



A common pathway for metabolism of 4-hydroxybenzylglucosinolate in *Pieris* and *Anthocaris* (Lepidoptera: Pieridae)

Niels Agerbirk^{a,*}, Caroline Müller^b, Carl Erik Olsen^a, Frances S. Chew^c

^a Department of Natural Sciences, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

^b Julius-von-Sachs-Institut für Biowissenschaften, Universität Würzburg, Julius-von-Sachs-Platz 3, D97082 Würzburg, Germany

^c Department of Biology, Tufts University, Medford, MA 02155, USA

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Abstract

Caterpillars of *Pieris rapae* L. (Lepidoptera: Pieridae) convert 4-hydroxybenzylglucosinolate (sinalbin) in brassicaceous plants into 4-hydroxybenzylcyanide sulfate (HBC sulfate), with 4-hydroxybenzylcyanide (HBC) as intermediate. This apparently serves as a detoxification, because alternative formation of a mustard oil is avoided. We confirmed the capacity of *P. rapae* to convert the intermediate HBC into HBC sulfate. Four additional Pieridae – *Anthocaris cardamines* L., *Pieris virginiensis* Harris, *Pieris napi oleracea* Edwards and *Pieris brassicae* L., likewise excreted HBC sulfate after ingesting leaves with topically added HBC or leaves naturally containing sinalbin and myrosinase, but not after ingesting control leaves devoid of HBC and sinalbin. We confirmed the capacity of the most distantly related pierid species (*A. cardamines*) for converting ingested (topically added) sinalbin into HBC sulfate. Larvae of two non-pierid Brassicaceae-feeding insects, the oligophagous sawfly *Athalia rosae* L. (Hymenoptera: Tenthredinidae) and the polyphagous moth *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), did not excrete HBC sulfate after ingesting sinalbin-containing leaves or topically added HBC.

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1. Introduction

The glucosinolate–myrosinase defence system is characteristic for plants of the Brassicaceae and related families in the order Capparales (Rodman et al., 1998). The system consists of β -thioglucosidases (myrosinases), stored spatially separated from their natural substrates, glucosinolates (Andréasson and Jørgensen, 2003). Upon tissue damage, the enzyme and substrate come into contact, and glucosinolates are hydrolysed into isothiocyanates (mustard oils) or other products. A number of insect species have developed metabolic adaptations that enable them to reduce the

* Corresponding author. Tel.: +45 35 28 24 38; fax: +45 35 28 23 50.
E-mail address: nia@kvl.dk (N. Agerbirk).

formation of mustard oils from the glucosinolate–myrosinase system (Müller et al., 2003; Rossiter et al., 2003; Wittstock et al., 2004, and references cited therein).

It was recently reported that *Pieris rapae* and *Pieris brassicae* possess a nitrile specifier protein (NSP), that enables them to avoid the formation of toxic mustard oils while feeding on host plants containing this defence system (Wittstock et al., 2004). In the presence of NSP, the formation of toxic mustard oils (Agrawal and Kurashige, 2003) is replaced by the formation of nitriles that are usually less toxic (Wittstock et al., 2003). The NSP appeared to be a crucial biochemical adaptation, allowing *P. rapae* and *P. brassicae* caterpillars to feed on a large number of plants containing the glucosinolate–myrosinase defence system. All other *Pieris* sp. also exclusively feed on glucosinolate-containing plants (reviewed by Chew and Renwick, 1995), suggesting that the remaining species in the genus might share this adaptation.

A taxonomically more distant pierid butterfly with a similar range of host plants is the orange tip, *Anthocharis cardamines* L. At least 37 plant species within the families Brassicaceae and Resedaceae have been recorded as host plants for *A. cardamines* in Europe (Courtney and Duggan, 1983). All these host plants are known or suspected to contain the glucosinolate–myrosinase system (Fahey et al., 2001), suggesting that *A. cardamines* also possesses a biochemical adaptation to this defence system.

Recent phylogenies based on two blocks from mitochondrial DNA have been proposed for the lepidopteran family Pieridae including *Colias* and its relatives (Pollock et al., 1998) and *Pieris napi* and its relatives (Chew and Watt, in press). These phylogenies show four points relevant to the current investigation: (1) within the subfamily Pierinae, the Anthocharini [(=Euchloini), including *A. cardamines*] and the Pierini are sister tribes; (2) *Pieris*, represented in that study by *P. napi*, *P. brassicae* and *P. rapae*, forms a monophyletic clade within the tribe Pierini; (3) all taxa of '*P. napi*' relatives in North America form a monophyletic clade that is sister to *P. napi* L. from Stockholm, Sweden; and (4) *Pieris virginianensis* and *Pieris napi oleracea* belong to distinct subclades of the "*P. napi*" complex in North America; despite extensive sympatry, they are not each other's closest relatives.

The nitriles resulting from the detoxification of the glucosinolate–myrosinase system by NSP are in at least two cases excreted without further metabolism (Wittstock et al., 2004). But the nitrile 4-hydroxybenzylcyanide (HBC) that is formed from detoxification of 4-hydroxybenzylglucosinolate (sinalbin) is apparently sulfated before excretion of the conjugate, HBC sulfate (Fig. 1; Müller et al., 2003; Wittstock et al., 2004). We confirmed the ability of *P. rapae* caterpillars to metabolise HBC into HBC sulfate, using HBC ingested with a leaf substrate or with an artificial diet.

