Phytochemical background matters for bioactivity of plant metabolites : a case study with pyrrolizidine alkaloids

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Chapter 7

Summary and discussion

To respond to and manipulate their natural environment, plants have developed various defense strategies, among which the chemical defenses are very powerful (Mithofer and Boland 2012). The plant kingdom has evolved an enormous number of chemically diverse metabolites (Fraenkel 1959; Hartmann 1996; Kliebenstein 2004; Mithofer and Boland 2012). The consequence of a large number of metabolites within a species is that there is a high probability of interactions. Such a co-occurrence of plant metabolites comprise a natural background where these metabolites have to function and this is often overlooked or ignored in ecology studies.

The main goal of this thesis is to understand the importance of the interactions between plant metabolites in a context of plant-insect herbivore interactions. To achieve this, I used *Jacobaea*, CGA and the pyrrolizidine alkaloids (PAs) as study objects. Here, I will first discuss the bioactivity of individual PAs. Secondly, I will discuss the interactions between plant metabolites on herbivore resistance, the effects of such interactions on herbivore resistance, and their significance in an ecological context. Third, I will briefly discuss the approach and the frame work I used in this thesis. Finally, I will draw the main conclusions of this thesis, along with perspectives for future research.

7.1 Bioactivity of individual metabolites

In Chapter 2, I found clear negative effects of PAs from *J. vulgaris* on thrips survival. This demonstrated the protective function of PAs against insect herbivores. The negative effects of individual PAs on thrips corroborate the findings of a previous correlative study with whole plants that showed that the total PA concentration was negatively correlated with herbivore performance (Cheng et al. 2011), and in particular that the jacobine-like PAs are negatively correlated with thrips damage (Leiss et al. 2009; Cheng et al. 2011).

Chapter 2 also shows that structurally related metabolites vary in their effects on thrips. Firstly, the free base PAs were found to be more toxic to thrips than their corresponding N-oxides. This finding is consistent with previous studies on the pea aphid (*Acyrthosiphon pisum*) (Dreyer et al. 1985), the snail *Arianta arbustorum* (Helicidae) (Speiser et al. 1992), the caterpillar *Spodoptera exigua* (van Dam et al. 1995), the locust *Locusta migratoria* (Macel et al. 2005), and *S. exigua* cell lines and larvae (Nuringtyas et al. 2014). The difference between the two forms of PAs in their effects on insect herbivores may be related to cell permeability. In general, a highly hydrophobic or lipophilic compound passes cell membranes easier because the membrane is composed of phospholipids. Most free base PAs are rather lipophilic and able to easily permeate bio-membranes, while N-oxides are polar hydrophilic compounds (Lindigkeit et al. 1997). Next, among the tested free base PAs, we found jacobine and erucifoline to be the most active against thrips while seneciphylline and senecionine were the least active ones. Different effects of individual PAs on herbivores have been reported for *S. littoralis* (Gonzalez-Coloma et al. 2002), thrips (Macel

et al. 2005), and *S. exigua* cell lines (Nuringtyas et al. 2014). In contrast, Dominguez et al. (2008) did not find any significant differences among the tested PAs on *Myzus persicae* or *Leptinotarsa decemlineata*. The various effects of PAs on insect herbivores could be attributed to the physiological processes in insects. For instance, the larvae of *S. littoralis* avoided the toxicity of senecionine by rapid and efficient excretion of this metabolite (Lindigkeit et al. 1997). However, knowledge about PA toxicity in herbivores is still rudimentary. We considered retrorsine, jacobine and erucifoline as more downstream in the biosynthetic pathway of PAs and senecionine and seneciphylline as PAs at the basis of the biosynthetic pathway. Senecionine N-oxide is the first PA in the biosynthesis from which the other PAs are derived (Hartmann and Dierich 1998; Pelser et al. 2005). Senecionine N-oxide and seneciphylline N-oxide co-occur in all *Senecio* species. From these two PAs, in one or two enzymatic steps, the other PAs including retrorsine N-oxide, jacobine N-oxide and erucifoline N-oxide are produced (Figure 1 in Chapters 2 and 3). In Chapter 2, I compared the effects of the more downstream free base PAs on thrips with that of the basic free base PAs. Although there was a trend that the more derived ones were more active against thrips than the senecionine and seneciphylline this was not significant. My results are partly in line with the Arms Race Hypothesis. However, to really test this hypothesis more herbivores should be tested, especially ones that co-evolved with *J. vulgaris*.

