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Origin of the defensive secretion of the leaf beetle *Chrysomela lapponica*

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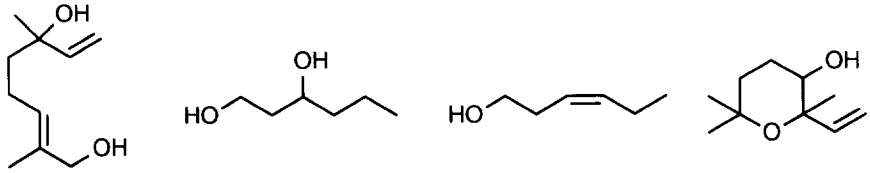
Abstract: The larvae of the European leaf beetle *Chrysomela lapponica* use esters of isobutyric acid and (*S*)-2-methylbutyric acid as defensive secretion. The acids are formed by the larvae from amino acids, as could be shown by experiments with labelled compounds. The alcohols taken up from the foodplant are either directly used for ester formation, like (*Z*)-3-hexenol (**3**) and 8-hydroxylinalool (**1**), or modified as 1-hydroxy-3-hexanone (**5**), which is reduced to the corresponding diol **2** prior to esterification. The cleavage of the respective glycosides or oxidation of fatty acids during feeding on the leaves leads to these alcohols. The ketol **5** and its isomer 6-hydroxy-3-hexanone have not been reported before from nature. © 1997 Elsevier Science Ltd.

The European leaf beetle *Chrysomela lapponica* feeds on both willow and birch trees. Its larvae use a defensive secretion exposed by 9 paired glands on its back when bothered. The volatile secretion can be retrieved after use. A population from Bavaria and Czech, which feeds naturally on birch leaves only, uses a mixture of more than 60 volatile components to deter predators.[1] This mixture is totally unrelated to the composition of the defensive secretion of other leaf beetles, which use either salicyl aldehyde or juglone, or iridoid monoterpenes as deterrents.[2] The only exception is the nearctic species *C. interrupta*, which secretion consists of a mixture of 2-phenylethyl isobutyrate and 2-phenylethyl 2-methylbutyrate.[3]

The bouquet of the glandular secretion of *C. lapponica* larvae feeding on birch consists of isobutyric and (*S*)-2-methylbutyric acids and esters of them with a wide variety of alcohols. Main components are esters with benzyl alcohol, 2-phenylethanol, and 8-hydroxylinalool (**1**) esterified at C-8. Diol diesters are also present, the most prominent of which are those with 1,3-hexanediol (**2**), accompanied by glycol, 1,2-propanediol, and 1,4-hexanediol esters. Other minor esters are those formed with (*Z*)-3-hexenol (**3**), and the pyranoid linalool oxides **4**. Larvae of the Bavarian and Czech population feeding on willow in a laboratory experiment show a



1 **2** **3** **4**

Figure 1. Alcohols occurring in esters of the defensive secretion of *Chrysomela lapponica*.

similar mixture, but contain additionally benzoic acid and the respective esters, while esters of the linalooloxides and 1,4-hexanediol are missing. In addition, esters of geraniol are present as minor constituents.[1] Surprisingly, larvae of a Finnish population feeding in the field exclusively on willow contain only small amounts of the esters, but large amounts of salicyl aldehyde, which is nearly absent in the secretion of Middle European individuals.[4] Salicyl aldehyde is the only or major component of the defensive secretion of many leaf beetles feeding on willow,[2] which form it by uptake of the glycoside salicin, enzymatic cleavage, and oxidation.[5]

In this paper we would like to present details on the origin and biosynthesis of the *C. lapponica* esters.