To investigate the occurrence of this two-step metabolism of sinalbin among other *Pieris* and insects that feed on Brassicaceae, we examined three other *Pieris* species in the tribe Pierini, *A. cardamines* in the tribe Anthocharini, and two phylogenetically distant species, a moth (Lepidoptera: Noctuidae) and a sawfly (Hymenoptera: Tenthredinidae).

2. Materials and methods

2.1. Plants, insects and chemicals

All plants used belonged to the Brassicaceae. Seeds of white mustard (*Sinapis alba* L.) cv. Salvo were from Advanta Seeds B.V.; The Netherlands. Seeds of wintercress [*Barbarea vulgaris* var. *arcuata* (Opiz.) Fries (G-type, accession B44, Agerbirk et al., 2003)] were a gift from J.K. Nielsen, Dept. of Nat. Sci., Royal Vet. and Agric. Univ., Denmark. Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr.) was obtained from local food stores. Siliques

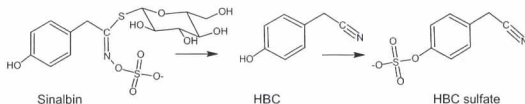


Fig. 1. Sinalbin and detoxification products. Reaction arrows indicate the proposed metabolic pathway (unbalanced) in the pierid caterpillars investigated. HBC, 4-hydroxybenzylcyanide.

of charlock (*Sinapis arvensis* L.) were collected from a natural population at Lake Utterslev Mose, Denmark. Foliage and siliques of garlic mustard (*Alliaria petiolata* (Bieb.) Cavara and Grande) were collected from natural populations at Lake Utterslev Mose, Denmark and at the river Main, Germany. Cabbage, *Brassica oleracea* L. cv. Primax was grown from seed (www.johnnyseeds.com, Albion, Maine 04910, USA) and hare's ear mustard *Conringia orientalis* (L.) Dumortier was collected in Wisconsin, USA from an apparently sterile vegetatively reproducing clone. For logistic reasons, plants were grown at three different localities, in all cases at long day conditions (16–18 h day length) at 18–24 °C, and used after 5–8 weeks. As the resulting leaves might differ in chemical composition, the locality of *S. alba* growth is indicated (with the initials of the relevant co-author) in Table 1.

P. rapae were reared from lab-collected eggs of wild females caught near Texas Falls (Hancock Co.), Vermont, USA, or from a laboratory colony kept for several generations in a 1 m³ cage at the same temperature and light conditions as used for plant cultivation, feeding the adults a 10% sucrose solution and using wintercress as oviposition substrate and larval food plant. The founding *P. rapae* individuals were obtained from eggs kindly supplied by Joop J. A. van Loon, Wageningen University, The Netherlands. Eggs of *P. brassicae* were collected on *Tropaeolum majus* in Copenhagen, Denmark, hatched caterpillars were reared on either *S. alba* or (for HBC-spiked exp.) on *T. majus*. Wild females of *P. napi oleracea* Harris and *P. virginensis* Edwards were collected from Lee (Berkshire Co.), Massachusetts, USA. Eggs oviposited in the lab were reared at ambient room temperature (19–23 °C), at 15:9 h l:d. Caterpillars of *A. cardamines* were collected in mid-June 2004 on siliques of solitary individuals of garlic mustard growing along the shore of Lake Utterslev Mose, Denmark, as well as along the river Main, Germany. Only one caterpillar was observed per plant. The identity of the caterpillars was confirmed by Peter Esbjerg, Dept. of Ecology, Royal Vet. and Agric. Univ., Denmark, based on comparison of caterpillar and pupal morphology with published illustrations (Spuler, 1910; Porter, 1997; Jacobi, 2004). Younger caterpillars appeared as illustrated by Spuler (1910), older appeared as illustrated by Porter (1997) and Jacobi (2004). Sawfly larvae were taken from a stock of *Athalia rosae* L. (Hymenoptera: Tenthredinidae) that was established from a field collection in Germany and kept in culture at 20 °C, a relative humidity of 70% and 16:8 h l:d. *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) were received as eggs from Bayer (Monheim, Germany) and kept in quarantine in the lab. Hatching larvae were kept on artificial diet. After 5 days, they were transferred to brassicaceous plants.

Sinalbin was from www.glucoisolates.com, Frederiksberg, Denmark. Sinigrin was from Karl Root, Karlsruhe, Germany. HBC was from Sigma–Aldrich, Steinheim, Germany. HBC sulfate was isolated as previously described (Wittstock et al., 2004).

2.2. Feeding experiments with artificial diet

"High wheat germ diet" (Bell et al., 1981) with or without HBC (5 µmol/g) was prepared as the previously described bioassay diet (Agerbirk et al., 2003), except that the wheat germ was from Naturdrogeriet, Hørning, Denmark. HBC was dissolved in boiling H₂O before addition of agar and other ingredients. In order to obtain naïve caterpillars able to accept a diet without glucosinolates (Renwick and Lopez, 1999), eggs were collected with a setup consisting of a 300-ml beaker covered by a cabbage leaf, with the leaf edges wrapped around the opening of the beaker with parafilm. Butterfly females oviposited sitting on the edge of the horizontal leaf surface and extending their abdomen downwards. Parafilm with eggs was transferred to artificial diet for rearing of naïve caterpillars.

