Recent Advances in Phytochemistry 43



David R. Gang *Editor*

50 Years of Phytochemistry Research

Volume 43



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Preface

Introduction to the 43rd Volume of the Recent Advances in Phytochemistry Series

This is the third volume since the reintroduction of the *Recent Advances in Phytochemistry* (*RAP*) series, an annual publication supported by the Phytochemical Society of North America. Topics appropriate for *RAP* include the biosynthesis of natural products and regulation of metabolism, the ecology of specialized metabolites and the evolution of their pathways, and the effects of natural products or plants on human health. Research appropriate for *RAP* involves genomics, proteomics, metabolomics, natural product structural determination and new technology development, medicinal chemistry and metabolic engineering, or any of the myriad of fields that are now closely associated with what may be called "traditional phytochemistry" and plant biochemistry. The advent of postgenomics-based ways of thinking, systems biology, synthetic biology, comparative genomics/proteomics/transcriptomics/metabolomics, and especially of the introduction and establishment of a mentality that leads to the support of large collaborative projects has opened up many new doors to scientists interested and versed in the (bio)chemistry of plants. The goal of *RAP* is to highlight these developments.

Two main types of articles are printed in *RAP*: Perspectives and Communications. Perspectives in *RAP* are expected to synthesize results from the primary literature and perhaps from new/novel results and place these in perspective relative to the broader field. These articles may be similar to review articles, but also are intended to present important ideas and hypotheses and may present proposals for interesting directions in the field. It is the hope of the Editorial Board that these articles will be of great value to a large audience. Communications are intended to present new advances in the field that will be of interest to a large audience. Articles of both types are typically solicited from the society membership based on the content of the annual meeting talks, but in keeping with the title "Recent Advances in Phytochemistry" the editorial board reserves the right to solicit additional Perspectives and/or Communications from non-attendees as well (e.g., where an editorial board member has knowledge of an interesting recent advancement that would be of general interest to the society membership).

All submissions to *RAP* go through a rigorous peer review process, overseen by the Editorial Board, which includes external review. *RAP* is indexed with Springer published journals. All *RAP* papers are available not only in the published volume form, but also electronically through Springer's online literature services. This marks a significant change from past volumes of *RAP* and it is the hope of the Editorial Board that this will lead to broader dissemination of the contents of and greater interest in *RAP*.

This 43rd volume of RAP includes a total of seven articles based on talks presented at the 50th Anniversary meeting of the PSNA, which was held at the Fairmont Orchid in Waikaloa, Hawai'i, USA. These seven Perspectives give a very good picture of the breadth of plant (bio)chemistry research in North America, which is also indicative of the state of the field worldwide. Each of these articles describes the integration of several different approaches to ask and then answer fascinating questions regarding the function of interesting plant metabolites, either in the plant itself or in interactions with the environment (natural setting or human health application).

Two perspectives outline very clearly the power of approaching biological questions from a modern "omics" or systems biology approach. Beale and Ward outline how metabolomics approaches can be brought to bear on plant biosynthetic questions and quickly lead to important advances in our understanding or how plants produce important metabolites. Zandkarimi et al. outline the integration of ion mobility spectrometry into mass spectrometry-based metabolomics investigations and show clearly how powerful those two spectrometric technologies can be when used together.

Plant biotechnology and its application to plant protection, pathogen/pest deterrence and drug production is discussed in three perspectives. Asano et al. describe the development of plant cell cultures and tissue culture techniques that lead to production of important indole alkaloid compound production, particularly of camptothecin production in cultures from *Ophiorrhiza* species. Mitchell Wise outlines how cereal crops can be protected from disease by application of plant defense activators, bioactive compounds that are typically derived from specific plants. Duke et al. provide an extensive review of the history and future prospects of prospecting within the plant kingdom for compounds that protect both crops and humans from insect pests.

The last two perspectives emphasize the role of plant-derived compounds in human health. Zhang et al. review the role of sulfhydryl-reactive compounds, such as the sulforaphanes from broccoli and related plants and several phenolics, including curcumin, in modulating two important pathways that are involved in protecting mammalian cells from oxidative and inflammation-induced damage, such as that which occurs in a number of degenerative diseases and cancer. Eggler and Savinov focus on phytochemicals that are involved in activating the Nrf2 pathway, and thereby help prevent disease.

As always, we hope that you will find these Perspectives to be interesting, informative, and timely. It is our goal that *RAP* will act not only as the voice of the

PSNA, but that it will serve as an authoritative, up-to-date resource that helps to set the gold standard for thought and research in fields related to plant biochemistry.

We welcome suggestions for future articles and comments on the new format.

The RAP Editorial Board

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Chapter 1
Metabolomics Reveals Hemiterpenoids as New Players in the Carbon–Nitrogen Economy

Michael H. Beale and Jane L. Ward

Abstract In plant metabolic engineering, a holistic view of plant metabolism can be provided by modern metabolomics. We discuss a recent application of combined nuclear magnetic resonance–mass spectrometry (NMR–MS) metabolomics to hydroponically grown *Arabidopsis thaliana* as a means to study metabolic reprogramming in plant shoots in response to root stress. This led to the discovery of a novel, specifically regulated overflow from the chloroplastic methylerythritol phosphate (MEP) pathway that resulted in the biosynthesis of hemiterpenoid glycosides (HTGs) from hydroxymethylbutenyl diphosphate. The induction of these compounds in leaves is quite specific to a few conditions that lead to low foliar nitrate, implicating the involvement of this anion in the molecular switch. The reprogramming of isoprenoid metabolism in shoots was also correlated with the production of the phenylpropanoids scopolin and coniferin in roots. These results are discussed against a survey of the literature on hemiterpenoids, including isoprene, in order to develop a unified model of metabolic switching of the MEP pathway that allows carbon overflow from a number of points, depending on the species.

1.1 Introduction

In living systems, the metabolites, whether in the free form, or as components of structural and functional macromolecules, are the end products of a complex self-replicating machinery (genes and proteins), which is itself also built from metabolic products. The complement of low-molecular-weight compounds in a living system is known as the metabolome [1]. Metabolomics is the study of the metabolome, usually in context with genetic, physiological, and environmental influences.

In plants, biochemistry is highly geared to assimilate sunlight, carbon dioxide, nitrate, and other nutrients into metabolites and ultimately into biomass. Most metabolic flux is thus dedicated to these primary processes. However, plants are also the source of a rich secondary metabolism, which is highly species dependent and

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has evolved to give plants an ecological advantage in the constant pressure from microbial pathogens and herbivores. One of the key areas of metabolic control is concerned with sensing and adapting to variable carbon (C) and nitrogen (N) supply. Both are crucial for the normal operation of fundamental biochemical and cellular activities, and internal monitoring of C/N balance is critical. It is recognized that C and N metabolism is coordinated. The C/N balance theory [2] was developed to explain the allocation of resources to secondary metabolism for defense. Over the years, there has been much discussion of the veracity of this hypothesis, for example, regarding plant herbivore interactions. Of particular concern has been the inability to predict quantities of individual secondary compounds [3]. However, outside of this discussion on the cost–benefits of plant defense, it is still widely recognized that the primary assimilation of C and N is monitored and balanced [4, 5] and this is supported by transcriptomic studies that demonstrate genome-wide reprogramming of metabolism in response to nitrate levels [6–9]. One of the key applications of plant metabolomics is in the assessment of this type of metabolic reprogramming that occurs when plants are subjected to environmental pressures and/or genetic alterations. An approach to this problem, at the whole plant level (roots and shoots), is described in this perspective.

1.2 Analyzing the Plant Metabolome

The technology used in metabolomics combines data collected using analytical spectroscopies, such as nuclear magnetic resonance (NMR)and mass spectrometry (MS)with that from genomic and/or phenotype studies. Application of multivariate and correlative statistics to these data allows discovery of metabolites, metabolic processes, and genes associated with developmental programming or environmental effects at the tissue or whole organism level. Increasing levels of sophistication in analytical technologies and our ability to generate and utilize data from large sample numbers, as well as in network modeling, have brought metabolomics to the forefront of plant systems biology research [10].

Obviously, metabolism is highly dynamic and the observed metabolome arises from the modulation of the network of biochemical pathways by genetic and environmental influences. Individual metabolite profiles are thus snapshots of the state of the biochemical network. The primary goal of metabolomics analysis is to assay as many metabolites as possible in order to maximize the view of the network. In reality, even when a panel of extraction, separation, and detection strategies is employed, not all metabolites can be detected and a truly holistic measurement of the metabolome cannot be obtained. However, technologies are improving rapidly and well-found laboratories, with access to high-field NMR and a range of MS techniques (especially LC–MS–MS and GC–MS; LC, liquid chromatography; GC, gas chromatography) and ever-growing spectral libraries of authentic metabolites, are able to measure an impressive number of metabolites, especially when the “lipidome”—the large group of homologous compounds for which specific

techniques have been developed—is included [11]. It is fair to say that metabolomics data are more meaningful, in terms of tracking the dynamics of the biochemical network in a systems biology approach, when they are collected from time-separated samples of tissue, as the network adjusts to genetic programming or environmental change, including stress, disease, and other predatory pressures.

In plants, there are significant challenges associated with simultaneous analysis of the primary metabolome, which is, qualitatively, fairly consistent across species, and the secondary metabolome which is generally species specific. However, closely related species contain related secondary metabolites (e.g., glucosinolates are associated with the Brassicaceae and isoflavonoids with legumes), and this forms the basis for much of the historical chemotaxonomic literature. The primary metabolome, in green parts of the plant, is intimately linked to photosynthesis and varies diurnally over the day–night cycle. There is also dependence on the growth or developmental stage of the plant. Secondary metabolism can be tissue specific (e.g., trichomes are rich sources of terpenoids) and is often induced by biotic and abiotic stresses. There is also dependence on developmental stage, e.g., older leaves can have different secondary metabolite compositions compared to younger leaves. Thus, plant metabolomics experiments have to be carefully designed to suit the biological question that is to be addressed, and the data interpreted against these background developmental and environmental effects.

In terms of the technologies utilized to measure the plant metabolome, the spectroscopic choices are no different to those faced in human and microbial metabolomics, or indeed in classical natural product discovery and identification. Whether collecting data directly on unpurified solvent extracts, or after separation using chromatography-linked spectroscopic techniques, there are pros and cons for every method in terms of metabolome coverage, dynamic range, accuracy of quantitation, and throughput. A summary of these considerations has been given for *Arabidopsis* [12], but is applicable to most plant systems. There are two different, but complementary, approaches to plant metabolomics. The first involves analysis of tissue extracts against a list of known compounds and, depending on the technique, can give quantitative data on several hundred metabolites, of types ranging from central metabolism to secondary products and lipids. The second approach is to compare metabolite signatures, containing thousands of signals from both known and unknown metabolites, by multivariate statistics to select discriminatory metabolite signals explaining the biology/biochemistry under test. Both approaches are hampered by the lack of standards of known compounds that can be used to annotate metabolite signals (i.e., the MS, NMR, or chromatography peaks). Thus, identification of unknowns and the building of spectral libraries have become major challenges in the quest for maximum coverage of the metabolome. This problem is of course much greater in plants and microbes, where there are large differences in secondary metabolomes.

Here, we describe the use of an *Arabidopsis* experimental setup that allows the rapid assessment of metabolic reprogramming in both roots and shoots when stresses are applied to the root or shoot. The data obtained have brought a root-to-shoot signaling perspective to plant metabolomics, and, thus, a more complete ‘systems’ view of the reallocation of metabolic resources.

1.3 The *Arabidopsis* Root–Shoot Metabolome Under Stress

A recent publication [13] describing our work on understanding the effects of different stresses on the *Arabidopsis* root and shoot metabolomes highlighted the importance of not confining studies to a single tissue type or time point. A series of experiments utilizing the commercially available Araponics system (http://www.araponics.com) allowed us to develop a reliable protocol for generating *Arabidopsis* root and shoot tissues in enough quantities to allow for a comprehensive metabolomics analysis to be carried out. The system also allowed for easy access of the root system allowing an investigation on the effect of root perturbation on the shoot metabolome and *vice versa*. Early experiments identified common discriminatory metabolites and also revealed key unknown metabolites, the identities of which were determined *via* a classical natural product chemistry approach. The biological provenance of these metabolites was established and results have highlighted a new link with C–N balance and the terpenoid pathway. The work is described in more detail in the subsequent sections of this perspective.

1.3.1 Effect of Total Nutrient Withdrawal

As a relatively simple initial experiment, the effect of total nutrient withdrawal was explored by switching healthy *Arabidopsis* plants, previously grown under full nutrient supply, to water. NMR–MS analysis of extracts of shoot tissue, harvested at two time points (3 days and 7 days), revealed striking and repeatable differences in metabolite fingerprints between nutrient-deficient and nutrient-supplied plants. Increases in common carbohydrates, flavonoids (kaempferol glycosides), and in particular the amino acid phenylalanine and decreases in sinapoyl malate and the amino acids alanine, threonine, aspartate, and glutamate were clearly evident in the ^{1}H NMR data. However, the most striking feature of the NMR data set was the presence of new signals for olefinic hydrogens and aliphatic methyl groups, which were structurally linked to each other and to other signals in the carbohydrate region, in the nutrient-deprived plants. In fact, further inspection of the data indicated that a small family of related compounds had been induced by the nutrient withdrawal, and that there appeared to be associated reprogramming of metabolism involving phenylalanine, flavonoids, and other primary metabolites. Direct infusion electrospray ionization–MS (DI-ESI–MS) data also displayed biomarkers for nutrient deprivation. A decrease in the ion *m/z* 640 (negative ion mode), a novel flavonoid species that was shown to be related to kaempferol 3,7-dirhamnoside (KRR), by MS–MS fragmentation was observed. However, the most prominent feature was in the positive ion fingerprints where two significant ions at *m/z* 287 and 303 were observed only in nutrient-deprived plants.

1.3.2 Identification of the "Unknowns"

MS data collected at higher resolution showed that the ions at *m/z* 287 and 303 actually arose from sodium and potassium adducts of a compound with the empirical formula $C_{11}H_{20}O_7$. Two-dimensional (2D)-NMR experiments on the nutrient-deprived plant extracts indicated that the discriminatory signals identified in the 1D-NMR fingerprints could be assigned to at least two closely related novel glycosides of a C_5-unsaturated diol, present in about a 4:1 ratio. Chemical shift, connectivity, and coupling data indicated that the major compound could be the hemiterpenoid (2*E*)-4-hydroxy-2-methyl-2-buten-1-yl-*O*-D-glucopyranoside (1) and further confirmation of the formula and the presence of two isomers were obtained from GC–MS analysis of the plant material.

The molecular ion was absent but fragments were characteristic of trimethylsilyl glycosides. The key fragment, *m/z* 157, present in both isomers, due to the aglycone had the empirical formula $C_8H_{17}OSi$ and corresponded to the structural fragment $[(CH_3)_3SiOCH_2CH=C(Me)CH_2]^-$ that was consistent with the C_5-enediol glycoside structure. 1D nuclear Overhauser enhancement spectroscopy (NOESY) NMR data of the major isomer indicated the *trans* (*E)* arrangement of the double bond and heteronuclear multiple bond correlation NMR spectroscopy (HMBC) placed the glycosidic linkage at the 1-position. The structures of this compound and the minor isomer were confirmed by synthesis. This was accomplished from (2*E*)-hydroxy-2-methylbut-2-enyl, 4-acetate, an intermediate previously utilized by us in the synthesis of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) [14]. The synthesis involved Koenigs–Knorr coupling of acetylbromoglucose to the 4-monoacetate, followed by deacetylation and, serendipitously, two isomers were produced that were identical to the two hemiterpenoids observed in *Arabidopsis*. The availability of synthetic material enabled comprehensive 2D-NMR investigations of both isomers to be completed in isolation from contaminating plant carbohydrate. This led to the definitive conclusion that the naturally occurring compounds were the two regional isomers of the hemiterpenoid glycoside (HTG)-*(2E)* -2-methylbut-2-en-1,4-diol glycoside, the major isomer being the 1-glycoside (1) and the minor isomer being the 4-glycoside structure (2) as shown in Fig. 1.1. 2-Methylbut-2-en-1,4-diol (3) was a by-product of the synthesis and was also available from separate deacetylation of the starting monoacetate. Small amounts of this compound were also detected in the NMR spectra of nutrient-deprived *Arabidopsis* extracts.

1.3.3 Nitrate, Nitrite, or N? Establishing the Link with HTG Production in Arabidopsis.

To explore the relationship between nutrient deprivation and the formation of HTGs, experiments were carried out where individual nutrients were omitted from the full growth medium. Using metabolomic screening to monitor the production of the identified biomarkers, it was demonstrated that the prime inducer of the HTGs

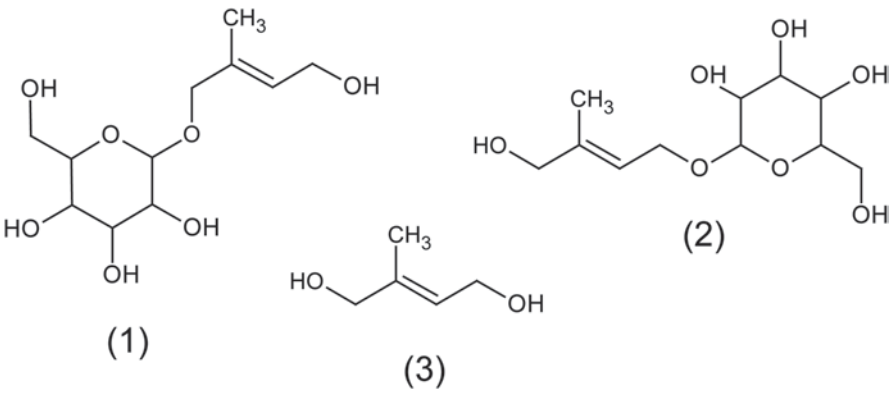


Fig. 1.1 Structures of hemiterpene glycosides identified in *Arabidopsis*

in shoots was root NO_3^- deprivation. The HTGs were still produced when $NH_4^+$was substituted for NO_3^- as a nitrogen source, indicating that nitrate ion sensing was the underlying mechanism behind the synthesis of these compounds. Alternating periods of nitrate deprivation and resupply led to a stepwise accumulation of HTGs under periods of starvation, rising to approximately 1 % dry weight. The HTGs did not appear to be substantially re-assimilated on nitrate resupply. Other primary metabolites also changed during nitrate resupply. Alanine, threonine, aspartate, asparagine, glutamine, and glutamate which were all depleted on nitrate deprivation recovered during the resupply period. Conversely, levels of metabolites that increased during nitrate deprivation (malate, phenylalanine, sucrose, glucose, stachyose, and maltose) were seen to drop during the resupply period, to a level similar to that observed in control plants. Thus, a clear synchronization of certain primary metabolites with nitrate availability and HTG production was observed.

An ion at *m/z* 640, routinely seen in untreated *Arabidopsis* leaf DI-ESI–MS negative ion spectra, was absent in the nitrate-deprived plants. From MS–MS experiments, it was determined that this was formed in the MS source and corresponded to a nitrate adduct of the major flavonoid glycoside KRR and that its intensity was dependent on the nitrate concentration in the leaf tissue. The titer of the *m/z* 640 ion thus serves as a second biomarker for NO_3^- starvation in *Arabidopsis* extracts, and independent measurement of nitrate in the samples by conventional means supported this hypothesis. A weak negative correlation of intensity of ions relating to the HTGs (*m/z* 287 and 303) and *m/z* 640 provided a semi-empirical means of relating high HTG levels directly with low nitrate ion concentration in the same tissue.

It was already apparent that ammonium ion was not involved in the metabolic switch to HTGs. However, there was a possibility that another product of nitrate assimilation—nitrite ion—was the signal. In order to investigate the possibility that levels of nitrite rather than nitrate may drive HTG production, HTG levels in extracts from tissue of nitrate reductase (NR) mutants of *Arabidopsis* were examined. Leaf tissue from these mutants, which cannot assimilate NO_3^-, typically

contain higher levels of free nitrate in the foliar tissue. Under starvation conditions the double (*nia1/nia2*) mutant, retaining only 0.5 % of wild-type NR activity [15], did not produce HTGs at all and the levels of nitrate in the foliar tissue remained high presumably as enough nitrate had built up prior to the starvation period (when the plants were growing on nitrate-containing media) which could not then be assimilated. Together, the results pointed to the fact that it was nitrate levels *per se* and not a product of assimilation, such as nitrite or ammonium ion, which were linked with the HTG formation.

1.3.4 Occurrence of the HTGs Under Alternative Stress Conditions and Evidence of Pathway Reprogramming

The Araponics system, and the ability to readily detect the HTG biomarkers, allowed the application of a number of different stresses within the same experiment and measurement of the concentration of HTGs produced. The results of the experiment are shown as a hierarchical cluster analysis in Fig. 1.2. Levels of the HTGs in leaves exceeded 0.5 % dry weight under nitrate deprivation. Wounding of the roots, but not of leaves, induced formation of the HTGs in leaves but other root stresses such as osmotic stress and salt treatment did not induce HTG biosynthesis despite showing characteristic metabolite changes illustrating that these plants were highly stressed. Likewise, cold treatment of the plants did not induce the new metabolite. Potassium deprivation induced HTGs, but the magnitude was less than that of nitrate starvation. However, oxidative stress induced by hydrogen peroxide treatment of roots induced the HTGs in leaves, to levels reaching approximately the same as those observed with nitrate starvation. Most importantly, we determined that high HTG production was always associated with low foliar nitrate levels. In contrast, no HTGs were detected in any of the root samples from this experiment but other major changes in root tissues were apparent. Increases in scopolin and coniferin were not only present in the same samples as those producing HTGs but were also very well correlated with HTG production. These metabolites are products of the phenylpropanoid pathway in *Arabidopsis* and were already known compounds in *Arabidopsis* roots [16].

Although previously associated with light treatment [17], in our study these metabolites were also evidence of a reprogramming of root metabolism that occurs on nitrate deprivation which is in agreement with observations of a scopolin increase in tobacco roots, in response to nitrogen starvation, as early as 1970 [18]. Analogous results on the redirection of carbon flow into phenylpropanoids under nitrate deficiency have also been reported in tobacco leaves [19]. In *Arabidopsis*, data from transcriptomic studies on nitrate limitation are available [6, 9, 20, 21] and these indicate a similar reprogramming, demonstrating that, as chlorophyll biosynthesis was repressed, phenylpropanoid biosynthesis genes were upregulated. Clearly, there is much more work needed to completely understand this relationship. Unpublished work from our laboratory has indicated that HTGs can be induced *via* a

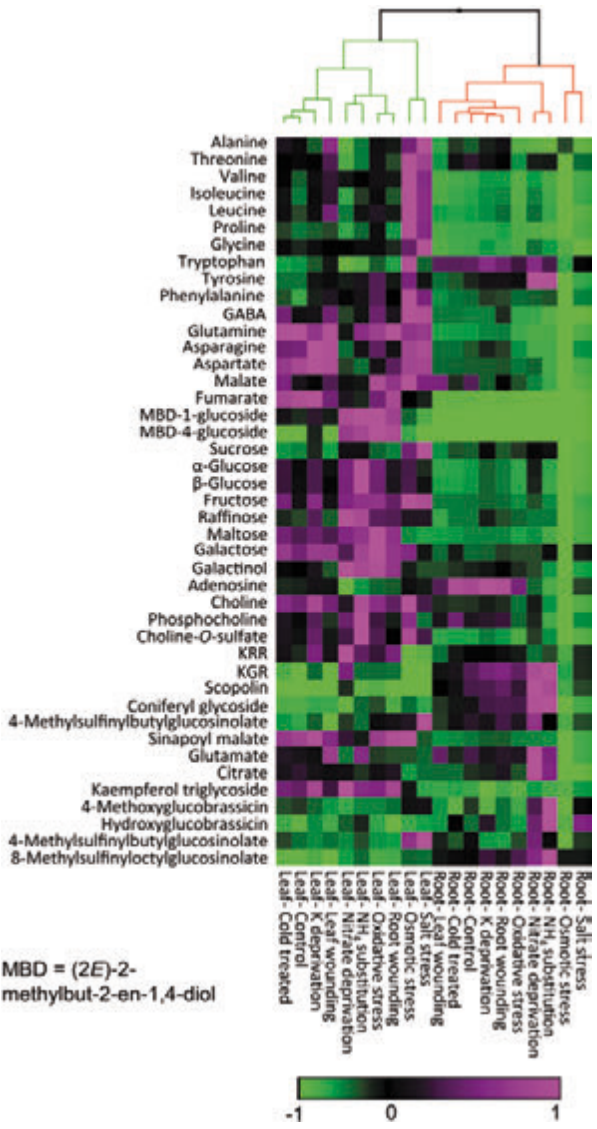


Fig. 1.2 Hierarchical cluster analysis of major metabolites in *Arabidopsis* leaf and root tissue as a result of perturbations from a range of abiotic stresses. Data from ^{1}H NMR (characteristic chemical shift regions) and ESI-MS (*m/z* ions) have been scaled to unit variance and mean centered

high light stress to leaves. Furthermore, despite being supplied with normal nitrate concentrations *via* the roots, foliar nitrate was again very low in the light-stressed plants. Regarding the order of molecular events leading to HTG induction, it is possible that high light induces oxidative stress and as a consequence low foliar nitrate status within the leaves. Alternatively, low nitrate levels caused by an acceleration of photoassimilation in high light induce a pseudo-oxidative stress which in turn triggers the production of HTGs.

