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ORIGINAL PAPER

How novel are the chemical weapons of garlic mustard in North American forest understories?

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Abstract The Novel Weapons Hypothesis predicts that invasive plants excel in their new ranges because they produce novel metabolites to which native species possess little resistance. We examined the novelty of the phytochemistry of the Eurasian invader, *Alliaria petiolata*, in North America by comparing its phytochemical profile with those of closely related Brassicaceae native to North America. We examined the profile and/or concentrations of glucosinolates, alliarinoside, flavonoids, cyanide, and trypsin inhibitors in cauline leaves of field-collected *A. petiolata*, *Arabis laevigata*, *Cardamine concatenata*, *C. bulbosa*, and *C. douglassii*. Cyanide and the glucosinolates and flavonoids produced by *A. petiolata* were detected only in *A. petiolata*. Trypsin inhibitor activity was highest in *A. laevigata*, intermediate in the *Cardamine* species, and lowest in *A. petiolata*. The phytochemical profile of *A. petiolata* was distinct from those of four closely related and/or abundant Brassicaceaeous species native to North America, providing support for the Novel Weapons Hypothesis.

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Introduction

Success of invaders is a complicated, multifaceted issue and several hypotheses have been advanced to explain the success of invasive plants. The Novel Weapons Hypothesis (NWH) provides an explanation for the success of some invasive plants, suggesting that native organisms in invaded ranges will be naïve and therefore especially sensitive to novel compounds produced by invasive plants (Callaway and Aschehoug 2000; Callaway and Ridenour 2004). These novel weapons may provide invaders with protection from herbivory (Carpenter and Cappuccino 2005), and can also directly inhibit other plants and/or their microbial symbionts through allelopathy. Presumably, some combination of these effects provides the invader with a competitive advantage over naïve native plants in the invaded range, while species that share a co-evolutionary history with the invader are expected to be more resistant to these effects. Some basic predictions of the NWH have been met for two Eurasian *Centaurea* species, which inhibit growth of North American grasses more than growth of European grasses (Inderjit et al. 2008). The invasive plants *Lepidium draba*, *Vaccinium myrtillus*, and *Alliaria petiolata* also more strongly inhibit growth of plant species from outside their native ranges than from within it (Mallik and Pellissier 2000; Prati and Bossdorf 2004; McKenney et al. 2007).

Alliaria petiolata (M.Bieb.) Cavara & Grande, Brassicaceae (garlic mustard), an herbaceous biennial, was introduced to North America from Europe in the 1860's and has since become invasive in much of North America (Nuzzo 2002). Allelopathic effects of *A. petiolata* have been demonstrated against other plants and arbuscular and ectomycorrhizal fungi (Vaughn and Berhow 1999; Roberts and Anderson 2001; Prati and Bossdorf 2004; Stinson et al. 2006; Wolfe et al. 2008; Barto and Cipollini 2009). Native mustard specialist herbivores naïve to this plant have difficulty consuming and completing development on it (Keeler and Chew 2008), and field surveys indicate that *A. petiolata* receives only minor herbivory from any species in the field (Evans and Landis 2007). Importantly, the negative effects of *A. petiolata* or its extracts on mycorrhizal fungi from North American or European soils follow biogeographical patterns predicted by the Novel Weapons Hypothesis (Callaway et al. 2008).

For predictions of the NWH to hold for *A. petiolata*, however, the suite of bioactive compounds and proteins produced by *A. petiolata*, or the way in which they are deployed, should be substantially novel relative to what native species may encounter in their habitat. Support for this aspect of the NWH was found for several invasive plants based on available literature (Cappuccino and Arnason 2006), but direct phytochemical surveys are lacking. Plants most likely to share similar phytochemical profiles with *A. petiolata* are other closely related Brassicaceous plants. Species within the Brassicaceae can be divided into 25 tribes, with *A. petiolata* in the Thlaspideae and the sole member of the genus *Alliaria*. Of the 564 Brassicaceous species native to North America, none are currently assigned to this tribe (USDA Plants Database, Al-Shehbaz et al. 2006). However, the closely related tribe Arabideae includes 183 species native to North America, at least five of which are found in the midwestern United States in forest understory habitats typically invaded by *A. petiolata* (USDA Plants Database, Al-Shehbaz et al. 2006). It is with species like these that biochemical comparisons with *A. petiolata* are most relevant.