Composition of leaves of *Betula pendula* and *Salix fragilis*

To elucidate which compounds are ingested by feeding *C. lapponica* larvae, leaves were analyzed for free and glycosidically bound alcohols present during the feeding process. In a first set of experiments, undamaged birch leaves were macerated and stirred with water. After centrifugation, the aqueous phase was either extracted with CH_2Cl_2 or supplied to solid phase extraction using Extrelut® (see experimental section for details). These extracts were analyzed by GC/MS. A second set of experiments were performed to determine glycosidically bound alcohols. For this purpose, leaves were macerated under liquid nitrogen and introduced into boiling water to destroy degrading enzymes. This could also be achieved by micro wave treatment of the leaves. The aqueous phase containing the glycosides was freed from alcohols and other lipohilic material by extraction.[6] Control experiments showed the absence of such compounds in the water phase after this treatment. The enzymes α- and β-glucosidase, as well as β-galactosidase were added and the mixture stirred overnight. Extraction of the liberated aglyca was performed by liquid/liquid extraction or use of Extrelut®. The results are shown in Table 1 and Figure 3.

During mechanical disruption of intact leaves several enzymatic degradation processes start which lead to the formation of typical green leaf components, such as (*E*)-2-hexenal or (*Z*)-3-hexenol (**3**), formed from unsaturated fatty acids.[7] Such common compounds are present in the extracts of macerated birch and willow leaves as well (see Table 1). In addition, a main component of such extracts could be identified as 1-hydroxy-3-hexanone (**5**). It is accompanied by small amounts of its isomer, 6-hydroxy-3-hexanone. These substances have not been reported from nature before and may be formed by oxidation of unsaturated fatty acids, followed by chain cleavage. The corresponding diol **2** could be observed in trace amounts in one experiment, only. Whether it is present in macerated leaves remains to be confirmed.

The analysis of extracts of leaves with inactivated enzymes showed, that free alcohols are present in the leaves in minor amounts, only. They are mostly bound as glycosides, as can be seen from the results of the enzymatic transformation. The principle aglyca of the glycosides are (*Z*)- and (*E*)-8-hydroxylinalool (**1**), components of the known birch glucosides betulalboside A and B.[8] The *R/S* ratio of **1** could be determined by GC using a chiral cyclodextrine column to be 41 : 59. To our knowledge, no other glycosides of low

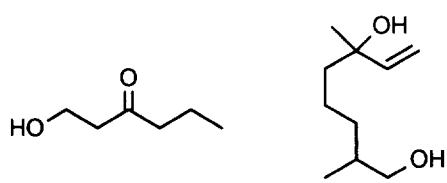
Table 1. Compounds identified in extract of macerated leaves (mac) or leaf extracts after glycosidase treatment (glu) of *Betula pendula* (birch) or *Salix fragilis* (willow).

		Birch		Willow	
Peak[1]	compound	mac	glu	mac	glu
1	hexanal	+		++	
2	(*E*)-2-hexenal	+++		+++	
3	(*E*)-2-pentenol	++		++	
4	hexanol	++	+	++	+
5	(*Z*)-3-hexenol (**3**)	++	++	++	+
6	(*E*)-2-hexenol	++		++	
7	acetic acid	++			
8	6-methyl-5-hepten-2-ol	+	+	+	+
9	1-hydroxy-3-hexanone (**5**)	++		++	
10	B95, M124[2]	++			
11	linalooloxide (**4**)	+	++	+	+
12	2-hexen-4-olide	+			
13	6-hydroxy-3-hexanone	+			
14	geraniol	+	+	+	++
15	benzyl alcohol	++	+++	+	++
16	2-phenylethanol	++	+++	++	++
17	B57, 85[2]	+++		++	
18	eugenol	++	+	++	++
19	8-hydroxy-6,7-dihydro-linalool (**6**)	++	++	+	+
20	(*Z*)-8-hydroxylinalool	++	+++	+	+
21	(*E*)-8-hydroxylinalool (**1**)	+++	+++	++	+++
22	6-hydroxy-2-cyclohexenone			+++	+++
23	1,2-cyclohexanediol			+	+++

[1]Peak numbers refer to Figure 3.