A 0.7–1.2 g lump of HBC-spiked or control diet was weighed and placed in a cage (25 ml transparent plastic container). To each cage with either HBC-spiked diet or control diet, one naïve late instar caterpillar was introduced and allowed to feed on the diet for two 24-h periods. On day 3, remaining diet was removed, weighed, and replaced with a lump of control diet to all caterpillars, and frass was collected for one additional 24-h period. The mass of diet ingested by each caterpillar offered HBC-spiked diet was calculated as the measured diet wt. loss (typically 0.1–0.2 g) corrected for the mean wt. loss in cages without caterpillars (0.018 g). The amount of HBC ingested was calculated from the mass of diet ingested multiplied with the known concentration. For the determination of excreted HBC sulfate and estimation of excreted HBC, collected frass was freeze dried and extracted as described below. Samples of control- and HBC-spiked diet were also incubated, weighed, frozen, freeze dried and extracted as described for frass samples, followed by determination of any HBC sulfate. Extracts of frass and diet from this experiment contained considerable amounts of a gel like material, which was avoided on columns by prolonged centrifugation and use of the resulting transparent supernatant.

2.3. Feeding experiments with leaves

Caterpillars were reared on the relevant host plants for at least 24 h, frass produced during this initial rearing was discarded. During continued rearing, frass was collected daily, frozen, and freeze dried. Dried frass was stored frozen until extraction (except brief periods at ambient temperature when mailed between laboratories). Spiking with HBC was carried out by distributing 200 μ l of HBC (25 mM in EtOH:H₂O (1:1)) on the upper surface of a leaf (either wintercress, Chinese cabbage, or garlic mustard, as indicated). After evaporation of the solvent, the spiked leaf was offered to a caterpillar (or a sawfly larva) or incubated in parallel without caterpillar. After complete or almost complete ingestion of the spiked leaf, the caterpillar was offered an unspiked leaf for an additional 24 h. Frass was collected and treated as above. Control experiments with leaves treated with solvent only and spiked leaves incubated without caterpillars were carried out in parallel. Siliques of garlic mustard were spiked with sinalbin or HBC by painting solutions (HBC as above, or 5 mM aq. sinalbin) on the siliques attached to short sections of the plant. After evaporation of the solvent, plant sections (in total seven siliques per caterpillar) were offered to *A. cardamines* caterpillars in 500 ml cages. Frass was collected as described above, and control experiments with solvent only or without caterpillars were conducted.

2.4. Chemical analysis

Determination of glucosinolates and HBC sulfate by the desulfoglucosinolate method was essentially carried out as previously described (Müller et al., 2003; Wittstock et al., 2004). However, sinigrin (allylglucosinolate) was used as internal standard, because the previously used benzylglucosinolate was present as an endogenous glucosinolate in *S. alba* leaves. The relative response factor of HBC sulfate relative to the internal standard sinigrin (the factor to be multiplied with the desulfo HBC sulfate area before comparison with the desulfo sinigrin area) was experimentally determined: solutions in D₂O of pure potassium sinigrin and pure potassium HBC sulfate were mixed, and subjected to ¹H NMR (at 400 MHz, instrumentation as in Agerbirk et al., 2003). The molar ratio of HBC sulfate to sinigrin was found to be 1.034 from peak areas of selected protons [1/4 of the combined area of the aromatic HBC sulfate protons (7.32 ppm, "d", 2H and 7.43 ppm, "d", 2H) divided by the area of the sinigrin H2 signal (6 ppm, m, 1H)]. Aliquots (approximately 300 nmol, 300 nmol, 600 nmol and 900 nmol of each compound) of the mixture were subjected to the desulfoglucosinolate analysis method, and the HPLC area (229 nm) of the desulfo HBC sulfate peak relative to the desulfo sinigrin peak was found to be 0.8101 ($N = 4$, s.d. = 0.0129). The response factor of HBC sulfate was calculated as $1.032/0.8101 = 1.28$. This response factor was independent of using methanol or acetonitrile as HPLC eluent, and of using crude sulfatase (Müller et al., 2003) or sulfatase further purified according to Bjerg and Sørensen (1987) (results not shown). Reported quantitative levels of HBC sulfate were obtained with the desulfoglucosinolate method. As explained by Müller et al. (2003), detection of HBC after sulfatase-treatment of extracted metabolites bound to an anion exchanger is not conclusive evidence of the presence of HBC sulfate in the extract, because the sulfatase enzyme preparation may hypothetically contain additional enzyme activities, such as phosphatases or glucuronidases. The fact that the HBC conjugate extracted from *P. rapae* frass was indeed HBC sulfate had previously been demonstrated (Wittstock et al., 2004). We confirmed the presence of HBC sulfate in representative frass extracts of *A. cardamines* and *P. rapae* by LC–MS, as will be indicated in the text (Sections 3.1, 3.2, 3.3 and 3.4). The stability of sinalbin incubated on *A. petiolata* siliques was likewise confirmed by LC–MS (Section 3.4). This direct determination of HBC sulfate or sinalbin was carried out as follows: freeze dried frass or plant material was extracted in boiling MeOH:water (7:3). Crude extract (typically equivalent to 6 mg frass or plant material) was passed through a 0.5 μ m Supelclean LC-18 SPE column (from Supelco, PA, activated with 2 ml MeOH, equilibrated with 2 ml MeOH:water (1:1)), which was further eluted with 2×1 ml MeOH:water (1:1). The eluate was taken to dryness under an air stream, and the residue dissolved in 0.02 ml MeOH and 0.98 ml 0.02 M sodium acetate pH 5. Of the resulting solution, 4 μ l was injected for LC–MS analysis. Analytical LC–MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). An XTerra MS C18 column (Waters, Milford, MA; 3.5 μ m, 2.1×100 mm) was used at a flow rate of 0.2 ml/min. The mobile phases were A, H₂O:HCO₂H (99.9:0.1) (v/v) and B, MeCN:H₂O:HCO₂H (80:19.9:0.1) (v/v). The gradient program was 0–4 min, isocratic 2% B; 4–10 min, linear gradient 2–8% B; 10–30 min, linear gradient 8–50% B; 30–35 min, linear gradient 50–100% B; 35–40 min, isocratic 100% B. The mass spectrometer was