Results of bioassays on pure PAs were not always consistent with correlative studies using whole plant bioassays. Especially, for metabolites which may be effective at low amounts in plants, correlative studies might not be sensitive enough to show their bioactivity. For instance, we found that retrorsine affected thrips survival in the bioassay. However, thrips performance was not significantly correlated with retrorsine using whole *Jacobaea* plants (Cheng et al. 2011), which may be due to the low concentrations present in plants. Jacobine N-oxide was found to be significantly correlated with thrips damage in the whole plant bioassay (Cheng et al. 2011) while jacobine N-oxide did not result in strong effects on thrips when tested as a pure compound (Chapter 2). The disadvantage of correlative studies is that correlation does not imply causation. The correlation between thrips damage and jacobine N-oxide found by Cheng et al. (2011) may have resulted e.g. from the correlation between jacobine N-oxide and free base. Another possible explanation is that jacobine N-oxide becomes active in the presence of other metabolites, emphasizing the importance of studying the interactions between plant metabolites.

7.2 The interactions between plant metabolites

The co-occurrence of plant metabolites provides a natural background for individual metabolites, where there is a high possibility for them to interact with each other. Plants may manage the interactions between metabolites by accumulating metabolites in organs, tissues, cells and even cell compartments. Such a compartmentalization can be used to keep antagonistically interacting metabolites apart but may also promote synergistic interactions

between plant metabolites by storing them in the same compartment. The interactions between plant metabolites are of vital ecological significance for plants. For instance, plants can benefit from synergistic interactions by increasing their bioactivity at a lower cost (Berenbaum and Zangerl 1998; Nelson and Kursar 1999). With respect to single metabolites, the modes of action in concert with other metabolites may differ from that as a single compound. As a consequence, potential interactions may provide new insights for the bioactivity of single metabolites that may have been overlooked when tested as pure compounds. If plant metabolites interact antagonistically, compartmentalisation is a solution to avoid antagonistic interactions. Indeed, metabolite content may differ largely between plant organs (Kuhlisch and Pohnert 2015) and even between different cell layers (Nuringtyas et al. 2014; Moussaieff et al. 2013). The importance of the interactions between plant metabolites in the context of plant-insect herbivore associations and the influence of the interactions on the bioactivity of individual metabolites will be presented below in detail. In spite of the great importance, interactions between plant metabolites and their effects have not been largely investigated in an ecological context (but see references below). The rare demonstrations could be due in part to the complexity of the interactions between plant metabolites, and to the difficulty of detecting and analyzing interactions in a proper manner (Nelson and Kursar 1999).

The complexity of interactions between metabolites presents a challenge both in terms of the enormous number of metabolites in a single plant and in terms of the infinite number of potential combinations. Plants have may have reduced the interaction between metabolites by dividing them over different plant parts, tissues, cell layers, cells and even within cells. Still, it is impossible to evaluate all combination of metabolites. Investigating interactions becomes even more complex if unidentified or even unknown metabolites are involved. The unknowns account for a large part of the total amount of metabolites in a plant (Trethewey 2004). In the light of these facts, it is a great challenge to measure the interactions between plant metabolites and the influence of interactions in plant-insect herbivore associations. In this thesis, in line with the level of complexity of chemical diversity, I studied the effects of the interactions between plant metabolites on insect herbivores on three levels, i.e. interactions within a structurally related class, interactions between metabolites of different classes, and interactions occurring in the natural phytochemical backgrounds of primary metabolites (PMs) and diverse classes of secondary metabolites (SMs). I used both a bottom-up approach and a top-down approach.