[2]Unknown compounds. Numbers denote characteristic ions. M: molecular ion; B: base peak.

molecular weight alcohols have been reported from birch before. Nevertheless, glycosides of (Z)-3-hexenol (**3**), geraniol, benzyl alcohol, and 2-phenylethanol are proposed to be common components of green plants,[9] while glycosides of the pyranoid linalooloxides occur only occasionally.[10] The monoterpene 8-hydroxy-6,7-dihydrolinalool (**6**)[11] has not been reported before from plants, but has been found by us in pheromone glands of *Danaus* butterflies.[13] Its absolute and relative configuration remains to be elucidated. Besides the compounds listed in Table 1, considerable amounts of phenols, accompanied by flavonoids and carotenoid degradation



5 **6**

Figure 2. New compounds from birch leaves.

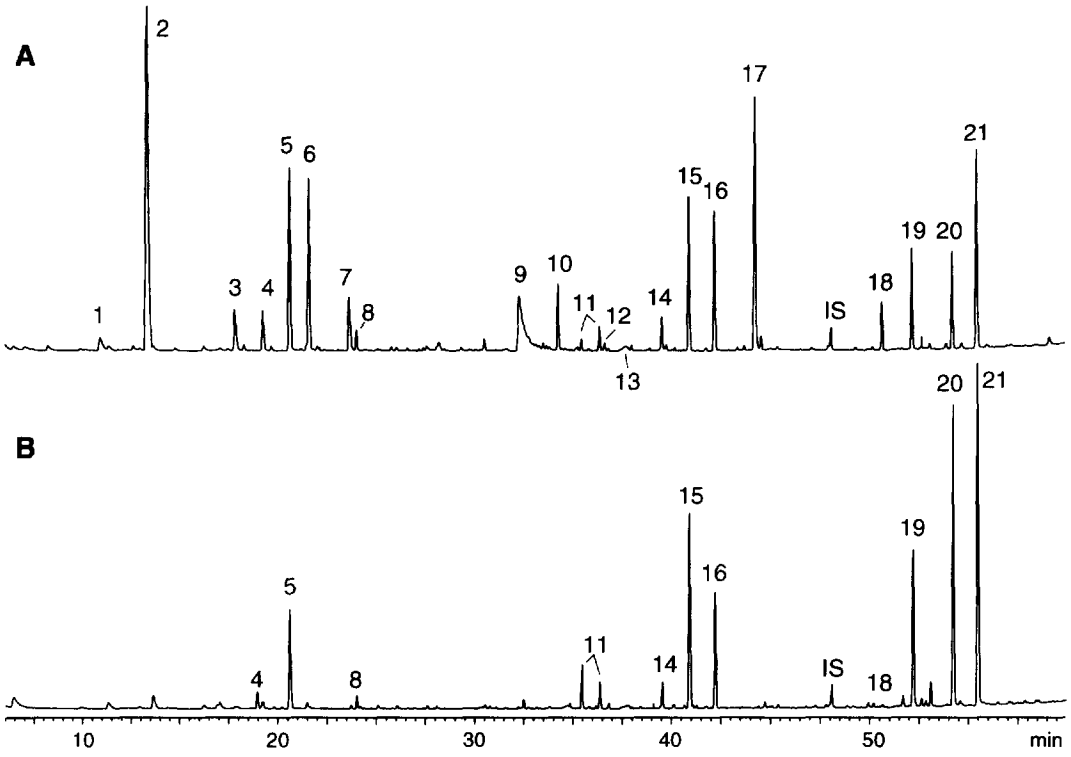


Figure 3. Gas chromatogram (30 m FS-FFAP, 5 min at 50 °C, then with 3 °C /min to 220 °C) of extracts form macerated *Betula pendula* leaves (**A**) and leaf extracts after glycosidase treatment (**B**). Peak numbers refer to Table 1. IS: internal standard methyl pentadecanoate

products were liberated during maceration and even more so after enzyme treatment. They have not been analyzed by us in detail, because they are mostly not transformed into defensive compounds by the beetle.[12]

Identical experiments were performed with *Salix fragilis* leaves. Surprisingly, nearly the same low molecular weight compounds could be identified in the samples (see Table 1), although in different proportions. The main differences in the macerated leaf samples are the higher amount of geraniol and the lower amount of the linalooloxides. By enzymatic cleavage, large amounts of **1** (*R/S* ratio = 31 : 69), together with salicyl alcohol and other phenolics, as well as oxygenated cyclohexane derivatives like 1,2-cyclohexanediol are liberated.

