# Modern approaches to study plant–insect interactions in chemical ecology

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Abstract | Phytochemical variation among plant species is one of the most fascinating and perplexing features of the natural world and has implications for both human health and the functioning of ecosystems. A key area of research on phytochemical variation has focused on insects that feed on plants and the enormous diversity of plant-derived compounds that reduce or deter damage by insects. Empirical studies on the ecology and evolution of these chemically mediated plant–insect interactions have been guided by a long history of theoretical development. However, until recently, such theory was substantially limited by inadequate data, a situation that is rapidly changing as ecologists partner with chemists utilizing the latest technological advances. In this Review, we aim to facilitate the union of ecological theory with modern chemistry by discussing important theoretical frameworks for studying chemical ecology and outlining the steps by which hypotheses on insect–phytochemical interactions can be advanced using current methodologies and statistical approaches. We highlight unique approaches to isolation, synthesis, spectroscopy, metabolomics and genomics relevant to chemical ecology and describe future areas for research that will bring an unprecedented understanding of phytochemical variation.

**Plant secondary metabolites**
Organic compounds not associated with primary metabolic functions; in plants in particular, these compounds have been the subject of research in biomedical fields and in chemical ecology, in which they have been found to have largely defensive functions (for example, anti-herbivore and antibacterial functions).

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The path to modern chemical ecology has a rich and interesting history (FIG. 1) and includes three unrelated scientific advances that are key to understanding the field: Fraenkel's recognition[1] that plant secondary metabolites are not metabolic waste products but rather contribute to defence against herbivores and pathogens; the presentation of co-evolution as a process that is capable of influencing the diversification of plants and associated insects[2]; and advances in the synthesis of natural products, including approaches enabled by the widespread application of Diels–Alder cyclization and retrosynthetic analysis by pioneers such as Corey and Woodward in the middle of the 20th century[3]. The third set of discoveries bolstered modern approaches to the total synthesis of natural products, and the first two advances created the impetus for formalizing the field of chemical ecology by studying the role of phytochemistry in plant–animal evolution. Although the field had a rapid start — with advances in experimental approaches[4] and systematics[5] despite relatively few collaborations between chemists and ecologists[6] — it has largely stagnated over the past few decades[7–9] owing to a failure to utilize advances in organic chemistry, a narrow focus on a few biologically active compounds and a lack of collaboration between ecologists and chemists[10]. Chemical ecology is again at a point of great potential for rapid growth and advancement owing to methodological innovations that enable the testing of classical hypotheses that still lack adequate investigation, such as the screening hypothesis[11] and the role of chemistry in geographic mosaics of co-evolution[12]. To date, the field has expanded considerably and now includes the investigation of plant volatiles, pheromones, insect cuticular hydrocarbons, detoxification enzymes and a myriad of other compounds that mediate interactions between populations of plants, microorganisms, animals and fungi that occur in both aquatic[9] and terrestrial ecosystems[7,10,13].

Here, we review the intersection between modern ecological theory and chemistry and present important theoretical developments and relevant hypotheses in chemical ecology. We highlight the most powerful quantitative approaches, experiments and observational studies that will advance chemical ecology, and we

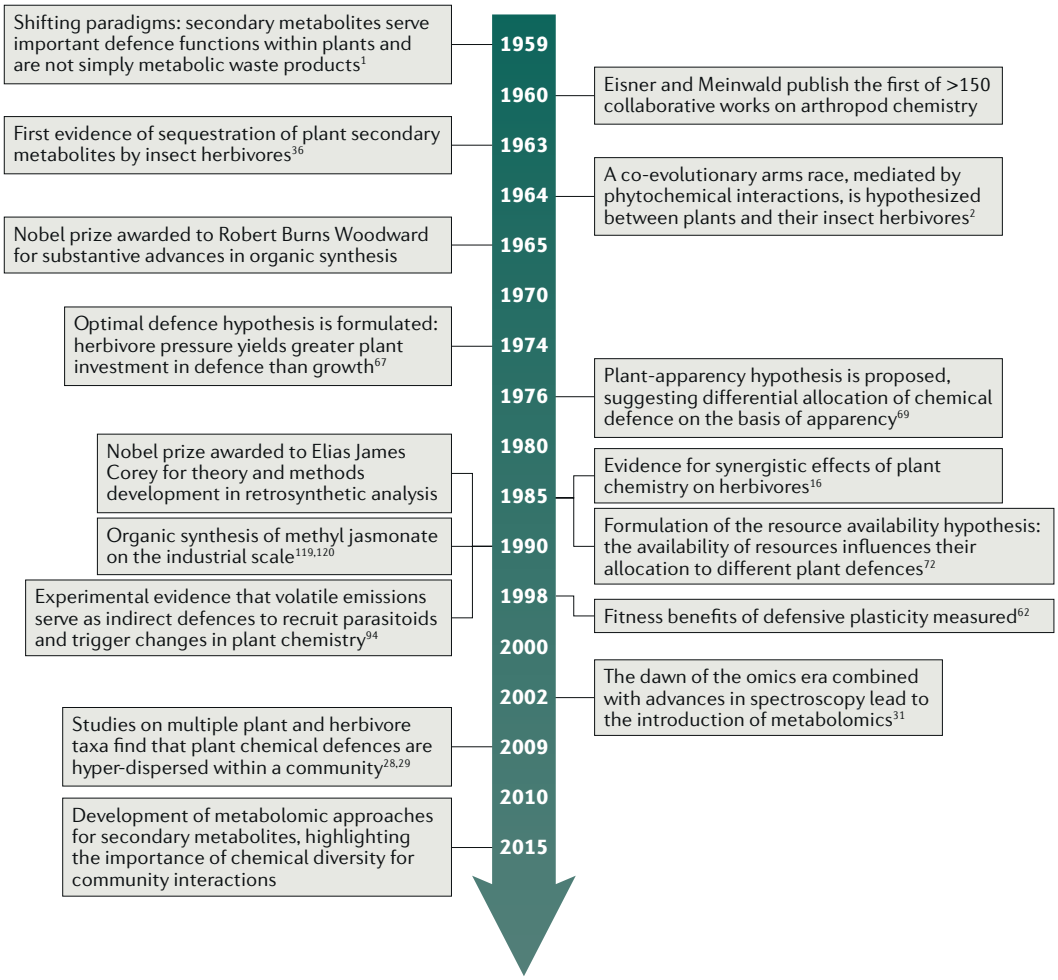



Fig. 1 | **Timeline of major breakthroughs in plant chemical ecology.**

provide recommendations for future research focused on the effects of phytochemical variation on insect herbivores. Rather than reviewing the entire discipline of chemical ecology, which is vast, we focus on the effects of plant secondary metabolites on plant–herbivore–enemy interactions, which have provided excellent case studies for chemical ecology. Nevertheless, our synthesis of the latest advances in chemistry, ecology and evolutionary biology is relevant to all areas of chemical ecology, and our discussion of advances in this field will be beneficial to chemists and ecologists alike.

## Guiding questions in chemical ecology

Four important lines of scientific inquiry have produced rich theory in chemical ecology, including questions as to why plants produce high diversities of secondary metabolites; the identity of the factors driving the spatial and temporal variation of phytochemicals; the physiological and ecological costs of phytochemical defences; and the mechanisms by which phytochemicals influence interactions among plants, insects and associated communities. In this section, we review research addressing these questions and discuss both traditional and modern methods used for developing theory in chemical ecology.

***Diversity of plant secondary metabolites.*** All plant species synthesize multiple secondary metabolites, many of which seem to have similar broad functions (such as anti-herbivore or antifungal effects); the diversity of these compounds can be quite high within a plant species[8]. This intraspecific phytochemical diversity (or redundancy) was not a central focus of early theory in chemical ecology but was acknowledged as an important factor[14,15]. However, as methods in analytical and organic chemistry have improved, phytochemical diversity has become a central variable of interest to answer the most important questions in chemistry, ecology and evolutionary biology, from the evolution of diverse metabolic pathways to understanding synergistic effects of multiple plant compounds in animals[16]. In fact, the causes and consequences of phytochemical diversity are relevant to all important theoretical advances in chemical ecology, including co-evolution, the phytochemical landscape, the cost of chemical defence and trophic interaction theory[17,18]. Two of the most important concepts for understanding phytochemical diversity as an adaptive response to herbivory are synergy and the screening hypothesis[19].