Biologically active secondary compounds produced by *A. petiolata* are qualitatively similar in European and North American populations (Cipollini et al. 2005), and include glucosinolates and their degradates, cyanoallyl and flavonoid glycosides, and cyanide (Haribal and Renwick 1998; Vaughn and Berhow 1999; Haribal and Renwick 2001; Haribal et al. 2001; Renwick et al. 2001; Cipollini and Gruner 2007). *A. petiolata* also produces many defensive proteins that are widely distributed among plant families, such as trypsin inhibitors (Cipollini and Bergelson 2001; Cipollini et al. 2005). The objectives of this study were to compare the presence and/or concentrations of flavonoids, glucosinolates, cyanide and trypsin inhibitors in leaves of *A. petiolata* with that of four Brassicaceous species common to forest understories in the Midwestern US. The NWH predicts that the profile of individual compounds or proteins produced by *A. petiolata* and/or their concentrations should be substantially distinct from that produced by relevant North American species.

Materials and methods

Study species and tissue collection

Cauline leaves were collected from *A. petiolata*, and from the North American natives *Arabis laevigata* (rock cress), *Cardamine concatenata* (cutleaved toothwort), *Cardamine bulbosa* (spring cress), and *Cardamine douglassii* (purple cress). These native species are abundant Brassicaceae in the habitats invaded by *A. petiolata*, and are therefore the ideal species to include in phytochemical surveys testing the novelty of *A. petiolata* metabolites. All plants were sampled in early spring while the plants were initiating flowering. *A. petiolata*, *C. concatenata*, and *C. douglassii* were collected in the Wright State University Forest Preserve, OH (39°48.0′N, 84°1.0′W). *A. laevigata* was collected in John Bryan State Park, OH (39°47.3′N, 83°51.1′W), and *C. bulbosa* was collected in Glen Helen Nature Preserve, OH (39°47.3′N, 83°52.8′W). *A. petiolata* also grows in each of these natural areas. Sampled individuals were chosen haphazardly from naturally growing populations in the forest understory of each natural area, avoiding individuals that were damaged by herbivores or other causes. Cauline leaves (those located on the flowering stem) were either placed in plastic centrifuge tubes on dry ice, or stems were cut and maintained fresh in water picks, for transport back to the laboratory.

Chemical analysis

Flavonoids, alliarinoside, and glucosinolates were analyzed in frozen leaves of three replicate plants of each species as in Callaway et al. (2008). Alliarinoside and the flavonoids produced by *A. petiolata* were initially identified using electrospray ionization (ESI)/MS and MS/MS on a liquid chromatography quadrapole (LCQ) ion-trap mass spectrometer (ThermoQuest, USA) (Cipollini et al. 2005). Subsequent identifications were based on retention time and UV spectra. The glucosinolates sinigrin and glucotropaeolin were identified by comparison with standards [sinigrin monohydrate from horseradish (Sigma–Aldrich, St. Louis, MO, USA), glucotropaeolin (POS Pilot Plant Corp, Saskatoon, SK, Canada)]. Compounds that could not be identified were distinguished from each other by retention time and UV spectra, but since our focus was on comparing phytochemical profiles we did not attempt to further identify unknown compounds. Cyanide concentrations were assessed in fresh leaves of ten to twelve individuals of each species as in Cipollini and Gruner (2007) and trypsin inhibitor activity was examined in soluble protein extracts of frozen leaves of ten to twelve individuals of each species as in Cipollini et al. (2005).