Experiments with labelled compounds

To test the ability of the larvae to synthesize their defensive compounds, several labelled precursors were injected into the larvae and the secretion analyzed after 24 h by GC-MS. Application of [D_8]valine furnished labelled isobutyrates. As an example, the mass spectrum of 1,3-hexanediyl diisobutyrate is presented (see Figure 4). Peaks shifted by the incorporation of seven deuterium atoms (one is lost during transformation of valine into isobutyric acid) are clearly visible. The incorporation rate is $\approx$ 10 %, as can be calculated from the ratio of

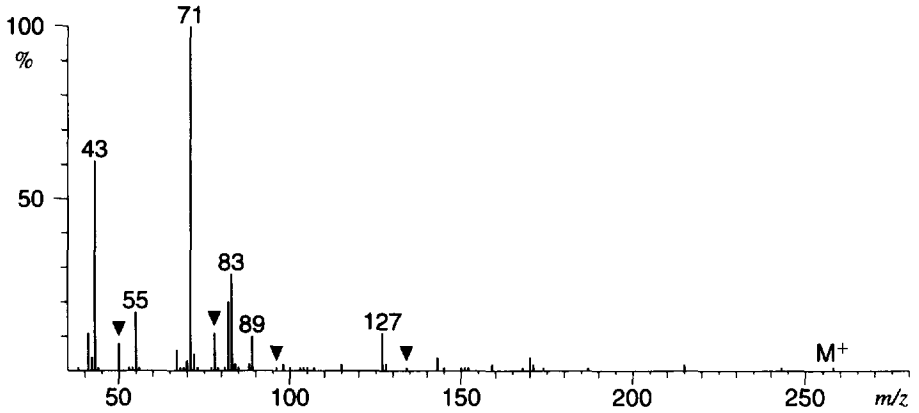


Figure 4. Mass spectrum of 1,3-hexanediyl diisobutyrate obtained from the larval secretion after application of [D_8]valine. Ions arising from deuterium incorporation are marked (▼).

the ions m/z = 71 and 78. Thus, valine is transformed into isobutyric acid and further into isobutyrates by the beetle. It can be assumed that (*S*)-2-methylbutyric acid is similarly formed from leucine, as has been shown for other insects.[14]

To test whether free alcohols as (*Z*)-3-hexenol (**3**), which is liberated during the disruption of green leaves,[15] are incorporated into the esters, a labelled form was synthesized. Thus, 3-butyn-1-ol was protected with dihydropyran. After lithiation, reaction with [D_5]ethyl tosylate furnished protected [D_5]-3-hexynol. This alkine was hydrogenated using Lindlar's catalyst and the protecting group removed under acidic conditions. The obtained pure [5,5,6,6,6-D_5]-(*Z*)-3-hexenol was injected into *C. lapponica* larvae. The analyses of the secretion revealed, that the alcohol is incorporated easily into the esters (incorporation rate ≈ 40 %, see Figure 5). The 1,3-hexanediyl diesters were not labelled. This shows, that **3** is not metabolized to 1,3-hexanediol in the larvae as has been suggested by us earlier.[1]

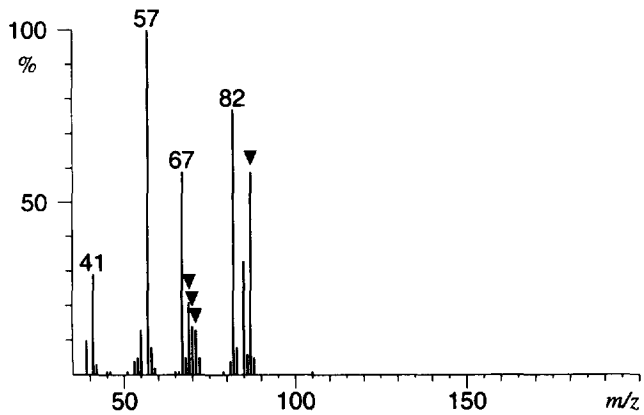


Figure 5. Mass spectrum (*Z*)-3-hexenyl 2-methylbutyrate obtained from the larval secretion after application of [5,5,6,6,6-D_5]-(*Z*)-3-hexenol. Ions arising from deuterium incorporation are marked (▼).