Individual plant compounds might have no or only a weak influence on higher trophic levels when tested in

Co-evolution
The evolution of reciprocal adaptation in response to reciprocal natural selection occurring with respect to a pair or complex of interacting species; often hypothesized to be associated with adaptive radiation and co-diversification.

Synergy
Combined effects of compounds in a mixture that are greater than the sum of effects for the individual compounds acting in isolation.

isolation, but those compounds can exhibit potent biological activities when part of a mixture[19,20]. Individual compounds can also function additively or might be antagonistic and counteract the effects of other compounds; although quantitative tests have been outlined to distinguish between independent joint action, additive effects, antagonistic effects and synergistic effects of compounds in mixtures[21,22], we focus on synergy in this Review because it seems to be widespread across most plant families and all classes of compounds[19]. Nevertheless, synergy as a defensive adaptation in plants has not been explored adequately, in part because gram quantities of isolated or synthetic compounds are required for synergy experiments. Access to pure compounds enables experiments with different concentrations of single compounds that can be compared with diets containing controlled concentrations of compounds of interest, while excluding confounding metabolites[23,24]. Of note, when isolated compounds are difficult to produce in gram quantities (owing to a prohibitive collection effort or a very low yield), synthetic compounds might be preferable[20,25]. Appropriate methods for both isolation and synthesis that could contribute substantively to current methods for detecting additive, antagonistic or synergistic effects of compounds in mixtures are outlined in a later section.

The screening hypothesis complements the synergy hypotheses and is a relatively untested alternative to co-evolution as a theoretical framework for understanding the evolution of secondary metabolites. The screening hypothesis suggests that many plant secondary metabolites have no adaptive value at any given time but that phytochemical variation and diversity are maintained because they increase the probability that a plant contains a potent compound (or a precursor) that is or will eventually be effective against a particular type of natural enemy (for example, insect or mammalian herbivores, pathogenic fungi and bacteria or viroids)[26,27]. This hypothesis is based on the assumptions that enzymes rarely produce a single product and subtle structural changes to an enzyme can result in substantial changes to the type of compound produced, and that most secondary compounds lack potent biological activity or seem to be redundant in their defensive ability when in isolation. The screening hypothesis might be interpreted to imply a teleological perspective that opposes the assumed costs of secondary metabolites, but this is not how it was formulated[11]. Key reactions within biochemical pathways can be affected by enzymatic promiscuity, generating different by-products owing to variations in enzyme–substrate stoichiometry, most of which might not influence plant fitness[11]. In contrast to macromolecules, secondary metabolites result from complex biosynthetic pathways — involving specific enzymes that produce certain classes of compounds — that diverge at different branching points to yield a large number of analogues that have small differences in their levels of oxidation, methylation and glycosylation. During the screening process, plant lineages characterized by biochemical reactions that inherently promote diversity might have a lower probability of extinction than linages without such mechanisms of producing diverse phytochemical mixtures owing to the exapted bioactivity of a previously extraneous or alternatively adapted metabolite. As variation is necessary for evolution, lineages that carry excess metabolic diversity possess the raw material for divergence and speciation; when coupled with the buffer against extinction, considerable diversification can occur.

Screening processes could give rise to adaptive divergence across populations and substantial intraspecific variation in phytochemical profiles. Divergent selection on the plant metabolome is generated by a diversity of different stimuli (including herbivory), and, furthermore, plant reproductive isolation can be linked to defensive compounds in plants when there are major differences in resource availability across geographically separated habitats[12]. The evolutionary patterns of plant defence documented for the genera *Bursera* (family Burseraceae) and *Inga* (family Fabaceae) have been interpreted by some as supporting the screening hypothesis, as the diversity of their secondary metabolites increases within diversifying clades[28,29]. By contrast, others have argued that plants that are not phytochemically diverse, such as *Plantago lanceolata* (family Plantaginaceae), are chemically variable across the landscape and are effective in their defence against most herbivores[30]; however, this assertion relies on the assumptions that the entire metabolome of such species has been characterized and that phytochemical diversity cannot vary when there are only a few compounds. The first of these assumptions is probably incorrect owing to incomplete phytochemical profiling for most species[31], and the second assumption is confounded by studies reporting that differences in relative abundances of only a few secondary metabolites produce biologically important differences in phytochemical diversity across the range of a tropical plant species[32]. The metabolomics[31] approaches outlined in a later section offer methods for testing such assumptions.

Very few tests of synergy or the screening hypothesis exist owing to a reduced focus on phytochemical diversity and inadequate methods in chemistry[19]. As the tools of organic chemistry have become more powerful, it has become possible to focus on a more complete picture of plant secondary metabolites, as well as the effects of phytochemical diversity on herbivores and higher trophic levels[15]. This approach expands the focus of ecologists beyond any one particular compound or class of compounds to encompass a more complete consideration of the role of phytochemical variation and synergistic effects of plant secondary metabolites on biotic communities[19,20] (FIG. 2). Phytochemical diversity can be quantified as the effective richness of compounds by using an entropy measure of the richness and relative abundance of spectroscopic peaks for a plant (FIG. 2). Thus, depending on the spectroscopy method used, diversity can be quantified as the effective richness of individual compounds (intermolecular diversity), effective richness of functional groups (intramolecular diversity) or both. Small changes in phytochemical diversity can have large biological effects on plant enemies, including specialist and generalist insect herbivores[33] (FIG. 2). An understanding of the evolutionary relationships between phytochemical diversity and

Speciation
The evolutionary process that results in the formation of new species by the divergence of an ancestral population into two genetically independent populations. This process is most often characterized by the evolution of reproductive isolation and the subsequent independent evolution of lineages.

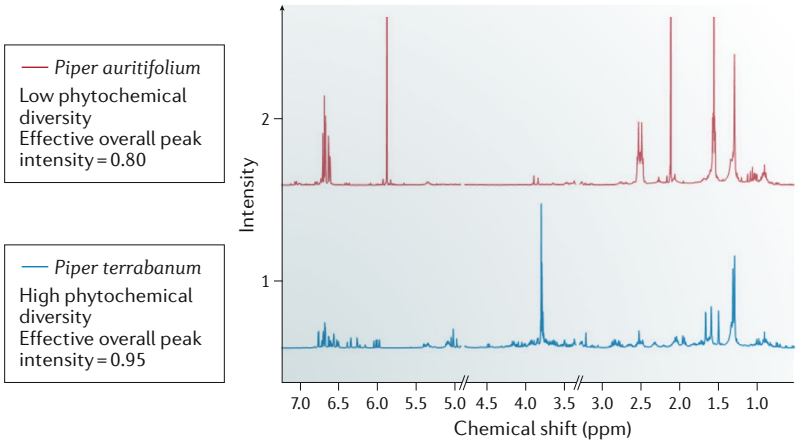



Fig. 2 | **Crude $^1$H NMR spectra from leaves of two *Piper* species.** Depiction of the crude $^1$H NMR spectra (400 MHz) from the leaves of two tropical shrub species of the genus *Piper* (family Piperaceae), *Piper auritifolium* and *Piper terrabanum*; the upper profile (*P. auritifolium*) is characterized by low phytochemical diversity, and the bottom profile (*P. terrabanum*) exhibits high phytochemical diversity. Peak diversity was measured as the richness (count of the peaks) and relative abundance (peak intensity) of binned integrals using the Simpson's entropy index, which was transformed to effective overall peak intensity, with a range from 0 to 1 and with higher values indicating greater phytochemical diversity. Differences of ±0.05 in the effective overall peak intensity are biologically meaningful, corresponding to differences in the presence or abundance of multiple functional groups or compounds. Richards and co-workers[33] linked chemical diversity in *Piper* (as characterized by these NMR peak diversities) to the overall diversity of herbivores and variation in the distribution of organismal traits, such as the proportion of generalist to specialist herbivores.

biodiversity will provide rich insight into the origin and function of secondary metabolites.