7.2.1 Approaches to interaction research and a statistical framework

A way forward would be to start with combinations of classes of metabolites of which we know that the potential of interactions is high such as saponins together with metabolites that not easily pass membranes (Gee et al. 1996; Herrmann and Wink 2011). Prior information of individual metabolites provided a starting point for Chapters 2, 3 and 4. In

Chapter 2, I studied the effects of the interactions between the most abundant PA N-oxides on thrips. I wanted to investigate if the predominance of PA N-oxides in plants despite their relatively weak activity against herbivores could be explained by interactions between them. In Chapters 3 and 4, I investigated the interactions between PAs and CGA on western flower thrips *Frankliniella occidentalis*, a generalist herbivore. We choose this combination of metabolites on the basis of existing knowledge of the two metabolites: PAs and CGA are differently distributed over plant cell layers and CGA is able *in situ* to form a complex with other alkaloids. Specifically, Nuringtyas et al. (2014) found that the mesophyll of *J. vulgaris* contained high amounts of PAs while CGA was accumulated in the epidermis. CGA forms a π-molecular complex with caffeine (a purine alkaloid) (Mösli Waldhauser and Baumann 1995). An earlier study with *S. exigua* cell lines also showed an antagonistic interaction between jacobine and CGA (Nuringtyas, PhD thesis, 2014). This finding may explain why PAs and CGA are distributed differently over cell layers. This would be most relevant for single cell feeders. For chewing insects such as *Spodoptera*, distribution of PAs and CGA over different cell layers will not prevent an antagonistic effect. I, therefore, studied the interaction between CGA and PAs in their effect on thrips which is a single cell feeder. Overall, for instance, in the case of PAs and CGA, prior information is useful in terms of forming a starting point for interaction studies.

In the case of the absence of prior knowledge about the metabolites that are involved, study on interactions between metabolites can be approached in a top-down direction, from a metabolome analysis down to component-interaction analysis. In Chapters 5 and 6, I did set the first step of a top-down approach to study the interactions between metabolites with two types of bioactivity, i.e. the effects on thrips as measured by *in vitro* bioassays (Chapter 5) and the mutagenicity as determined by the Ames test (Chapter 6). I studied the bioactivity of individual PAs when they were added to their natural phytochemical background (fractions from plant extracts). This starting step provided a general impression of the importance of interactions between metabolites from a metabolome perspective. When the bioactivity of a metabolite is increased when it is present in plant fractions, this points to the importance of interactions among plant metabolites for plant defence. Further sub-fractionation and recombining sub-fractions may narrow down the candidate compounds that are involved in these interactions.

The reason that only few studies on interactions between metabolites were carried out is probably because a theoretical framework is lacking (Nelson and Kursar 1999). Here, I describe a method to calculate the expected interaction effects of two or more components, which then allows for a proper statistical testing both of the pattern and the magnitude of interaction effects. With regard to thrips survival, I formulated a multiplicative null model. This model is applicable to calculate survival, given that an individual cannot die twice (Chapter 5). Regarding to the mutagenicity as measured in the Ames test, an additive model was applied because the probability of a revertant colony is very small compared to the

initial number of cells (Chapter 6). The interaction effects then can be calculated by comparing the observed results and the expected results. Such a framework not only allows testing for the existence of interaction effects but also for differences in their magnitude. The applications of the theoretical frame work that is put forward in this thesis can be extended to other aspects of plant-environment interactions, other multi-component materials, and to the design of bioactivity studies in fields as toxicity, mutagenicity, health and plant protection.

7.2.2 **The effects of the interactions between plant metabolites and their ecological significance**

Interaction effects of metabolites within a structural related class

In plant-insect herbivore associations, examples of metabolite interactions within a structural related class include the synergistic effects of two potato glycoalkaloids on the snail *Helix aspersa* (Smith et al. 2001) and on the Khapra beetle *Trogoderma granrium* (Nenaah 2011), the antagonistic effects of two furanocoumarins on *S. littoralis* (Diawara et al. 1993; Calcagno et al. 2002), the synergistic effects of piper amides on the mosquito *Aedes atropalpus* (Scott et al. 2002) and on *S. frugiperda* caterpillars (Dyer et al. 2003; Richards et al. 2010), the synergistic effects of two iridoid glycosides on a specialist caterpillar *Junonia coenia* (Richards et al. 2012). Only few studies have been carried out on interactions among PAs. Macel (2005) found a weak synergistic effect of a mixture of the free bases of senecionine, seneciphylline and senkirkine on *S. exigua* and of a mixture of the free bases of senecionine and seneciphylline on *L. migratoria*. Yet, Siciliano et al. (2005) did not find any synergistic effects of two PAs from *Anchusa strigosa* on *S. exigua*. These two studies have only tested the interaction effects of free bases. As PAs in plants occur mainly as PA N-oxides, it is therefore ecologically relevant to study the interaction between PA N-oxides and their effects on insect herbivores or pathogens, which however have not been studied yet. In Chapter 2, the most abundant PA N-oxides of *J. vulgaris*, senecionine N-oxide, jacobine N-oxide and erucifoline N-oxide (Joosten et al. 2011) were combined and showed no synergistic (or antagonistic) effects on thrips survival. As such, our results about the effects of interactions between PA N-oxides on thrips do not provide an explanation for why PA N-oxides are the dominant form in which PAs occur. Neither does our study explain why there are so many different PA N-oxides present in a single plant.