Nevertheless, not all alcohols are taken up from the foodplant during feeding. After application of [D_5]phenylalanine, a low incorporation of deuterium into 2-phenylethyl esters could be observed (0.5 %). The larvae is obviously able to transform the amino acid into 2-phenylethanol which is then transformed into the respective esters. As indicated by the low incorporation rate, only a small amount of 2-phenylethanol may be formed by this pathway. The major amount stems obviously from the foodplant.

1-Hydroxy-3-hexanone (**5**) is produced when birch or willow leaves are macerated. It may be the precursor of 1,3-hexanediol and we needed a labelled form to test this hypothesis. The synthesis starts from ethyl 3-oxohexanoate, which was protected as a dioxolane and the resulting ester reduced using $LiAlD_4$. After removal of the protecting group under acidic conditions, the pure [1,1-D_2]-1-hydroxy-3-hexanone could be obtained by column chromatography. Injection into the larvae leads to highly labelled 1,3-hexanediyl esters in the secretion (incorporation rate $\approx$ 75 %, see Figure 6). The larvae possess reducing activity and are able to convert **5** into 1,3-hexanediol, the precursor of the respective esters. The other components of the secretion were present in low amounts, only, in these samples. It indicates that the relative proportion of the esters depends largely on the availability of the alcohol precursors.

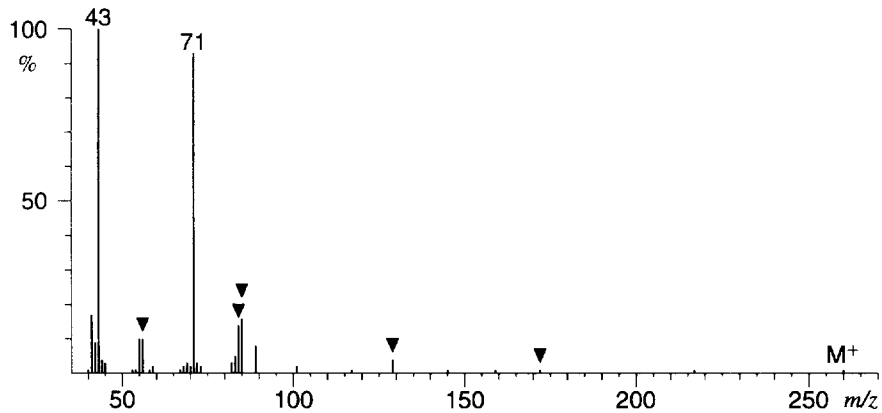


Figure 6. Mass spectrum 1,3-hexanediyl diisobutyrate obtained from the larval secretion after application of [1,1-D_2]-1-hydroxy-3-hexanone. Ions arising from deuterium incorporation are marked (▼). The characteristic double peak at *m/z* = 82/83 (see Figure 4) shifts largely to *m/z* = 84/85 due to the incorporation of two D-atoms.

Discussion

From the described results a general scheme for the formation of the defensive compounds of the larvae could be established (see Figure 7).