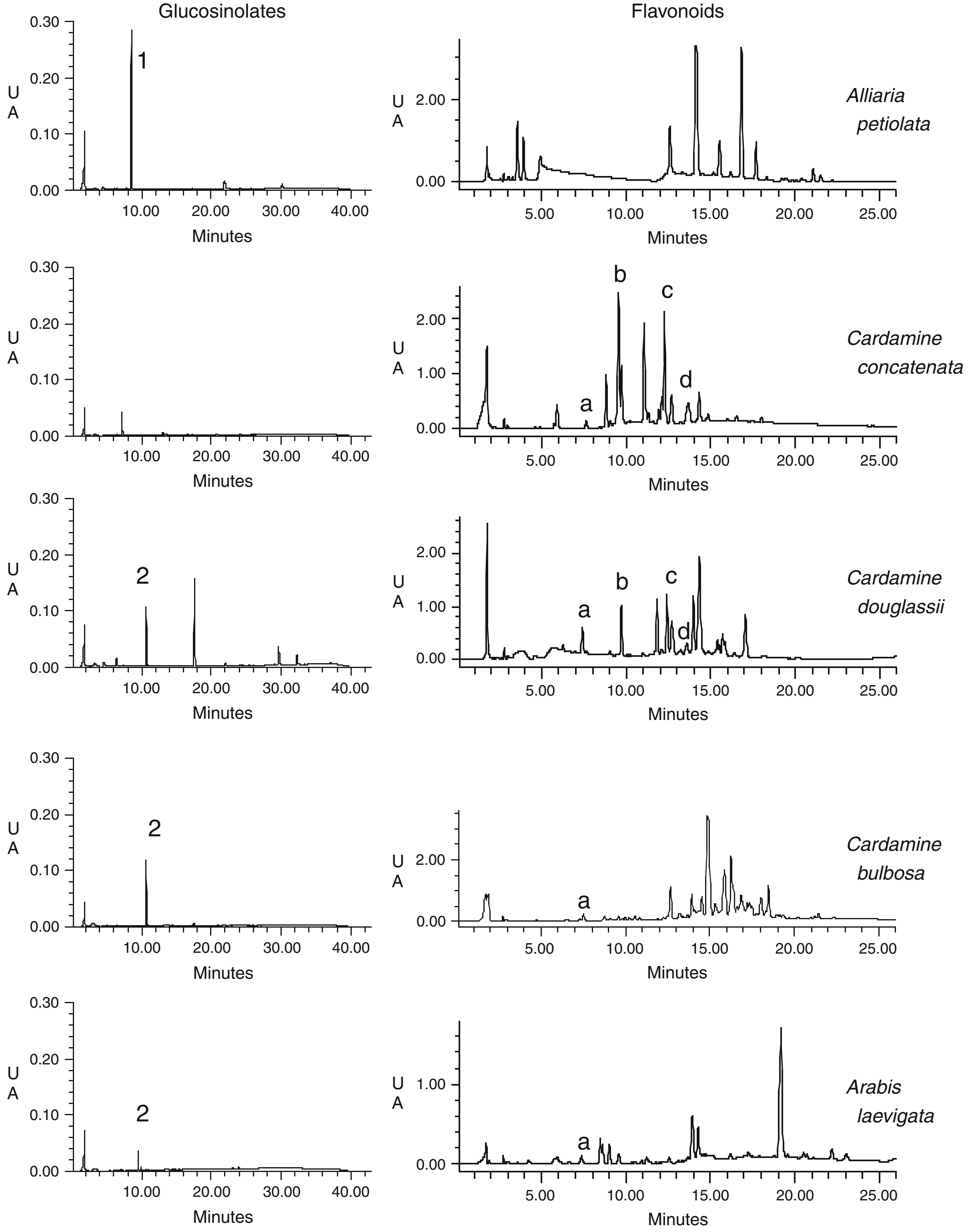
Statistical analysis

Representative chromatograms are shown in Fig. 1. Cyanide was only detected in *A. petiolata*. Trypsin inhibitor activities (mg TI/g protein) were compared among species using one-way ANOVA on SAS (Version 9.1, SAS Institute, Cary, NC). Differences in chemical profiles among the plant species were evaluated with Principal Components Analysis (PCA) using the “ade4” package (Version 1.4-10, Dray and Dufour 2007) in R (Version 2.8.0, R Development Core Team 2008). We estimated the contribution of species identity (representing classes in the model) to inertia decomposition in the model using the methods of Dolédec and Chessel (1987). Briefly, the method estimates the variation between the classes (from the centroid of data associated with each class relative to that of all other classes) relative to variation within the classes (from the individual data points within a class to the centroid of that class) and represents this between-classes inertia as a percentage of the total inertia in the data. Statistical significance is determined by Monte-Carlo test, estimating the between-class inertia after randomization of the data matrix. Due to the small amount of plant material available, response variables were not collected from the same individual samples. To account for this, we employed a resampling approach to generate the matrix. The matrix consisted of three rows for each plant species (the minimum number of independent replicates for each estimated response variable) and the cells for each row were filled by a random draw from the pool of estimates for that species. We evaluated the sensitivity of this approach by generating 1,000 independent bootstrap samples and estimating the distance between the points in each ordination.

Results

In the tissues that we examined (cauline leaves of flowering plants), phytochemical profiles were generally consistent within a species, accounting for 58% of inertia decomposition in the PCA when including these classes (range of 52–68% for each bootstrap replicate; $P < 0.008$). Patterns in the expression of glucosinolates, alliarinoside, flavonoid glycosides, cyanide, and trypsin inhibitor by *Alliaria petiolata* were distinct from that in four common North American understory Brassicaceous species (Fig. 2). The native species generally shared more similarity with each other than with *A. petiolata*; on average, this contrast accounted for 21% of inertia decomposition (range: 18–26%; $P < 0.018$).