Interactions between metabolites of different chemical classes

Antagonistic interactions between free base PAs and CGA on thrips

In Chapter 3, I found antagonistic interactions between free base PAs and CGA on their effects on thrips for all five PAs tested. Although the strength of the interaction differed among these PAs, it suggests that this is a general pattern. From a plant's point of view, antagonistic interactions in most cases would not be an advantage to plant fitness. Therefore, from an ecological perspective, such an interaction is not easily explained. Together with a previous study, in which PAs and CGA were found to be differentially distributed over different cell layers of *Jacobaea* leaves (Nuringtyas et al. 2014), the antagonistic effects of free base PAs and CGA on thrips suggest that antagonistic interactions may represent a constraint caused by the accumulation of metabolites in plants (Nelson and Kursar 1999). It has been reported that caffeine and CGA *in situ* formed a complex at a ratio of 1:1 (Sondheimer et al. 1961; Horman and Viani, 1972; Chapman and Miller 1974). If similar complexes would be formed between free base PAs and CGA, we expect that antagonistic interactions will depend on the ratio of two components. We indeed found that the effects of the antagonistic interactions between free base PAs and CGA depended on their ratio. However, there was too much variation in the measured effects to reliably determine the optimal ratio.

The fact that all the tested free base PAs showed interactions with CGA suggest that the necine part of the PA molecule involved in the interaction. It is the retronecine base which all tested free base PAs have in common, rather than the macrocyclic ring that varies in structure. This fits very well with the observation that the quinic acid part of the CGA molecule interacted antagonistically with retrorsine while the caffeoyl part did not. In Chapter 3 I only set the first steps but the results do show that that modification of functional groups, addition/elimination of specific groups, or changing the substitution pattern can be used to further reveal the mechanism from a chemical perspective.

Synergistic interactions between PA N-oxides and CGA on thrips

In Chapter 3 I studied the interaction between free base PAs and CGA on thrips survival. However, as mentioned before, in plants PAs occur mostly as N-oxides (Hartmann et al. 1989). PA N-oxides were less active than the corresponding free bases when tested alone on thrips survival (Chapter 2). Therefore, it is of ecological significance to study the interactions between PA N-oxides and other SMs such as CGA on thrips survival. In Chapter 4 I found synergistic interactions between PA N-oxides and CGA on thrips survival. Although the strength of the interaction varied, the pattern was similar for all PA N-oxides tested which again suggests a general pattern, I then compared the interaction effects of the combinations between free base PAs and CGA with that of the combinations between the corresponding PA N-oxides and CGA on thrips survival. Surprisingly, I found that the thrips survival of the two forms of PAs was reversed when they were in combination with CGA. With respect to the CGA molecule, it suggests a dual role of alleviating the bioactivity of free base PAs and of enhancing the bioactivity of PA N-oxides

on thrips survival. These findings may give an alternative explanation for the fact that PA N-oxides are more abundant than free base PAs. However this was not completely backed up by the results of Chapter 5 in which I found synergistic interactions on thrips survival between both retrorsine and retrorsine N-oxide with two fractions of a methanol extract of *Jacobaea* plants. In the case of PA N-oxides, the findings of synergistic effects on thrips survival provide a new insight into the defensive functions of PA N-oxides. In the same way, these results supply an ecological driven alternative of the predominate storage of PA N-oxides in plants, in addition to a physicochemical explanation that N-oxides are better soluble in water and are therefore more easily stored in the vacuoles and transported through the plant (von Borstel et al. 1986).