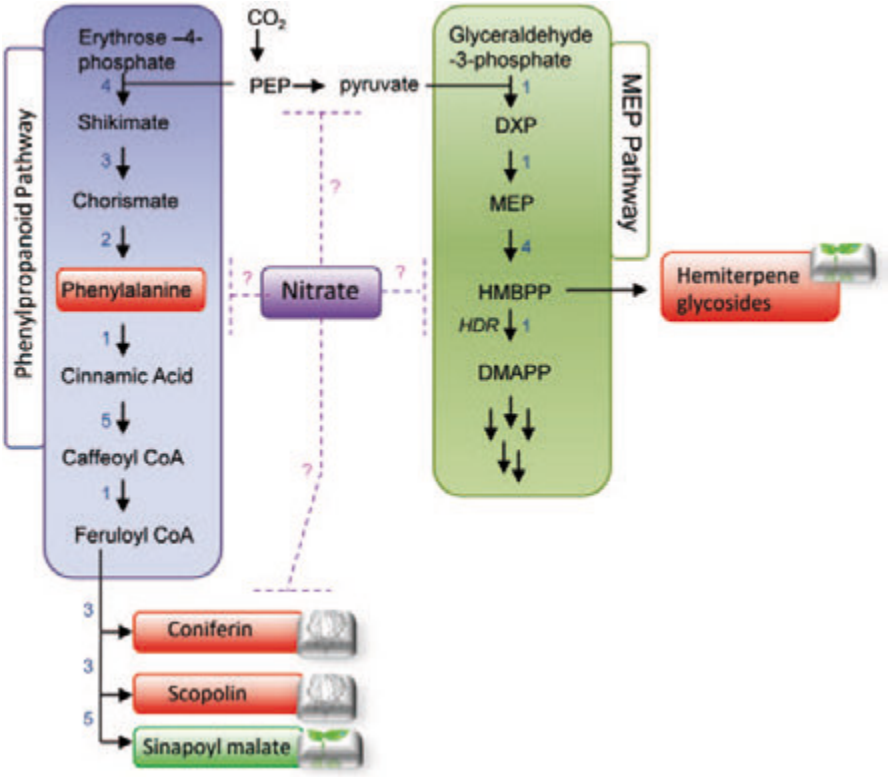
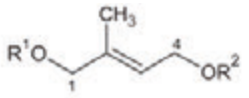


Fig. 1.3 Model of putative pathway interactions in *Arabidopsis*, under foliar nitrate control. Metabolites boxed in red increased under nitrate starvation while those in green decreased. Arrow numbers represent the number of reaction steps between metabolites

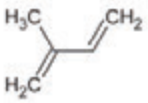
The HTGs are products of the chloroplast terpenoid pathway, known as the methylerythritol phosphate (MEP) pathway (see later). The relationship between nitrate and the co-regulation of MEP and phenylpropanoid pathways is shown in Fig. 1.3. The common link between the pathways involves photoassimilates such as phosphoenol pyruvate (PEP) and partitioning of this type of primary precursor may also be part of the nitrate sensing process.

1.4 Hemiterpenoid Diversity

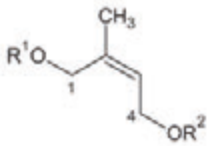
Terpenoids are one of the largest known groups of secondary metabolites. They are incredibly diverse in their structures and biological activities. Despite their structural complexity, however, they all share a common building block, that of the C_5 unit isoprene (4) (Fig. 1.4). While monoterpenes are comprised of two such building



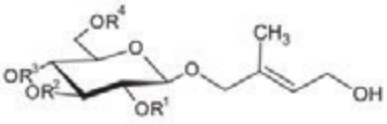
(1) R^1= Glc, R^2 = H
(2) R^1= H, R^2 = Glc
(3) R^1= R^2 = H



(4) Isoprene

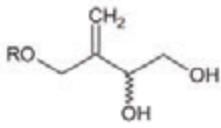


(5) R^1= Glc, R^2 = H
(6) R^1= H, R^2 = Glc
(7) R^1= R^2 = H

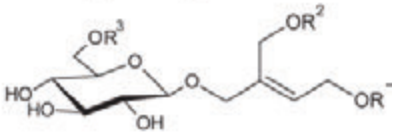


(8) R^1 = R^2 = 4-HPA, R^3 = R^4 = H
(9) R^1= R^2 = 3,4-DHPA, R^3 = R^4 = H
(10) R^1= 3,4-DHPA, R^2 = 4-HPA, R^3 = R^4 =H
(11) R^1= 4-HPA, R^2 = 3,4-DHPA, R^3 = R^4 = H
(12) R^1= R^4 = 3,4-DHPA, R^2 = R^3 = H
(13) R^1= R^4 = 3,4-DHPA, R^2 = 4-HPA, R^3 = H
(14) R^1= 3,4-DHPA, R^2 = R^3 = R^4 =H
(15) R^2= 3,4-DHPA, R^1 = R^3 = R^4 =H
(16) R^4= 3,4-DHPA, R^1 = R^2 = R^3 =H
(17) R^1 = 4-HPA, R^2 = R^3 = R^4 = H
(18) R^4 = 4-HPA, R^1 = R^2 = R^3 = H

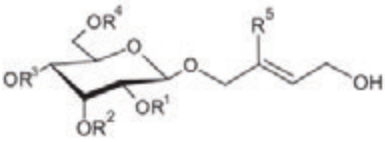
HPA = hydroxyphenylacetyl
DHPA = dihydroxyphenylacetyl



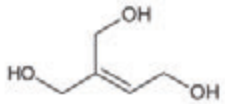
(27) R = glucopyranose
(28) R = allopyranose



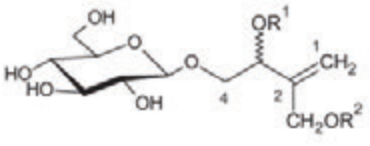
(29) R^1 = caffeoyl, R^2 = R^3 = H
(30) R^1 = R^3 = H, R^2 = caffeoyl
(31) R^1 = R^3 = caffeoyl, R^2 = H



(19) R^1 = R^2 = R^4 = H, R^3 = caffeoyl, R^5 = CH_3
(20) R^1 = R^2 = R^3 = H, R^4 = 3,4-DHPA, R^5 = CH_2OH
(21) R^1 = R^3 = R^4 = H, R^2 = caffeoyl, R^5 = CH_2OH
(22) R^1 = R^2 = R^4 = H, R^3 = caffeoyl, R^5 = CH_2OH
(23) R^1 = R^2 = R^3 = H, R^4 = caffeoyl, R^5 = CH_2OH
(24) R^1 = R^2 = R^3 = R^4 = H, R^5 = CH_2OH
(25) R^1 = R^2 = R^4 = 3,4-DHPA, R^3 = H, R^5 = CH_2OH
(26) R^1 = H, R^2 = R^3 = R^4 = 3,4-DHPA, R^5 = CH_2OH



(32)



(33) R^1 = H, R^2 = caffeoyl
(34) R^1 = caffeoyl, R^2 = H

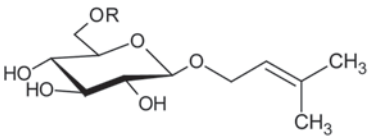
Fig. 1.4 Structures of plant hemiterpenoids

blocks and sesquiterpenes three isoprene units, hemiterpenes are based on a single C_5 structure. These basic units may of course be saturated or unsaturated, isomerized, or bear functional groups which may themselves allow for conjugation to an array of other structural moieties. The family of hemiterpenoids is quite large but here we discuss key HTG structures which have been previously reported.

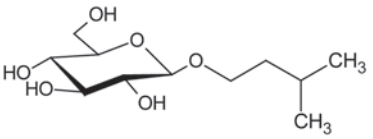
The simplest of all hemiterpenes is the compound isoprene (4) which is emitted from the leaves of many plants. How this emission arises and whether it is as a result of a specific function has, over recent years, been the subject of much discussion and speculation [22, 23]. *Arabidopsis*, however, is a non-emitter of isoprene but our results have demonstrated that, under certain conditions, reasonably high levels of the related conjugated hemiterpenoids can be produced in the aerial tissue of the plant. The compounds described earlier had not previously been detected in *Arabidopsis* but have been reported, mostly at low levels, earlier in a number of less-tractable plant species as demonstrated in Fig. 1.4. The 1-*O*-glycoside (1) was initially reported in 1992 [24] and was isolated from the aerial tissue of *Ornithogalum montanum*, a perennial herbaceous bulb found in Italy and the Balkans. The identical compound was reported in the *Torillis japonica* fruit some years later, along with the simple diol (3) in [25]. The 4-*O*-glycoside (2) has also been previously reported as a deterrent of the bean aphid in stems and leaves of *Vicia hirsuta* [26].

Until our work in *Arabidopsis*, the two positional isomers of the glycosylated hemiterpene had not been reported in the same species. This is unlike the corresponding *cis* isomers found in *Vitis vinifera* leaves where both the *cis*-1-*O*-glycoside (5) and the *cis*-4-*O*-glycoside (6) were detected and found to exist in a 3:1 ratio [27]. The *cis*-1-*O*-glycoside (5) was also reported in leaves of the South American cangorosa plant *Maytenus ilicifolia* where it was described as one of three ilicifolinosides isolated [28]. Unlike the *trans* diol (3), the *cis* diol (7) does not appear to have been isolated from plants, but has been synthesized as part of structure determinations of compounds above [26].

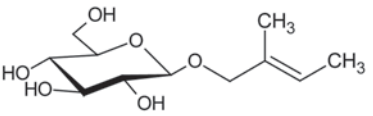
Clearly, the common factor with the compounds discussed so far is the aglycone, 2-methyl-but-2-ene-1,4-diol moiety, whether in the *cis* or *trans* configuration. Further, more complex analogs based on the same agylcone skeleton have also been reported from methanolic extracts of the Japanese Fern, *Hymenophyllum barbatum* [29]. These analogs (8–18) include a *trans* 1-*O*-substituted glucose moiety. The family of compounds isolated, named hymenosides, included a mixture of mono- and dihydroxyphenylacetylated glucose substituents with the position of the substitution varying around the carbohydrate ring. A much wider selection of these so-called hymenosides, bearing a hydroxylated aglycone skeleton and a substituted allose moiety, were also isolated from the same plant species [29–31]. These compounds bore a mixture of caffeoyl- or dihydroxyphenylacetyl-substituted allose arrangements and were conjugated to either 2-methylbut-2-ene-1,4-diol (19), 2-hydroxymethyl-but-2-ene-1,4-diol (20–26) or 2-methylene-butane-1,3,4-triol (27–28). Caffeoyl-substituted hemiterpene glycosides have also been isolated from the bark and roots of two different *Ilex* (holly) species within the Aquifoliaceae family. Three new caffeoyl-substituted HTGs were isolated from methanolic extracts of the dried bark of *Ilex macropoda* [32]. These metabolites (29–31), based on the triol structure (32), bear caffeoyl substituents at one (C-4 or C-5) or two (C-4 and C-5) positions alongside the glucose substitution at C-1. Two further HTGs, named pubescenosides, were isolated from the roots of *Ilex pubescens* [33]. These compounds (33, 34) possessed potent antiplatelet aggregation activities and were based on the aglycone structure 2-hydroxymethyl-3,4-dihydroxy-but-1-ene.



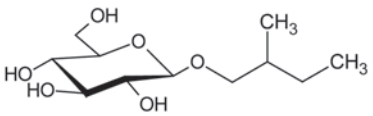
(35) R = H
(39) R = apiofuranose



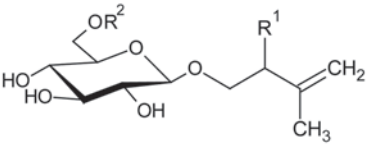
(36)



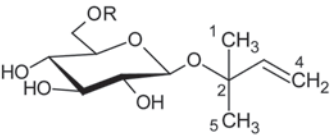
(37)



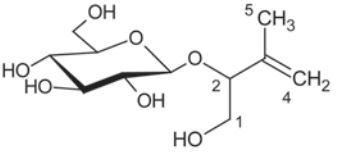
(38)



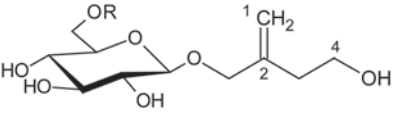
(40) R^1 = H, R^2 = apiofuranose
(42) R^1 = OH, R^2 = H



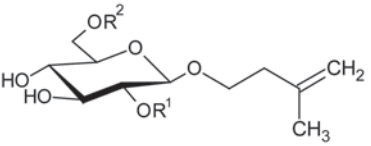
(41) R = apiofuranose



(43)



(44) R = H
(45) R = CO-C(CH_2)CH_2CH_2OH



(46) R^1 = R^2 = H
(47) R^1 = glucopyranose, R^2 = H
(48) R^1 = H, R^2 = xylopyranose
(48) R^1 = H, R^2 = xylofuranose

Fig. 1.5 Structures of plant hemiterpenoids, continued

Further HTGs (Fig. 1.5) have been reported in a number of diverse species. Perhaps the most relevant of these is a prenyl glycoside based on a 3-methylbut-2-en-1-ol (dimethylallyl alcohol) (35). Originally reported as a fragrance precursor

in flower buds of *Citrus unshiu* [34] and subsequently in 1977 [35] *via* Soxhlet extraction of the aerial parts of *Ferula loscosii,* a prevalent species in Spain, this metabolite was again reported as being part of a collection of metabolites present in the water-soluble fraction of the methanol extract of fennel fruit (*Foeniculum vulgare,* Umbelliferae) alongside the fully saturated analog (36) and the positional isomer, 2-methyl-but-2-en-1-ol glucoside (37), and the analog (38) [36]. Early reports of these saturated HTGs generally associated these types of compounds with a variety of medicinal properties such as antimicrobial and antifungal activities. Such an example [37] was the isolation from leaves of the Canarian shrub *Bystropogon plumosus* of 2-methylbutan-1-yl-β-D-glucoside (38) to levels of around 0.01 %.

An apiofuranose analog (39) of the dimethylallylalcohol glycoside (35) was isolated from *Vitis vinifera* wine [38] alongside the similar analog from the 3-methylbut-3-en-1-ol glycoside (40). An apiofuranose analog (41) of 2-methylbut-3-en-2-ol glycoside has also been isolated from the water-soluble extract of the root and rhizome tissue of *Glehnia littoralis* [39]. The position of the double bond within the aglycone structure varies and there have been a number of reports of such metabolites with a terminal double bond structure. The position of the double bond and subsequent substitution are presumably as a result of the biosynthetic route utilized in particular tissues. Aerial tissue of *Lamium album* afforded the 2-*O*-glucoside of 3-methylbut-3-en-1,2-diol (43) named by the authors as hemialboside [40] while the extraction of fresh rhizomes of *Coptis japonica* var. *dissecta* allowed the isolation of a group of close analogs (42, 44–45) [41]. Finally, a series of hemiterpene glycosides (46–49) based on the 3-methylbut-3-en-1-ol parent structure were isolated recently from fruits of noni (Indian mulberry) [42]. This set of metabolites included the 1-*O*-glucoside (46) and conjugates further extended with an additional glucopyranose (47), xylopyranose (48), or xylofuranose (49).

In summary, we have highlighted examples of compounds that appear to be structurally related to the main C_5 building blocks of isoprenoid biosynthesis. Other related structures have been summarized by Dembitsky [43]. Of particular interest in metabolomics and carbon flow is the provenance of the variety of skeleta observed, and this is discussed in the next section.

1.5 Hemiterpenes—Products of Internal Regulation of the Chloroplast Isoprenoid Pathway?

The structural resemblance of the hemiterpene glycosides to HMBPP, a key intermediate in the chloroplastic MEP pathway, indicates a biosynthetic pathway involving hydrolysis of the diphosphate group followed by glycosylation at either end of the 1,4-diol. (Fig. 1.6). Support for this route was provided [13] by feeding intermediates—HMBPP, desoxyxylulose phosphate (DXP), and 1,4-diol—to leaves excised from nitrate-depleted *Arabidopsis* plants. Although, in feeding studies such as these, phosphorylated compounds are unlikely to be taken up into plant chloroplasts, the results indicated that the distal precursor DXP was taken up and

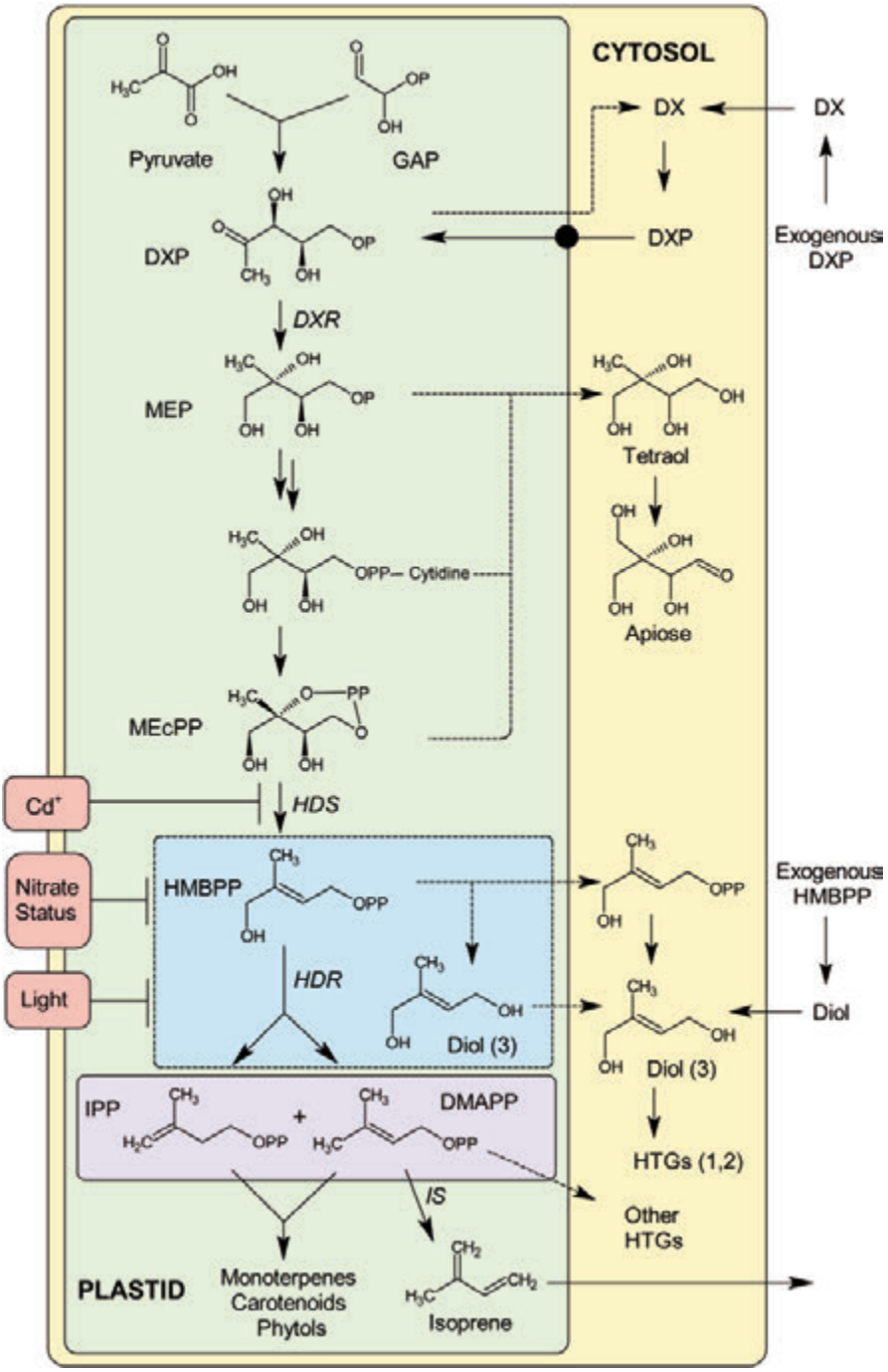


Fig. 1.6 The chloroplast MEP pathway and proposed provenance of hemiterpene alcohols and derivatives *via* stress-induced pathway shunts

resulted in the formation of some 3–4 times more hemiterpene glycosides in nitrate-depleted tissue when compared with controls. There are precedents for DXP uptake and turnover in plant tissue and the mechanism is thought to involve extracellular dephosphorylation to the alcohol and then uptake into the cell, followed by re-phosphorylation to DXP which is then actively transported into the chloroplast by a carrier protein [44, 45]. When either HMBPP or the 1,4-diol was fed to cut leaves of nitrate-depleted *Arabidopsis*, Ward et al. [13] observed high turnover of both compounds to the hemiterpene glycosides, providing firm evidence for the proposed pathway. However, there was no difference in turnover between nitrate-depleted and control plants.

Given that HMBPP has been shown not to be transported across chloroplast membranes [46] and it can be assumed that it also was dephosphorylated outside of the cell, this result indicated that the glycosylation of the diol occurred outside of the chloroplast. Together, the feeding results from nitrate-depleted plants support the hypothesis that nitrate signaling decreased flux through the enzyme hydroxymethylbutenyl diphosphate reductase (HDR; Fig. 1.6) causing HMBPP to build up and overflow to the HTGs. HMBPP generated *de novo* within the chloroplast (from the endogenous pathway or from exogenous DXP) was subject to the nitrate-regulated shunt mechanism. HMBPP added exogenously does not appear to reach the chloroplast but is dephosphorylated and glycosylated irrespective of nitrate levels. The experiments could not distinguish between low-nitrate induction of an HMBPP phosphatase in the chloroplast and the alternate mechanism of low-nitrate-induced translocation of excess HMBPP from chloroplast to cytosol (Fig. 1.6). The results observed in *Arabidopsis* parallel those observed in virus-induced gene silencing of HDR reductase activity in *Nicotiana benthamiana* [47]. In this system, silencing of HDR led to the conversion of HMBPP to the diol, via the monophosphate HMBP, indicating similar regulatory mechanisms to those observed in *Arabidopsis*.

Many of the known hemiterpene structures depicted in Figs. 1.4 and 1.5 can be accommodated by similar biosynthetic conversions from HMBPP, dimethylallyl diphosphate (DMAPP), or isopentenyl diphosphate (IPP). Some structures (e.g., 41, Fig. 1.5) can arise from allylic rearrangement of the double bond in the dephosphorylation step or at the alcohol stage. Most difficult to rationalize is the biosynthesis of the dihydro analogs (e.g., 36, Fig. 1.5) and *cis*-diol-based structures (e.g., 5–7, Fig 1.4). Both appear to arise out of the downstream modification of dimethylallyl alcohol by either hydrogenation or hydroxylation.