***Factors driving variation in plant chemical defences.*** Co-evolution and co-diversification — when interacting taxa exert and evolve in response to reciprocal natural selection[12,34,35] — have shaped the chemical interface and outcome of species interactions across a variety of evolutionary scales and are central processes in chemical ecology theory. Indeed, much of the literature on chemical ecology has focused on adaptations that are both causes and consequences of co-evolution; these adaptations mediate bitrophic and multitrophic ecological interactions[13]. The co-evolutionary adaptations that are most relevant to chemical ecology include the production of plant secondary metabolites that are toxic to parasites and pathogens, the feeding specialization of animals on plants that produce specific compounds, the detoxification or sequestration of plant secondary metabolites by insects[36], the production of phytochemical attractants to predators and parasitoids, the development of antennal or ovipositor sensilla that are sensitive to various volatile or non-volatile compounds and the production of variable mixtures of secondary metabolites[37–40]. The concept of a co-evolutionary arms race between plants and herbivores — yielding variation and increasing the diversity of plant secondary compounds along with variation in herbivore adaptation and diversity — has long been an appealing theoretical framework for chemical ecology[2,12,34,41–44]. For chemically mediated plant–herbivore interactions, escape-and-radiate co-evolution[2,45] has been commonly invoked to explain patterns of evolutionary diversification in plants and their herbivores. The escape-and-radiate process includes the evolution of novel phytochemical chemical defences in response to insect-induced selective pressures, yielding an adaptive radiation of the plant taxa, which is matched by the evolution of insect adaptations to those defences and associated diversification of those insect lineages.

Studies of the influence of geographic variation on the form and outcome of chemically mediated co-evolution have contributed to our understanding of co-evolution as a microevolutionary processes that is capable of driving geographic variation in chemically mediated phenotypes[41,42,46,47]. Thompson's geographic mosaic theory of co-evolution[12] recognizes the inherent geographic variation in the form and outcome of species interactions and posits that the dynamics of co-evolutionary interactions are shaped by selection mosaics arising from spatial variation in reciprocal selection and trait remixing across the mosaic. Indeed, some thorough studies on geographic variation in the phenotypic interface of chemically mediated co-evolution[41,48] have illustrated that the form and outcome of chemically mediated interactions vary geographically and that a thorough understanding of how co-evolution shapes chemical phenotypes might often require an understanding of geographic variation in the process. As new methods enable the rapid and thorough characterization of chemical phenotypes across individuals and populations, chemical ecology research exploring geographic variation in plant–herbivore co-evolution has perhaps the greatest potential to increase our understanding of the processes generating and maintaining phytochemical variation across landscapes. A remaining challenge for chemical ecology (and co-evolutionary biology in general) is establishing links between co-evolutionary processes (microevolution) and patterns of biological diversification (macroevolution)[49,50]. In Supplementary Box 5, we outline methods in genomics and comparative phylogenetics and work with non-model systems that will enable researchers to establish such links for understanding co-evolution, co-diversification and the origins of phytochemical diversity.

For the chemical ecology of plant–herbivore interactions, the co-evolutionary process is predicted to yield high herbivore specialization owing to evolutionary trade-offs in physiological responses to different plant chemical defences[2]. Thus, a major focus in chemical ecology has been to utilize quantitative genetic approaches to uncover trade-offs in herbivore performance across different hosts or diets with different secondary metabolites[51]. Although genetic evidence for antagonistic pleiotropy has been notoriously elusive[52–54], a study from 2016 by Gompert and Messina[55] utilized experimental evolution and genomic re-sequencing to demonstrate that for a seed beetle, genetic variants that were selected on an experimental novel host plant were selected against in populations that had been returned to the original host plant. Thus, the possibility that genetic trade-offs affect the evolution of specialization remains and should be investigated further with modern genomic and chemical approaches. Furthermore,

Parasitoids
Organisms characterized by a unique form of parasitic lifestyle, in which the host is killed by the developing juvenile stage; the most diverse taxa, the wasps (insect order Hymenoptera) and flies (insect order Diptera), have a dramatic influence on the ecology of terrestrial ecosystems.

Antagonistic pleiotropy
A type of genetic architecture in which a single genetic locus affects more than one trait (which can include performance or fitness in more than one environment), with effects of one trait (or in one environment) being positive and effects of the other trait (or environment) being negative.

it is unknown if a small number of trade-off loci are important within a large, polygenic system or in the face of multiple, shifting selection pressures, which is an area requiring both theoretical and empirical work. Tests of the hypothesis that trade-offs constrain the evolution of plant–insect interactions should be careful not to confound evidence for local adaptation (to a particular mix of compounds or species of host plant) with evidence for genetic trade-offs (which must be tested within populations[56]) or with the possibility that the loss of alleles (through mutation or drift) could also lead to local specialization[57,58]. Finally, we note that much is unknown about sensory systems and phytochemistry[59] and that the host finding and handling behaviour of adult insects is an area in which host-associated trade-offs might indeed be the most likely reason for herbivore specialization[60,61].

***Physiological and ecological costs of defence.*** The assumption that there are physiological and ecological costs to chemical defence has yielded a rich literature on plasticity in defence, and theory related to plasticity has helped to elucidate these costs[62]. In particular, numerous hypotheses have been developed on factors that influence the allocation of resources to chemical defence in plants[63–66] (Supplementary Box 1 and Supplementary Box 2). The most prominent of these hypotheses include the optimal defence hypothesis, which postulates that plant defensive strategies are associated with variation in selective pressure from various plant herbivores and pathogens as well as fitness consequences of damage to tissues[67,68]; the plant-apparency hypothesis, which posits that the predictability of plants in space and time determines plant chemistry and herbivory[66,69,70]; the carbon–nutrient balance hypothesis, which theorizes that the production of plant secondary metabolites is determined by the availability of carbon and nitrogen[71]; the resource availability hypothesis, which focuses on the adaptation of plants to poor soils via chemical defence of tissues that are difficult to replace[72]; and the growth–differentiation balance hypothesis, which speculates that resource allocation between different physiological processes depends on environmental conditions and constraints[64,73]. All of these hypotheses focus on defences as adaptations to both abiotic and biotic selective pressures; however, the carbon–nutrient balance and growth–differentiation balance hypotheses are primarily plasticity hypotheses, focused on ecological responses of plant chemistry to environmental conditions. These five hypotheses have inspired hundreds of studies and reviews, but few generalities have emerged across plant taxa or classes of secondary metabolites. For the plasticity hypotheses, resource availability substantially alters chemical defence for different plant taxa and classes of compounds[63–66], but the relative utility of any one hypothesis depends on the compounds being studied — for example, phenolics are more positively influenced by increasing carbon:nitrogen ratios than other compounds[63,74–77]. However, it is clear that more empirical tests are needed[66–71] (Supplementary Box 2).

A useful approach for the continued investigation of these different hypotheses is to consider variation in plant defensive compounds as integrated layers, for which each hypothesis provides different insight into the distribution and natural variation of secondary compounds, including insights on ontogenetic processes, physiological mechanisms, evolutionary origins and functional consequences[78].