run in negative ion mode. Total ion current and ion traces for specific $[M - H]^-$ ions were used for locating compounds.

3. Results

3.1. Metabolism of HBC into HBC sulfate by *P. rapae*

To test the hypothesis of HBC as an intermediate in the metabolism of sinalbin by *P. rapae* caterpillars, we offered the caterpillars an artificial diet with and without added HBC. The presence and stability of HBC in the spiked diet was confirmed analytically (results not shown). Only "naïve" caterpillars that had never been exposed to glucosinolates accepted the wheat germ diet. Such caterpillars were reared to their fourth or fifth instar on the diet, and offered the HBC-spiked diet. HBC sulfate in the resulting frass accounted for 36 mol% of ingested HBC ($N = 5$, s.d. = 15 mol%). Considerable amounts of free HBC were also detected in the frass extracts by HPLC of the initial run-through from the anion exchange columns (results not shown). HBC sulfate was not excreted after ingestion of the artificial diet without added HBC ($N = 3$). The presence of HBC sulfate in frass from caterpillars offered the HBC-spiked diet was confirmed by LC-MS analysis of the extract: a peak with the correct retention time and m/z ratio (212) was detected, confirming the identity of the excreted metabolite. Taken together with the results reported by Wittstock et al. (2004), this result confirmed the capacity of *P. rapae* caterpillars to metabolise sinalbin in two steps: formation of the nitrile HBC, followed by sulfation of the phenol group and excretion of the resulting HBC sulfate.

3.2. Effects of food plants with or without sinalbin

Caterpillars of all five pierid species (*Pieris* spp., as well as *A. cardamines*), excreted appreciable amounts of HBC sulfate when reared on sinalbin-containing brassicaceous plant tissue (Table 1). But when reared on various brassicaceous plants (found to be devoid of sinalbin, results not shown), HBC sulfate was not detected in the frass. This suggested that the five pierid species share the pathway for sinalbin metabolism previously demonstrated in *P. rapae*. In the case of *A. cardamines* reared on white mustard, the presence of the intact sulfate ester HBC sulfate was directly confirmed by LC-MS of the native extract: a peak with the correct retention time and m/z ratio for HBC sulfate was detected (results not shown). Caterpillars of the non-pierid generalist herbivorous moth *S. frugiperda* also accepted

Table 1

Concentrations ($\mu\text{mol/g dw}$, mean (s.d.)) of HBC sulfate in frass from caterpillars reared on white mustard leaves or charlock siliques, which both contained sinalbin, or on Chinese cabbage, which was devoid of sinalbin

Species	Origin	N	HBC sulfate	Plant source
A: reared on white mustard leaves				
<i>P. rapae</i>	Vermont	2	33 (21)	FC
	Laboratory culture	4	35 (18)	NA
<i>P. napi oleracea</i>	Massachusetts	5	17 (32)	FC
<i>P. virginensis</i>	Massachusetts	3	26 (14)	FC
<i>P. brassicae</i>	Denmark	4	41 (17)	NA
<i>A. cardamines</i>	Denmark	1	25	NA
	Germany	3	8.0 (2.7)	CM
<i>S. frugiperda</i>	Laboratory culture	5	<0.1 (tr. or n.d.)	CM
B: reared on Chinese cabbage leaves				
<i>S. frugiperda</i>	Laboratory culture	5	<0.2 (tr. or n.d.)	Store
C: reared on charlock siliques				
<i>P. rapae</i>	Laboratory culture	1	35	Field
<i>A. cardamines</i>	Denmark	3	23 (1.3)	Field

The column "Origin" indicates the origin of the tested caterpillars. In the column "Plant source", initials (FC, NA or CM) indicate the exact conditions of *S. alba* cultivation, "Field" indicates field collected material, whereas "Store" indicates material from a food store (see "Section 2"). HBC sulfate was not detected after rearing *P. rapae* on cabbage ($N = 2$), *P. brassicae* on wintercress ($N = 2$), *P. napi* on cabbage, wintercress or hare's ear mustard ($N = 4$), or *A. cardamines* on garlic mustard ($N = 6$), and the mentioned food plants were likewise found to be devoid of sinalbin (s.d. = standard deviation; tr. = trace; n.d. = not detected).