The isobutyric and 2-methylbutyric acids are formed by the insects. In contrast, benzoic acid, which esters occur only in larvae feeding on willow, is likely to be taken up from the leaves, because it can be found in them.[16] The alcohols used originate mostly from the hostplant. Except for the green leaf compounds, all alcohols are

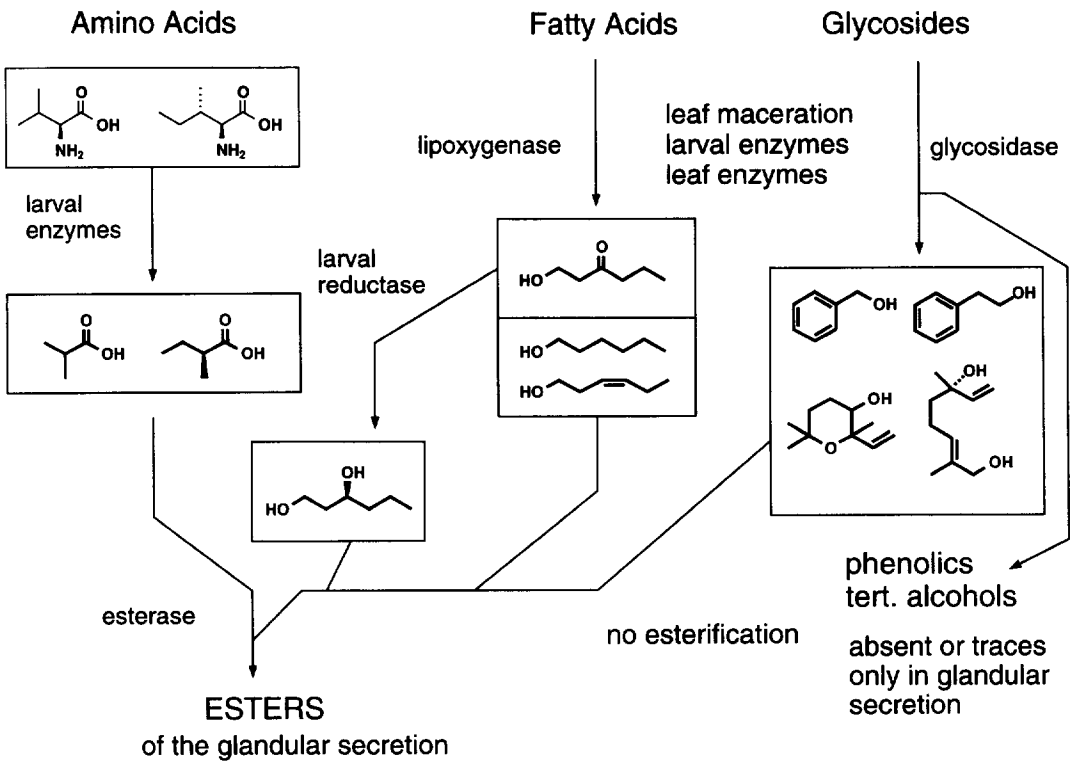


Figure 7. Metabolic transformations of leaf compounds by *Chrysomela lapponica*

present in the leaves as glycosides. Only the occurrence of glycosides of **1** in birch and of **3**, benzyl alcohol, 2-phenylethanol, 1,2-cyclohexanediol, and geraniol in willow have been described earlier.[9,16] The larvae takes up the alcohols or glycosides during feeding. As shown by our results, mechanical disruption of the leaves cleaves some glycosides. Whether the larvae itself possesses glycosidases is unknown, but seems likely because other Chrysomelidae are known to contain glucosidases.[17,18] *Plagiodera versicolora* and *Gastrophysa viridula* also store 8-hydroxygeraniol-8-*O*-β-D-glucoside as precursor for the synthesis of defensive iridoids.[18,19] This glucoside is closely related to the betulalbosides containing **1** as aglycon. Different families obviously use the same group of compounds, but metabolize them differently. In addition, some alcohols like **5** are reduced by the larvae to a suitable alcohol precursor, and other alcohols can be synthesized de novo, as shown by the formation of 2-phenylethanol. Enantiomeric discrimination may also take place, because in the leaves (*S*)-**1** predominates, while its larval esters are predominantly formed from the (*R*)-enantiomer.[1]