***Plant secondary metabolites in ecological interactions.*** Plant secondary metabolites mediate ecological interactions across different spatial, temporal and organizational scales[79–81]. A myriad of useful hypotheses exist about how plant secondary metabolites, accumulated metals[82] or poor nutritional mixes of primary metabolites affect herbivores[83,84] and their natural enemies[81]. The main hypotheses relevant to chemically mediated trophic interactions are focused on sequestration, specialization and community diversity. A traditional view of tritrophic interactions is that insect herbivores either excrete, biotransform or sequester plant secondary metabolites and that any of these responses potentially influence their physiology, population dynamics and interactions with natural enemies[85]. These responses are generally partitioned by diet breadth, and sequestration is the most common response associated with specialized species[86]. Sequestration can have negative effects on predators and parasitoids[87] or positive effects on parasitoids via disruption of herbivore physiological defences such as encapsulation and melanization[88]. A current paradigm is that specialist herbivores are more toxic to predators than generalists but that specialists host higher numbers and diversities of parasitoids[89]. Thus, well-defended plant communities should be characterized by greater compartmentalization and more reticulate local food webs if there are greater abundances of specialist herbivores than generalist herbivores and parasitoids. The question remains, however, as to whether such effects of secondary metabolites on consumers can structure entire arthropod communities. Few studies have demonstrated that entire plant-associated communities are influenced by phytochemicals. For example, higher concentrations of tannin in various genotypes of cottonwood trees (*Populus* spp.) and saponins in alfalfa plants (*Medicago sativa*) were linked to increased arthropod diversity[90–92].

Another paradigm in the chemical ecology of trophic interactions is that plant secondary compounds are beneficial to plants because they enhance the activity of secondary consumers, either by facilitating the effectiveness of predation and parasitism or by providing chemical cues used by predators and parasitoids to find their prey and hosts[93]. For example, after being attacked by a herbivore, plants might synthesize and emit volatile organic compounds (VOCs) that serve as host location cues to the natural enemies of the herbivore[94–96]. Studies using several model systems, including tomato, tobacco and maize, have shown that in some cases the VOCs released by the plant can vary depending upon the number and types of herbivores present, thereby providing accurate information to predators and parasitoids regarding the suitability of available prey[93]. Whether this phenomenon is common in natural systems or in high-diversity systems (such as tropical forests) is unclear; thus, this

Box 1 | **Metabolomics in chemical ecology**

Advances in metabolomics[144,146] along with measures of phytochemical diversity[19,33] are integral to the understanding of insect–phytochemical interactions, and the 'phytochemical landscape' is one of the most powerful predictive frameworks for understanding the chemical ecology of insect communities. Hunter[17] describes the phytochemical landscape as variation in secondary metabolites of primary producers across biotic communities in both terrestrial and aquatic systems. This variation drives variation in consumer abundance and diversity, which in turn feeds back to alter phytochemical diversity across the landscape[17,19,33]. Methods developed in the past 5 years have provided the necessary tools to characterize this landscape.

**Sampling**

The sampling of plant tissues for metabolomics starts with a natural community of multitrophic interactions (see figure below, part **a**). Typically, consumer abundance and diversity are known for plants in a biotic community, and leaf material (or other plant tissue) is collected from plants for which ecological data have been collected, then dried in the field and prepared for analyses[33].

**Processing**

High-throughput processing of plant tissues produces NMR spectra for all of the plants in the community, which can be quantified as peak entropies and associated diversity values, representing the number and relative abundances of different functional groups (see figure below, part **b**).

**Downstream analysis**

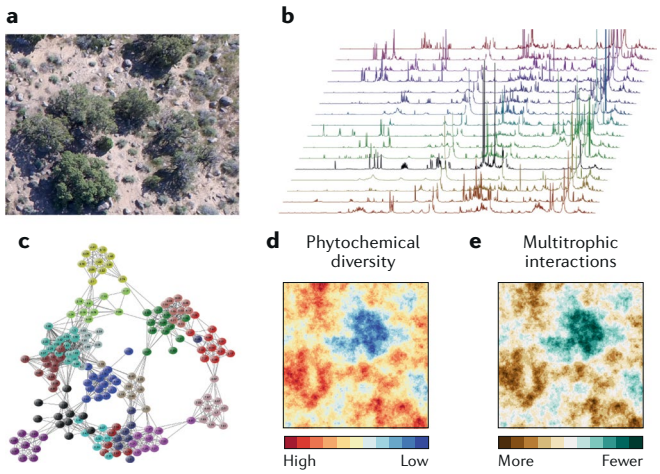
Many downstream options for analysis exist, from ordination to network analyses. Both network analyses and ordination provide exploratory clustering approaches to multivariate data. Network analyses with metabolomics data produce nodes (or vertices) representing peaks from chromatography, and these nodes are connected by edges (or links) that are determined by shared peaks among samples (see figure below, part **c**). Useful ordinations for metabolomics data include factor analysis, principal components analysis and nonmetric multidimensional scaling. Ordination methods produce multiple factors or components, which are linear combinations of peak data from chromatography and indicate similarity among samples (based on shared metabolites or functional groups). In the network analysis schematic, the nodes (or vertices) represent functional groups, and the edges (or links) represent correlations between nodes on the basis of co-occurrence in secondary metabolites.

**Phytochemical landscape**

Phytochemical diversity indices based on network modules can be projected back into space for a new visualization of the original phytochemical landscape, as envisioned by Hunter[17] (see figure below, part **d**).

**Consumer landscape**

Multitrophic interactions are superimposed on this phytochemical landscape to examine the metabolomic influence of phytochemistry on arthropod communities. The heatmap of multitrophic interactions (see figure below, part **e**) depicts lower herbivory (blue) and higher predator loads (red) where phytochemical diversity is high.




hypothesis remains important to test across selected taxa and VOC systems.

Great potential exists for understanding the role of plant chemistry in mediating interactions in complex biological communities, particularly by quantifying a phytochemical landscape (BOX 1). The integration of all of the methods (discussed below) will contribute substantially towards this goal, particularly metabolomic and genomic techniques for analysing data from natural systems combined with isolation and synthesis techniques for conducting careful experiments in the laboratory to quantify the effects of controlled phytochemical mixtures on insect herbivores and predators.

## Methodological advances in chemistry

Many tests of the aforementioned prominent hypotheses in chemical ecology require comparative methods in analytical chemistry applied across large taxonomic and geographic scales. Thus, the field benefits from all advances in analytical chemistry and spectroscopy, which facilitate the study of increasingly complex mixtures of phytochemicals. Furthermore, organic synthesis methods and analytical chemistry can provide useful insight into the biosynthetic pathways and mechanisms that lead to structural diversity in plants[97]. Understanding these biosynthetic pathways and how they generate phytochemical diversity is also quite relevant to the screening hypothesis.

A major hurdle for chemical ecology and natural products chemistry has been the lack of accuracy of techniques to fully characterize natural products because of the myriad of different and uncharacterized structures and the unavailability of pure compounds for further study. Isolation methods are still quite valuable for chemical ecology, but if gram or kilogram quantities of natural products are required for analytical standards, feeding studies or structural confirmation, synthesis might be the most economical source. The synthesis approach involves the examination of metabolite transformation in vitro, enabling the thorough exploration of biosynthetic hypotheses and the potential chemical space of natural products[98].

Another challenge has been the characterization of the full phytochemical phenotype of individual plants, but advances in non-targeted metabolomics approaches have been increasingly applied to ecological systems over the past decade, replacing single-component or multi-component targeted approaches (Supplementary Box 2 and Supplementary Table 2). The initial expectation in the field that metabolomics would rapidly provide as much data as other omics approaches (genomics, transcriptomics and proteomics) has still not been attained owing to the great complexity of secondary

Dereplication methods
Fast identification of compounds using orthogonal physicochemical characteristics to compare spectroscopic data with molecular features gleaned from libraries of known compounds and to confirm identifications.

Macrolides
Phytochemicals that have antibacterial or antifungal properties.

compounds that cannot be determined properly by any of the dereplication methods that are based on mass spectrometry (MS) or spectroscopic analysis of crude plant extracts.