Other naturally occurring C_5 polyols such as apiose and the related tetraol (Fig. 1.6) are also potentially formed from MEP pathway intermediates as shown [25]. This opens up the possibility of other overflow mechanisms from different places in the MEP pathway. It has also been reported that 2-C-methyl-D-erythritol 2,4 cyclodiphosphate, a potential precursor of such tetraol structures, builds up in high light, high temperatures, and also on cadmium stress in spinach tissue [48]. The production of isoprene itself is well established from chloroplastic DMAPP *via* the action of a specific enzyme isoprene synthase and has been suggested to be a shunt product when DMAPP pool size becomes too large [22]. There has been much discussion concerning the raison d'être for isoprene emission from plants. This

occurs in many tree species and contributes to greenhouse gases as well as the summer haze over forests. There is evidence that isoprene emission has a function in the protection of plant membranes under heat and light stress [23], but recent results of field trials with poplar trees where isoprene synthase genes have been suppressed by RNA interference (RNAi) techniques indicate that there is no growth and development advantage from isoprene emission, and perhaps the only benefit of isoprene emission may come from interactions with herbivores [49]. Despite the different routes of formation of isoprene (induction of a specific chloroplast enzyme) and the HTGs (potentially via nonspecific extra-chloroplastic phosphorylases and glycosyl transferases), there are parallels that can be drawn. Both processes appear to be governed by the relevant isoprenoid diphosphate pool size [13, 50] and furthermore appear to be a consequence of plant nitrate status [13, 51] as well as induced by high light [52, Ward et al. unpubl.].

Obviously more work needs to be done on flux through the MEP pathway, but this work and increasing evidence in the literature indicate that hemiterpenoid production is the result of a multilevel intermediate pool size management system that is responsive to a number of external and internal signals.

1.6 Future Prospects

This work has demonstrated the power of unbiased metabolomics not only to discover new natural products but also to provide a "systems" overview of metabolism as it adjusts to pressure from environmental and nutritional stress. Until this work, flux through the terpenoid pathway in *Arabidopsis* was thought to be low, relative to many plants that accumulate large amounts of secondary terpenoids. Despite the presence of greater than 30 mono- and sesquiterpene synthase genes [53], oxidized terpenes have not been reported in the model plant, although volatile mono- and sesquiterpene hydrocarbons are emitted at low levels by flowers and roots [54]. Aside from the extensive research on isoprene, hemiterpenoids have been a somewhat neglected research area. However, the discovery of HTGs in the model plant and the specific nature of the induction of their biosynthesis will stimulate renewed interest in these molecules. The possibility that the HTG formation in *Arabidopsis* is a paradigm for isoprene production in trees will bring a greater range of genetic tools and resources to bear on carbon overflow mechanisms. This will be of direct interest to terpene engineering whether the strategic outlet is the accumulation of valuable compounds or for manipulation of interactions with predators. The production of isoprene and other linear terpenoids as biofuels is also a growing research topic. Genetic engineering is beginning to shed light on the 'raison d'être' for isoprene production by tree species [49], and the debate on carbon overflow versus thermoprotectant continues. Delineation of the regulatory mechanism of HTG production in *Arabidopsis* will surely inform on this discussion.

The use of the metabolomics screen for HTGs will allow a detailed genetic, temporal, and environmental study of the induction of the low foliar nitrate state

that leads to HTG synthesis. Nitrate sensing and signaling in leaf biochemistry has long been mooted as a key factor in metabolic control in plants [7]. The work described here presents a new opportunity to explore this, using the extensive genetic resources available for *Arabidopsis*. The links between high light, oxidative stress, and low foliar nitrate revealed in this study can also now be explored by using HTG production as readout. The high correlation between HTG production in the shoots with diversion of phenylpropanoid flow to coniferin and scopolin in the roots perhaps indicates that aspects of the plant defense response cross over with the nitrate response, and that nutrient limitation may activate the flow of carbon into secondary defense metabolites.

In the discussion, we have suggested that a palette of regulatory mechanisms modulate the chloroplastic MEP pathway. We suggest that different plants use different routes to off-load excess flux in stress conditions. The determinant appears to be the pool size of the relevant isoprenoid diphosphate. *Arabidopsis* and tobacco [47] produce HMBPP-derived products. Isoprene is emitted by many tree species, and DMAPP pool size appears to be a factor [50] in isoprene emission. Similarly, hemiterpenoids apparently derived from DMAPP (and IPP) have been widely reported (Figs. 1.4 and 1.5). Also of interest are the tetraols that could be derived from a number of the higher precursors (Fig. 1.6). Future developments in metabolomic analysis of the MEP pathway metabolites will be necessary as an aid to understanding the extent of this form of regulation. Also of interest, in the biosynthesis of HTGs, are the dephosphorylation and glycosylation steps. Evidence for the presence or absence of specific and inducible enzymes for these steps needs to be sought and combined transcriptomic–metabolomic studies of stressed plants will aid in this endeavor.

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Chapter 2
Electrospray Ionization Traveling Wave Ion Mobility Spectrometry Mass Spectrometry for the Analysis of Plant Phenolics: An Approach for Separation of Regioisomers

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Abstract The use of ion-mobility spectrometry (IMS) coupled to mass spectrometry (IMS–MS) for biomolecule analyses has steadily increased over the past two decades, and is now applied to both proteomic and metabolomic investigations. This chapter describes the application of traveling-wave ion-mobility spectrometry–mass spectrometry (TWIMS–MS) to the analysis of a selection of bioactive phytochemicals used in dietary supplements. Applications include the analysis of grape seed proanthocyanidins and the structural characterization of bioactive constituents of dietary supplements using TWIMS-MS in conjunction with tandem mass spectrometry. We also discussed is the application of TWIMS-MS for the gas-phase mobility separation of structural isomers and the estimation of collision cross sections for a small selection of phenolic compounds from hop. Recent applications of IMS–MS to a broad range of biomolecule measurements have demonstrated that IMS–MS has emerged as a powerful analytical technique capable of providing the separation space necessary to analyze highly complex samples. We give a perspective on emerging applications of IMS–MS for small molecule and biopolymer applications. The combination of devices that allow real-time monitoring of living systems using IMS–MS is an exciting avenue of facilitating system-biology experiments. The future of IMS–MS is bright and full of opportunities.

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2.1 Introduction

There is increasing evidence that plant phenolics have health benefits which may, at least partially, stem from their antioxidant and radical scavenging activity [1–3]. Considering the increasing interest in plant phenolics as nutraceuticals, comprehensive profiling methods for plant extracts are highly needed. We report on the characterization of plant phenolics using traveling-wave ion-mobility spectrometry–mass spectrometry (TWIMS–MS) and emphasize the structural analysis of plant secondary metabolites that are commonly found in over-the-counter dietary supplements.

The use of ion-mobility spectrometry (IMS) coupled to mass spectrometry (IMS–MS) for biomolecule analyses has steadily increased since the 1990s. Many applications describe IMS–MS for studying peptides and proteins and their folding behaviors in the gas phase [4–10]. IMS–MS has also been used for assessing synthetic polymers [11, 12]. More recently, IMS–MS has been described as a powerful addition to the arsenal of tools for the structural analysis of small molecules including drugs, metabolites, lipids, carbohydrates, phytochemicals, and other natural products [13–19]. Comprehensive reviews are available that describe in detail the principles and applications of IMS–MS [20–22]. Briefly, in IMS ions are separated according to their charge state, shape, and size. IMS systems function as gas-phase separation devices. IMS uses nondestructive low-energy collisions to separate ions predominately on the basis of ion-neutral collision cross sections. Ion-mobility separations in the gas phase have considerably lower resolution compared to the resolution that can be achieved with modern condensed phase chromatographic separation technologies. However, the separation of ions occurs several orders of magnitudes faster than separations based on liquid chromatographic techniques; ion-mobility separations usually occur on a time scale of milliseconds compared to the seconds to hours in chromatographic separations. MS measurements occur in the microsecond range and, as such, are nested within the IMS experiments [23]. IMS–MS experiments allow real-time separations of the components of complex mixtures and provide access to three-dimensional (3D) analytical information, namely shape, mass, and abundance. The combination of IMS with MS results in two-dimensional plots of drift time (t_d) versus *m/z*. The three-dimensionality of TWIMS–MS datasets is best captured in so-called driftscope images that contain information regarding the drift time (t_d, in ms) and *m/z* values displayed in a nested fashion, t_d as function of (*m/z*), with ion abundances given in a color-coded style. These images enable the extraction of distinct features that otherwise would get lost or overlap in crowed spaces of traditional mass spectra. A unique feature of IMS–MS is the ability to conduct drift time measurements that allow the calculation of collision cross sections (CCSs) for low- and high–molecular-weight molecules and the possible separation of isomeric analytes, which is not possible solely with MS-based techniques [13–15].

IMS–MS systems are available in many different instrument configurations. In the traditional configuration, the drift tube-based IMS device is placed between the ionization source and the mass analyzer [20]. The recent advent of a commercial

IMS–MS system, in which a traveling-wave IMS device has been integrated into a hybrid quadrupole-orthogonal acceleration time-of-flight (TOF) mass spectrometer, opens new possibilities for the structural characterizations of small molecules and biopolymers [24]. In this contribution, we describe the application of TWIMS–MS for the analysis of a selection of bioactive phytochemicals used in dietary supplements. We first give a brief description of the TWIMS–MS instrument used. Then, we discuss several applications of TWIMS–MS: (1) the analysis of a biopolymer mixture, namely grape seed proanthocyanidins; (2) the structural characterization of bioactive constituents of dietary supplements using TWIMS in conjunction with tandem mass spectrometry; and (3) the gas-phase mobility separation of structural isomers and the estimation of collision cross sections for a small selection of phenolic compounds from hop. We conclude with a brief review of recent developments and applications, and provide a perspective on the emerging application of IMS–MS for small molecule and biopolymer applications.

2.2 Ion-Mobility Mass Spectrometry Using an Electrospray Ionization Quadrupole Traveling-Wave Ion-Mobility TOF Instrument

Many different instrument designs have been described that combine IMS with different types of MS analyzers [20]. The availability of a commercial hybrid system that integrates traveling-wave ion-mobility separation with a quadrupole TOF analyzer makes the technology accessible to a wider research community. For the applications described here, we used a Waters Synapt G2 HDMS instrument equipped with an electrospray ionization (ESI) source. This instrument has the following configuration: a quadrupole mass filter, the TriWave™ section consisting of three traveling-wave (T-wave) devices (Trap T-wave, Ion mobility separation (IMS) T-Wave, and Transfer T-wave) and an orthogonal acceleration (oa) TOF analyzer (Fig. 2.1). The traveling-wave IMS device is a radio-frequency (RF) ion guide based on a stack of ring electrodes. The RF voltage confines the ions radially. By applying a continuous series of DC voltage pulses traveling along the stacked ring electrodes, ions are moved through the gas-filled guide. The ability of an ion to travel along with the DC pulse through the gas-filled T-wave ion guide will depend on the ion's size, charge, shape, and the interaction cross section between the ion and background gas. Instrumental parameters that affect the ability of an ion to move with the traveling wave are the amplitude of the wave, the travel velocity of the wave, and the type and pressure of the background gas. Applying an optimized set of parameters, high-mobility ions will travel with the wave and pass through the ion guide faster than ions with low mobility, which roll over the top of the wave and as a result spend more time in the device [24].

The TriWave™ section of this instrument consists of three traveling-wave-enabled stacked ring ion guides. This configuration allows for unique fragmentation experiments (Table 2.1). The trap ion guide is used for the accumulation of ions and

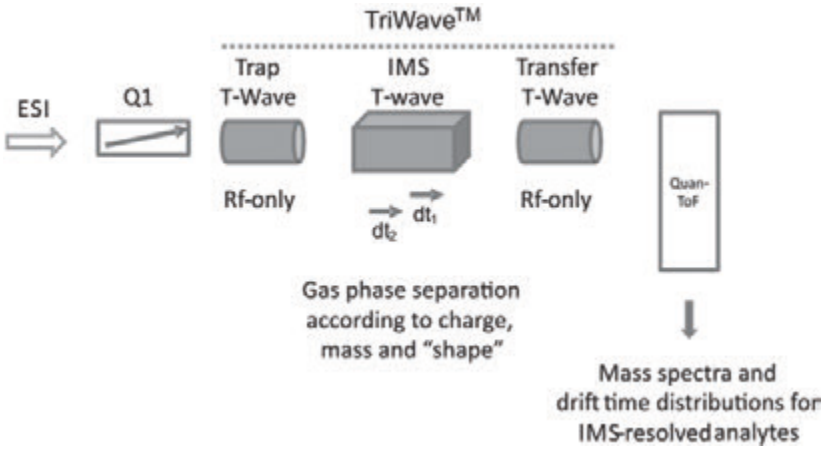


Fig. 2.1 Conceptual diagram of the commercially available TWIMS–MS instrument (Waters Synapt G2 HDMS instrument) operated in the mobility time-of-flight (TOF) mode. This operating mode is used for gas-phase mobility separation of ions in combination with high-resolution mass spectrometry. This mode also enables the extraction of drift times for the estimation of collision cross sections after calibrating the traveling-wave ion-mobility separator. In mobility-TOF mode, the quadrupole (Q1) analyzer is operated in the transmitting mode, the trap and transfer devices serve at radio-frequency (Rf)-only ion guides. *T-wave*, traveling wave; *IMS*, ion mobility spectrometry; *dt*, drift time

release of ions as packets into the ion-mobility separation device. The transfer ion guide conveys the mobility-separated ions to the orthogonal acceleration (oa) TOF analyzer. Fragmentation can take place either in the trap, in the transfer device, or in both devices. A detailed description of the working principle and the design of the traveling-wave ion-mobility separator have been published previously by Giles et al. [24, 25]. Details on the theoretical background on classical IMS–MS and the adaption to traveling TWIMS–MS have also been described in several recent papers [24–27]. In the following sections, we describe the use of TWIMS–MS experiments for the structural characterization of plant phenolics.

2.3 TWIMS–MS Analysis of Biopolymers: Application to Grape Seed Proanthocyanidins

Grape seed extracts have been extensively studied by diverse mass spectrometric techniques. In most cases, the mass spectrometric analyses were accompanied by laborious and extensive chromatography of the highly complex grape seed proanthocyanidin mixtures [28–30]. Considering the current interest in grape seed proanthocyanidins as nutraceuticals in biomedical applications, comprehensive and fast profiling of grape seed extracts is highly desirable. The analysis of proanthocyanidins by ESI–MS is challenging due to the overlapping of ion signals of constituents

Table 2.1 Operating modes of the TWIMS–MS instrument utilizing the TriWave™ section

Mode	TriWave™ usage			Products
	Trap	TWIMS device	Transfer	
Mobility-ToF	Ion guide only	Ion-mobility separation	Ion guide only	Ions are separated according to their mobility; drift time measurements enable collision cross-section estimations after calibration of the TWIMS device
Trap fragmentation	Elevated energy	First-generation fragment ions	Ion guide only	First-generation fragment ions are ion mobility separated
Transfer fragmentation	Storage device only	Precursor ions	Elevated energy	Precursor ions are separated according to their mobility. First-generation fragment ions align with precursor ion drift time
Time-aligned parallel (TAP) fragmentation	Elevated energy	First-generation fragment ions	Elevated energy	First-generation fragment ions are separated according to their mobilities. Activation in the transfer device results in second-generation product ions which are time-aligned to the respective first-generation product ion precursor

of the highly complex mixtures of proanthocyanidins oligo- and polymers differing in length (i.e., degree of polymerization or DP), subunit composition, and type of linkage between flavanol units [29–33].

The three-dimensionality of TWIMS–MS dataset is highlighted in Fig. 2.2a–d. ESI–TWIMS–MS driftscope images (drift time versus m/z; color coding: white most to blue least abundant ions) of a typical grape seed proanthocyanidin preparation are shown (Figs. 2.2a, c). What makes these images remarkable is (i) the separation of the ions into distinct charge state groups which are denoted as (+1) and (+2) and (ii) the separation of the proanthocyanidin oligomers into distinct ion clusters. The sodium adducts of the proanthocyanidin oligomer ions displayed shorter drift times than the corresponding protonated molecular ions (Fig. 2.2a).

In the TWIMS–MS driftscope image depicted in Fig. 2.2a, the gas-phase mobility separation of singly protonated proanthocyanidin ions from the doubly protonated proanthocyanidin ions is highlighted. The two charge state groups are denoted with (+1) and (+2). In Fig. 2.2b, a section of the ESI mass spectrum (*m/z* 500–1,400) is depicted. Singly charged ion signals dominate the mass spectrum. Due to the mobility separation of singly and doubly charged ions, it is possible to extract distinct ion clusters, which helps with the analysis of overlapping ion signals. For instance, extraction of the singly protonated ions of the procyanidin trimer (PC3, $[M+H]^+$, m/z 867.2) and the doubly protonated ions of the procyanidin hexamer (PC6, $[M+2H]^{2+}$, m/z 866.2) results in mass spectra that show baseline resolved isotope clusters (Fig. 2.2c).

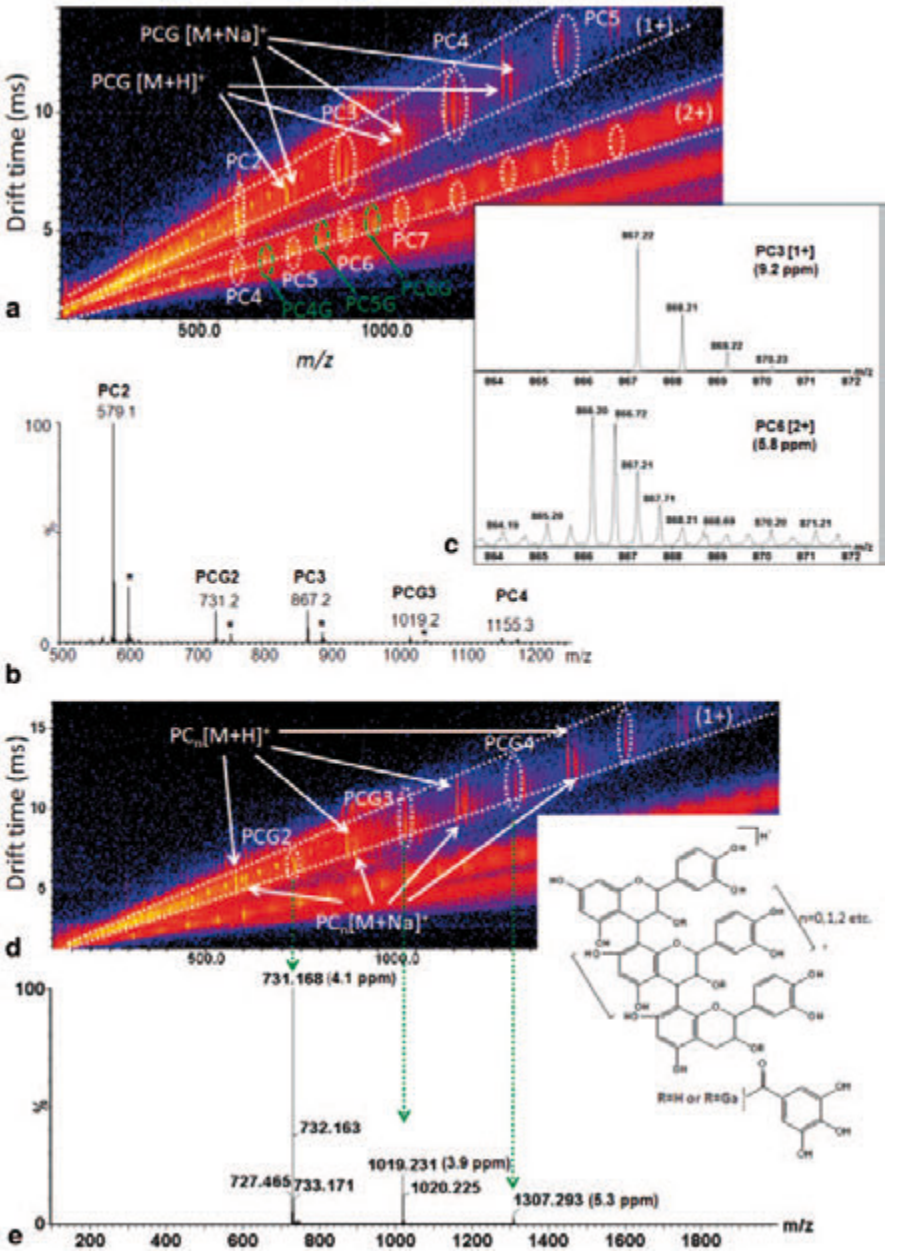


Fig. 2.2 TWIMS–MS analysis of grape seed proanthocyanidins. **a** TWIMS–MS driftscope image (drift time versus *m/z*) of a grape seed extract. PC ions separate in the TWIMS cell into charge groups labeled with (+1) and (+2) representing singly protonated and doubly protonated ions. **b** ESI mass spectrum of grape seed proanthocyanidins (depicted is range from *m/z* 500–1,450). The *asterisk* indicates the sodium adduct, $[M+Na]^+$, of the respective protonated molecular ion,

In the image depicted in Fig. 2.2d, arrows mark the singly protonated molecular ion clusters of procyanidins at *m/z* 579, 867, 1,155, and 1,443 corresponding to procyanidins with increasing degree of polymerization, namely DP2, DP3, DP4, and DP5. The sodiated molecular ions $[M+Na]^+$ are marked as well. The procyanidin monogallate ion clusters are encircled in this plot. Extraction of selected ion clusters allows the detailed analysis of ion signals that belong to a distinct proanthocyanidin series. For instance, the ion clusters of the procyanidin monogallates (PCG) were extracted and the respective mass spectrum of the extracted ion clusters is shown in Fig. 2.2b. The spectrum shows only the ion signals of procyanidin monogallates with DP2 (MH^+, *m/z* 731.17), DP3 (MH^+, *m/z* 1,019.23), and DP4 (MH^+, *m/z* 1,307.29).

Although the present TWIMS–MS spectra were obtained in the positive ionization mode, proanthocyanidins give information-rich TWIMS–MS plots in the negative mode. Matrix-assisted laser desorption ionization (MALDI)-MS analysis has been described as a powerful approach for the characterization of PC mixtures [28, 30]. Therefore, it would be interesting to see if the combination of MALDI with TWIMS–MS would further advance the analysis of these highly complex biopolymer mixtures. The above example attempts to demonstrate some of the features that TWIMS–MS offers as an analytical platform for the in-depth interrogation of proanthocyanidin preparations. We anticipate that TWIMS–MS will emerge as measurement technology for the comprehensive analysis of other biopolymers and bio-inspired plastics as well.

Experimental Details The instrument was operated in positive ionization mode with an ESI capillary voltage of 2.75 kV and a sampling cone voltage of 30 V. The other conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas flow rate, 500 L/h; and cone gas flow rate, 40.0 L/h. Ion-mobility separation conditions included: ion-mobility gas flow rate, 75.30 mL/min, IMS wave velocity, 542 m/s, and wave height, 40.0 V. Argon was used as collision gas in the trap and transfer cells, while nitrogen (N_2) was used as IMS cell gas. Data acquisition was carried out using Waters MassLynx (V4.1), and for IMS data processing DriftScope software (V 2.1, Waters) was used. Positive ion mass spectra were acquired in the resolution mode over a mass range of 100–2,500 *m/z* using continuum mode setting. Mass calibration in positive mode was performed by infusing sodium iodide solution (2 µg/µL, 1:1 (v/v) water:2-propanol).

$[M+H]^+$. **c** The inset shows the extracted mass spectra of the singly protonated ions of procyanidin trimers, PC3, (upper mass spectrum) and the doubly protonated ions of procyanidin hexamers, PC6 (lower mass spectrum). Note the baseline-resolved isotope cluster for the doubly protonated PC6 ions; TWIMS–MS enables the gas-phase separation of the singly protonated ions from the doubly protonated PC oligomers avoiding overlapping of the isotope clusters on the *m/z* scale. **d** Distinct ion clusters can be individually extracted and exported to display the respective mass spectra. The extracted mass spectrum depicting singly protonated procyanidin (PC_n) oligomers with DP 2–4 is shown. Sodiated proanthocyanidin ion $[M+Na]^+$ has been annotated as well. Procyanidin monogallate (PCG) ion clusters are seen in between procyanidin clusters. **e** Extracted ion signals for procyanidin monogallates

2.4 Tandem Mass Spectrometry Approaches for the Structural Analysis of Plant Phenolics Using Dried Spot Analysis in Combination with TWIMS–MS

The need for high-throughput techniques for the analysis of dietary supplements and active ingredients encouraged us to explore the combination of dried spot analysis using thin-layer chromatography (TLC) plates in combination with ESI–TWIMS–MS. A combination of TLC, desorption electrospray ionization, and TWIMS–MS has been described previously for the direct analysis of pharmaceutical formulations [34]. The combination of ion-mobility separation with MS allows gas-phase ions to be separated by their mobility and then to be analyzed according to their mass-to-charge ratio in the TOF analyzer. Analysis specificity is further increased by combining ion-mobility separation with collision-induced fragmentation in the transfer region of the Synapt G2 instrument, thus, enabling the extraction of structural information and high resolution accurate mass measurements in one experiment. The combination of TLC-based spot analyses and ESI–TWIMS–MS resembles a multidimensional separation approach that results in high-content mass spectral information for the analytes of interest. Here, we describe the application of TWIMS–MS with tandem mass spectrometry for the analysis of bioactive flavonoids in dietary supplements, namely rutin (quercetin-3-O-rutinoside, $C_{27}H_{30}O_{16}$, M_{mono} 610.1534 Da) and hesperidin (hesperitin-7-O-rutinoside, $C_{28}H_{34}O_{15}$, M_{mono} 610.1898 Da).