The only glucosinolate detected in cauline leaves of *A. petiolata* was sinigrin, although high levels of glucotropaeolin have been reported in roots (Vaughn and Berhow 1999). Sinigrin was not detected in cauline leaves of the four North American species in this study, although it has been reported in other tissues of *A. laevigata* and *C. concatenata* (Feeny and Rosenberry 1982; Fahey et al. 2001). Among other glucosinolates, compound 2 was present in two of the three *Cardamine* species surveyed (Fig. 1). Four glucosinolates were detected in *C. douglassii*, while only one was found in each of the other species surveyed.


Glucosinolates
Flavonoids
Alliaria petiolata
Cardamine concatenata
Cardamine douglassii
Cardamine bulbosa
Arabis laevigata
UA
Minutes
0.00
0.10
0.20
0.30
10.00
20.00
30.00
40.00
1.00
2.00
5.00
15.00
25.00
1
2
a
b
c
d

◀**Fig. 1** Representative HPLC chromatographs showing glucosinolate and flavonoid profiles of five Brassicaceae. Only peaks labeled with the same letter or number were present in more than one species. Compound 1 is sinigrin, no other labeled compounds were identified

All of the North American species produced more individual flavonoids than *A. petiolata*, and all produced compound a, which was not found in *A. petiolata* (Fig. 1). *C. concatenata* and *C. douglassii* had very similar flavonoid profiles, but completely distinct glucosinolate profiles. Isovitexin-6''-*O*-*β*-D-glucopyranoside (IVG) and 6'''-*O*-sinapoylisovitexin 6''-*O*-*β*-D-glucopyranoside are examples of bioactive flavonoid glycosides produced by *A. petiolata* (Haribal and Renwick 1998; Haribal et al. 1999), that were found in no other species. Only *A. petiolata* produced measurable amounts of cyanide (mean ± SE; 142.2 ± 25 ppm) from its leaves, which has largely been the case for all comparisons to date between *A. petiolata* and other Brassicaceous species (Cipollini and Gruner 2007). Trypsin inhibitor (TI) activity was highest in *A. laevigata* (56.8 ± 7.7 mg TI/g prot), intermediate in the *Cardamine* species [*C. bulbosa* (28.5 ± 2.31 mg TI/g prot), *C. douglassii* (20.21 ± 2.8 mg TI/g prot), *C. concatenata* (15.9 ± 1.3 mg TI/g prot)], and lowest in cauline leaves of *A. petiolata* (5.86 ± 0.8 mg TI/g prot) ($F_{4,51} = 62.95, P < 0.0001$). Despite having low constitutive activity in cauline leaves, TI activity is known to be highly inducible by wounding or jasmonic acid application in rosette leaves of *A. petiolata* (Cipollini 2002; Cipollini et al. 2005). Activity of this protein has never been examined in the other species to our knowledge.

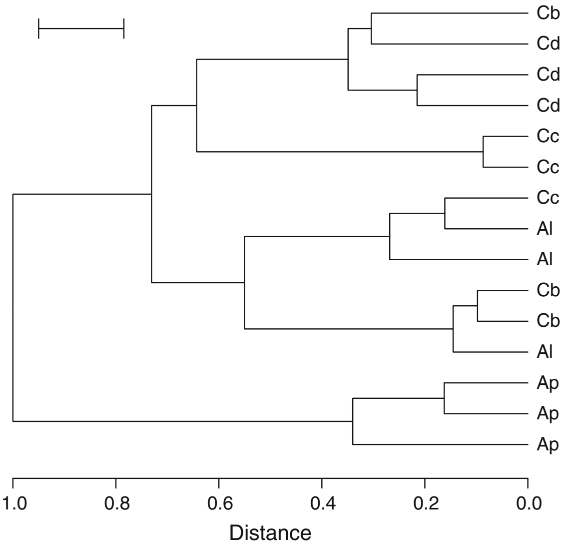


Fig. 2 Distance matrix derived from principle components of chemical profiles for five Brassicaceae. The line represents the variation (standard deviation) in distance estimates between all tips across all bootstrap samples (see methods). Ap: *Alliaria petiolata*, Cb: *Cardamine bulbosa*, Cc: *Cardamine concatenata*, Cd: *Cardamine douglassii*, Al: *Arabis laevigata*

Discussion

We tested the assumption of the NWH that invasive plants produce secondary metabolites not found in their invaded ranges using *A. petiolata*. The phytochemical profile of *A. petiolata* shared almost no overlap with Brassica species native to North America, suggesting that the allelopathic compounds produced by *A. petiolata* are indeed novel in its invaded range. The phytochemical profile of *A. laevigata* clustered within those of the *Cardamine* species, but these species are no more phylogenetically similar to each other than to *Alliaria*. The tribes containing *Arabis* (Arabideae), *Cardamine* (Cardamineae), and *Alliaria* (Thlaspideae) probably diverged from each other at a similar point in the evolutionary history of the family (USDA Plants Database, Al-Shehbaz et al. 2006), within the last 26 million years (Koch et al. 2000). This further underscores the importance of biogeography in explaining some plant invasions. Based on literature surveys, this pattern of novel secondary compounds in invasive plants appears to be widespread (Cappuccino and Arnason 2006), but targeted phytochemical surveys of other invasive plants are still lacking.