white mustard leaves, but only a trace of a peak possibly corresponding to HBC sulfate was observed, and the trace peak had apparently no relation to sinalbin detoxification, because a similar peak was formed when the species was reared on Chinese cabbage, which was devoid of sinalbin (Table 1). Sinalbin was not sequestered intact in body tissue or haemolymph in *S. frugiperda* (results not shown). However, only traces of sinalbin were detected in frass from *S. frugiperda* reared on white mustard, suggesting that sinalbin was indeed metabolised (by plant myrosinase or caterpillar enzymes). Sinalbin was likewise absent or nearly absent in frass from the pierid species.

For logistic reasons, sinalbin-containing plants reared at different conditions were used in the experiments (Table 1). Appreciable levels of sinalbin (usually between 5 and 100 $\mu\text{mol/g}$ dry wt.) were observed in white mustard leaves grown at all three conditions, but as sinalbin levels vary significantly between individual plants and leaves, we did not attempt to correlate excreted HBC sulfate to sinalbin contents in ingested tissue. Levels of excreted HBC sulfate were similar for all the pierid caterpillars reared on white mustard grown at the conditions "FC" and "NA". Similar levels of HBC sulfate were also seen for *P. rapae* and *A. cardamines* reared on charlock siliques. There was a tendency for lower levels of HBC sulfate excreted from *A. cardamines* reared on white mustard grown at a third set of conditions (CM), but this could be due to lower levels of sinalbin at these conditions of growth. All in all, the data suggest quantitatively similar metabolism of sinalbin in all pierid species.

Later instars of *A. cardamines* often feed on siliques (Courtney and Duggan, 1983). As all our experiments with *A. cardamines* had to be initiated during the brief period where caterpillars were available, we decided to offer leaves to some individuals (for comparison with the other tested species), and siliques to other individuals. The caterpillars readily accepted both food items.

3.3. Effects of ingestion of HBC added to leaves

In the three plant species devoid of sinalbin, wintercress, garlic mustard, or Chinese cabbage, there was apparently a slight conversion of HBC to HBC sulfate when HBC-spiked leaves were incubated without caterpillars at the bioassay conditions (Table 2). However, levels of HBC sulfate in caterpillar frass were much higher than could be explained by plant-catalysed sulfation. The two tested species of *Pieris* metabolised a high proportion of ingested HBC into HBC sulfate, as previously found for *P. rapae* using artificial diet as substrate (Section 3.1). *A. cardamines* seemed to convert a lower proportion of ingested HBC into the sulfate ester, but this was still significantly higher than the plant-catalysed proportion, which was negligible with garlic mustard leaves (Table 2). When a solution of HBC was painted on siliques of garlic mustard, conversion to HBC sulfate was not detected in the absence of *A. cardamines* caterpillars, but after ingestion by the caterpillars, HBC sulfate was readily detected in the frass by both direct LC-MS and by the desulfoglucosinolate method (results not shown). In contrast, larvae of two non-pierid insects, *A. rosae* and *S. frugiperda*, did not excrete HBC sulfate after ingestion of HBC painted on leaves.

3.4. Confirmation of a causal relationship between sinalbin and HBC sulfate for *A. cardamines*

We considered it likely that excretion of HBC sulfate after ingestion of white mustard was due to metabolism of sinalbin. In the case of *P. rapae*, this had previously been shown using radiolabelled sinalbin (Wittstock et al., 2004).

Table 2
HBC sulfate (mol% of added HBC, mean (s.d.)) excreted by caterpillars (or produced by leaves in control experiments) after addition of 625 nmol HBC to leaves fed to caterpillars

Caterpillar	Leaf	N	Sample	HBC sulfate (mol%)
None	Wintercress	4	Leaf	2.7 (1.8)
<i>P. rapae</i>	Wintercress	4	Frass	45 (8)
<i>P. brassicae</i>	Wintercress	3	Frass	28 (10)
None	Garlic mustard	3	Leaf	tr. (mean = 0.1)
<i>A. cardamines</i>	Garlic mustard	3	Frass	5.1 (2.7)
None	Chinese cabbage	3	Leaf	tr. (mean = 0.3)
<i>S. frugiperda</i>	Chinese cabbage	2	Frass	tr. (mean = 0.2)
<i>A. rosae</i>	Chinese cabbage	4	Frass	n.d.

In the absence of a caterpillar ("None" in column 1), HBC sulfate was determined in an extract of the substrate leaf after incubation for the same time and at the same conditions as used for experiments with caterpillars. For this table, *P. rapae* were from the laboratory culture in Denmark, and *A. cardamines* were collected in Germany (s.d. = standard deviation; tr. = trace; n.d. = not detected).