Synergistic interactions are assumed to be of ecological and evolutionary significance in the plant-environment associations. Results of Chapter 4 and other studies suggest that the bioactivity of single SMs can be enhanced in concert with others. This is meaningful for metabolites which may not be active by themselves. For instance, rutin by itself did not have a negative effect on the growth rate of the caterpillar *S. exigua*, while CGA had a slight negative effect, but together they had a strong negative effect (Stamp and Yang 1996). Synergistic interactions can also provide an advantage to plant defense in terms of producing a greater toxicity at a lower cost (Nelson and Kursar 1999; Ryabushkina 2005). In this regard, it is expected that natural selection selects for synergistic interactions.

Interactions between plant metabolites occurring in natural backgrounds

In the first chapters I studied interactions between known plant metabolites by combining them, which can be seen as a bottom-up approach. In the remaining chapters I used a top-down approach and investigated interactions between plant metabolites within the natural phytochemical background in which both PMs and SMs occur. In Chapter 5 I studied the effects on thrips survival. In Chapter 6 I used the mutagenicity as determined by the Ames test as a measure of bioactivity. Fractions of a methanol extract of *J. vulgaris* leaves differed in their bioactivity in the Ames test. Especially the chloroform fraction showed a strong mutagenicity. Upon (sub-)fractionation, this bioactivity was largely gone. Recombining the sub-fractions in their original proportion restored the bioactivity. This suggests that (sub-) fractionation led to the loss of synergistic interactions among metabolites. For thrips too, fractionation led to the loss of bioactivity. Such effects are often observed in phytochemical studies (Williamson 2001; Herrera and Amor 2011; Labuschagne et al. 2012; Inui et al. 2012).

The fractions of *J. vulgaris* plants differed in their anti-herbivore and mutagenic effects. Nevertheless, the order of bioactivity was not consistent with the order of the natural PA content of these fractions, suggesting that besides PAs, other metabolites also contributed directly or indirectly, through interacting effects, to the overall activity of plant fractions.

With the generally increasing polarity of solvents used in the fractionation process, the fractions contain different types of metabolites (Sasidharan et al. 2011). Although beyond the scope of this thesis, further sub-fractionation and identification may narrow down the potential metabolites that are responsible for the bioactivity.

In Chapters 5 and 6, I set the first step of a top-down approach by adding individual PAs to various fractions to study the interactions between plant metabolites at a whole-metabolome level, as well as to determine how natural phytochemical backgrounds shape the bioactivity of individual metabolites. In Chapter 5, both retrorsine and retrorsine N-oxide interacted synergistically with the chloroform and the *n*-butanol fractions in their effects on thrips survival. In Chapter 6, the chloroform and ethyl acetate fractions of *J. vulgaris* leaves significantly increased the mutagenicity of retrorsine while the hexane, *n*-butanol and aqueous fractions decreased the mutagenicity of retrorsine. Not surprisingly, this also suggests that the type of interaction in this case was dependent on the type of bioactivity. Considering the PA amount in these fractions, the interactions between PAs and other SMs may dominate the ultimate effects of the interactions between PAs and fractions. Altogether, the results of Chapters 5 and 6 suggest that natural backgrounds influence the bioactivity of individual SMs in both positive and negative patterns. Moreover, the strength of the interaction depended on the fractions, suggesting that the effects of plant metabolites may vary depending on the phytochemical background.

The importance of natural backgrounds

Plant extacts and fractions offer an effective approach to measure the interactions between plant metabolites as a natural combination treatment and as a natural background, without requiring prior knowledge about the metabolites involved. To study the overall efficacy of a mixture of plant metabolites is of ecological significance because insect herbivores always encounter mixtures of metabolites in nature. To study the bioactivity of individual plant metabolites against a natural background of other metabolites can assist in understanding plant-insect herbivore interactions, because a given plant metabolite is likely not to be the sole agent, but rather is likely to be imbedded as a participant in multitude of interactions that naturally occur in plants. Still, we lack sufficient evidence for the importance of natural phytochemical backgrounds in an ecological context. This thesis sets an important first step.