As an overall pattern, primary and secondary alcohols are esterified, while tertiary and phenolic hydroxyl groups remain free. Compounds containing both phenolic and aliphatic hydroxyl groups, like rhododendrol (4-(4-hydroxyphenyl)-2-butanol) are transformed into esters to a minor extent, only.[1] Despite the fact that relatively large amounts of salicyl alcohol occur in the secretion of larvae feeding on willow, no esters of it

could be detected. Also, some prominent aliphatic alcohols of willow leaves, like 1,2-cyclohexanediol, are not esterified, and do not even occur in the defensive secretion. Thus, certain ways of uptake of host components into the glands must exist, which excludes some compounds, and leads to similar mixtures of defensive esters when different foodplants are used.

EXPERIMENTAL

General Methods

High and low resolution EI mass spectra (70 eV) were recorded with a VG 70/250 S mass spectrometer coupled to a Hewlett-Packard HP 5890 A gas chromatograph. For determination of incorporation rates, all scans of the respective compounds were summarized (Figures 4-6 show such spectra). Deuterated compounds eluted earlier than unlabelled compounds. For example, diesters like 1,3-hexanediyl diisobutyrate eluted as three partly separated peak, which were summarized. ^{1}H NMR spectra were obtained with Bruker WM 400 and AC 250P instruments in $CDCl_3$ and TMS as internal standard if no other solvent is specified. Analytical GLC analyses were carried out with a Carlo-Erba Fractovap 2101 gas chromatograph with a flame-ionization detector and split/splitless injection. Enantiomer separations were performed using a 25 m fused silica column with octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrine (50 % in OV-1701) as chiral phase.[20]

Leaf maceration experiments

Undamaged leaves were collected from *Betula pendula* and *Salix fragilis* trees near the Elbe river or in the Botanical Garden in Hamburg and stored at -40 °C until use. Freeze drying prior to storage did not furnish other results in the following experiments and was therefore not applied. Portions of 5 g leaves were macerated under liquid nitrogen cooling and stirred with 15 ml H_2O overnight in a closed 100 ml vial. The resulting slurry was centrifuged and 3.5 ml of the water phase applied to a column filled with Extrelut® of corresponding capacity. The volatiles were collected with 9 ml CH_2Cl_2. Finally, the solvent was evaporated at 42 °C to a volume suitable for GC/MS analyses.

Enzymatic transformations

A portion of 10 g of birch or willow leaves were macerated under liquid nitrogen and added to 20 ml boiling water. After 1 h, the mixture was centrifuged, and the water phase extracted three times with CH_2Cl_2. The water phase was subjected to a vacuum for a short time to remove dissolved CH_2Cl_2 and divided into two parts. To one part β-glucosidase (5 mg, 25 units, Sigma, G-0395), α-glucosidase (5 units, Sigma, G-7256), and β-galactosidase (10 units, Sigma G-6008) were added. The mixture was stirred at 30 °C overnight. The resulting suspension was treated as described above to yield an CH_2Cl_2 extract suitable for GC-MS analysis. No enzymes were added to the other part which served as a control. Alternatively, inactivation of enzymes was reached by the treatment of whole leaves (5 g) in a household microwave for 1 minute at 800 Watt prior to maceration.

Experiments with labelled compounds

The exocrine secretion of second and third instar larvae of the Czech population of *C. lapponica* feeding on birch were "milked" with bits of filter paper in order to empty their dorsal gland reservoirs. They were stored in CH_2Cl_2 as control. Groups of 15 larvae with emptied gland reservoirs were used for each labelled precursor ([D_8]-L-valine (Cambridge Isotopes); [D_5]-(Z)-3-hexenol; [D_5]-L-phenylalanine (Cambridge Isotopes); [D_2]-1-hydroxy-3-hexanone). About 1 μl of aqueous solutions of each precursor were injected into the ventral intersegmental folds of the abdomen of the larvae by use of a micromanipulator with microcapillaries. The larvae were allowed to feed on birch leaves for 24 h. After this time, the newly produced secretion was collected on filter paper which were stored in CH_2Cl_2. The pooled samples were analyzed by GC-MS.