However, in the case of the remaining species it might also be an effect of another difference between white mustard and the plant species without sinalbin used as controls. For this reason, we tested whether application of pure sinalbin to garlic mustard would likewise result in excretion of HBC sulfate. The experiment was carried out with the more distantly related of the pierid species; *A. cardamines*. Indeed, sinalbin was absent in the produced frass, while HBC sulfate was present (Fig. 2E–H). Control experiments showed that sinalbin painted on the siliques could be recovered after incubation for several days (Fig. 2B and D), that HBC sulfate was not formed in the absence of caterpillars (Fig. 2C), and that HBC sulfate was not excreted in control experiments where the caterpillars were offered siliques painted with solvent (water). The experiments based on painted siliques were only semiquantitative, as we could not control how much of the solution was painted onto the siliques, but we attempted to apply quantitatively similar volumes of painting solution to experiments with and without caterpillar feeding. We concluded that the excretion of HBC sulfate was due to ingestion of sinalbin, presumably metabolised by nitrile formation followed by sulfation.

The dominant endogenous glucosinolate in garlic mustard was sinigrin (dominant peak at 3 min in Fig. 2A, with $m/z = 258$). This glucosinolate was present in about 25-fold molar excess of topically added sinalbin, and was essentially absent from frass (Fig. 2E).

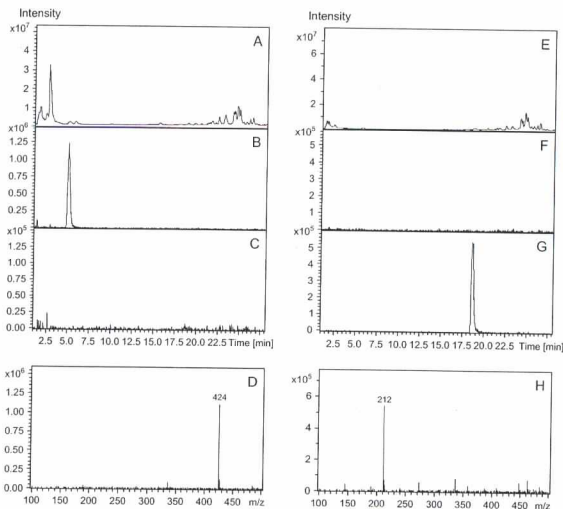


Fig. 2. Demonstration by LC–MS of conversion of sinalbin to HBC sulfate in *Anthracis cardamines*. A–D: analysis of an extract of garlic mustard siliques painted with sinalbin, incubated for two days in the absence of caterpillar in order to test the stability of sinalbin. E–H: analysis of an extract of *A. cardamines* frass after ingestion (during two days) of garlic mustard siliques painted with sinalbin. A and E: total ion LC–MS chromatogram. B and F: as A/E, but monitoring only $m/z = 424$ (sinalbin). C and G: as A/E, but monitoring only $m/z = 212$ (HBC sulfate). D: mass spectrum at time 5.0 min (sinalbin) from the chromatogram in A. G: mass spectrum at time 18.6 min (HBC sulfate) from the chromatogram in F.

4. Discussion

At the subfamily level, butterflies are associated with plant orders (Ehrlich and Raven, 1964). The subfamily Pierinae (including the tribes Pierini and Anthocharini) is mainly associated with Brassicaceae and related families in the Capparales containing glucosinolates (Ehrlich and Raven, 1964; Rodman et al., 1998; Fahey et al., 2001). Our finding, that exemplars of both tribes excrete a nitrile as metabolic product of a glucosinolate, suggests that detoxification by nitrile formation is (so far) coincident with the Capparales specialist habit in the Pierinae. This suggests that the nitrile formation trait evolved in a common ancestor of the subfamily Pierinae.

Other Lepidoptera do not share the nitrile formation trait. Although some other lepidopterous larvae do accept plants with the glucosinolate–myrosinase system, NSP was not detected in gut extracts of the generalist *Spodoptera littoralis* (Wittstock et al., 2004) reared on plants with this defence system. The diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) overcomes the defence system by a biochemically different mechanism, a sulfatase (Ratzka et al., 2002). Still other Lepidoptera are devoid of any biochemical adaptations to this defence system: when *Papilio polyxenes* F. (Lepidoptera: Papilionidae) larvae were fed sinigrin-infused leaves of their normal host celery, *Apium graveolens* L., feeding rates were not significantly affected but growth and development rates were substantially reduced; larval mortality was 100% at concentrations of 0.01% (of fresh weight) or higher (Erickson and Feeny, 1974; Blau et al., 1978).

A biochemically different adaptation to the glucosinolate–myrosinase system, namely sequestration, is known for several non-lepidopteran insect species: the cabbage aphid *Brevicoryne brassicae* L. (Homoptera: Aphididae) (Bridges et al., 2001), the turnip sawfly *A. rosae* (Müller et al., 2001; Müller and Wittstock, 2005) that was included in this investigation as a control, and the harlequin bug *Murgantia histrionica* (Hahn) (Heteroptera: Pentatomidae) (Aliabadi et al., 2002), but has so far not been shown for any Lepidoptera (Müller et al., 2003).