***Natural product characterization.*** The approaches and goals of natural product drug discovery differ from those of chemical ecology, but both fields exist at the interface of biology and small-molecule chemistry and rely on the efficacy of compound isolation and synthesis. Improvements in extraction and isolation methods facilitate structure identification and the generation of biosynthetic hypotheses, whereas advances in synthetic chemistry facilitate further in situ, in vivo and in vitro study of individual compounds and mixtures when isolated compounds are scarce. Similar to natural product drug discovery, individual constituents must be isolated or synthesized for the following three reasons. First, spectroscopic techniques for complex natural products (gas chromatography (GC)–MS, liquid chromatography (LC)–MS, MS, NMR spectroscopy and Fourier transform infrared (FTIR) spectroscopy are summarized in Supplementary Table 2) might be insufficient to determine structure, and revisions to the proposed structure are often necessary following total synthesis[98] A classic example in which the absolute stereochemistry was undetermined and synthetic methods were required to determine the correct absolute stereochemistry is the synthesis of periplanone, an insect sex pheromone, by Still and colleagues[99] (FIG. 3a). An example of structural revision can be found in the macrolides mandelalides A–D after completing the total synthesis of mandelalide A[100] (FIG. 3b). Similar to many other natural products, the structural complexity of the mandelalides has inspired advances in synthetic chemistry[100–102] as well as computational chemistry[103]. In metabolomic approaches, the annotation and identification of metabolites is contingent on the availability of synthetic and isolated standards for direct comparison. As the need for diverse metabolite libraries increases, technologies for synthesis, biosynthesis and purification will become increasingly important[104]. Second, determination of the biological function and metabolic fate of natural products in consumer trophic levels is dependent on the availability of synthetic material for laboratory experiments[105]. The development of a semi-synthetic biomimetic toolkit for the generation of common metabolites from natural product standards could greatly facilitate studies investigating the metabolic fate of phytochemicals in primary and secondary consumers. Furthermore, studies of synergy in the biological activity of phytochemicals cannot be performed without varying the levels of the individual constituents of a mixture, which are obtained via isolation or synthesis. Third, isolated and synthetic compounds are utilized as analytical standards in targeted chemical ecology analyses, a method that has been used for the analysis of *Piper* amides[20,25,106–108]. A few notable examples of these approaches are summarized in TABLE 1 (REFS[24,105,109–112]).

Despite the tremendous advances that have been made in chemical ecology research, the extraction of phytochemical mixtures and the isolation of individual components from complex matrices remains a challenge in the field. Modern extraction and isolation methods are similar to the more traditional methods employed in chemical ecology research, but all techniques have been substantially improved over the years[113]. Over the past decade, developments in extraction technology have reduced the need for and the use of organic solvents, resulting in 'green' methods that minimize the environmental influence of chemical ecology research[114]. An understanding of the most appropriate extraction or isolation method for a given study system is crucial to conducting the most productive research possible[115] (Supplementary Table 1).

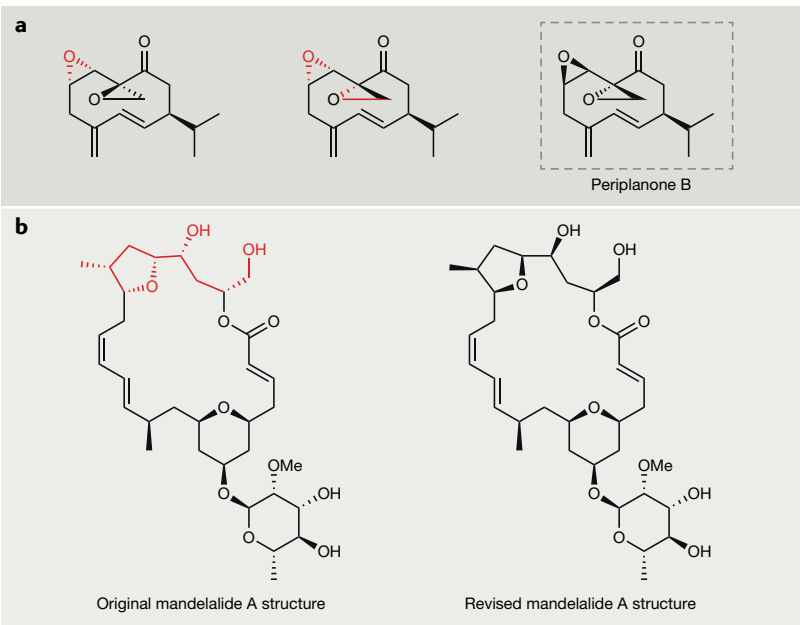
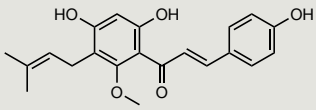
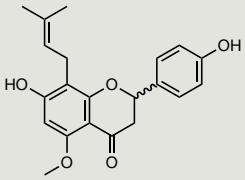
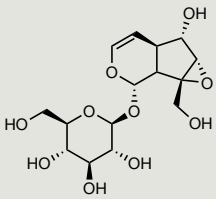
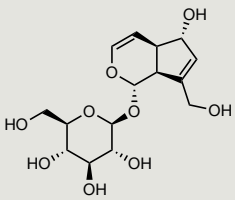
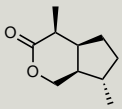
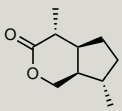



Fig. 3 | **Periplanone and mandelalide A structure determination by total synthesis.** **a** | Two periplanone diastereomers synthesized by Still[99] are shown on the left. The structure of naturally occurring periplanone B is highlighted in the box on the right. **b** | Original proposed structure for mandelalide A (left) and revised structure based on total synthesis (right).

***Organic synthesis.*** The evolution of organic synthesis over the past century was largely driven by the need for new methodologies to accomplish natural product syntheses, and the widespread application of the Diels–Alder reaction has had a particularly positive effect on total synthesis. More generally, methods have progressed from simple bond formation to diastereoselective and stereoselective bond formation. Methyl jasmonate, an important plant hormone and defensive compound, serves as just one example of the history and potential of ecology-inspired natural product synthesis (FIG. 4). After the isolation and characterization of methyl jasmonate in 1962 (REF. 116), the first racemic synthesis was reported by Sisido in 1969 (REF. 117). In the intervening years, the synthesis of methyl jasmonate has progressed with the state of organic chemistry; for instance, Negishi[118] produced racemic methyl jasmonate with a 70% yield from cyclopentenone via nickel-catalysed

Table 1 | **Chemically mediated trophic interactions studied using isolated or synthetic phytochemicals**

| Phytochemical | Structure | Source | Trophic interactions | Refs |
|---|---|---|---|---|
| Xanthohumol |  | Isolation from natural source | Anti-feedant activity against peach-potato aphid *Myzus persicae* (Sulzer) | 105 |
| Isoxanthohumol |  | Synthetic or semi-synthetic | | |
| β-Caryophyllene |  | Synthetic or semi-synthetic | • Quantification in maize stimulated by *Diabrotica virgifera* beetles<br>• Attractant of the *D. virgifera* parasitoid *Heterorhabditis megidis* in maize | 110 |
| Catapol |  | Isolation from natural source | Synergistic *Plantago* spp. (family Plantaginaceae) chemical defences against *Junonia coenia* (family Nymphalidae) caterpillars | 112 |
| Aucubin |  | Isolation from natural source | | |
| Iridomyrmecin |  | Synthetic or semi-synthetic | • Headspace quantification and stereoselectivity of *Leptopilina heterotoma* (order Hymenoptera, family Figitidae) chemical defences were measured in the presence and absence of *Myrmica rubra* ants<br>• Isoiridomyrmecin causes *Drosophila* avoidance of the parasitoid *L. heterotoma* via a dedicated olfactory circuit | 24,109 |
| Isoiridomyrmecin |  | Synthetic or semi-synthetic | | |