[5,5,6,6,6-D_5]-(Z)-3-hexenol To a stirred solution of 2.25 g (14.6 mmol) 1-(2-tetrahydroypyranyloxy)-3-butyne (prepared from 3-butyn-1-ol and tetrahydropyran by standard procedures[21]) were added 10.7 ml of a 1.6 M solution of *n*-butyllithium in hexane at –40 °C. After 1 h, the mixture was warmed to –10 °C and 3 g (14.6 mmol) [D_5]ethyl tosylate (prepared from [D_6]ethanol and tosyl chloride by standard procedures[21]) added. The mixture was heated to 60 °C for 4 h. After cooling, water was added and the mixture extracted three times with diethyl ether. The etheral phase was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. The crude product was purified by chromatography on silica to yield 600 mg [5,5,6,6,6-D_5]-1-(2-tetrahydropyranyloxy)-3-hexyne (23 % yield). This product was subjected to hydrogenation using Lindlar's catalyst[21] and the product immediately treated with acidic methanol to cleave the acetal.[21] ^{1}H NMR (CD_2Cl_2, 400 MHz) σ 2.29 (quart, 2H, J = 1.5 Hz, J = 6.8 Hz, H-2), 3.59 (m, 2H, H-1), 5.33 (dt, 1H, J = 10.8 Hz, H-3), 5.53 (bd, 1H, H-4); MS *m/z* 41 (29), 42 (37), 43 (87), 44 (100), 45 (45), 46 (19), 47 (15), 57 (32), 58 (14), 59 (13), 60 (21), 61 (5), 68 (24), 69 (28), 70 (29), 71 (32), 72 (11), 74 (30), 75 (15), 85 (13), 86 (39), 87 (12), 105 (3, M^+); exact mass, *m/z* 105.1222 (calcd for $C_6H_7D_5O$, *m/z* 105.1202).

[1,1-D_2]-3,3-ethylenedioxy-hexanol Ethyl 3-oxohexanoate was converted into its 1,3-dioxolane derivative by heating with ethyleneglycol and *p*-toluenesulfonic acid in toluene for three days under reflux according to standard procedures[21] (yield 15 %, b. p. 95-98 °C at 0.1 torr). Reduction of the product with $LiAlD_4$ in diethyl ether[22] furnished [1,1-D_2]-3,3-ethylenedioxy-hexanol in 72 % yield. ^{1}H NMR (400 MHz) σ 0.92 (t, 3H, J = 6.2 Hz, H-6), 1.39 (sex, 2H, J = 6.6 Hz, H-5), 1.62 (t, 2H, J = 6.6 Hz, H-4), 1.92 (s, 2H, H-2), 3.99 (m, 4H, CH_2–O); exact mass, *m/z* 162.1241 (calcd for $C_8H_{14}D_2O_3$, *m/z* 162.1225).

[1,1-D_2]-1-hydroxy-3-hexanone A mixture of [1,1-D_2]-3,3-ethylenedioxy-hexanol (250 mg, 1.54 mmol), water (3.8 ml), and acetic acid (17.5 ml) was stirred for 4 h at room temperature. The solvent was removed in vacuo after neutralization with satd. $NaHCO_3$ and the residue was triturated three times with diethyl ether. After drying ($MgSO_4$) and removal of the solvent the product was obtained by chromatography on silica. ^{1}H NMR (250 MHz) σ 0.92 (t, 3H, J = 6.2 Hz, H-6), 1.64 (sex, 2H, J = 6.6 Hz, H-4), 2.45 (t, 2H, J = 6.6 Hz, H-4), 2.67 (s, 2H, H-2); MS *m/z* 41 (17), 42 (9), 43 (58), 44 (6), 47 (8), 57 (100), 58 (13), 71 (28), 72 (17), 75 (27), 83 (6), 90 (7), 99(4), 100 (5), 118 (0.5, M^+); exact mass, *m/z* 118.0997 (calcd for $C_6H_{10}D_2O_2$, *m/z* 118.0963).

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