Dietary supplements were extracted with methanol and the extracts were spotted on cellulose TLC plates. A CAMAG TLC MS interface combined with an LC system was used for eluting the analytes from the TLC plate and subsequent infusion into the mass spectrometer. Under the experimental conditions used, this approach enabled the interrogation of dietary supplement spots for a time period of approximately 1.5 min. A typical total ion response is shown in Fig. 2.3a. By extracting all ion signals at the plateau of the total ion chromatogram, a 3D driftscope image is generated (Fig. 2.3b). Note the relatively broad ion distribution at *m/z* 611 indicating insufficient resolution to separate the protonated ions of hesperidin and rutin under the TWIMS conditions used. However, two well-separated ion signal distributions were observed for the sodiated ions, $[M+Na]^+$, of those two flavonoid glycosides (*m/z* 633). The selected ion signals with *m/z* 633 show better signal-to-noise ratios than the protonated molecules $[M+H]^+$ at *m/z* 611 (Fig. 2.3d, e). Under the TWIMS conditions used, hesperidin ($[M+Na]^+$, *m/z* 633.18) and rutin

measured under the current conditions (*m/z* range 100–1,200 Da). Note the broad ion assemblies at *m/z* 611 and the two ion distributions with similar *m/z* values at *m/z* 633 but clearly different drift time distributions. **d** and **e** Comparison of drift time distributions of ion signals observed for two over-the-counter dietary supplements that contain both flavonoid diglycosides, hesperidin ($[M+Na]$ *m/z* 633.18, dt 5.21 ms), and rutin ($[M+Na]$ *m/z* 633.14, dt 5.97 ms). Drift time distributions were obtained by selecting the ions at *m/z* 633 with the quadrupole device Q1 followed by gas-phase separation of the ions in the TWIMS cell. In **e**, the mass spectrum has a different *m/z* scale than in **d** to illustrate that the ion signals for both flavonoid diglycosides ($[M+Na]^+$) are observable at the *m/z* scale

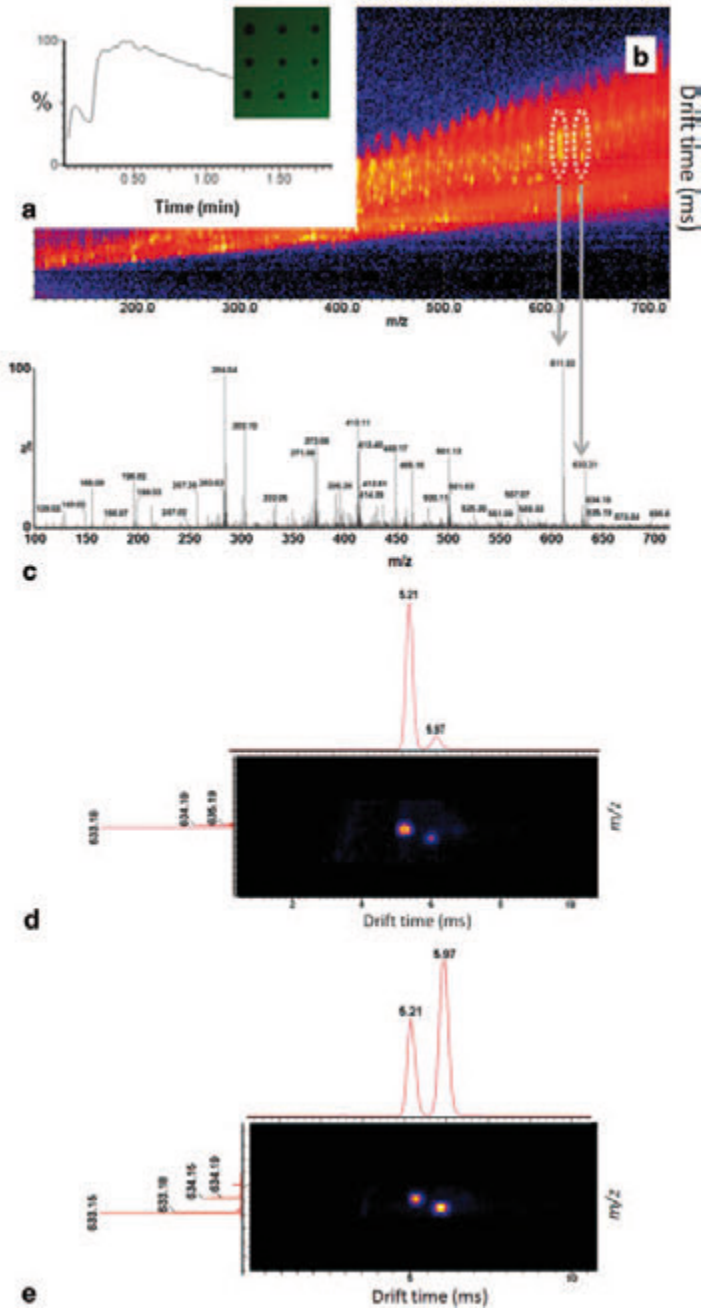


Fig. 2.3 Dried spot analysis of phytochemicals in dietary supplements with electrospray ionization TWIMS–MS. **a** Total ion chromatogram of a dietary supplement spotted on cellulose TLC plate, extracted from the plate, and infused into the mass spectrometer. **b** Driftscope image (drift time versus *m/z*) and **c** the integrated mass spectrum over the entire range of drift time distributions

($[M+Na]^+$, *m/z* 633.14) had drift time distributions centered around 5.21 and 5.97 ms, respectively.

In order to obtain structural information, the ions at *m/z* 633 were subjected to tandem mass spectrometry using the transfer region of the TriWave™ device. Transfer fragmentation was conducted by selecting the ions at *m/z* 633 using the quadrupole device. Ions were then separated in the T-wave ion-mobility cell and subsequently subjected to collision-induced fragmentation in the transfer region (Fig. 2.4a). Applying elevated collision energy to the transfer device causes fragmentation of the mobility-separated precursor ions. Because the fragment ions preserve their velocity of the precursor ion, the fragment ions align with the drift times of the precursor ions. The ions at *m/z* 633 were selected in the quadrupole region, separated in the T-wave cell, and subsequently fragmented by collisions in the transfer region. The integrated fragment ion spectrum is shown in Fig. 2.4b. In Figs. 2.5 and 2.6, the fragment ion mass spectra of the sodiated molecular ions of hesperidin and rutin are shown, respectively. The time-aligned and compound-specific ions were extracted for each of the flavonoid glycosides separately. Because transfer dissociation experiments were conducted using sodiated precursor ions, the fragment ions are sodiated as well. Fragment ions of the rutinoside moiety dominated the spectrum for both species. The observed fragment ions are indicated in the schematic presentation of the two flavonoid glycoside structures.

Experimental Details The analytes were extracted using a CAMAG TLC interface from cellulose TLC plates using 75% acetonitrile/25% water. A Shimadzu LC-10AD pump was used for solvent delivery. The flow rate was 0.1 mL/min. Mass spectral experiments were performed using a Waters Synapt G2 HDMS mass spectrometer (Manchester, UK) equipped with TWIMS. Mass spectra were acquired in positive mode. The instrument was operated in the resolution mode with a capillary voltage of 3.0 kV and a sampling cone voltage of 30.0 V. The other conditions comprise the following: extraction cone voltage, 4.1 V; ion source temperature, 80 °C; desolvation temperature, 250 °C; desolvation gas flow rate, 500 L/h; and cone gas flow rate, 5.0 L/h. Ion-mobility separation conditions included ion-mobility gas flow rate; 90 mL/min, wave velocity ramping from 480 to 556 m/s; and wave height, 40.0 V. Argon was used as collision gas on the trap and transfer cells, while nitrogen (N_2) was used as IMS cell gas. Data acquisitions were carried out using Waters MassLynx (v4.1). IMS–MS data were processed with DriftScope software (v2.1, Waters). All analyses were conducted in the positive ionization mode. Mass spectra were acquired over the mass range of 50–1,200 *m/z* in continuum mode. A 0.1 ng/μL solution of leucine enkephaline ($[M+H]+556.2771$) was infused at 5 μL/min as the reference mass (lock mass) for accurate mass measurements. Mass calibration in positive mode was performed by infusing sodium formate (5 mM, prepared in 1:1 (v/v) CH_3CN:H_2O).

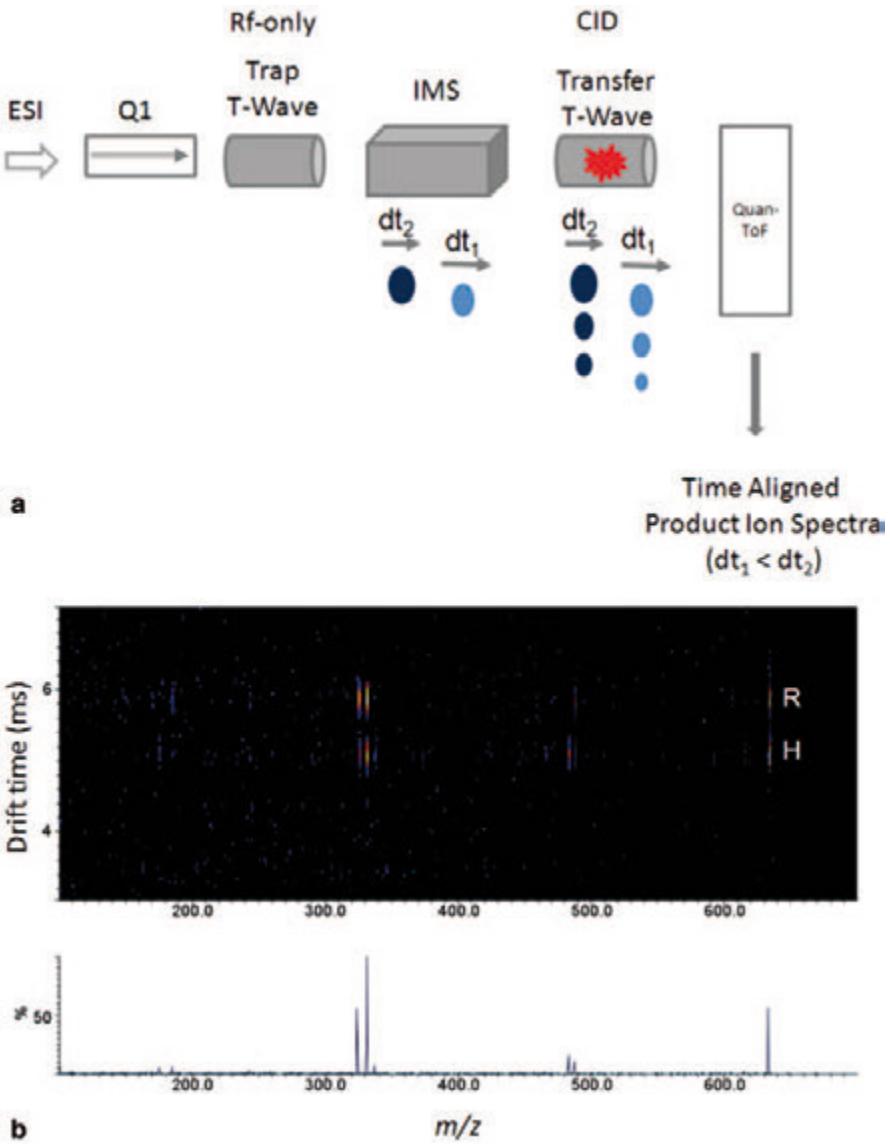


Fig. 2.4 a–b Transfer fragmentation mode for the characterization of bioactive ingredients in dietary supplements. **a** Transfer fragmentation mode—conceptual presentation. In this mode, fragment ions generated in the transfer region can be correlated to their time-aligned precursor ions. This mode of operation is particularly useful for distinguishing isobaric precursor ions as long as the precursor ions have different interaction cross sections; *v*, velocity; *dt*, drift time. **b** TWIMS–tandem mass spectrometry analysis of a supplement that contains both flavonoid glycosides, hesperidin and rutin. Transfer dissociation was conducted by selecting the ions at *m/z* 633 in the quadrupole device Q1, separation of the ions in the TWIMS region, and by collisional activation in the transfer region. *Upper panel*: drift time plot displaying the time-aligned product ions for the precursor ions with drift times 5.21 ms (hesperidin, $[M+Na]^+$) and 5.97 ms (rutin, $[M+Na]^+$). *Lower panel*: extracted tandem mass spectrum after integration of the ion signals that contribute to both time-aligned drift time distributions at 5.21 and 5.97 ms. *H*, hesperidin; *R*: rutin

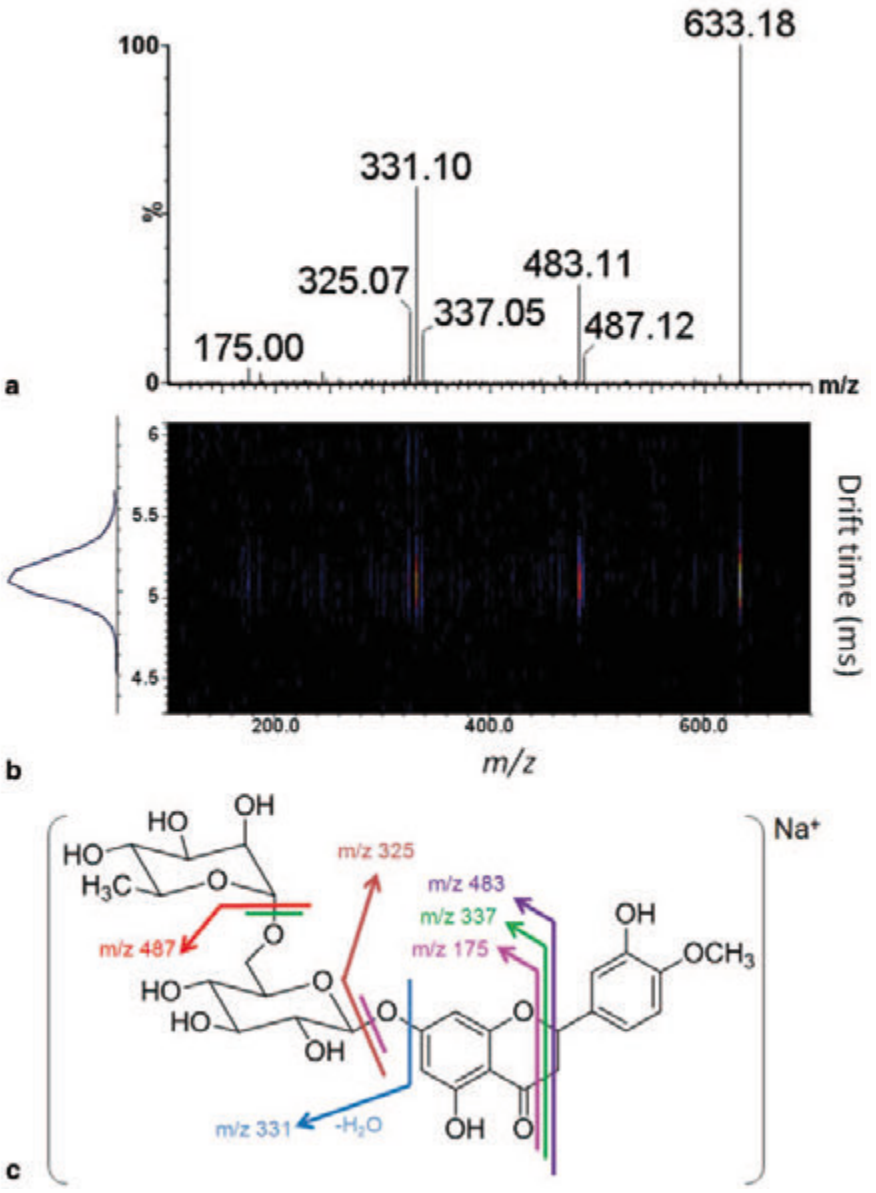


Fig. 2.5 ESI q-IMS–MS/MS transfer dissociation analysis of the sodiated hesperidin ions ($[M+Na]^+$, *m/z* 633). **a** Fragmentation spectrum, **b** drift time distribution, and **c** the proposed transfer dissociation pathways of sodiated hesperidin

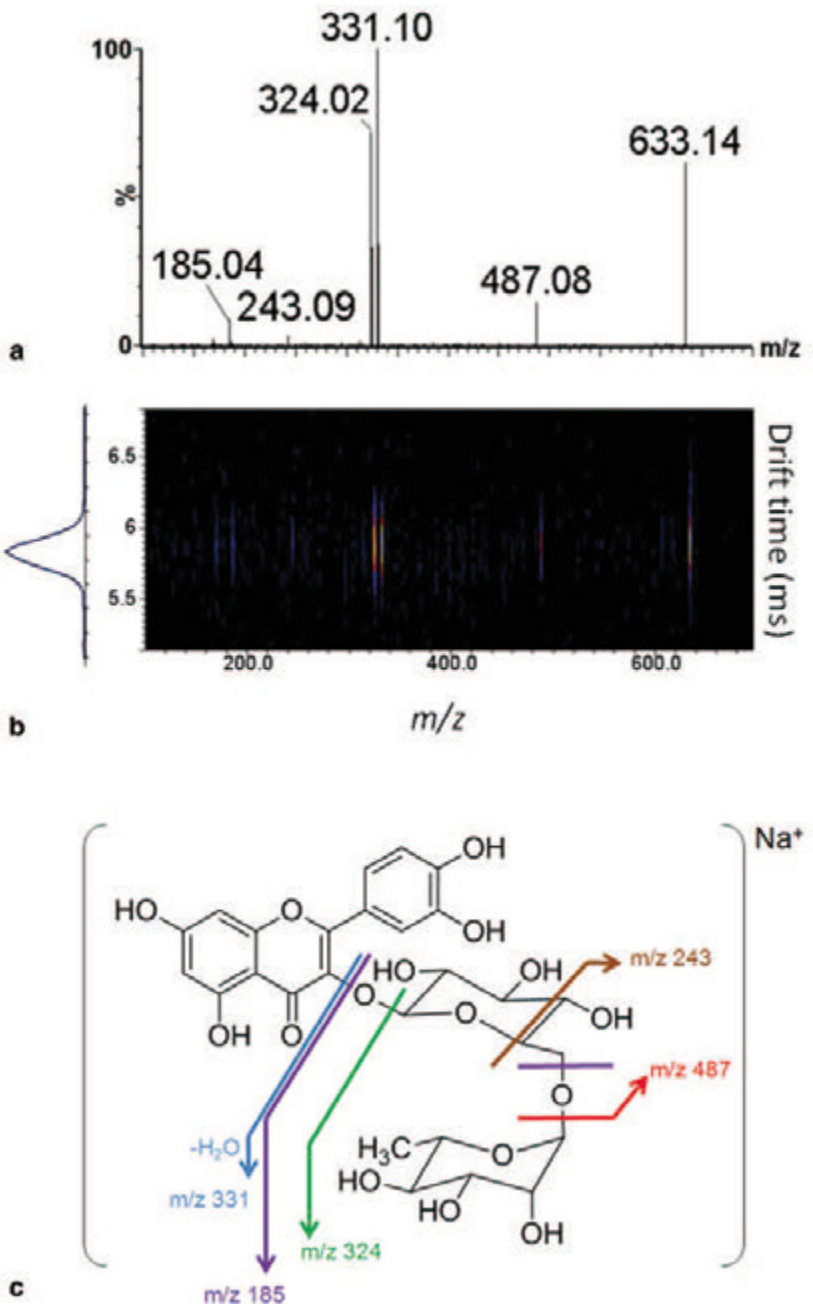


Fig. 2.6 ESI q-IMS–MS/MS transfer dissociation analysis of the sodiated rutin ions ($[M+Na]^+$, *m/z* 633). **a** Fragmentation spectrum, **b** drift time distribution, and **c** the proposed transfer dissociation pathways of sodiated rutin

2.5 TWIMS–MS for Obtaining Collision Cross Sections as an Additional Parameter for Plant Metabolite Characterization

In metabolomics, the characterization of a metabolite, e.g., a plant natural compound, is currently based on accurate mass, fragment ion spectra, and retention time comparison with a standard compound. Collision cross sections are independent of chromatographic conditions and therefore collision cross-section determinations would add an additional dimension to the characterization of a metabolite. Collision cross-section determinations have the potential of distinguishing structural isomers which is usually not possible solely by MS-based methods. We have started to build up a database for plant metabolites that compiles classical MS data (accurate mass, tandem mass spectral data) and collision cross sections. Here, we report on the determination of collision cross sections for a selection of hop phenolics. Hop phenolics have recently attracted public attention because of their health-promoting effects. These compounds show antiproliferative activity, cancer chemopreventive, and antioxidant properties [2]. Specifically, we were interested in applying TWIMS–MS for the characterization of isobaric hop phenolics, namely (i) the hop chalcone xanthohumol (XN)and the isobaric prenylated flavonoid isoxanthohumol (IX), and (ii) the two geometric isomers 6- and 8-prenylnaringenin (6-PN, 8-PN).

TWIMS separates ions according to their mobility through a continuous sequence of transient voltage pulses (traveling waves). The mobility of an ion through the T-wave cell depends on the charge on the ion, the mass of the ion and the buffer gas, the identity, temperature and pressure of the buffer gas, and its collision cross section. Hence, the collision cross section, Ω, of an ion can be expressed as given in Eq. 2.1. Since the separation of ions in the TWIMS section of the instrument is more complex than a classical drift tube, additional parameters (A and B) need to be included to account for the nonlinear effects of the TWIMS device [24, 27]:

$$\Omega = Z\,e\left[\frac{1}{m_1}+\frac{1}{m_N}\right]^{1/2} A t_D{}^{B} \tag{2.1}$$

Ω = Collision cross section
Z = Number of charges on the analyte ion
e = Charge on an electron
m_1 = Mass of the analyzed ion
m_N = Mass of the buffer gas
A = Correction factor for the electric field parameters
B = Correction factor for the nonlinear effect of the TWIMS device
t_D = Drift time

Since the separation of ions in the TWIMS section of the instrument is more complex than a classical drift tube, which uses a constant electric field, the T-wave mobility separation device needs to be calibrated. For this purpose, a drift time calibration procedure was applied that uses absolute cross-section values of polyglycine (Poly-Gly) and polyalanine (Poly-Ala) peptide ions known from classical

Table 2.2 Estimated collision cross-section values of hop phenolic compounds which were obtained by TWIMS–MS

Compound	Estimated CCS[a] (Å^2)			Theoretical CCS[a], (Å^2)
	Poly-Ala[b]	Poly-Gly[b]	Poly-Ala[b] and Poly-Gly[c]	
XN[d]	128.3	128.9	125.4	127.7
IX[e]	123.5	122.8	120.4	120.3
6-PN[f]	122.9	n.d.	120.2	118.7
8-PN[g]	119.9	n.d.	117.2	122.1

Experimentally estimated cross sections were derived by comparison with poly-DL-alanine mixture (Poly-Ala) and oligo-glycine (Poly-Gly) mixture following the procedure described by Williams et al. [32]. For comparison, theoretical cross sections are listed in the right column of the table

[a] Collision cross section
[b] Poly-DL-alanine
[c] Polyglycine
[d] Xanthohumol
[e] Isoxanthohumol
[f] 6-Prenylnaringenin
[g] 8-Prenylnaringenin

drift-tube ion-mobility studies [26, 35, 36]. The experimentally estimated cross-section values were then compared to the calculated cross sections (Table 2.2). The theoretical cross sections of the compounds were obtained by using the DriftScope software (v4.1). This software uses the projection approximation (PA) approach which is also one of the models in the MOBCAL software, a program to calculate mobilities which was developed by Martin Jarrold's group [37]. Briefly, MOBCAL calculates the collision cross-sections based on three different models. These models are the exact hard sphere scattering (EHSS), projection approximation (PA), and the trajectory model™. PA calculates cross sections in regard to a collision between the buffer gas and the analyte atoms [38–40].