or palladium-catalysed allyl cross-coupling (FIG. 4a), whereas Tsuji[119] produced racemic methyl jasmonate with a 60% yield via palladium-catalysed decarboxylative dehydration from the precursor diallyl adipate (FIG. 4b). Eventually, the Tsuji chemistry was commercialized for industrial-scale synthesis by Yoshioka and Yamada at the Nippon Zeon Company[120], which coincided with the publication of the first instance of methyl jasmonate-induced defences by Farmer and Ryan[121]. Methyl jasmonate research has accelerated rapidly in the years since the publication of these two studies, demonstrating how advances in synthesis can facilitate advances in chemical ecology. Although racemic methyl jasmonate is readily available commercially, enantiopure material is much more expensive and difficult to synthesize. Several asymmetric syntheses of methyl jasmonate have been reported[122–124] since the first report by Quinkert et al.[125]. However, the approaches of Negishi and Tsuji could be revisited using modern asymmetric techniques to produce (−)-methyl jasmonate from inexpensive racemic starting materials. An asymmetric product could be produced with chiral ligands in conjunction with reactions catalysed by palladium[126] or copper[127] using the Negishi approach (FIG. 4c) or with an asymmetric organocatalyzed Michael addition[128] (FIG. 4d) incorporated into the Tsuji approach[126,127]. Owing to its importance in the perfume industry[129], its function as a signal for paclitaxel (Taxol) production in cell cultures[130] and its role as a possible chemical mediator of plant interactions in ecology[131], new synthetic routes to methyl jasmonate are still under development[132]. Hopefully, further synthetic advances and the demand for material will make enantiopure methyl jasmonate more readily available for ecological research. Continued improvements to natural product synthesis will certainly benefit from other chemical ecology-inspired metabolites.

Natural products have continually inspired the development of new methodologies in organic chemistry.

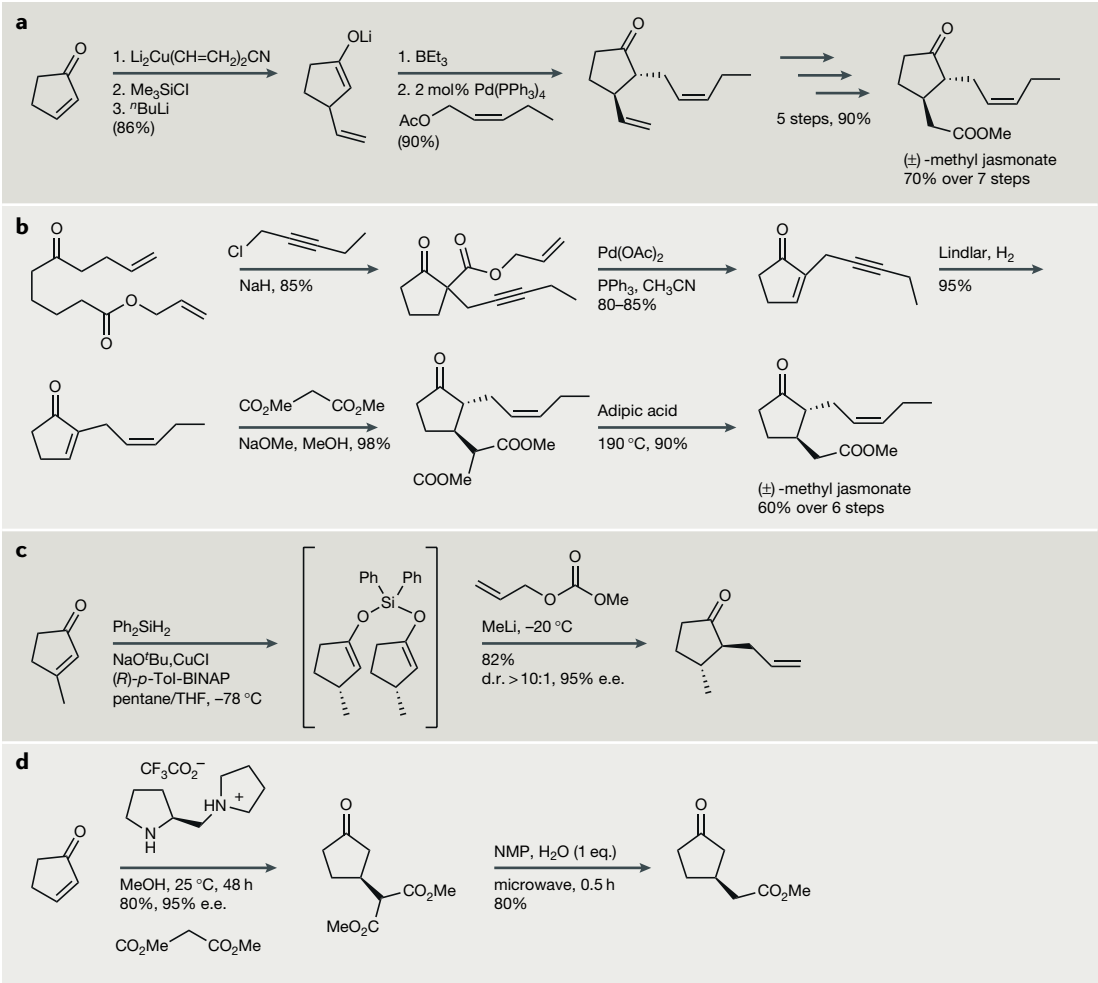



Fig. 4 | **Syntheses of methyl jasmonate and proposed modifications for asymmetric synthesis. a** | Negishi's synthesis of racemic methyl jasmonate employing palladium-catalysed cross-coupling[118]. **b** | Tsuji's synthesis of racemic methyl jasmonate via palladium-catalysed decarboxylative dehydration[119]. **c** | A modification of the Negishi approach using a chiral ligand is used to produce an enantioenriched product. **d** | An asymmetric organocatalyzed Michael addition step is incorporated into the Tsuji approach[128].

One area of organic synthesis in which this phenomenon is most apparent is biomimetic synthesis, wherein a biosynthetic hypothesis is tested synthetically — a retrosynthetic analysis is developed in the context of biologically available precursors and is subsequently performed to investigate the plausibility of biosynthesis. The first example of biomimetic synthesis was the Robinson tropinone synthesis (FIG. 5a), in which a synthesis was undertaken using primary metabolite precursors[133,134]; this approach serves as the basis of retrosynthetic analysis and biomimetic chemistry, two essential tools for synthetic organic chemistry. The cycle of innovation resulting from biomimetic synthesis and biosynthetic discovery[135] can also be seen in the development of the Stork–Eschenmoser hypothesis, which posits that sesquiterpenes can be derived from a concerted cyclization of linear polyenes[136,137] (FIG. 5b). Although Robinson and Stork are among the earliest and most enduring examples of biomimetic syntheses, many additional advances in synthesis and the understanding of biosynthesis have been accomplished using biomimetic approaches[138]. In addition, the way in which nature creates diversity in stages, such as the cyclase–oxidase sequence (FIG. 6a), has inspired a new generation of combinatorial chemistry — diversity-oriented synthesis (DOS). Inspired by natural product scaffolds and using the build–couple–pair approach[139], DOS builds in functional and stereochemical diversity in a step-wise manner, creating relatively small libraries of compounds that represent large swaths of chemical space[140–143] (FIG. 6b).