The phytochemical profiles seen here may have been influenced by several factors that we did not measure, and it is possible that broader sampling would reveal different qualitative phytochemical profiles, even within a species. Environmental variables, such as light and nutrient levels, could have influenced the expression of defensive compounds, but the nearly identical qualitative patterns within a species and similarities among closely related species (e.g., flavonoids in *C. concatenata* and *C. douglassii*) suggest strong genetic control. We have seen no qualitative differences in phytochemical profiles of *A. petiolata* populations sampled throughout North America and Europe (Cipollini et al. 2005; Cipollini and Gruner 2007), but see Haribal and Renwick (2001).

Concentrations of these compounds are likely affected by herbivory or other forms of damage (Cipollini 2002), but we selected plants with limited visible sign of folivory. Seasonal and or tissue-specific differences in production of glucosinolates, alliarinoside, and flavonoids have also been reported (Vaughn and Berhow 1999; Haribal and Renwick 2001; Gols et al. 2007), emphasizing the importance of incorporating seasonal sampling of multiple tissue types into future surveys. However, the native species studied here have a relatively ephemeral spring phenology and do not retain leaves throughout the year like *A. petiolata*. These effects could be studied under controlled conditions in the future, but the results here represent what can be observed in a single tissue type in naturally growing plants in the field, standardized for developmental stage (flowering).

While we do not claim that all of the compounds examined here are biologically active in interactions between *A. petiolata* and other species, evidence suggests that this is the case for several of them (Haribal and Renwick 1998; Haribal et al. 2001; Roberts and Anderson 2001; Callaway et al. 2008). For example, extracts from *A. petiolata* enriched in flavonoids, glucosinolates, or both were shown to more greatly impact mycorrhizal fungi from North American soils than those from European soils, as predicted by the NWH (Callaway et al. 2008). In addition to allelopathic effects on plants or mycorrhizal fungi, some of the same compounds may contribute to the low levels of insect herbivory observed on *A. petiolata* in North America (Lewis et al. 2006; Evans and Landis 2007). For example, *A. petiolata* is almost completely undamaged by the native mustard specialist flea beetle of understory habitats, *Phyllotreta bipustulata* (Hicks and Tahvanainen 1974), while all of the native species examined here are subject to damage by this insect in habitats containing *A. petiolata* growing nearby at the same time (D. Cipollini, personal observation). This effect is almost certainly chemically mediated through a balance of feeding stimulants and deterrents. Glucosinolates deter feeding by generalist herbivores, but attract specialist insects that are resistant to their effects (Renwick 2002). *Pieris napi oleracea* is a native North American mustard specialist that feeds on native Brassicaceae, including *Cardamine* and *Arabis* species (Renwick 2002). This butterfly is attracted to oviposit on *A. petiolata*, but alliarinoside and IVG (and perhaps other phytochemicals) inhibit its feeding and growth (Renwick et al. 2001). While well known as an inhibitor of aerobic respiration, the ecological relevance of the amount of cyanide produced by *A. petiolata* has yet to be determined.

Strong chemically mediated effects of *A. petiolata* on populations should select for resistance in native populations, given enough time for the "novelty" to wear off. *Pieris napi olearceae* collected from sites with a 35–55 year invasion history of *A. petiolata* (which equates to 60–100 insect generations) oviposited more often on *A. petiolata* than insects collected from *A. petiolata*-free sites (Keeler and Chew 2008). In addition, larvae from sites with *A. petiolata* survived to pupation more often on *A. petiolata* than did larvae from sites without *A. petiolata*, although pupation rates were still lower on *A. petiolata* than on native host plants. Reduced production of allelochemicals, as observed in established *A. petiolata* invasions (Lankau et al. 2009), would further limit inhibition of native plants and herbivores in areas with long invasion histories.

Our results provide the best chemical evidence to date in support of the Novel Weapons hypothesis for *A. petiolata*. Similar phytochemical surveys targeting other invasive plants would more empirically test the conclusion of Cappuccino and Arnason (2006) that novel weapons are widespread among invasive plants. Such studies would help determine whether or not chemically mediated interactions involving those weapons are important in the field relative to other mechanisms that may contribute to the success of invasive plants.

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