The second step in the metabolism of sinalbin, sulfation of HBC into HBC sulfate, was observed for all the investigated Pierinae. In contrast to the NSP controlled first detoxification step, the second step might not be vital, as HBC is a fairly stable compound compared to an isothiocyanate, and might be expected to be less toxic. HBC is structurally similar to benzylcyanide, which is an anti-aphrodisiac that is transferred from *P. brassicae* males to females during mating (Andersson et al., 2003), and which is attractive to the parasitic wasp *Trichogramma brassicae* (Fatouros et al., 2005). However, HBC is non-volatile, and is therefore unlikely to show any of those activities. Sinalbin is a relatively rare glucosinolate in nature, and a specific detoxification reaction for this glucosinolate might be expected to have evolved in only some species. For these reasons, we a priori considered it likely that one or more species with ability for nitrile formation would have been deficient in sulfation of HBC, but so far we have not encountered such a species within the Pierinae we sampled.

Sulfation is a common phase 2-detoxification reaction (Brattsten, 1992), and sulfation of phenols of comparable structure to HBC (e.g. 3-aminophenol and 4-nitrophenol) has been reported for several non-pierid Lepidoptera as well as other insect species (Smith, 1955; Yang and Wilkinson, 1973). The demonstration of sulfation of ingested HBC by the examined pierid species is therefore not necessarily an indication of an adaptation to sinalbin- or HBC detoxification, it could also be a side reaction of one (or more) sulfotransferase(s) with another physiological function in the investigated Pierinae. However, sinalbin-containing white mustard and charlock are known host plants for *P. rapae* (Courtney and Chew, 1987; Garzia, 1988; Porter, 1997) and charlock for *A. cardamines* (Courtney and Duggan, 1983), so the two-step metabolism of sinalbin in both *Pieris* and *Anthocharis* reported here can be expected to take place under natural conditions. The two *Sinapis* spp. are encountered by *P. brassicae*, but are not to our knowledge common host plants (Courtney and Chew, 1987). The two *Sinapis* spp. are not commonly encountered by *P. virginensis* or *P. napi oleracea* (FC, unpublished observations).

It is well known that *Pieris* spp. are frequently parasitised in nature, in contrast to a laboratory situation. However, when a number of *P. rapae* caterpillars were collected in nature and tested individually, all were found to excrete HBC sulfate after ingesting white mustard. Subsequently, it was found that some of the caterpillars were parasitised (at the time of the collection) by *Cotesia* (= *Apanteles*) *glomerata* (L.) [Hymenoptera: Braconidae] and by an unidentified fly species [Diptera: Tachinidae] (results not shown). So parasitised *P. rapae* likewise metabolise sinalbin into HBC sulfate.

In conclusion, a common two-step metabolism of sinalbin, by nitrile formation and sulfation, was demonstrated for both *Pieris* (tribe Pierini) and *Anthocharis* (tribe Anthocharini) in laboratory experiments, and is likely to occur in nature as well for both tribes. While the advantage of the first step, nitrile formation as an alternative to isothiocyanate formation is obvious, any physiological advantage of the second step is not yet fully understood.