***Metabolomics.*** In addition to the aforementioned methods, non-targeted metabolomic approaches to drug discovery[144] and dereplication[145] might also have utility for chemical ecologists, and the promise of exploiting the same data set to explore drug discovery and chemical ecology is extremely enticing (BOX 1). Advances in metabolomics are also presenting new possibilities to improve our understanding of insect–phytochemical interactions, such as the importance of synergy and phytochemical diversity in shaping insect communities associated with different plants[19,33]. Historically,

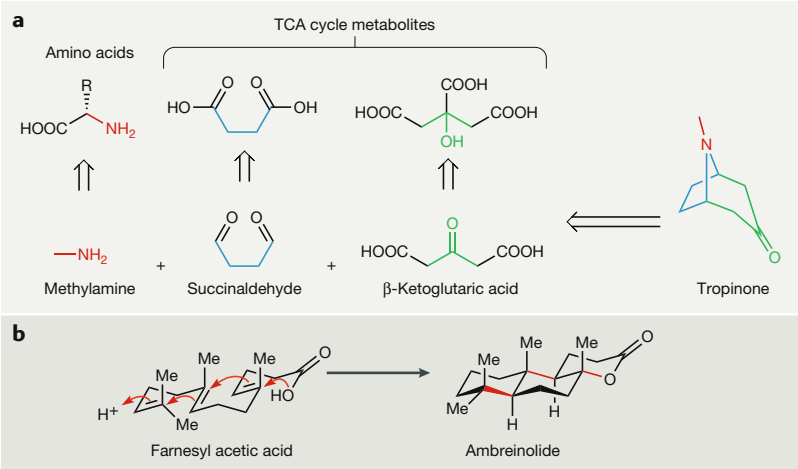



Fig. 5 | **Early approaches to the biomimetic synthesis of natural products.** **a** | Robinson's tropinone synthesis[133] serves as the basis for both retrosynthetic analysis and biomimetic chemistry. The proposed primary metabolite precursors of the tropinone structure are highlighted. **b** | Stork's polyene cyclization[136] hypothesis shows how the concerted cyclization of linear polyenes can lead to complex sesquiterpene structures. TCA, tricarboxylic acid.

Next-generation sequencing
Modern DNA sequencing platforms that leverage direct sequencing by synthesis technologies to simultaneously determine the DNA sequences of millions or hundreds of millions of DNA fragments. Also known as high-throughput or massively parallel sequencing, these methods have revolutionized genomics.

RNA sequencing
The use of next-generation DNA sequencing approaches to characterize and quantify RNA from biological samples. RNA extracted from tissue is converted into cDNA and directly sequenced on next-generation sequencing platforms such as Illumina. These approaches allow for efficient characterization of the coding regions of genomes (for example, transcriptome sequencing) and for analysis of differential gene expression.

Genome-wide association studies
Observational studies of a genome-wide set of genetic variants in a sample of phenotypically variable individuals aimed at detecting specific variants in which genotypic variation is associated with phenotypic variation.

the process of characterizing bioactive compounds has been tedious, tremendously time consuming and has required substantial quantities of starting material[146]. Furthermore, the ecological effects of plant secondary compounds are usually quantified in isolation, rather than in mixtures with other naturally occurring compounds[19]. In addition to metabolomics, other technologies — including gene expression profiling techniques, targeted knockout of candidate genes, proteomics and genomics — have improved approaches to examine the ecological effects of phytochemicals[146].

The three metabolomics techniques that are particularly promising combine analytical instrumentation with statistical analysis — namely, the comparative metabolomics approach, the systems biology approach and the comprehensive metabolome approach[146]. The comparative metabolomics approach aims to reduce the complexity of the entire metabolome by pre-selecting target compounds from a subset of the metabolome and comparing the presence or absence of these metabolites found in study units from different experimental or observational treatments[146]. For this approach, multivariate analyses are used to discriminate key differences in mixtures between treatments, rather than isolating each compound and conducting synergy or redundancy bioassays. The systems biology approach[147] combines metabolomics with other omics techniques, such as proteomics, genomics or transcriptomics. The ultimate goal of this approach is to identify metabolites, the biochemical pathways associated with their production or the biosynthetic role of specific genes or enzymes and to link this information with physiological processes or ecological interactions. Finally, the comprehensive metabolome approach[146] characterizes the entire metabolome of model species to create a global metabolome library, which is useful for assessing metabolic potential and inferring possibilities for chemical signalling. Such data sets exist for *Arabidopsis thaliana*[148], *Escherichia coli*[149] and *Saccharomyces cerevisiae*[150]; however, the development of these data sets remains a major challenge for non-model plants, which have not been as intensively studied as *A. thaliana*.

## Methodological advances in ecology

Over the past 20 years, ecology has benefited from advances in methodologies that allow for addressing less studied disciplines within chemical ecology, such as the importance of fungal endophytes (Supplementary Box 1), as well as the mainstays of chemical ecology, such as the plasticity of chemical defences (Supplementary Box 2). These advances include more sophisticated mathematical, computational and statistical models (Supplementary Box 3,4) and comparative phylogenetics (Supplementary Box 5), but the most rapid advances that will transform chemical ecology are the methodologies of genomics for non-model organisms.

***Genomics and the genetic architecture of metabolic phenotypes.*** An understanding of how plant chemistry evolves in response to environmental variation and selection from herbivory requires an understanding of the genetic architecture of metabolic phenotypes. Despite long-term interest in plant chemical defences and natural product discovery, very little is known about the genetic basis and architecture of metabolic phenotypes or even the extent to which such phenotypes are heritable. This paucity of knowledge is set to change as technological advances within the past decade in DNA sequencing have revolutionized our ability to generate genome-level data across natural populations[151,152]. Next-generation sequencing technologies have enabled inexpensive and rapid DNA sequencing at the whole genome or whole transcriptome levels[151,153]. Draft whole genome references are becoming easier to generate, and whole genome re-sequencing studies in large numbers of individuals are beginning to emerge[152,154]. Cost-effective RNA sequencing (RNA-seq) approaches are facilitating transcriptome-wide studies that can detect differential gene expression between organisms in different environments and with differing chemical phenotypes[155]. Finally, restriction-enzyme-guided reduced representation approaches that allow for the economical generation of population genomic data for a subset of genomic regions in large numbers of individuals are leading to unprecedented advances in our ability to understand the relationship between geographic, ecological, phenotypic and genetic variation[152,156,157].

The marriage of new methods for metabolomic profiling with genome-wide association studies (GWAS) should facilitate improvements in our understanding of the genetic basis, complexity and evolution of metabolomic variation[158,159]. Currently, knowledge of the genetic architecture of metabolic phenotypes is limited to model organisms, such as *Arabidopsis*[160–162], rice[163], tomato[164] and corn[165,166]. In model organisms, metabolomic GWAS are revealing the genetic basis for the production of key metabolites and hold promise for elucidating the link between genomic and metabolomic variation[158]. These GWAS have also facilitated the discovery and subsequent reworking of our understanding