XN and IX (M^-, *m/z* 353.15) were detected after TWIMS–MS in negative mode with slight difference in their drift time distributions (t_{DTD} ~0.2 ms). As shown in Fig. 2.7, infusion of the IX solution resulted always in two peaks with drift times differing by 0.2 ms. The peak ratio and drift times were the same as those detected for a mixture of IX and XN. This may indicate that isomerization of IX to XN occurred in the source prior to ion-mobility separation. A similar behavior was observed for carotenoids [15]. The isomeric PNs (M^-, *m/z* 339.12) showed distinct peaks that showed only marginally different drift time distributions under the conditions used. The experimentally estimated cross sections were in good agreement with the theoretical values. This example demonstrates that TWIMS–MS is a powerful technique that is capable of providing access to an additional analytical parameter for the in-depth characterization of phytochemicals, namely experimentally estimated collision cross sections besides exact mass determination and fragment ion information. However, more research needs to be done on structurally diverse sets of small molecules to explore the full potential and limitation of TWIMS–MS.

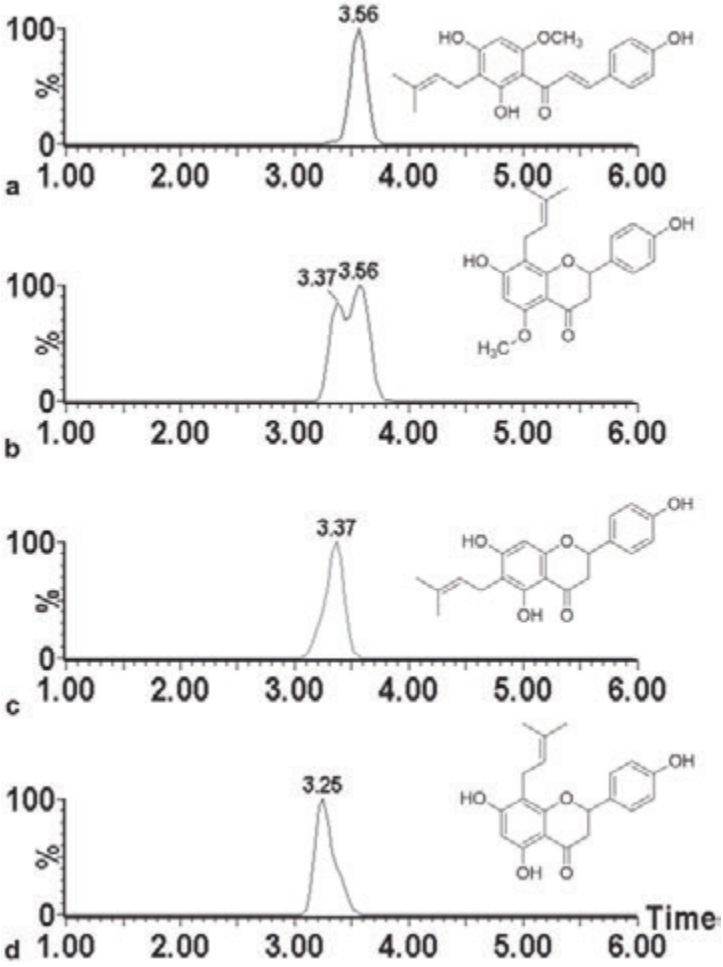


Fig. 2.7 **a**–**d** Drift time distributions and structures of the hop phenolics XN, IX, 6- and 8-PN. Negative ion electrospray TWIMS–MS analysis of the $[M-H]^-$ ions of the isomeric flavonoids XN and IX (both *m/z* 353.15, $C_{21}H_{21}O_5^-$) and the regioisomers 6- and 8-PN (*m/z* 339.12, $C_{20}H_{19}O_5^-$) was conducted following infusion. Drift time distributions were recorded for **a** XN at 3.56 ms, **b** IX at 3.37 and 3.56 ms, and **c** 6-PN at 3.37 ms and **d** 8-PN at 3.25 ms. Further work is needed to rationalize the possible isomerization of IX during the electrospray process. Calibration of the TWIMS device enables the estimation of the collision cross section (see Table 2.2). *XN*, xanthohumol; *IX*, isoxanthohumol; *6-PN*, 6-prenylnaringenin; *8-PN*, 8-prenylnaringenin; *time*, drift time in milliseconds (ms)

Experimental Details Stock solutions of XN, IX, 6-PN, and 8-PN were prepared separately (10 μg/mL) in methanol from solid standards. Equal amounts of XN and IX solutions (1:1 v/v) were mixed with 50% water before use to minimize degradation and isomerization, and then immediately infused into the ESI source at a flow rate of 5 μL/min. A solution containing both prenylated naringenins, 6-PN

and 8-PN, was prepared and infused. Mass calibrations in both modes (positive and negative) were done by infusing a solution of sodium formate (5 mM; 1:1, v/v, acetonitrile:water). The instrument was operated in the resolution mode. Source parameter settings used for negative ion acquisition were a capillary voltage of 2.0 kV and a sampling cone voltage of 25.0 V. The other optimized negative ion electrospray conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas flow rate, 500 L/h; and the cone gas flow was off. Ion-mobility separation settings were ion-mobility gas flow rate of 110 mL/min, wave velocity ramping from 500 to 570 m/s, and wave height of 40.0 V. Data acquisitions were carried out using Waters MassLynx (v4. 1) and ion-mobility spectra were processed using DriftScope software (v2.1, Waters).

The calibration of the T-wave cell was achieved by infusing a solution of polyalanine (Poly-Ala, 0.1 mg/mL, 1:1 acetonitrile:water) and a solution of six oligoglycines (Poly-Gly, 0.1 mg/mL in 1:1 acetonitrile:water). The flow rate was 5 µL/min. The electrospray source was operated in the positive mode and the settings were a capillary voltage of 2.25 V and a sampling cone voltage of 25 V. The ion-mobility separation conditions used for the calibration of the T-wave device were identical to those used for the analytes.

2.6 Conclusion and Perspectives

In summary, we discussed several applications of TWIMS–MS for the analysis of plant phenolics and demonstrated that combining ion-mobility separation and gas-phase fragmentation adds an extra level of selectivity and specificity for the structural characterization of plant metabolites.

We explored the use of ESI TWIMS–MS for the examination of highly complex biopolymers, namely grape seed proanthocyanidins. The use of ion-mobility separation prior to high-resolution accurate MS resulted in reduced spectral complexity compared to ESI–MS acquisitions alone. In particular, the presentation of TWIMS–MS data in a 2-dimensional contour plot, drift time vs. m/z, allows the deconvolution of many spectral features associated with the inherent heterogeneity of proanthocyanidin mixtures. We were able to extract proanthocyanidin oligomers that differ in their compositions. The capability of TWIMS–MS to separate proanthocyanidin ions with different charge groups enabled the analysis of proanthocyanidins with higher degree of polymerization compared to ESI–MS alone. There is an increasing interest in proanthocyanidins as dietary supplements, but the methods for characterizing proanthocyanidin preparations are sparse and mainly limited to gel permeation chromatography and acid hydrolysis in combination with liquid chromatography [30, 41]. Traditionally, MALDI MS has been the method of choice for the characterization of proanthocyanidin oligomers and higher-molecular-weight tannins [28, 30]. ESI–MS, in particular in combination with liquid chromatography, shows promise for the characterization of proanthocyanidins. Its broad use has been hampered by the limitation that the extraction of oligomer distribution

information is complicated due to overlapping charge state distributions of the different oligomers [30, 33]. The possible inclusion of TWIMS may provide the means to advance the analysis of proanthocyanidins. It would be interesting to evaluate MALDI TWIMS−MS for the analysis of proanthocyanidins, as it is likely that this will further improve the analysis of this class of compounds. The additional insight into the structural complexity of proanthocyanidin preparations may ultimately lead to standardized proanthocyanidin preparations for research purposes and the dietary supplements market. Beyond the current application to proanthocyanidin oligomers, IMS–MS has been used for the characterization of synthetic polymers [11, 12]. We foresee that TWIMS–MS and alternative IMS–MS techniques will significantly impact the way we characterize other biopolymers and bio-inspired polymeric materials.

TWIMS–MS technology provides access to drift time information and experimentally estimated collision cross sections, analytical parameters that are not available on MS/MS-only instruments. The potential separation and assignment of positional isomers of natural products and metabolites is an emerging application of TWIMS–MS. The potential of TWIMS of distinguishing structural isomers that differ in the site of substitutions has been proven to be a particularly powerful technique for drug metabolism studies [13, 14]. The combination of molecular modeling studies for deriving theoretically derived cross sections with TWIMS measurements of cross sections provides an exciting new strategy for the assignment of metabolite isomers. Here, an ensemble of energy-minimized metabolite structures is generated “in silico.” For the ensemble of energy-minimized structures, the theoretical cross sections are calculated. The experimentally derived cross sections are then compared with the theoretical cross sections to support the assignment of discrete metabolite structures. For instance, this approach has been successfully demonstrated for the elucidation of hydroxylation sites of ondansetron metabolites [13]. In the ondansetron metabolite study, TWIMS–MS was capable of reproducibly measuring drift time distribution differences between metabolites of 20 μs corresponding to a difference of only 0.3 Å in experimentally derived cross sections. Similarly, the capability of measuring small differences in drift times (50 μs corresponding to 0.6 Å) with TWIMS–MS was also shown for organoruthenium anticancer complexes where the arene is ortho- or meta-terphenyl [35]. Due to the increasing interest in applying TWIMS–MS characterization to small molecule studies, a small database of TWIMS-derived and validated collision cross sections for pharmaceutically relevant compounds has become available recently, which will certainly further broaden the use of TWIMS–MS for small molecule analyses [42]. Since the advent of the first commercially available TWIMS–MS instrument in 2006, technology has advanced significantly resulting in increased resolution of the traveling-wave ion-mobility separator [25], new applications [19, 43–47], and much enthusiasm in the scientific community. We foresee that TWIMS–MS and alternative IMS–MS techniques will significantly impact and advance our measuring capabilities in the phytochemical and pharmaceutical sciences, and in the emerging field of metabolomics [17, 18, 48, 49].

Advancements in system biology research will depend on tools that are capable of deconvoluting highly complex systems. The analysis of complex systems requires the availability of a new generation of separation and measurement tools. Recent applications of IMS–MS to a broad range of biomolecule measurements have demonstrated that IMS–MS has emerged as a powerful analytical technique capable of providing the separation space necessary to analyze highly complex samples [17, 23]. The combination of devices that allow real-time monitoring of living systems using IMS–MS is another exciting avenue of facilitating system-biology experiments [50]. The future for IMS–MS is bright and full of opportunities.

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Chapter 3
Camptothecin Production and Biosynthesis in Plant Cell Cultures

Takashi Asano, Kazuki Saito and Mami Yamazaki

Abstract Camptothecin, a well-known monoterpenoid indole alkaloid originally identified in the extracts of the Chinese tree *Camptotheca acuminata* (Nyssaceae), exhibits antitumor activity due to its ability to kill cancer cells via topoisomerase I poisoning. Other plant species have since been shown to produce camptothecin and related compounds. In particular, *Ophiorrhiza* species (Rubiaceae) are important resources for the production of various alkaloids, including camptothecin. This chapter describes the production of camptothecin-related alkaloids and the elucidation of the mechanisms of camptothecin biosynthesis using plant cell and tissue cultures. In particular, aseptically grown plants, callus cultures, and hairy root cultures were established for several species, *O. liukiuensis, O. kuroiwai*, and *O. pumila*, which were then evaluated for production of camptothecin and related alkaloids. The metabolite profiles differed between the species, and between tissues of the same species; for example, profiles from hairy roots were not identical to those of aseptic plants. The complementary DNAs (cDNAs) for strictosidine synthase, tryptophan decarboxylase, and cytochrome P450 reductase were cloned from *O. pumila* and evaluated for involvement in production of camptothecin in this species. RNA interference (RNAi)-mediated knockdown of gene expression indicated that

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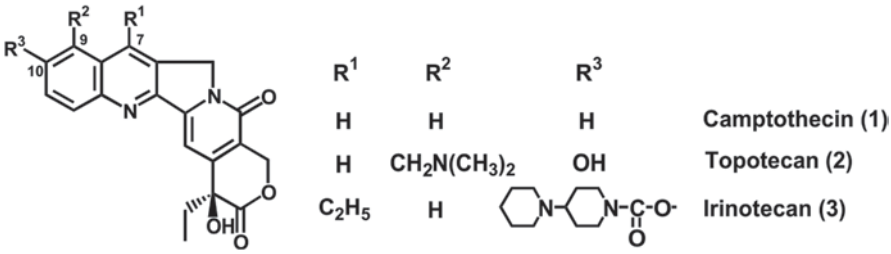


Fig. 3.1 Camptothecin (*1*) and its clinically used derivatives, topotecan (*2*) and irinotecan (*3*). (With permission from Ref. [38])

the production of camptothecin, strictosidine, and camptothecin-related alkaloids was suppressed in a *TDC* expression-dependent manner in RNAi hairy roots.

3.1 Introduction

Alkaloids are nitrogen-containing basic compounds known from about 20% of all plant species. Many alkaloids are pharmacologically active and have been used traditionally in the form of medicinal plant extracts as treatments for various diseases [1]. A few dozen pharmacologically active alkaloids, including camptothecin, are widely used in modern medicine, and worldwide sales of alkaloid-containing drugs were projected to exceed US$ 4 billion in 2002 [2].

Camptothecin (1) is a well-known monoterpenoid indole alkaloidand was originally identified in the extracts of the Chinese tree *Camptotheca acuminata* (Nyssaceae) [3]. Camptothecin exhibits antitumor activity, which is due to its ability to kill cancer cells via topoisomerase I poisoning [4]. At present, the semi-synthetic water-soluble camptothecin derivatives, topotecan (2) and irinotecan (3), are used worldwide as clinical antitumor agents against cancers of the lung, cervix, ovaries, colon [5], and other organs [6] (Fig. 3.1). In addition, a number of reports are available announcing the therapeutic values of camptothecin derivatives against acquired immunodeficiency syndrome (AIDS) [7] and falciparum malaria [8]. Consequently, the demand for camptothecin will continue to increase in the future.

Despite the rapid growth of the pharmaceutical market for this compound, camptothecin is still supplied exclusively from intact plants, mainly *C. acuminata* and *Nothapodytes foetida* [9]. However, the extraction of this compound from intact plants is problematic because of the shortage of natural resources and the resultant environmental concerns. Thus, the production of secondary metabolites by genetically engineered plant cell cultures, particularly for compounds such as camptothecin, has become a keen issue [10].

Camptothecin-related alkaloids have been reported to be produced in a relatively wide array of plant species, besides *C. acuminata* and *N. foetida* [11]. For instance,

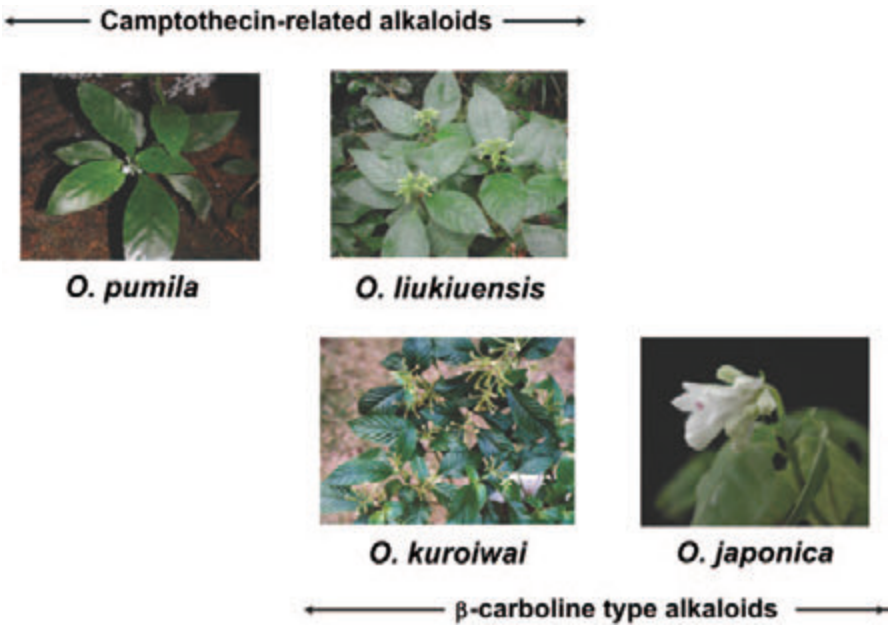


Fig. 3.2 The genus *Ophiorrhiza* species distributed in Japan

Merrilliodendron megacarpum [12], *Pyrenacantha klaineana* (Icacinaceae) [13], *Ervatamia heyneana* (Apocynaceae) [14], *Mostuea brunonis* (Loganiaceae) [15], *Ophiorrhiza mungos* [16], and *O. filistipula* (Rubiaceae) [17] have been reported to produce camptothecin-related compounds. Moreover, the results of phytochemical studies of the genus *Ophiorrhiza* have shown that camptothecin also accumulates in some *Ophiorrhiza* species (e.g., *O. pumila*) distributed in Japan [18, 19].

The genus *Ophiorrhiza* is widely distributed around tropical and subtropical Asia and comprises about 150 species [20]. Moreover, some of these species produce indole alkaloids [21]. With regard to the chemical constituents of *Ophiorrhiza* species distributed in Japan, *O. pumila* accumulated camptothecin and related alkaloids [18, 22] and *O. japonica* accumulated β-carboline-type alkaloids, such as lyalosidic acid and harman [23, 24]. Meanwhile, *O. liukiuensis* [25] and *O. kuroiwai* [26], which was shown to be an interspecies hybrid of *O. pumila* and *O. liukiuensis*, accumulated both camptothecin-related alkaloids and β-carboline-type alkaloids (Fig. 3.2). Therefore, these *Ophiorrhiza* species are important as resources for the production of various alkaloids, including camptothecin.

In this chapter, we describe the production of camptothecin-related alkaloids and the elucidation of the mechanisms of camptothecin biosynthesis by use of plant cell and tissue cultures.

3.2 In Vitro Cultures of Camptothecin-Producing Plants

3.2.1 Establishment of In Vitro Cultures

Cell and tissue cultures of several camptothecin-producing plants have been investigated as alternative sources for camptothecin production [27]. Sakato et al. [28] reported the first establishment of a rapidly growing cell suspension culture of *C. acuminata*, although the camptothecin productivity was insufficient (0.002 mg g^{-1} dry weight) for practical use. Callus cultures of *C. acuminata* established by Wiedenfeld et al. [29] produced comparatively adequate amounts of camptothecin (2 mg g^{-1} dry weight). These callus cultures were also reported to contain 10-hydroxycamptothecin, from trace amounts up to 0.08–0.1 mg g^{-1} dry weight [29]. Callus cultures of *N. foetida* were found to accumulate small amounts of camptothecin and 9-methoxycamptothecin [30–32], but the level of alkaloid production was 100- to 1000-fold lower than that from soil-grown plants. Callus cultures of *O. pumila* produced no camptothecin-related alkaloids but accumulated only anthraquinones [33, 34].

Since alkaloid biosynthesis and accumulation are under the strict control of cell developmental and environmental factors [35], establishing cultures of cell types suitable for the production of the camptothecin is important. Accordingly, aseptic plants and hairy roots of *Ophiorrhiza* species have been established as an effective means of producing camptothecin (Fig. 3.3) [36–38].

3.2.2 Camptothecin Production and Metabolite Profiles in Tissue Cultures of Ophiorrhiza Species

In shoots and roots of established aseptic plants of *Ophiorrhiza* species, camptothecin production per tissue weight was the highest in the roots of *O. pumila*. On the other hand, the production per tube was the highest in *O. kuroiwai* because it showed the higher growth rate of the two species. The concentration and total amount of camptothecin in *O. liukiuensis* were lower than those of *O. kuroiwai* and *O. pumila*.

Camptothecin accumulated to higher levels in hairy root lines of *O. pumila* than in those of *O. liukiuensis* and *O. kuroiwai* [38]. Camptothecin accumulation and increased growth rate of *O. pumila* hairy roots have the best results in the reports of camptothecin production by *in vitro* tissue cultures [37, 39].

The patterns of secondary metabolite production in the aseptic plants and hairy roots of *Ophiorrhiza* species were profiled by high-performance liquid chromatography–diode array detection–electrospray ion trap tandem mass spectrometry (Fig. 3.4 and Table 3.1) [38]. The metabolite profiles of *O. liukiuensis* and *O. kuroiwai* were highly similar in the shoot and root. 10-Methoxycamptothecin (5) and lyalosidic acid (6) were detected in the roots and shoots, respectively, of both *O. liukiuensis* and *O. kuroiwai* but not in those of *O. pumila*. Moreover, 3(*S*)- and

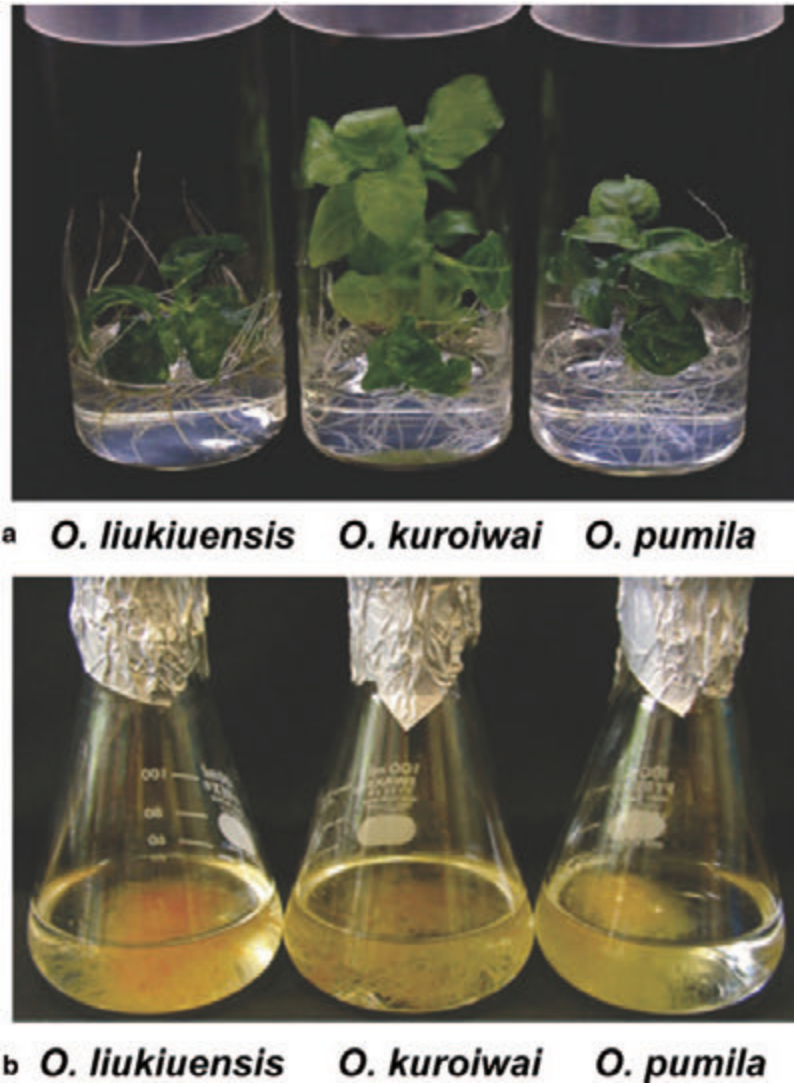


Fig. 3.3 Established tissue cultures of *Ophiorrhiza liukiuensis, O. kuroiwai*, and *O. pumila*. **a** Aseptic plants cultured for 5 weeks on 1/2 MS medium containing 1 % sucrose and 0.2 % gellan gum in test tubes. **b** Hairy roots cultured for 4 weeks in B5 liquid medium containing 2 % sucrose. (With permission from Ref. [38])

3(*R*)-deoxypumilosides (9, 10) were detected only in *O. pumila*. Camptothecin (1), 9-methoxycamptothecin (4), strictosamide (7), pumiloside (8), strictosidinic acid (11), and 3-*O*-caffeoylquinic acid (13) were detected in all three species. The metabolite profiles of the hairy roots were not identical to those of aseptic plants.