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References

- Agerbirk, N., Olsen, C.E., Bibby, B.M., Frandsen, H.O., Brown, L.D., Nielsen, J.K., Renwick, J.A.A., 2003. A saponin correlated with variable resistance of *Barbarea vulgaris*, to the diamondback moth, *Plutella xylostella*. *J. Chem. Ecol.* 29, 1417–1433.
- Agrawal, A.A., Kurashige, N.S., 2003. A role for isothiocyanates in plant resistance against the specialist herbivore *Pteris rapae*. *J. Chem. Ecol.* 29, 1403–1415.
- Aliabadi, A., Renwick, J.A.A., Whitman, D.W., 2002. Sequestration of glucosinolates by harlequin bug *Murgantia histrionica*. *J. Chem. Ecol.* 28, 1749–1762.
- Andersson, J., Borg-Karlson, A.-K., Wiklund, C., 2003. Antiaphrodisiacs in pierid butterflies: a theme with variation. *J. Chem. Ecol.* 29, 1489–1499.
- Andréasson, E., Jørgensen, L.B., 2003. Localisation of plant myrosinases and glucosinolates. In: Romeo, J.T. (Ed.), *Recent Advances in Phytochemistry*, vol. 37. Pergamon, Amsterdam, pp. 79–99.
- Bell, R.A., Owens, C.D., Shapiro, M., Tardif, J.R., 1981. Mass rearing and virus production. Development of mass-rearing technology. In: Doane, C.C., McManus, M.L. (Eds.), *The Gypsy Moth: Research Towards Integrated Pest Management*. Forest Service Technical Bulletin 1584. USDA, Washington, DC, pp. 599–655.
- Bjerg, B., Sørensen, H., 1987. Quantitative analysis of glucosinolates in oilseed rape based on HPLC of desulphoglucosinolates and HPLC of intact glucosinolates. In: Wathelet, J.-P. (Ed.), *Glucosinolates in Rapeseeds: Analytical Aspects*. Martinus Nijhoff Publishers, Dordrecht, pp. 125–149.
- Blau, P.A., Feeny, P., Contardo, L., 1978. Allylglucosinolate and herbivorous caterpillars: a contrast in toxicity and tolerance. *Science* 200, 1296–1298.
- Brattsten, L.B., 1992. Metabolic defences against plant allelochemicals. In: Rosenthal, G.A., Berenbaum, M.R. (Eds.), *Herbivores and Their Interactions with Secondary Plant Metabolites*, vol. 2. Academic Press, New York, pp. 175–242.
- Bridges, M., Jones, A.M.E., Bones, A.M., Hodgson, C., Cole, R., Bartlett, E., Wallsgrove, R., Karapapa, V.K., Watts, N., Rossiter, J.T., 2001. Spatial organization of the glucosinolate–myrosinase system in *Brassica* specialist aphids is similar to that of host plant. *Proc. R. Soc. Lond. B* 269, 187–191.
- Chew, F.S., Renwick, J.A.A., 1995. Host plant choice in *Pteris* butterflies. In: Cardé, R.T., Bell, W.J. (Eds.), *Chemical Ecology of Insects*, vol. 2. Chapman and Hall, New York, pp. 214–238.
- Chew, F.S., Watt, W.B., The Green-veined White (*Pteris napi* L.), its Pierine relatives, and the systematics dilemmas of divergent character sets (Lepidoptera, Pieridae). *Biol. J. Linn. Soc. Lond.*, in press.
- Courtney, S.P., Chew, F.S., 1987. Coexistence and host use by a large community of Pierid butterflies: habitat is the templet. *Oecologia* 71, 210–220.
- Courtney, S.P., Duggan, A.E., 1983. The population biology of the orange tip butterfly *Anthocharis cardamines* in Britain. *Ecol. Entomol.* 8, 271–281.
- Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants: a study in coevolution. *Evolution* 18, 586–608.
- Erickson, J.M., Feeny, P., 1974. Singing: a chemical barrier to the black swallowtail butterfly *Papilio polyxenes*. *Ecology* 55, 103–111.
- Fahey, J.W., Zalcman, A.T., Talalay, P., 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56, 5–51.
- Fatouros, N.E., Huigens, M.E., van Loon, J.J.A., Dicke, M., Hilker, M., 2005. Butterfly anti-aphrodisiac lures parasitic wasps. *Nature* 433, 704.
- Garzia, E.F., 1988. Spring and summer hosts for *Pteris rapae* in southern Spain with special attention to *Capparis spinosa*. *Entomol. Exp. Appl.* 48, 173–178.
- Jacobi, B., 2004. Drüsenhaare und Wehrsekrete bei Raupen des Aurorafalters *Anthocharis cardamines* (Linnaeus, 1758) – fast eine Entdeckung (Lep., Pieridae). *Melanargia* 16, 29–31.
- Müller, C., Agerbirk, N., Olsen, C.E., Boevé, J.-L., Schaffner, U., Brakefield, P.M., 2001. Sequestration of host plant glucosinolates in the defensive hemelymph of the sawfly *Athalia rosae*. *J. Chem. Ecol.* 27, 2505–2516.
- Müller, C., Agerbirk, N., Olsen, C.E., 2003. Lack of sequestration of host plant glucosinolates in *Pteris rapae* and *P. brassicae*. *Chemoecology* 13, 47–54.
- Müller, C., Wittstock, U., 2005. Uptake and turn-over of glucosinolates sequestered in the sawfly *Athalia rosae*. *Insect Biochem. Mol. Biol.* 35, 1189–1198.
- Pollock, D.D., Watt, W.B., Rashbrook, V.K., Iyengar, E.V., 1998. Molecular phylogeny for *Colias* butterflies and their relatives (Lepidoptera: Pieridae). *Ann. Entomol. Soc. Am.* 91, 524–531.
- Porter, J., 1997. *The Colour Identification Guide to Caterpillars of the British Isles*. Viking, Penguin Books, London.
- Ratzka, A., Vogel, H., Kliebenstein, D.J., Mitchell-Olds, T., Kroymann, J., 2002. Disarming the mustard oil bomb. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11223–11228.

- Renwick, J.A.A., Lopez, K., 1999. Experience-based food consumption by larvae of *Pieris rapae*: addiction to glucosinolates? *Entomol. Exp. Appl.* 91, 51–58.
- Rodman, J., Soltis, P.S., Soltis, D.S., Sytsma, K.J., Karol, K.G., 1998. Parallel evolution of glucosinolate biosynthesis inferred from congruent nuclear and plastid gene phylogenies. *Am. J. Bot.* 85, 997–1006.
- Rossiter, J.T., Jones, A.M., Bones, A.M., 2003. A novel myrosinase–glucosinolate defense system in cruciferous specialist aphids. In: Romeo, J.T. (Ed.), *Recent Advances in Phytochemistry*, vol. 37. Pergamon, Amsterdam, pp. 127–142.
- Smith, J.N., 1955. Comparative detoxification 4. Etheral sulphate and glucoside conjugation in insects. *Biochem. J.* 60, 436–442.
- Spuler, A., 1910. *Die Schmetterlinge Europas IV*. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.
- Wittstock, U., Kliebenstein, D.J., Lambrix, V., Reichelt, M., Gershenzon, J., 2003. Glucosinolate hydrolysis and its impact on generalist and specialist insect herbivores. *Recent Adv. Phytochem.* 37, 101–125.
- Wittstock, U., Agerbirk, N., Stauber, E.J., Olsen, C.E., Hippler, M., Mitchell-Olds, T., Gershenzon, J., Vogel, H., 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4859–4864.
- Yang, R.S.H., Wilkinson, C.F., 1973. Sulphotransferases and phosphotransferases in insects. *Comp. Biochem. Physiol.* 46B, 717–726.