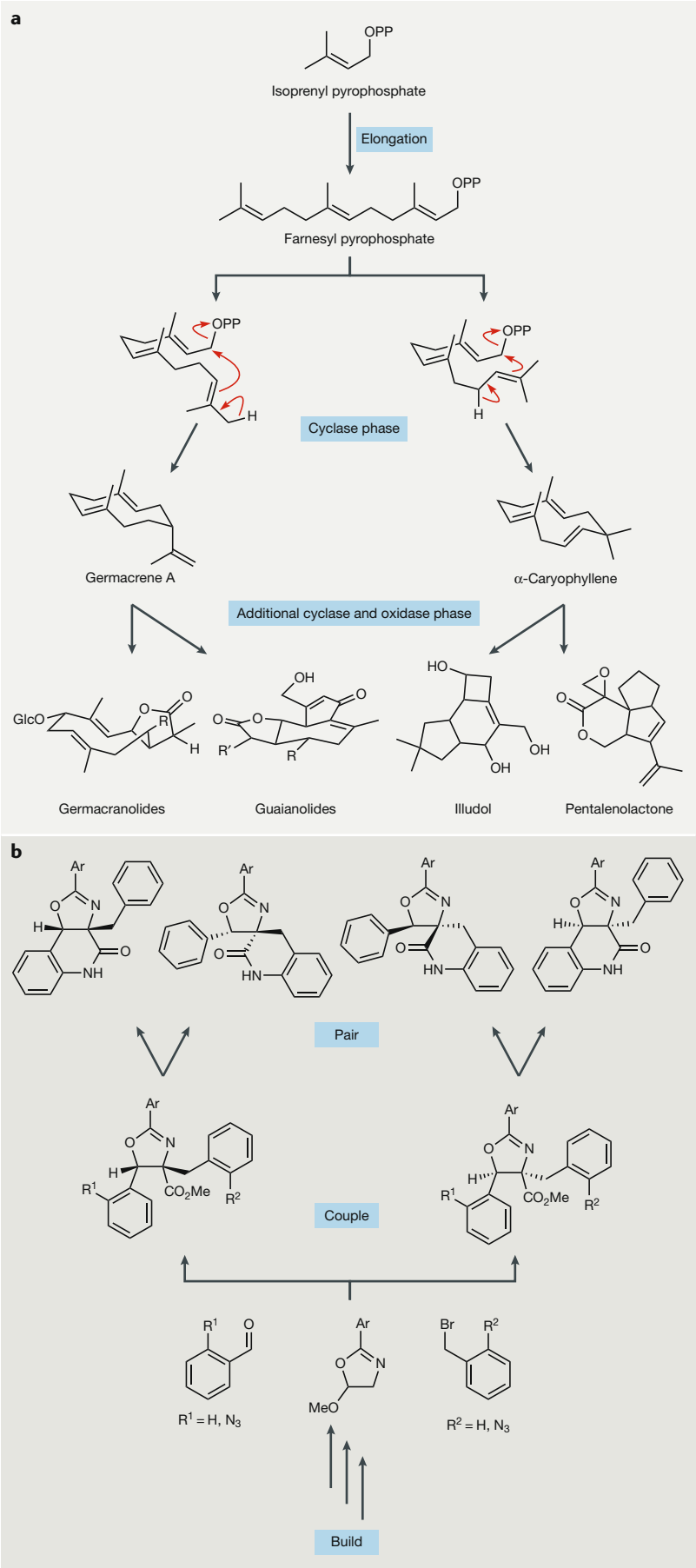



Fig. 6 | **Building diversity via biosynthesis and diversity-oriented synthesis. a** | The diversity of natural products is created in stages[183–186]. Here, a cyclase phase that produces the basic carbon skeleton of the natural product is followed by an oxidase phase that introduces various oxygen functionalities. **b** | Mimicry of this diversity-building process resulted in the development of diversity-oriented synthesis[139], which has been used to rapidly build libraries of compounds that can be used, for example, in medicinal chemistry.

of biochemical pathways, although knowledge is mostly limited to a select subset of pathways[160,166,167]. In addition, gene expression analyses of RNA-seq data coupled with metabolomic profiling across different life stages and environments has led to the discovery of candidate genes and pathways in the production of key natural products, such as etoposide, used in chemotherapy[168]. The rapid growth of genomic resources will increase our ability to identify and characterize enzymes and biosynthetic pathways underlying key metabolite production and could revolutionize natural product discovery[169]. More generally, these approaches are poised to make major contributions to our understanding of the genetic basis and evolution of plant phytochemistry.

***Beyond old concepts of model systems.*** The analysis of the genome sequence of *A. thaliana* (family Brassicaceae) along with subsequent metabolomics analyses and associated research have provided substantial insight into the chemical ecology of this species and its wild relatives (that is, species of *Arabidopsis* and *Arabis*)[146,170]. As argued in another review of chemical ecology[13], model species and their non-model relatives should continue to provide useful data for existing chemical ecology theory. Nevertheless, the methods we have reviewed are increasingly available for application outside of model systems, yielding promising opportunities to discover and investigate the phytochemistry of important non-model systems. Other study systems could be considered important in many ways, including demonstrations of keystone effects in communities, high abundances, broad distributions, unique phytochemistry or diverse interactions.

Several plant genera and their associated arthropods have emerged as promising non-model study systems for chemical ecology, including (but certainly not limited to) *Asclepias* (family Apocynaceae), *Inga*, *Piper* (family Piperaceae), *Nicotiana* (family Solanaceae) and *Solanum* (family Solanaceae). All of these plant genera have been used for careful and thorough tests of important hypotheses in chemically mediated plant–insect interactions. Most notably, the cardenolides and bufadienolides produced by the genus *Asclepias* have been the focus of studies in chemical ecology for more than 50 years[171], and this genus has provided clear examples of plasticity in secondary metabolites, particularly with respect to changes in chemistry following herbivory[39]. The tropical genera *Inga* and *Piper* have been used to test various predictions of escape-and-radiate co-evolution[29,33,172,173] as well as hypotheses about phytochemical diversity within communities[19,174–176].

Wu and colleagues[177] describe an exemplary metabolomics approach for examining the evolution of biosynthetic pathways in the genus *Solanum* that uses most of the spectroscopic methods outlined in Supplementary Table 2. Finally, studies with another genus of family Solanaceae, *Nicotiana*, have contributed substantially to chemical ecology (FIG. 1), including elucidation of the ecological importance of methyl jasmonate, demonstration of the costs of chemical defence and support for the optimal defence hypothesis, but have not provided evidence for the carbon–nutrient balance hypothesis[67,178,179]. Similar systems could be easily developed using the tools described in this Review, and the field of chemical ecology will benefit from integrative research programmes that focus on such non-model systems[180].

## Conclusions

The current era of omics techniques (genomics, transcriptomics and proteomics) has the potential to yield an unprecedented mechanistic understanding of model organisms as well as of important crops, medicinal plant species and ecological interactions. These techniques enable the determination of regulatory processes involved in the adaptation of plants to abiotic and biotic stresses and have guided the development of plant species that are resistant to or unaffected by such stresses and have increased productivity. Nevertheless, the metabolomics step is still a major bottleneck because of the high diversity of plant secondary compounds and the myriad of unresolved biosynthetic pathways. The development of powerful analytical tools based on high-resolution MS or high-field NMR spectroscopy combined with bioinformatics promises to translate metabolomic information into usable data to merge with other omics analyses. However, an enormous amount of work is still required for the characterization of individual compounds within a chemical profile. These challenges necessitate a combination of traditional approaches focused on structure determination and examination of the bioactivity of pure compounds, combined with characterization (via network parameters or other statistical approaches) and examination of the biological activities of mixtures containing a large number of unknown compounds. Thus, the best modern approach for investigating the chemical ecology of plant–insect interactions is to comprehensively characterize the chemical profiles of host plants using the data-rich outputs from different gas chromatography and high-performance liquid chromatography techniques coupled to high-resolution MS and NMR spectroscopy, while also creating specific databanks and large sources of standards for naturally isolated or synthetized compounds to enable the testing of specific mechanistic hypotheses of chemically mediated interactions.

Advances in theory and methodological improvements over the past decade will contribute substantively to answering long-standing questions in chemical ecology, and chemical ecology research programmes should include strong cross-discipline collaborations with integrative training for students in modern chemistry, genomics and mathematics. Most importantly, chemical ecologists should recognize that a strong theoretical framework has already been established in the field and that the field needs empirical data, modern approaches, sophisticated statistical approaches (Supplementary Box 3), mathematical models (Supplementary Box 4) and a phylogenetic framework utilizing current methods (Supplementary Box 5) as much as it needs new ideas. Nevertheless, novel ideas or hypotheses can still be useful, particularly those that emerge from examination of hyper-dimensional data from complex systems. However, none of the existing theories or hypotheses described in this Review should be rejected or dropped[181]. A hypothesis that yields a plethora of citations from a traditional or Internet-based literature search is not necessarily a well-tested hypothesis, particularly if the investigative methods are poor. Despite the thousands of publications in chemical ecology, none of the aforementioned hypotheses have amassed sufficient empirical data from techniques utilizing combinations of the modern approaches outlined in this Review. The modern chemical ecologist should consider this issue when faced with the temptation of developing a completely novel theory that might gather more attention but might not be as productive for the field in general. We recommend an approach in which chemical ecologists continue to collect high-quality data[182], modify existing hypotheses based on synthesis and meta analysis[66] and contribute to answering the most interesting questions in chemistry, ecology and evolutionary biology.

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