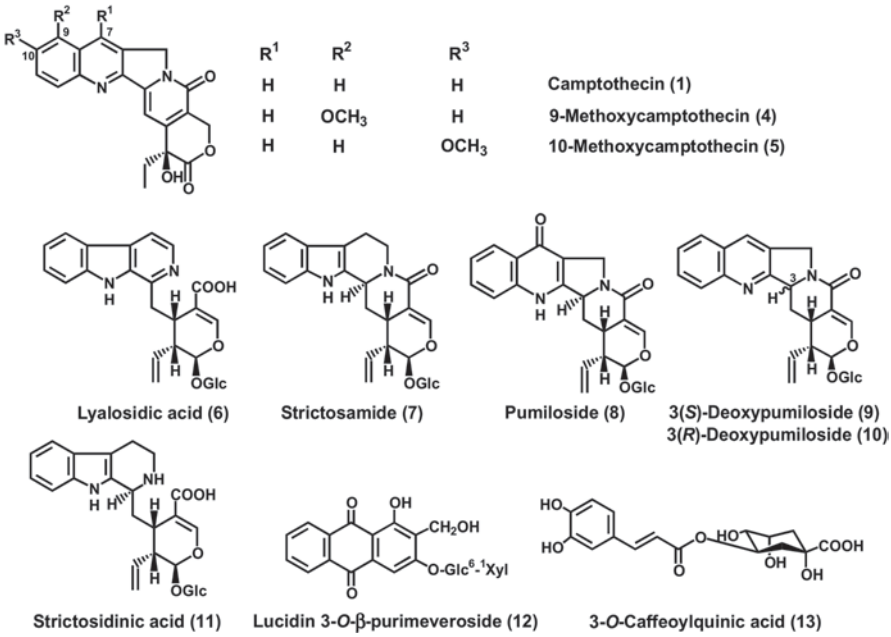


Fig. 3.4 Chemical structures of secondary metabolites detected in tissue cultures of *Ophiorrhiza* species. (With permission from Ref. [38])

Table 3.1 Alkaloids and anthraquinones detected in tissue cultures of *Ophiorrhiza* species

Compound	*O. liukiuensis*			*O. kuroiwai*			*O. pumila*		
	Shoot	Root	Hairy root	Shoot	Root	Hairy root	Shoot	Root	Hairy root
1 Camptothecin	+	+	+	+	+	+	+	+	+
4 9-Methoxycamptothecin	+	+		+	+		+	+	
5 10-Methoxycamptothecin		+			+				
6 Lyalosidic acid	+			+					
7 Strictosamide		+	+		+	+	+	+	
8 Pumiloside	+	+	+	+	+	+	+	+	+
9 3(S)-Deoxypumiloside[a]							+	+	
10 3(R)-Deoxypumiloside[a]									
11 Strictosidinic acid	+			+			+		
12 Lucidin 3-O-β-purimeveroside									+
13 3-O-Caffeoylquinic acid	+			+	+		+	+	+

[a] 3(*S*)-Deoxypumiloside (9) and 3(*R*)-deoxypumiloside (10) cannot be separated in this condition

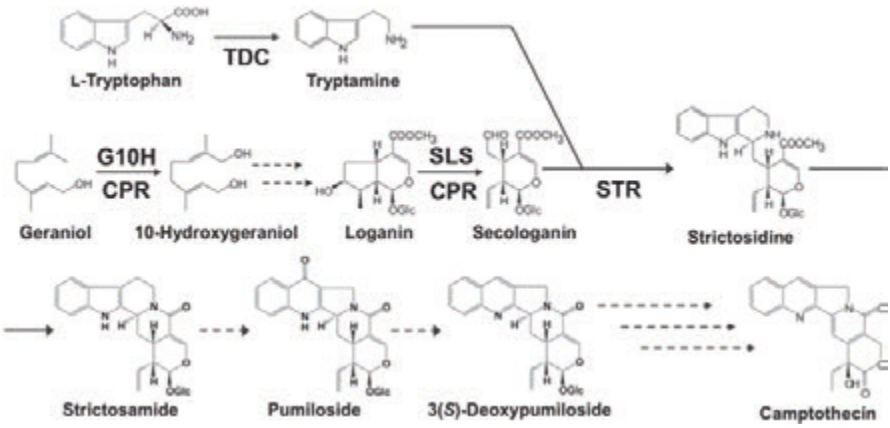


Fig. 3.5 Predicted camptothecin biosynthetic pathway in *O. pumila*. The enzymes are as follows: *TDC*, tryptophan decarboxylase; *G10H*, geraniol 10-hydroxylase; *CPR*, NADPH:cytochrome *P450* reductase; *SLS*, secologanin synthase; *STR*, strictosidine synthase. Plausible intermediates of camptothecin biosynthesis are provided in *parentheses*

3.3 Biosynthesis of Camptothecin

3.3.1 Camptothecin Biosynthetic Genes

Monoterpenoid indole alkaloids, including camptothecin, are derived from strictosidine, which is a common intermediate formed by condensation of the indole tryptamine with the iridoid glucoside secologanin by the enzyme strictosidine synthase (STR) [40–42] (Fig. 3.5). The intramolecular cyclization of strictosidine results in strictosamide, which is an intermediate peculiar to camptothecin biosynthesis, as proven by the incorporation of radiolabeled precursor [43]. The remaining details between strictosamide and camptothecin are not completely defined. However, camptothecin has been postulated to be formed potentially from strictosamide by three transformations: (1) oxidation–recyclization of the B- and C-rings, (2) oxidation of the D-ring and removal of the C-21 glucose moiety, and (3) oxidation of ring E [43]. Plausible camptothecin precursors, such as pumiloside and 3(*S*)-deoxypumiloside, were isolated from *Ophiorrhiza* species [18, 19]. Pumiloside has been found also in *C. acuminata* [44].

The cloning of complementary DNAs (cDNAs) from *O. pumila* hairy roots has been successfully performed to isolate the genes encoding the biosynthetic enzymes involved in the upper part of camptothecin biosynthesis, including STR, tryptophan decarboxylase (TDC) [45], and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH):cytochrome P450 reductase (CPR), in this species [46] (Fig. 3.5). The full-length STR cDNA sequence isolated from *O. pumila* (*OpSTR*) contained a 1,056-bp open reading frame (ORF) encoding a protein of 351 amino acids with a molecular mass of 38.9 kDa. The deduced amino acid sequence of

OpSTR exhibited 55% and 51%identities with STRs from *Rauwolfia serpentina* [41] and *Catharanthus roseus* [47], respectively. OpSTR most likely localizes to the vacuole, as predicted by the PSORT program. Southern blot analysis suggested that a single STR-encoding gene is present in the genome of *O. pumila*. The highest *OpSTR* expression occurred in hairy roots, followed by the root, and the stem, whereas *OpSTR* was apparently not expressed in leaves. STR enzymatic activity was detected in the protein extracts of stems, roots, and hairy roots; however, no activity was detected in leaf and callus extracts. The distribution of STR activity correlated with the messenger RNA (mRNA) accumulation pattern and the camptothecin concentrations in *O. pumila* tissues, with the exception of the young leaves, suggesting that roots and stems are the main tissues for camptothecin biosynthesis [34].

Tryptamine, a precursor of strictosidine, is formed by the decarboxylation of tryptophan by the enzyme TDC. The cDNA clone encoding TDC was first isolated from *C. roseus* [48]. The full-length TDC cDNA sequence isolated from *O. pumila* (*OpTDC*) contained a 1,521-bp ORF encoding a protein of 506 amino acids with a molecular mass of 56.6 kDa. The deduced amino acid sequence of OpTDC showed high identity to TDCs from *C. acuminata* [49] and *C. roseus* [48] (71 and 67%, respectively). Southern blot analysis suggested that at least TDC-encoding genes are present in the genome. The expression patterns of *OpSTR* and *OpTDC* were nearly the same.

The enzyme CPR is essential for the activity of cytochrome P450 monooxygenases, such as geraniol 10-hydroxylase (G10H) and secologanin synthase (SLS), which are involved in camptothecin biosynthesis [50] (Fig. 3.5). The full-length CPR cDNA sequence isolated from *O. pumila* (*OpCPR*) contained a 2,073-bp ORF encoding a protein of 690 amino acids with a molecular mass of 76.6 kDa. The deduced amino acid sequence of OpCPR showed high identity with *Arabidopsis thaliana, Petroselinum crispum*, *Pisum sativum*, and *Triticum aestivum* CPRs (72, 66, 65, and 67%, respectively). Southern blot analysis suggested that only a single CPR-encoding gene was present in the genome of *O. pumila*. Mirroring the general importance of the enzyme, *OpCPR* was expressed in all tissues.

Studies have been performed to investigate the effects of stress compounds, such as methyl jasmonate (MeJA), salicylic acid (SA), and yeast extract (YE), on the expression of *OpSTR*, *OpTDC*, and *OpCPR* in *O. pumila* hairy roots [46]. The changes in the expression patterns of *OpSTR* and *OpTDC* in response to these various compounds were highly similar. In particular, *OpSTR* and *OpTDC* expression was repressed by SA and YE treatments but unaffected by MeJA. Meanwhile, no treatment resulted in the induction or repression of *OpCPR* transcripts. In addition, no change in STR activity was observed after treatment with either stress compounds or phytohormones.

3.3.2 In Silico and In Vitro Tracer Studies with [1-13C] glucose

Both the mevalonate (MVA) pathway [51] and the 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway [52–54] have been recognized for their role in the formation

of isopentenyl diphosphate, the precursor of terpenoid biosynthesis. Yamazaki et al. [55] investigated the incorporation of [1-^{13}C]glucose into camptothecin in the hairy roots of *O. pumila* by *in silico* computation using the Atomic Reconstruction of Metabolism (ARM) [56] program and by *in vivo* tracer experiments. The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) analysis clearly showed that the secologanin moiety of camptothecin was synthesized via the MEP pathway. Furthermore, in *O. pumila* hairy root cultures, treatment with fosmidomycin, a specific inhibitor of the MEP pathway, resulted in a significant decrease in camptothecin production. These results support the conclusion that the secologanin moiety of camptothecin is derived from the MEP pathway.

3.4 Metabolic Modification in Hairy Roots of *O. pumila* by RNA Interference

A detailed understanding of camptothecin production, including the enzymatic pathway for its biosynthesis, will be essential to the ultimate goal of the metabolic engineering of this compound. In *Papaver somniferum* (opium poppy), genetic approaches using antisense RNA [57, 58] or RNA interference (RNAi)-mediated silencing [59] of biosynthetic enzymes have been performed, leading to rapid progress in the metabolic engineering of benzylisoquinoline alkaloids. Therefore, it is considered that RNAi technology is an effective strategy for investigating camptothecin biosynthesis. In our study, the production of camptothecin, strictosidine, and camptothecin-related alkaloids was suppressed in a *TDC* expression-dependent manner in RNAi hairy roots. Among the hairy root-specific peaks correlated with *TDC* expression in the liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS) analysis, two unknown peaks with a positive correlation were annotated as alkaloids and six unknown peaks with a negative correlation, as flavonoids. The exact mass of several non-annotated peaks was similar to those of predicted intermediates in camptothecin biosynthesis, suggesting that most peaks that positively correlated with *TDC* expression could be intermediates in camptothecin biosynthesis [60].

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Chapter 4
Plant Defense Activators: Application and Prospects in Cereal Crops

Mitchell L. Wise

Abstract Cereal grains are one of the primary sources of food products in the world. Increased productivity in crop yield, particularly for cereal crops, is absolutely essential for future food security, but is impeded by disease, with annual estimates ranging from 10 to 30 % crop loss due to disease alone. There have been remarkable advances in understanding pest and disease resistance in plants in the past three decades, with the application of chemical plant defense activators (PDAs) being of particular interest. The advances in recent years in understanding the molecular basis for systemic acquired resistance (SAR), induced systemic resistance (ISR), priming, and next-generation immunity portend a wider role for PDAs. These agrochemicals are gaining some acceptance in Europe where there is a strong interest in curtailing the use of more traditional fungicides and pesticides. Much work, however, is needed to understand the effects of nutrition, dose rates, timing of application, and genotypic effects in the application of PDAs. This review addresses the current understanding of plant immunity, particularly with respect to cereal crops and the potential for PDAs to enhance the potential yield and nutritional quality of cereal crops.

4.1 Introduction

As the world population increases from the current estimate of 7 billion to a projected 9 billion by 2050 [1] and as greater demand on land usage for activities other than agriculture increases, food supply will become an issue of even greater importance. Cereal grains are one of the primary sources of food products in the world today [2]; this is unlikely to change in the foreseeable future. Thus, increased productivity in crop yield, particularly for cereal crops, is absolutely essential for future

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food security. One of the major impediments to crop yield is disease, with annual estimates ranging from 10 to 30% crop loss due to disease alone [1]. In addition to yield loss, pathogens can also contaminate food crops with toxins, rendering them useless, when detected, and dangerous for human consumption when left undetected. Disease loss, however, can be mitigated by informed agricultural practices.

There have been remarkable advances in understanding pest and disease resistance in plants in the past three decades. Much of this research has been conducted on dicots, *Arabidopsis* and tobacco being the two principal model organisms. After the physical barrier of the leaf cuticle or outer cell wall of other tissues, the fundamental disease resistance mechanism in plants is its basal resistance. Our understanding of this phenomenon has undergone dramatic changes in the last two decades, leading to a much keener understanding of the molecular events and signaling mechanisms involved in plant "immunity." As a result, improved methods to elicit this response have come to fruition. One of these methods is the application of chemical plant defense activators (PDAs). This review addresses the current understanding of plant immunity, particularly with respect to cereal crops and the potential for PDAs to enhance the potential yield and nutritional quality of cereal crops.

4.2 Pathogen Recognition

The prevailing model in plant disease resistance for most of the past century has been the gene-for-gene theory, based on the pioneering genetic studies of Harold H. Flor [3]. This theory posits that plants recognize microbial pathogens by their avirulence factors and combat them through expression of resistance genes, termed "R" genes. More recently, a "zigzag" model (Fig. 4.1) of pathogen resistance has emerged [4]. This paradigm rationalizes that plants recognize a pathogen invader first by interaction of pathogen-associated molecular patterns (PAMPs) (sometimes called microbe-associated molecular patterns (MAMPs)). The presence of these molecules at the plant cell membrane suggests attack by a potential pathogen. Bacterial flagellin, certain lipopolysaccharides, and chitin (polymeric *N*-acetyl glucosamine, a component of many fungal species cell walls and, coincidentally, crustacean shells) are well-documented examples of MAMPs. Plant cell surface receptors called pattern recognition receptors (PRR) interact with MAMPs to trigger the initial stage of plant defense, termed PAMP-triggered immunity (PTI). PRRs are transmembrane proteins consisting of an extracellular leucine-rich repeat motif and, typically, an intracellular protein kinase. Only a few PRRs have been characterized for their specific binding mechanisms. Perhaps the most thoroughly studied examples are the *Arabidopsis* receptor for bacterial flagellin peptide [5] and the receptor for bacterial elongation factor-Tu [6, 7]. For the most part, these PRRs recognize highly conserved pathogen-derived molecules. MAMPs appear to be essential to the survivability and pathogenicity of the offending organism, thus not being readily adaptable to mutation. Equally important is the ability of the plant to discern these MAMPs from beneficial microbes and even its own molecular patterns, thus

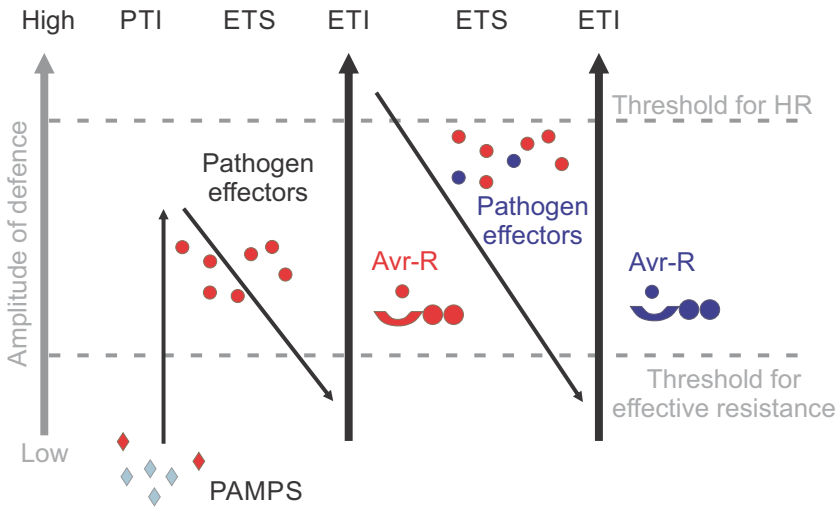


Fig. 4.1 The "zigzag" model of pathogen resistance

avoiding complications arising from autoimmunity [8]. Plants do, however, have the ability to detect self-antigens in the form of damage-associated molecular patterns (DAMPs) typically resulting from herbivore damage or microbial pathogen-mediated lytic enzymes (see [9] for an excellent review of the biochemistry of plant PRRs).

Upon activation, the PRRs initiate a plethora of defense mechanisms, including callose deposition in the cell wall, increased ion flux, particularly the influx of H^+ and Ca^{++} and efflux of K^+ ions, activation of plant mitogen-activated protein kinases (MAPKs) with concurrent phosphorylation of numerous signaling proteins, the generation of reactive oxygen species, biosynthesis of pathogenesis-related (PR) proteins,and the production of phytoalexins.

To overcome the basal immunity elicited through PTI, pathogenic microbes have evolved another strategy to circumvent the signaling mechanisms triggered by their MAMPs. This second phase of the zigzag model involves the delivery of effectors into the plant cell. Effectors are, to a large extent, what was previously termed virulence (or avirulence) factors in Flor's gene-for-gene theory of disease resistance. In essence, effectors are an array of pathogen-derived metabolites and proteins that interfere with host defense mechanisms. Pathogenic bacteria typically inject their effectors into the host cytoplasm through a type-three secretory system [10]. Introduction of effectors by other eukaryote pathogens is not as well understood; however, evidence points to RxLR-EER protein motifs, similar to those employed by *Plasmodium* (malarial) parasites in mammals [11]. This motif binds to phosphatidylinositol-3-phosphate moieties in the cell membrane [12], whereupon they are translocated into the cytoplasm. This likely represents a mechanism for both fungal and oomycete effectors [12, 13]. Effectors work through a wide range

of mechanisms. Small molecule effectors like coronatine, a non-host-specific phytotoxin produced by pathovars of the bacterial pathogen *Pseudomonas syringae*, appear to mimic the action of jasmonic acid (JA) [4, 14, 15], thus suppressing the effect of salicylic acid (SA) [16, 17]. Effectors also abrogate the defense response through other, largely unknown, mechanisms. Many clearly inhibit the host defense signaling pathways [18, 19].

4.3 Salicylic Acid Signaling

Recognition of pathogen or herbivore invasion results in what is now considered two separate signaling pathways that elicit an enhanced resistance response at locations distal to the site of infection. The first of these pathways was described by Ross in 1961 in which he demonstrated that tobacco leaves inoculated with tobacco mosaic virus (TMV) produced a lasting immunity in other portions of the plant against this virus as well as other viral pathogens. He termed this response systemic acquired resistance (SAR) [20]. This phenomenon has subsequently been demonstrated in numerous host–pathogen relationships and appears to be a characteristic of most, if not all, terrestrial plants [21]. The nature of the mobile signaling molecule(s) has been a subject of intense research and not inconsiderable controversy since SAR was initially proposed. Acetylsalicylic acid along with salicylic and benzoic acid were demonstrated to induce resistance against TMV in tobacco plants in 1979 [22]. Subsequently, SA and its methyl ester have been presented as the likely candidate as the mobile messenger in SAR [23, 24] and, in fact, a chemical analog for SA, 2,6-dichloroisonicotinic acid (INA), can replace SA in eliciting SAR in *Arabidopsis* and tobacco plants deficient in SA biosynthesis [25]. At present, SA is generally accepted as a key molecular component in SAR signaling [26]. Indeed, a chemical rationale for SA activation of defense responses has been demonstrated. Xinnian Dong and her colleagues' pioneering work has revealed important relationships between SA accumulation, PR protein expression, and activation of the nonexpressor of the PR (NPR1) protein [27]. NPR1 is so named because *Arabidopsis* mutants deficient in this protein do not respond to the normal signaling mechanisms for PR gene expression as well as a number of other pathogen defense-related genes [28]. This phenotype has also been called NIM1 and SAI1 [29]. NPR1 is now recognized as an essential regulator of plant defense mechanisms that normally resides in the cytosol of plant cells as a multimeric complex. This complex is maintained through redox-sensitive disulfide bonds [30, 31] that, under reducing conditions resulting from, for example, high concentrations of SA, partially disassociate into monomers [32]. The monomeric form of NPR1 is subsequently transported to the cell nucleus where it serves as a gene transcription coactivator [31]. Interestingly (and almost paradoxically), Spoel et al. [31] have recently shown that the full expression of SAR requires that NPR1 be imported into the nucleus and then be ubiquitinylated and degraded by nuclear proteasomes. This process is hypothesized to facilitate clearance of the NPR1-transcription factor (TF)/polymerase complex to allow fresh NPR1-TF

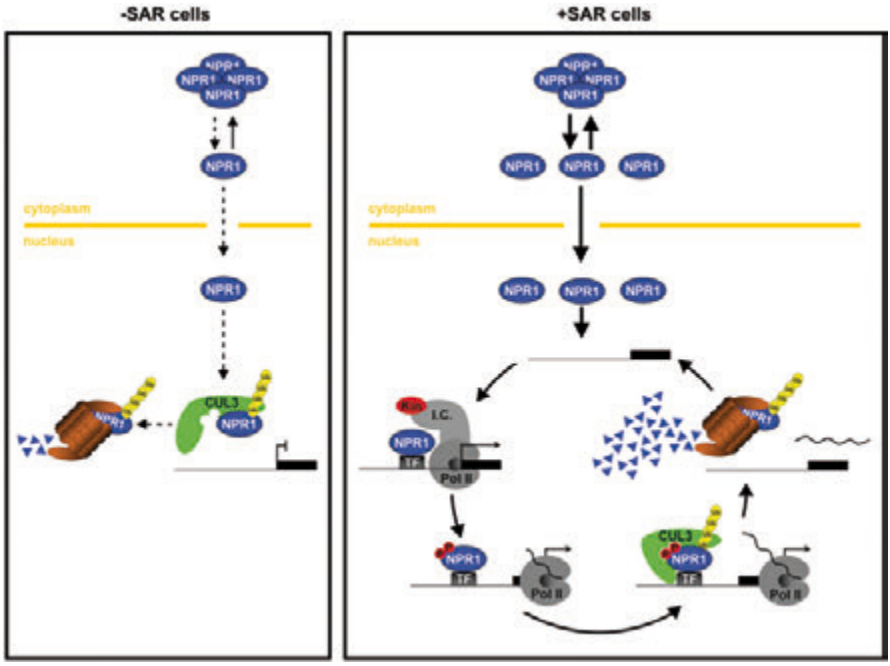


Fig. 4.2 The role and activation of NPR1 in SAR + and SAR − cells

access to the promoter region of affected genes in order to maintain transcription activity. Ubiquitinylation appears to rely on phosphorylation of specific serine residues in the NPR1 protein (Fig. 4.2).

Additional mobile signals are being recognized as mediators of SAR. Azelaic acid [33] and glycerol-3-phosphate [34] are two small molecules recently associated with SAR. Both of these also require expression of DIR1 (defective in induced resistance) protein [35]. There appears to be additional, as yet undetermined, mobile signals in the phloem exudates from *Arabidopsis* associated with azelaic acid signaling [34].

