched aliphatic alcohols were tentatively identified in active extracts. Nine compounds belonging to either of these classes of compounds were tested, and seven were antifeedants.

Apart from the 11 new active compounds and the four known antifeedants identified in non-host plants (Table 1), the contrasting activity of polar and nonpolar extracts is a striking finding. Activities of extracts are related to the polarity of the solvent used for extraction (Fig. 1). Thus, only four out of ten plant barks furnished active inhibitory extracts (*Populus*, *Ilex*, *Aesculus*, and *Taxus*), and all of these were methanol extracts. In contrast, the pentane extracts of *Fagus*, *Viburnum*, *Aesculus*, and *Syringa* were strongly feeding stimulatory. Only the bark of one species, *Aesculus*, produced extracts that were both stimulatory (pentane) and inhibitory (MeOH).

We previously showed that some aliphatic carboxylic acids (in the range C6–C10) act as antifeedants against the pine weevil (Månsson et al. 2005, 2006). Of these, nonanoic acid was found in a dichloromethane/methanol bark extract of *Tilia* (Månsson 2005; Månsson et al. 2005). In addition, hexanoic, heptanoic, octanoic, and nonanoic acids have been found in *Fagus* wood (Guillén and Ibargoitia 1996). We found only hexanoic acid in the pentane bark extracts of *Ilex* and *Alnus*, and nonanoic acid in the pentane bark extract of *Ilex* (Table 1). Thus, the feeding avoidance of *H. abietis* on the ten woody species investigated in this work is not fully explained by the presence of these carboxylic acids, as it was for linden (Månsson et al. 2005, 2006).

Fractionation of the *Aesculus* methanol extracts led to the discovery of two new groups of pine weevil antifeedants, i.e., esters of hexanoic acid and branched alcohols. Additionally, the aromatic compounds benzyl alcohol and 2-phenylethanol were identified as new antifeedants. Eugenol had earlier been found in a polar extract of the feces of *H. abietis* females. Similar to our results, the compound exhibited antifeedant activity against the weevil when tested in a feeding bioassay (Borg-Karlson et al. 2006). In accordance with the decreased antifeedant activity found for carboxylic acids with chains longer than C10 (Månsson et al. 2006), no antifeedant activity was found for hexadecanoic-, linoleic-, or linolenic acids that were present in almost all extracts (Table 1).

The heteroaromatic aldehyde 5-(hydroxymethyl)-2-furaldehyde showed feeding stimulating properties. Previously, 5-(hydroxymethyl)-2-furaldehyde was reported as a constituent of the essential oil and head space extract of

Aesculus flowers (Buchbauer et al. 1994). However, both 5-(hydroxymethyl)-2-furaldehyde and 2-furaldehyde, which was also found in some extracts, could be artifacts formed by carbohydrate dehydration (Kallury et al. 1986; Antal Jr. et al. 1990), either during the extraction procedure or in the injector of the GC.

In contrast to the high feeding-deterrent activity found for some methoxy-substituted benzaldehyde derivatives (Eriksson 2006), benzaldehyde itself showed no antifeedant activity. However, the inactivity of cinnamic acid and benzoic acid was not surprising since earlier studies have shown that methoxy-substituted benzoic- and cinnamic-acid derivatives are less active antifeedants than the corresponding aldehydes (Eriksson 2006) or methyl esters (Unelius et al. 2006).

All extracted species, except holly, contained β -sitosterol, which strongly stimulated feeding of *H. abietis*. This compound has been identified as a feeding stimulant for the obscure root weevil, *Sciopithes obscurus* Horn (Doss et al. 1982), an effect that was synergistically enhanced by adding sucrose (Shanks and Doss 1987). In our tests of β -sitosterol, sucrose was always added to the test plate.

Most extracts contained a complex mixture of antifeedant and feeding stimulatory compounds. Whether the final outcome of an extract was a feeding deterrent or a stimulatory effect was dependent on the mixture of compounds therein and on their concentrations. The feeding stimulant β -sitosterol, occurring in almost all extracts, was a minor constituent in the antifeedant active extracts, whereas in the feeding stimulatory and inactive extracts, it was a major constituent. Hence, the deterrent activity of the extracts appears to be partially dependent on the efficiency by which β -sitosterol is removed during the non-polar pentane extraction. The concentration dependence was exemplified by the fact that when the eight fractions of the active methanol extract of *Aesculus* were combined, deterrent activity was lost. Probably, this can be ascribed to the fact that the concentrations of the specific deterrents became too low relative to that of β -sitosterol.

Even after extensive fractionation of extracts, the difficulty of identifying antifeedants in the presence of feeding stimulating compounds remains. After fractionation of the first methanol extract of *Aesculus*, fractions M1:2 and M1:3 were found to have a similar composition. However, only fraction M1:3 (Fig. 2) showed feeding deterrent activity. This is possibly due to the absence of the feeding stimulant, 5-(hydroxymethyl)-2-furaldehyde, which was detected only in the less active fraction 2 (M1:2, Fig. 2).

In conclusion, eleven new active compounds were found in this study. Two of these, β -sitosterol and 5-(hydroxymethyl)-2-furaldehyde, are the first reported feeding stimulants for the pine weevil, while another two compounds, benzyl alcohol and 2-phenylethanol, were

found to be new antifeedants identified from several non-hosts of the weevil. Seven compounds belonging to either of the two classes of aliphatic esters of hexanoic acid or branched aliphatic alcohols were found to be active antifeedants. In addition, four earlier known antifeedant compounds were identified from four non-host plants. Our work suggests that further active compounds in the bark of these woody species remain to be identified.

Acknowledgment Elisabeth Marling is acknowledged for technical assistance and Dr. Tessa Pocock for comments on the manuscript. We are also grateful for financial support to the research program “Pheromones and kairomones for control of pest insects” from Mistra, the cross-disciplinary grant “Formulation of semiochemicals for plant protection against forest pest insects” from Formas, and from EU, “Objective 1: the region of south forest counties.”

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