This study supports a commonly held notion that plant chemical defence is dependent on a variety of metabolites, which together shape the outcome of the defensive efficacy. Interactions between plant metabolites may provide additional or novel information for the bioactivity of individual SMs. Interaction effects of plant metabolites could also explain why some SMs show a certain activity in particular species while they do not show the same activity in others. For instance, CGA in *chrysanthemum* was negatively correlated with the feeding damage of thrips *Frankliniella occidentalis* (Leiss et al. 2009), while no

effect of CGA on thrips was detected in tomato *Solanum lycopersicum* (Mirnezhad 2011). Knowledge about interactions of metabolites can also be applied in other multi-component materials. For instance, the anti-proliferative activity of quercetin 3-β-D-glucoside against human breast cancer cell was increased 4 fold by combination with apple extracts (Yang and Liu 2009).

7.3 Final conclusions and future perspectives

From this thesis, the importance of interactions between plant metabolites in plant-insect herbivore associations is evident. Several conclusions emerge. First, PAs significantly decreased thrips survival, indicating the role of PAs as a plant defence against insects. Different PAs affect thrips differently with some PAs being more active than others. Secondly, combinations of plant metabolites affected thrips survival both in synergistic and antagonistic patterns. It suggests that interactions between plant metabolites should be taken into consideration in their effect on herbivore performance. Thirdly, the interactions on thrips survival and mutagenicity between plant metabolites are also observed in complex mixtures of metabolites (i.e. plant extracts and fractions). I showed that the bioactivity of individual metabolites is strongly influenced by their natural biochemical backgrounds that may potentiate or mitigate their efficacy. Therefore, the phytochemical backgrounds should be taken into account when designing bioassays. Taken as a whole, it can be argued that the bioactivity of a given metabolite is not merely dependent upon the amount and chemical structure of that metabolite, but also on the co-occurring metabolites of the natural phytochemical backgrounds.

A number of points should be considered in future studies with regard to the interactions between plant metabolites. First, there is shortfall in our current knowledge of the interaction mechanisms. Further study on physiochemical and physiological processes will likely be fruitful. From a physiochemical perspective, metabolites can alter solubility and/or the resorption rate and thereby their bioavailability (Amin et al. 2015). Physiological processes can also be critical in determining the interaction effects e.g. digestive metabolism or detoxification enzyme activity (Stermitz et al. 2000; Scott et al. 2002). Secondly, the importance of paying attention to antagonistic effects and of understanding its ecological significance should be taken into account. As evolution of plant defences occurs under selection pressures from other factors such as abiotic stresses, pathogens, other herbivores, testing the interaction effects on varied plant enemies would also be an avenue of future work. Thirdly, compartmentalization of plant metabolites between different cell organelles, cells, cell layers and organs may contribute to a plant's defence through promoting synergistic and avoiding antagonistic interactions. Therefore, as a first step it is important that chemical analysis of SMs of such compartments are carried out to identify which metabolites co-occur and which not. Finally, demonstrations of interaction effects of plant metabolites in the context of plant-herbivore associations are rare. This

likely holds true for other ecological relationships as well. To better have a better understanding of mechanistic and ecological interaction between plants and their environment a concerted and combined effort of various research fields is required. To follow up the first steps that were set in this thesis, both the bottom-up and the top-down approach are important. Considering the enormous number of plant metabolites, one of the challenges would be to measure a large number of possible combinations that are of interest. This is an impossible task with the bioassays that were used in this thesis. Development of high-throughput screening, as often applied in pharmacological studies especially in drug discovery, is essential in this respect. For example, receptor-based approaches have been widely applied in drug discovery. From a mechanistic perspective, the effects of interactions between compounds can be caused by blocking or disturbing the membrane-bound receptor function. For instance, ramipril inhibits the angiotensin receptor, thereby facilitating the antihypertensive effect of candesartan-cilexetil (Raasch et al. 2004). As such, approaches based on receptor activity would be a screening strategy. Another approach is the use of cell lines for bioactivity screening. While cell lines of thrips are presently not available, we can start using cell lines of other herbivores, e.g. the beet armyworm, *S. exigua*. Such cell lines have been used in our group (Nuringtyas et al. 2014). The effects of the interactions between free base PAs and CGA on *S. exigua* cell lines (Nuringtyas et al. 2014) were consistent with that on thrips larvae (Chapter 3). Bioassays on cell lines, however, cannot account for metabolomic changes that may occur during digestion and absorption (see a review by Yoon et al. 2012). Despite this disadvantage, cell lines can still be employed for initial screening and selecting candidate metabolites or combinations of interest to be further used in bioassays with living organisms.

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