Airborne signals can also contribute to plant defense against pests and pathogens [36]. Notably, JA and related jasmonates are known for their role in systemic responses to wounding by insect herbivores [37]. The methyl ester of JA and certain other jasmonates are also quite volatile and have been implicated in airborne signaling to nearby plants [36]. Other volatile metabolites, such as short-chain oxylipins and terpenoids, also function in plant-to-plant defense signaling as well as complex tritrophic interactions involving plant pests' predators [38].

The second of the two major defense pathways is termed induced systemic resistance (ISR)and is a systemic immune response elicited primarily by plant growth-stimulating rhizobacteria [39] and certain rhizosphere-associated fungi [40]. Similar to SAR in many respects, ISR, however, does not require SA; it is

more dependent on JA and ethylene signaling. ISR does not typically result in PR accumulation, although both pathways depend on NPR1 activation [41, 42].

Thus, plants have evolved intricate systems to defend themselves against herbivorous pests and microbial pathogens. These defenses involve signaling mechanisms to alert distal parts of the plant, or even other plants, of impending attack. Because plants must not only respond to invading pest and pathogens but also avoid autoimmune responses, or unnecessarily responding to a plethora of non-harmful (or even beneficial) microorganisms, multiple and complex signaling mechanisms should be expected.

4.4 Priming in Plant Defense

Another defense mechanism related to induced resistance is "priming." This is defined as a condition in which plants respond faster or more strongly than unprimed plants in the activation of defense responses when subsequently challenged by microbial pathogens, herbivorous insects, or abiotic stresses [43]. This phenomenon occurs after an initial encounter with a pathogen or chemical elicitor but without a display of the typical phenotypes of induced resistance such as upregulation of PR proteins or phytoalexins. Because the molecular mechanisms of priming are just now being determined (phenotypic analysis of priming has relied on tedious post-challenge defense responses [44]), this phenomenon has likely been overlooked in many SAR studies. Uwe Conrath and coworkers demonstrated that cultured parsley cells, pretreated with low concentrations of INA, responded to subsequent elicitation with a known fungal MAMP with dramatically higher levels of coumarins and phenylalanine ammonia lyase (PAL) activity, SAR biomarkers [45]. Similar results were observed using benzo (1,2,3) thiadiazole (BTH), another synthetic SA analog [46]. It is important to note that this "priming" was dependent on the dose rate of INA or BTH, with relatively low doses resulting in priming, and higher doses resulting in elicitation of SAR [43]. Thus, priming appears to potentiate the plant for subsequent pathogen or pest challenge. This phenotypic difference is important because direct activation of plant defense mechanisms appears to extract a fitness cost that may, for example, reduce seed set [47] (discussed later).

Over the past 3–5 years, inroads have been achieved in determining the molecular mechanisms responsible for priming. *Arabidopsis* plants treated with BTH under priming conditions were shown to upregulate the levels of two mitogen-activated protein kinases, MPK3 and MPK6. These are cytosolic elements that transmit and amplify extracellular stimuli from external receptors into intracellular responses through a series of protein phosphorylation reactions. This study convincingly demonstrated that *Arabidopsis* plants primed with BTH responded far more strongly to biotic and abiotic stress in producing SAR biomarkers such as PR proteins and PAL. The primed plants also proved significantly more refractive to bacterial infection. Use of mpk3- and mpk6-deficient mutants conclusively demonstrated the involvement of these two enzymes in priming [48, 49].

Priming was also shown to modify chromatin associated with the promoter regions of certain WRKY genes in *Arabidopsis*. Methylation and acetylation of histones are instrumental in gene regulation [50, 51] and can result in long-term activation (or suppression) of the associated gene [52, 53]. WRKYs are transcription factors closely allied with many defense-related genes in plants [54–56]. Using chromatin immunoprecipitation, Jaskiewicz et al. [57] recently demonstrated that histones bound to certain WRKY promoter regions are methylated and or acetylated in response to priming by either BTH treatment or exposure to a pathogen. Thus, chromatin modification appears to be involved in priming.

Epigenetic control of plant immunity can also be manifested through DNA methylation, which, in turn, can even result in enhanced pathogen resistance in progeny plants. This was recently demonstrated in *Arabidopsis* which the investigators termed "next-generation SAR" [58]. These investigators found that when *Arabidopsis* plants were repeatedly inoculated with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) their first-and even second-generation progeny were more resistant to infection with the oomycete pathogen *Hyaloperonospora arabidopsidis*. Use of *Arabidopsis* mutant lines further demonstrated that this transgenerational resistance was dependent on functional NPR1 activation. Additionally, triple mutants deficient in DNA methylation were constitutively more resistant to *Pst*DC3000 infection, leading these investigators to suggest that hypomethylation plays a role in next-generation resistance [58]. Hypomethylation has been observed previously in *Arabidopsis* in response to *P. syringae* attack [59] and was specifically associated with defense-related gene expression in tobacco infected with TMV [60]. Similar transgenerational enhancement of SAR response, as determined by PR-1 biosynthesis and resistance to bacterial and oomycete challenge, was observed in *Arabidopsis* treated with β-aminobutyric acid (BABA), another of the chemical SA mimics [61]. Although in this case the next-generation priming only appeared to last through one generation with respect to PR-1 expression, second-generation BABA-treated plants did retain some resistance to pathogen challenge.

4.5 Commercial Plant Defense Activators

Advances in our understanding of the plant immune response have resulted in, and in some cases resulted from, the development of numerous synthetic compounds that appear to mimic the effect of SA in stimulating plant immunity. Some of these are currently marketed for commercial field application. Interestingly, the first of these commercial products was probenazole (3-allyoxy-1,2-benzisothiazole-1,1-dioxide) marketed under the trade name Oryzemate®, which proved effective in reducing rice blast infection. Developed in the mid-1970s, it was almost 20 years later that this compound was recognized as being effective against certain bacterial pathogens as well. Only due to advances in understanding SAR and ISR was the mechanism of resistance revealed [62, 63]. A systematic investigation of synthetic

chemical inducers of SAR by investigators at Ciba-Geigy in the early 1990s resulted in the discovery of INA and derivatives [64]. Although highly effective in some plants, INA was not well tolerated by others and was never developed commercially [65]. Subsequently, benzothiadiazole (BTH) derivatives were found to be just as effective in eliciting SAR but better tolerated in a broader range of crops. Formulations of these compounds have been marketed under the trade names BION® in Europe and Actigard® in the USA [62]. A more recent addition among the thiadiazole derivatives is 3′-chloro-4,4′-dimethyl-1,2,3-thiadiazole-5-carboxanilide, common name tiadinil, marketed by Nihon Nohyaku Co, Led with the trade name V-GET® for use against rice blast [66]. A bacterial pathogen effector protein, harpin, has been used to successfully combat blue mold in apples [67]. Fragments of the harpin protein also stimulated plant growth and disease resistance in rice in field trials [68]. A commercial formulation of harpin was originally marketed by Enden Bioscience as Messenger®; it has recently been replaced by Employ® from Plant Health Products. A de-acetylated form of chitin (chitosan) has been produced as a commercial product named Elexa® in a 4% chitosan formulation. Greenhouse as well as field trials of Elexa® on pearl millet (*Pennisetum glaucum*) administered either as a seed treatment or as a foliar spray or in combination showed it to be highly effective at inducing resistance to downy mildew disease caused by *Sclerospora graminicola* [69]. Although chitin and chitosan are well-known elicitors of SAR, chitosan also shows some fungicidal activity [70]. BABA is a nonprotein amino acid that also induces systemic resistance in plants. The amino acid has been used extensively in experimental systems to induce SAR as well as priming. Its commercial application has not been realized.

4.6 Induced Resistance in Cereal Crops

The bulk of the research on induced resistance has been performed on dicots, particularly the model plants *Arabidopsis* and tobacco. Nevertheless, monocots are capable of generating SAR and ISR and thus likely possess all the requisite signaling and defense activators found in dicots [71–74]. Indeed, BTH was originally developed to protect wheat from a variety of fungal pathogens. It proved effective in both growth chamber and field experiments [65]. These results were somewhat telling because although an important role of SA in dicot innate immunity is well established [26], an analogous role in monocots was not obvious at the time of these experiments. Rice (*Oryza sativa*) had been shown to constitutively produce dramatically higher levels of SA than healthy tobacco plants and these levels did not appear to be affected by infection with avirulent or virulent pathogens [75]. Thus, early investigations into the mechanism of SAR in cereals cast doubt on the role of SA. In addition, while INA and BTH proved effective in eliciting SAR-like responses in cereals [65, 76], the suite of defense-related gene expression appears to differ from dicots. Specifically, PR-1-related protein expression was not observed in wheat treated with INA or BTH [77]. Thus, some investigators did not consider PR gene expression a particularly reliable biomarker for SAR in monocots, although other

"chemically inducible" genes have been described in wheat, rice, and barley [78]. Nevertheless, as more research on defense mechanisms in monocots is published, the similarity to dicots becomes more apparent [72–74, 79]. Rice, for example, has now been shown to have an SA/NPR1-mediated defense network similar to *Arabidopsis* [80]. Thus, employment of PDAs on cereal crops appears to be perfectly feasible, including those that mimic SA, at least in terms of eliciting a defense response. The method of PDA application on cereals, however, can be a critical factor. Initial efforts to induce resistance to *Fusarium* head blight (FHB) in wheat, through the application of BTH as a foliar spray, proved ineffective [81]. More recently, both SA and BTH proved highly effective in protecting wheat against the same pathogen (*Fusarium graminearum*) in greenhouse trials when applied as a root soak [82].

Another aspect of PDA application that warrants consideration is their ability to elicit phytoalexin biosynthesis. Oat (*Avena sativa*) plants treated with SA or BTH were recently shown to dramatically increase their production of avenanthramides. Avenanthramides are phenolic alkaloid compounds produced, among food crops, exclusively by oats. They are known to function as phytoalexins in the vegetative tissue in response to crown rust (*Puccinia coronata*) infection [83, 84]. These metabolites are also potent antioxidants that, in laboratory trials, show potential as phytonutrients [85]. Unfortunately, the levels of avenanthramides found in the grain are highly variable and subject to strong environmental influence [86], and there appears to be an association between crown rust infection and avenanthramide content in the mature grain under field conditions [87]. The means of enhancing the levels of grain avenanthramides is of interest. BTH treatment in the form of Actigard® was recently shown to strongly induce avenanthramide biosynthesis in vegetative tissue of oat seedlings when administered as a root soak in greenhouse experiments [88]. RNA hybridization (Northern) analysis also showed elicitation of an RNA message hybridizing with a barley PR-1 probe, suggesting that the avenanthramide production was part of a SAR response. Moreover, when mature plants were treated with BTH just prior to heading, certain cultivars showed a statistically significant increase in avenanthramide content in the filling grain [89]. Indeed, all of the treated oat cultivars were higher in grain avenanthramide content than the untreated controls. However, since oat constitutively produces avenanthramides in their grain, and these levels show high variability, it can be difficult to establish a statistically significant difference. Nevertheless, these findings portend the utility of PDAs to upregulate the biosynthesis of avenanthramides in oat and, by extension, may be of use in other crops whose phytonutrient content is augmented by natural phytoalexins. Harpins, for example, were shown, in field trails, to increase yield and catechol levels in green tea [90].

4.7 Fitness Costs

The evolutionary rationale for induced resistance holds that plants cannot afford to biosynthesize the pest and pathogen defense metabolites on a constitutive basis because of either detrimental allocation of nutrient resources, production of autotoxic

metabolites, or negative effects on beneficial microorganisms [67, 91]. Thus, it is better to resort to these biosynthetic pathways only when they are in dire need. The application of PDAs circumvents this "just-in-time" approach evolved in plants. In most cases, field studies have focused on the efficacy of PDAs to reduce disease pressure. One study specifically aimed at determining the "fitness" cost of BTH application was conducted with spring wheat (*Triticum aestivum* cv 'Hanno') by treating them with BION® under a variety of treatment regimens and growing them either hydroponically with carefully controlled nutrient conditions or in a field environment with added fertilizer and fungicide treatment to ensure no disease pressure [47]. The investigators reported a statistically significant reduction in seed production and growth rates in the BTH-treated plants relative to uninduced control plants when nitrogen availability was limited. They suggested that this likely represented an allocation cost to chemically induced resistance in the absence of disease pressure. It should be noted, however, that in those plants treated late in their growth cycle or provided adequate nitrogen no significant differences in the measured parameters were observed between the BION®-treated and the untreated controls [47]. Several studies comparing the yield of various cereals treated with BTH versus standard pesticides provide little evidence for increased yield from BTH treatment (summarized in [42]). Treatment of plants with PDAs mimicking the SA elicitation pathway can also prove antagonistic to JA signaling. A study conducted with two barley (*Hordeum vulgare*) cultivars, 'Celler' and 'Optic', treated with a combination of BION®, BABA, and *cis*-jasmone under field conditions resulted in a marked decrease in infection levels by the biotrophic pathogen *Blumeria graminis* and the hemibiotroph *Rhynchosporium secalis*, etiological agents of powdery mildew and leaf scald, respectively. Infection by *Ramularia collo-cygni* (*Ramularia* leaf spot), another hemibiotroph, however, was significantly higher in the treated plants. Analysis of PR-1 and lipoxygenase (LOX2, an enzyme involved in JA biosynthesis) showed that elicitor treatment upregulated PR-1 expression, whereas LOX2 expression was downregulated. The combination of PDAs was used to specifically target *R. secalis*, making the interpretation of the results somewhat complicated. Note, also, that the grain yield from the elicitor-treated cultivars was slightly higher than the controls in both years of this study, although the authors speculated that the mixed result in protection might be due to suppression of the JA signaling, this pathway possibly being more important in defense against *R. collo-cygni* infection [92]. BTH-treated tomato plants have shown enhanced resistance to *Pseudomonas syringae* pv. *tomato* but compromised resistance to herbivore attack by *Spodoptera exigua* and, conversely, treatment with JA enhanced herbivore resistance at the expense of bacterial infection [93]. Thus, antagonism between SA and JA signaling may result in tradeoffs in the protective effects of PDAs in some situations [67].

In contrast to PDA-induced resistance, priming seems to have minimal allocation costs. Laboratory trials using *Arabidopsis* treatment with BABA in a range of concentrations resulted in induced direct defense response (as determined by PR-1 biosynthesis) at higher concentrations and priming of the plants at lower treatment levels. The primed plants were only slightly less resistant to subsequent challenge with

either the bacterial pathogen *P. syringae* or the fungal pathogen *Hyaloperonospora parasitica* but did not demonstrably increase PR-1 levels prior to the challenge [94]. All the PDA-treated plants, including control plants fully induced with BTH, were significantly more resistant to infection than the mock-induced controls. Moreover, fitness costs, evaluated in terms of seed set and relative growth rates, were only marginally affected in the BABA-primed plants; plants induced to direct defense levels demonstrated significantly lower fitness levels.

Saccharin is a metabolic by-product of probenazole (Oryzamate®) that can induce priming in barley [95]. In a study on fitness costs of saccharin-induced priming in barley (*H. vulgare* cv 'Celler'), Walters et al. found that, in greenhouse experiments, priming did not incur significant costs under low disease pressure by the hemibiotrophic fungal pathogen *R. secalis* and that it provided significant benefits under high disease pressure [96]. Use of saccharin-primed barley, in a field environment subject to low disease pressure from three fungal pathogens, *R. secalis, B. graminis, and R collo-cygni*, similarly showed little or no fitness costs, although the application of commercial fungicide resulted in significantly higher grain yield in adjacent plots [96].

4.8 Plant Defense Activators, Prospects for Cereal Crops

The commercial application of PDAs in cereal crops has been met with limited enthusiasm [67, 97]. Probenazole, as Oryzemate®, has been used for over three decades and remains one of the major fungicides used for seedling box treatment of rice [98]. It is noteworthy that no resistance to this product has yet developed [62]. Other commercial formulations such as BION®/Actigard®, Messenger®, and Elexa® have found use primarily on vegetable crops [62]. Some of the major drawbacks to PDAs are their unreliability under field conditions [99]. Abiotic and biotic factors affecting induced resistance in commercial/field application are still poorly understood. Numerous parameters must be further investigated. For example, the timing of application may be critical [99, 100]. Recent evidence has even shown that plant immune responses, particularly those associated with SA, are sensitive to light intensity and circadian rhythms [101]. Genotypic effects have also received little attention. Oat cultivars, for example, show dramatically different responses in both the magnitude and kinetics of avenanthramide biosynthesis in response to BTH and INA treatment [89]. When seven cultivars of spring barley were assessed for the efficacy of induced resistance to *R. secalis* and powdery mildew (*B. graminis*) induced by treatment with a suite of PDAs (BION®, BABA and *cis*-jasmone), significant differences were observed between cultivars [102]. A few additional examples are outlined by Walters and Fountaine [67].

PDAs are not curative; they must be administered prior to pathogen or pest invasion; thus, any fitness costs incurred may, in fact, be as detrimental to crop yield as the pathogen itself. However, an integrated crop management approach where PDAs are used in concert with more traditional fungicides/pesticides or other

agents such as biocontrol or plant growth promoting rhizobacteria might have some merit, particularly in reducing fungicide treatment levels [103]. Certainly more research on fertilizer augmentation to ameliorate allocation costs is warranted. PDAs can elicit volatile signals yielding protective effects against bacterial infection on neighboring plants as was recently demonstrated in lima bean (*Phaseolus lunatus*) [104]. This suggests the possibility of treating border rows, for example, to enhance resistance in the larger field. Maize is well known to produce volatile organic compounds (VOCs) in response to herbivore attack [105, 106]. There is evidence that these airborne signals can prime neighboring plants to respond to subsequent herbivore attack [107, 108]. Cereal crops also release VOCs in response to fungal infection [109], although the chemical ecology of these emissions is poorly understood. Indeed, in spite of an extensive literature on the generation of VOCs in response to herbivore attack, where cereal crops such as corn and rice are well represented [110], there is little research on the chemical ecology of VOC emission resulting from pathogen infection, especially in cereal crops.

The advances in recent years in understanding the molecular basis for SAR, ISR, priming, and next-generation immunity portend a wider role for PDAs. These agrochemicals are gaining some acceptance in Europe where there is a strong interest in curtailing the use of more traditional fungicides and pesticides. Much work, however, is needed to understand the effects of nutrition, dose rates, timing of application, and genotypic effects in the application of PDAs. Exploitation of plant immunity can and should be a useful tool in our collective arsenal for combating plant disease.

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Chapter 5
Phytochemicals for Pest Management: Current Advances and Future Opportunities

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Abstract As with pharmaceuticals, a significant proportion of commercial pesticides are natural molecules or are derived from natural compounds. This review describes some of the past commercial successes of phytochemicals as pesticides by pesticide class as well as current work and future prospects for development of pesticides from plant-derived natural compounds. For example, two compounds isolated by assay-guided fractionation of the essential oil of American beautyberry (*Callicarpa americana* L.) (Verbenaceae), callicarpenal and intermediol, were found to have very potent insect repellent properties. An analysis of the number of new phytochemicals being discovered yearly and the relatively few bioassays for potential pesticidal activity that most of the known phytochemicals have been subjected to, indicates that this area still has a bright future. Furthermore, chemical modification of these compounds and their use to discover new modes of action greatly expand the scope for future work. In addition, the use of transgene technology holds great promise, not only to protect crops from pests, by imparting production or manipulation of production of pest management phytochemicals, but also for crop/weed allelopathy, as success in this effort would greatly decrease the most used form of synthetic pesticides, herbicides.

5.1 Introduction

A large fraction of phytochemical secondary compounds owe their existence to the coevolution of the producing plant with its biotic threats, such as herbivorous arthropods and mollusks, plant pathogens, and competing plant species. At least one of their functions in nature is to repel, inhibit, kill, or otherwise avoid damage from

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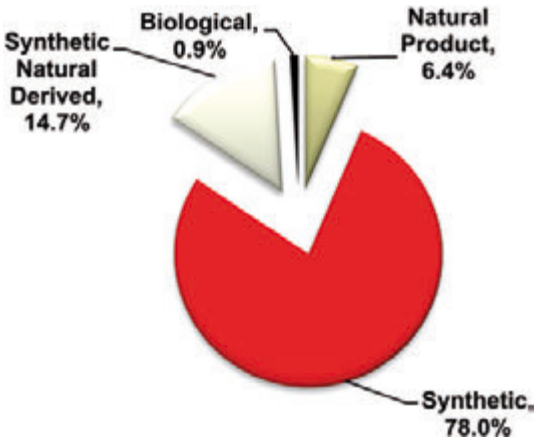


Fig. 5.1 Proportions of pesticides approved by the United States Environmental Protection Agency (USEPA) for use in the USA that are synthetic, natural compounds, derived from natural compounds, and biological agents [1]

these biotic hazards. Thus, these compounds are much more likely to have utility as a pesticide or as a molecular scaffold for pesticide design than compounds in synthetic libraries that have not been designed around compounds with known biological activity. Indeed, as with pharmaceuticals, a significant proportion of commercial pesticides are natural molecules or are derived from natural compounds [1]. From 1997–2010, about 20 % of the new pesticide active ingredients approved for use in the USA were natural products or natural product derivatives (Fig. 5.1). Some of the purely synthetic pesticides were discovered after the discovery of the molecular target site of natural inhibitors. These are not counted in the proportions in Fig. 5.1.

Historically, most of the natural products that have been useful as pesticides or as leads in pesticide discovery have come from plants. Yet, the pesticide industry seems to have focused recently on microbes as sources of leads. Our group spends most of its efforts on discovering potential pesticides from plants. We are also interested in the genetics and synthesis of these compounds as transgene technology allows us to impart production of these natural pesticides into crops.

Several aspects of natural products have reduced interest in them for pesticide discovery. The structural complexity of many natural products is too great for economically feasible production on a commercial scale. Much effort can be wasted in rediscovering known compounds [e.g., 2]. Obtaining enough of some phytochemicals for adequate evaluation can be time consuming and expensive. Sustainable harvest of botanical sources for a compound is often problematic. Natural does not equal nontoxic. We do not cover the mammalian toxicity of the compounds discussed in this short review. Except for materials used in traditional Chinese medicine (TCM), there is very little of this type of information available for the compounds that we mention. The half-lives of many natural compounds are often very short in the environment. This is an environmental advantage, but pesticides must persist sufficiently long to have their desired effects. Patenting can be more complex with natural products for several reasons. Legal complexities with countries or even populations of origin have grown, especially with plant species. This is one

reason that even the interest of pharmaceutical companies in phytochemical sources has waned [3]. Lastly, the physicochemical properties of natural compounds are often unsuitable for agricultural use.

Still, there are numerous advantages to phytochemicals in pesticide discovery. They are generally more environmentally benign. They are often sources of new molecular target sites, an aspect that is increasingly important as evolution of pesticide resistance to current modes of action increases [4]. They are often a source of novel chemical structures that differ from those more likely to be devised by traditional pesticide chemists. In some cases, pesticidal phytochemicals have evolved useful selectivity. In addition, modern technology has made discovery of these compounds and their biological activity simpler, faster, and less expensive than a few years ago. Finally, production of these compounds can be transferred from one plant species to another via transgene technology. Crops have sometimes been bred for phytochemical-based pest resistance in the past, but this process has been limited by the phytochemical makeup of related species with which the crop can interbreed.

In this chapter, we briefly describe some of the past commercial successes of phytochemicals as pesticides by pesticide class. Then, we discuss some of the promising work from our group within these pesticide classes.

5.2 Insecticides and Arthropod Repellents

Of all commercial pesticide classes, insecticides have the highest fraction of natural product or natural product-derived products [1]. Slightly more than 30 % of the conventional arthropod pesticides and repellent new active ingredients registered and approved for use by the United States Environmental Protection Agency (USEPA) from 1997 to 2010 were natural products or natural product-derived products [1]. The biggest classes of these compounds, the pyrethroids and the neonicotinoids, originated from phytochemicals. There are also quinoline- and pyrrole-derived commercial insecticides. A very important new class of insecticides are those that target the ryanodine target site in insects. Ryanodine is a compound from the plant *Ryania speciosa* that binds Ca channels of insect muscles [5, 6]. This phytochemical provided the clue for a much-needed new insecticide target site with which to fight evolution of insecticide resistance. Veratridine sulfate from the sabadilla lilly (*Schoenocaulon officinale*) is sold as an insecticide [7]. The scientific literature is full of reports of insecticidal phytochemicals that have not been widely or successfully commercialized.

Several formulations of plant extracts such as neem (*Azadirachta indica*) containing the insect-active compound azadirachtin are sold. There are numerous reviews of neem and azadirachtin as an insecticide [e.g., 8]. Many plant essential oils are available as bioinsecticides, a category of pesticides that does not require the stringent toxicological and environmental testing required of conventional pesticides [9].

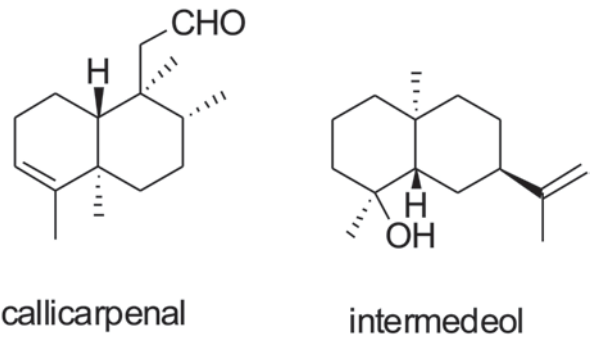


Fig. 5.2 Structures of two insect repellent compounds from *Callicarpa americana*

Our laboratory has focused on insect repellents. One of the more potent repellents is a constituent of the essential oil obtained from American beautyberry (*Callicarpa americana* L.) (Verbenaceae), a common shrub in the US southeast. In Mississippi, crushed leaves of *C. americana* were placed under the harnesses of draft animals as a traditional means to protect the animals from hematophagous insects [10, 11]. Specific identification of the compounds responsible for the mosquito (*Aedes aegypti*) biting deterrence in the leaves of this folk remedy was recently completed using a bioassay-directed fractionation approach. Ultimately, the study identified the compounds callicarpenal and intermediol as those responsible for the biting deterrence from the leaves and hence the folk remedy (Fig. 5.2). Both compounds were evaluated in laboratory bioassays for repellent activity against host-seeking nymphs of the blacklegged tick, *Ixodes scapularis*. Callicarpenal and intermediol, at 155 nmol/cm^2 of cloth, repelled 98 and 96% of *I. scapularis* nymphs, respectively. Dose–response tests with *I. scapularis* nymphs showed no difference in repellence among callicarpenal, intermediol, and *N, N*-diethyl-*m*-toluamide (DEET) [12]. Callicarpenal, at 155 nmol/cm^2 of cloth, repelled 100 and 53.3% of *I. scapularis* nymphs at 3 and 4 h, respectively. Both compounds also repel imported fire ants (*Solenopsis* spp.) [13].

More recently, two additional arthropod repellent folk remedies, breadfruit (*Artocarpus altilis*) and *Jatropha* sp., were investigated by Cantrell and colleagues [14, 15]. These two folk remedies are administered traditionally as spatial arthropod repellents by both burning seed-pressed oil in the case of *Jatropha* sp. and burning the dried male inflorescence of breadfruit.

A systematic bioassay-directed study of *Jatropha* sp. oil using adult *Aedes aegypti* females indicated that oleic, palmitic, linoleic, and stearic acids were all active at 25 nmol/cm^2 above a solvent control and were partially responsible for the activity of the oil itself. Evaluation of the triglycerides containing each of these fatty acids revealed that tripalmitin, tristearin, trilinolein, and triolein all demonstrated significant activity above a solvent control at 10 μg/cm^2, with tripalmitin the most active. This study was the first report on the insect repellent activity of triglycerides.

A similar approach to that used for *Jatropha* sp. identified capric, undecanoic, and lauric acids as primary deterrent constituents from the male inflorescence of

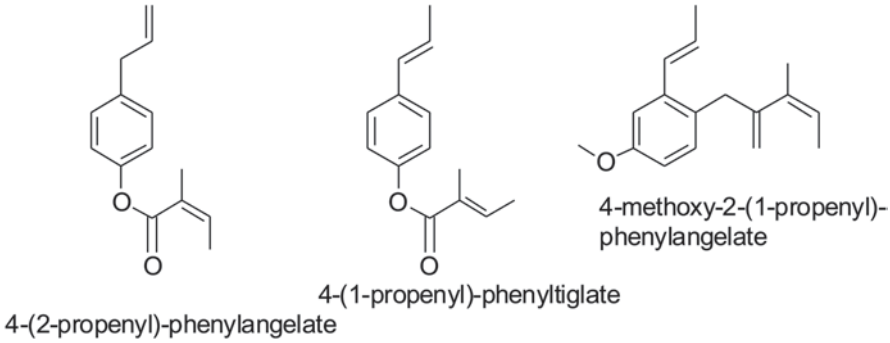


Fig. 5.3 Structures of three major phenylpropanoid constituents of *P. isaurica* oil that were bioassayed against *Liphaphis pseudobrassicae*

breadfruit. A synthetic mixture of fatty acids present in the most active fraction and individual fatty acids was significantly more active than DEET.

Essential oils and plant extracts from plants used in traditional medicines worldwide still continue to provide us with new and unique biological activity. Our group evaluated essential oils from 23 plant species comprising 14 genera and 4 plant families obtained from 26 locations in Turkey [16]. Essential oils obtained by Clevenger distillation were mixed with dimethyl sulfoxide and evaluated for insecticidal activity against adult turnip aphids (*Lipaphis pseudobrassicae* Davis). Aphids were quickly incapacitated by aliphatic aldehydes, phenols, and monocyclic terpenes contained in *Biflora* and *Satureja* species at concentrations as low as 0.3–1.0 mg/ml. *Pimpinella isaurica* essential oil and its three pure phenylpropanoids were tested at a single concentration of 10 mg/ml. Individually, the three major phenylpropanoids—4-(2-propenyl)-phenylangelate, 4-(1-propenyl)-phenyltiglate, and 4-methoxy-2-(1-propenyl)-phenylangelate (Fig. 5.3)—were not toxic to turnip aphids; however, when they were combined they killed aphids. The intact *P. isaurica* oil killed aphids faster than a mixture of the three phenylpropanoids.

We are studying TCM plants to find new agrochemicals with exceptionally low mammalian and environmental toxicity. *Angelica sinensis* (Apiaceae) is one such plant. Dong quai is the Chinese name for the roots of *A. sinensis*, which is a TCM treatment for gynecological disorders. Bioassay-guided fractionation of *A. sinensis* root extract led to the isolation of (*Z*)-ligustilide as an effective insect repellent [17]. This compound had previously been found as an insecticidal constituent of the essential oil of *Ligusticum mutellina* [18]. A mosquito biting deterrence assay showed that (*Z*)-ligustilide (Fig. 5.4) was more potent than the commercial standard DEET to *Ae. Aegypti* and *Anopheles stephensi.*

Essential oils of *Cupressus funebris*, *Juniperus communis*, and *J. chinensis* were evaluated for repellence against adult yellow fever mosquitoes, *Ae. Aegypti*; host-seeking lone star tick nymphs, *Amblyomma amerincanum*; the blacklegged tick, *I. scapularis*, and for toxicity against *Ae. aegypti* larvae and adults [19]. All oils were repellent to both species of ticks. The EC_{95} values of *C. funebris*, *J. communis,*

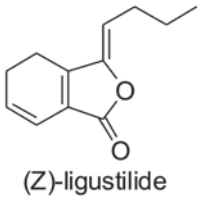


Fig. 5.4 Structure of a mosquito repellent compound from *Angelica sinensis*

and *J. chinensis* oils against *A. americanum* were 0.43, 0.51, and 0.92 mg oil/cm^2 filter paper, respectively, compared to 0.68 mg DEET/cm^2 filter paper. All *I. scapularis* nymphs were repelled by 0.10 mg oil/cm^2 filter paper of *C. funebris* oil. At 4 h after application, 0.83 mg oil/cm^2 filter paper, *C. funebris* and *J. chinensis* oils repelled ≥ 80 % of *A. americanum* nymphs. The oils of *C. funebris* and *J. chinensis* did not prevent female *Ae. aegypti* from biting at the highest dosage tested (1.50 mg/cm^2). However, the oil of *J. communis* had a minimum effective dosage (estimate of ED_{99}) for repellence of 0.029 ± 0.018 mg/cm^2; this oil was nearly as potent as DEET. The oil of *J. chinensis* showed a slight ability to kill *Ae. aegypti* larvae, at 80 and 100 % at 125 and 250 ppm, respectively.

5.3 Fungicides

Almost 30 % of the new fungicide active ingredient registrations in the USA from 1997 to 2010 were either natural products (11.4 %) or natural product-derived synthetic compounds (17.1 %) [1]. Several of the latter are derived from phytochemicals, such as benzothiazdiazole, acibenzolar-S-methyl, and the alkaloid sanguinarine (Fig. 5.5).

During the past 15 years, we have learned that biological activity of plant extracts against filamentous plant pathogenic fungi does not parallel activity against human pathogenic fungi. We have found that fungicidal chemistry from plants is more common in plants obtained from tropical, moist environments. Medicinal and aromatic plants used in traditional medicine often provide rich sources of novel activity against fungi. A discovery strategy based on this information has led to patenting of sampangine and novel cyclopentenedione compounds for the control of agriculturally important fungal plant pathogens [20, 21].

As part of our ongoing studies on the essential oils, we evaluated *Pimpinella* essential oils that are characterized by high concentrations of pseudoisoeugeneol-type phenylpropanoids. Trinorsesquiterpenes (geijerenes and azulenes) were also found to be characteristic constituents of *Pimpinella* oils [22]. Of the 22 isolated compounds during this investigation, two phenylpropanoids, 4-(3-methyloxiranyl) phenyl 2-methylbutyrate and epoxypseudoisoeugenyl 2-methylbutyrate, showed better antifungal activity than the trinorsesquiterpenes, 4-(6-methylbicyclo[4.1.0]

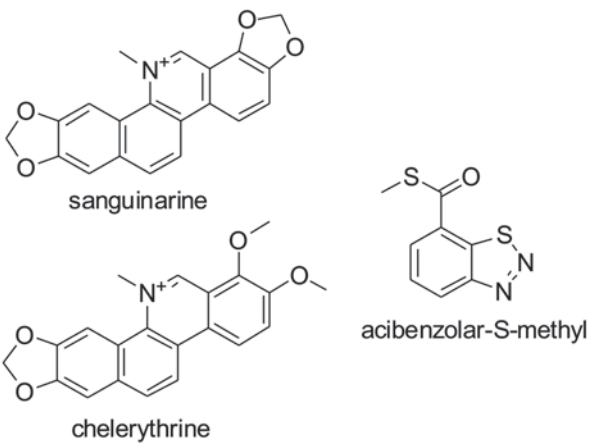


Fig. 5.5 Phytochemical-derived commercial fungicides

hept-2-en-7yl)butan-2-one (tragione) and dictamnol (Fig. 5.6), using direct bioautography against *Collectotrichum acutatum*, *C. fragariae*, and *C. gloesporioides*. The compounds were subsequently evaluated in a 96-well microtiter assay that showed that 4-(3-methyloxiranyl)phenyl 2-methylbutyrate and epoxypseudoisoeugenyl 2-methylbutyrate (Fig. 5.6) produced the most significant growth inhibition in *Phomopsis* spp., *Colletotrichum* spp., and *Botrytis cinerea* [23].

The peanut plant (*Arachis hypogaea* L.), when infected by a microbial pathogen, is capable of producing stilbene-derived compounds that are considered antifungal phytoalexins. In addition, health benefits of some stilbenes from peanuts, including resveratrol and pterostilbene, have been and are being established. Since peanut stilbenoids appear to play roles in plant defense mechanisms, they were evaluated for their effects on economically important plant pathogenic fungi of the genera *Colletotrichum*, *Botrytis*, *Fusarium*, and *Phomopsis*. The results of these studies reveal that peanut stilbenoids, as well as related natural and synthetic stilbene derivatives, display a diverse range of biological activities against fungal plant pathogens [24].

A preparative overpressure layer chromatography (OPLC) method was used for the separation of two new natural compounds, 4-hydroxy-5,6-dimethoxynaphthalene-2-carbaldehyde and 12,13-didehydro-20,29-dihydrobetulin, together with nine known compounds from the acetone extract of the roots of *Diospyros virginiana*. All isolated compounds were evaluated for their antifungal activities against *Colletotrichum fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phomopsis obscurans*, and *P. viticola* using an *in vitro* micro-dilution broth assay. The results indicated that the compounds methyl-juglone and isodiospyrin (Fig. 5.7) were highly active against *P. obscurans* at 30 μM with 97.0 and 81.4% growth inhibition, respectively, and moderate activity against *P. viticola* (54.3 and 36.6%, respectively). OPLC is a rapid and efficient method of exploiting bioactive natural products [25].

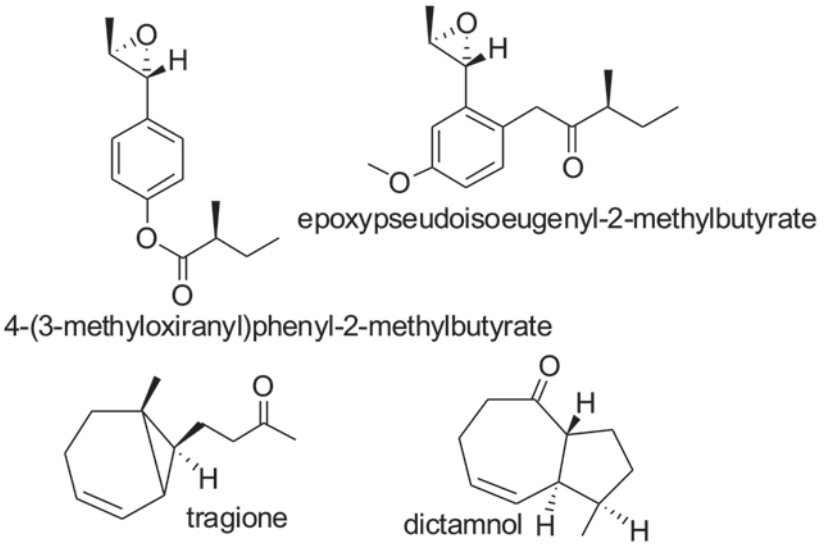


Fig. 5.6 Active antifungal compounds from the essential oil of *Pimpinella* species

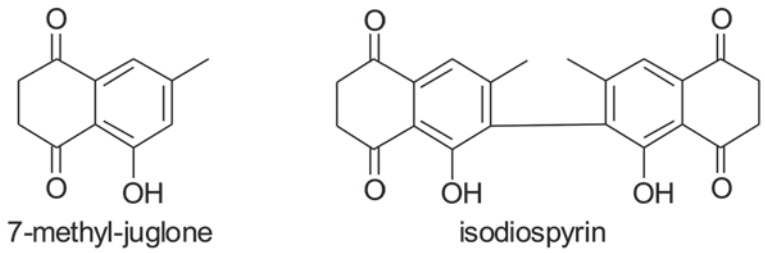


Fig. 5.7 Active antifungal compounds from the roots of *D. virginiana*

5.4 Molluscicides

There are relatively few effective, commercial molluscicides available. However, there are many reports of molluscicidal effects of crude extracts of plants and phytochemicals. For example, a crude butanol extract of *Phytolacca dodecandra* (endod) is effective against *Biomphalaria* snails [26]. Many of these are reviewed by Marston and Hostettmann [27].

Development of the berries of endod as a molluscicide to control schistosomiasis has been very successful in Ethiopia [28]. The active constituents have been isolated and identified as saponins [27]. *Lonicera nigra, Hedera helix, Cornus florida,* and *Asparagus curillus* are among some other plants that have been investigated

for molluscicidal saponins [27]. *H. helix* contains a hederagenine glycoside with an LC_{100} of 3 ppm against *B. glabrata* snails.

Our efforts are geared toward development of natural product-based molluscicides to control snails that are harmful for agricultural commodities such as channel catfish (*Ictalurus punctatus*), rice, taro, and orchids. Catfish is one of the main farm-raised fish in the USA. The ram's horn snail (*Planobdella trivolvis*) is an intermediate host for the trematode *Bolbophorus confusus* that was discovered to be a significant problem in 1999 in commercial channel catfish production ponds in the Mississippi Delta region [29, 30], and it has been reported from other states (Arkansas, Louisiana, Alabama, and California). These trematodes have a digenetic life cycle that involves two intermediate hosts, the snail and the catfish, and the American white pelican (*Pelecanus erythrorhynchos*). Catfish infested by the parasitic metacercariae develop cysts, have impaired growth, and are prone to other diseases that can weaken and kill the catfish. The annual economic loss to the catfish industry in the USA due to the trematode problem is estimated to be in millions of dollars. At present, there is no cure or treatment for infected fish. One practical approach to eradicate or control this problem is to interrupt the life cycle of the parasite by eliminating the snails, which are essential to the life cycle.

We have shown that vulgarone B (Fig. 5.8), isolated from the steam distillate of the aerial parts of the plant *Artemisia douglasiana* (Asteraceae), is active toward the snails with an LC_{50} of *ca* 24 μM [31]. The snails showed severe hemolysis associated with lethality when treated with vulgarone B. Channel catfish toxicity studies indicate an LC_{50} of *ca* 207 μM. Thus, vulgarone B may be an environmentally acceptable alternative for snail control in aquaculture when applied within the margin of safety [31]. 2*Z*,8*Z*-matricaria methyl ester (Fig. 5.8) isolated from *Erigeron speciosus* (Asteraceae) has also shown molluscicidal activity against *P. trivolvis* with a LC_{50} of 50 μM associated with marked hemolysis of the snail [32]. In laboratory experiments, Yucca extract at 10 ppm caused 100 % mortality of *P. trivolvis*, but ethanol extracts of *Phytolacca americana* (American poke weed) berries and *Lonicera nigra* (black-berried honeysuckle) were inactive (unpublished data). On the other hand, hederagenin 3-O-β-D-glucopyranoside (Fig. 5.8), isolated from English ivy (*H. helix*, an invasive plant in the Northwestern states, showed activity against *P. trivolvis* with an LC_{50} of 30 μM in laboratory studies (unpublished data). This compound has also shown activity against *B. glabrata* snails [27].

The golden apple snail (GAS), *Pomacea canaliculata* (Lamarck), is a major pest of rice in all rice-growing countries outside the USA, where it was either intentionally or accidentally introduced [33]. In the Philippines, the government promoted GAS production for human consumption [34, 35]. However, the demand dropped because GAS was found to transfer the rat lungworm parasite (*Angiostrongylus cantonensis*) to humans if undercooked GAS was consumed. Thus, snail farmers growing GAS abandoned their cultures, and the snails were disposed of without precautions. GAS soon invaded the rice fields, where it found an ideal habitat and abundant food supply. The economic losses were estimated to be up to $ 1.2 billion by 2003 [36]. GAS is also a problem in taro plantations in Hawaii, where $CuSO_4$ is currently used to control the snail population [37].

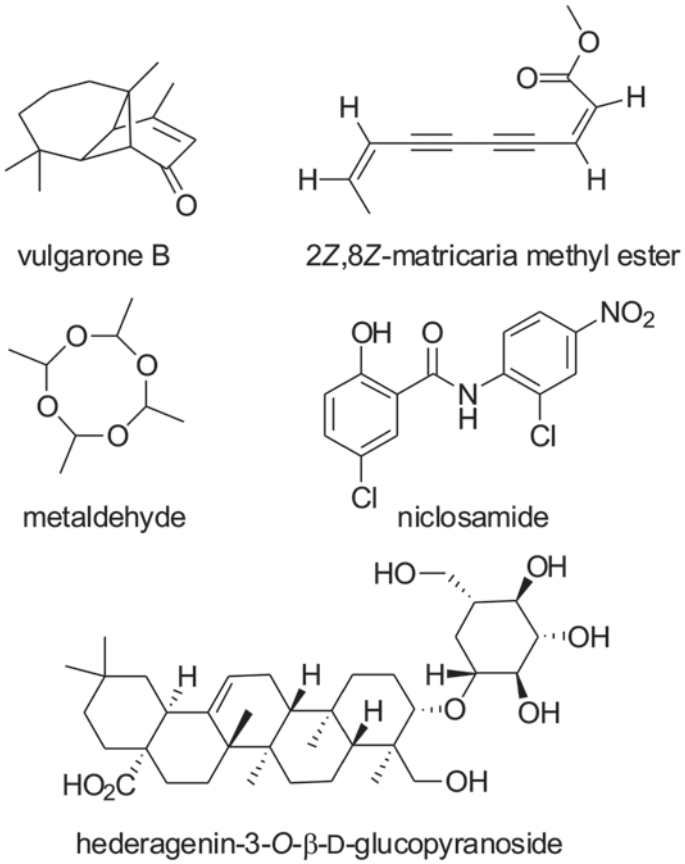


Fig. 5.8 Molluscicidal compounds mentioned in the text

Integrated management methods are recommended for GAS, but many farmers depend on commercially available synthetic molluscicides (niclosamide and metaldehyde—Fig. 5.8). There are numerous cases of poisoning caused by metaldehyde [38, 39]. Therefore, cost-effective, target-specific, and environmentally friendly molluscicides are needed, due to the economic burden and undesirable effects of currently available commercial molluscicides. Vulgarone B is a potential molluscicide with an LC_{50} value of about 30 μM at 24 h for GAS [40]. In the same bioassay, the standard commercial molluscicide, metaldehyde, also had an LC_{50} of about 30 μM. This corresponds to about 6.5 and 4.4 mg/L of the vulgarone B and metaldehyde, respectively. The concentrations needed for 100% mortality at 24 h were about 75 and 200 μM, respectively, for vulgarone B and metaldehyde. In practical terms, a rice farmer who uses about 250 liters of water for spraying one hectare will require 4.8 g of pure vulgarone B for GAS control [40]. Vulgarone B did not cause further mortality at 48–96 h after treatment, unlike the observed increased mortality

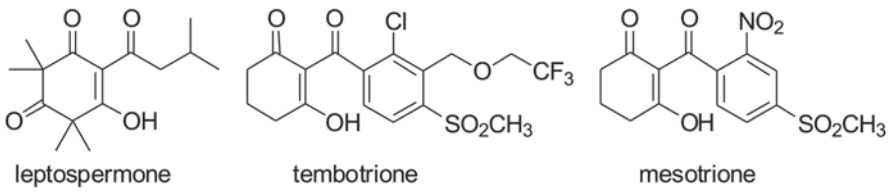


Fig. 5.9 Structures of the natural triketone leptospermone and two synthetic analog that are sold as commercial herbicides

with time with metaldehyde. This indicates that the vulgarone B is fast acting, unlike metaldehyde.

There was no phytotoxicity 10 days after treatment to 14-day-old rice plants at concentrations of vulgarone B that cause complete or nearly complete mortality of GAS. However, when incorporated into agar growth medium, pronounced chlorosis occurred after 14 days of growth. Therefore, vulgarone B should be used after the germination of rice seeds. Field and laboratory experiments have also shown the potential of vulgarone B as a molluscicide in taro paddies in Hawaii (unpublished data).

5.5 Herbicides and Algicides

5.5.1 Herbicides

Only about 8 % of the new active ingredient registrations for conventional herbicides from 1997 to 2010 in the USA have been synthetic, natural product-derived compounds [1]. These have all been triketones that were partially inspired by the plant allelochemical leptospermone (Fig. 5.9) [41]. The discovery and development of these herbicides resulted from a convergence between astute chemical ecology observations made by Reed Gray of the Western Research Center in California (Stauffer Chemical at the time) and independent chemical synthesis efforts in the same laboratory. In 1977, Gray observed that the bottlebrush plant (*Callistemon citrinus*) appeared to repress the growth of plants in its surroundings. Crude extracts from this plant caused the bleaching of grass weeds. He identified the active component as leptospermone, a natural triketone structure with no known biological activity, although it had been reported in a number of Australasian shrubs several years earlier [42]. Leptospermone was moderately active in greenhouse tests, controlling mostly small-seeded grass weeds. This natural product and a small number of synthetic structural analogs were patented as herbicides in 1980 [43]. A few years later, a separate group at the Western Research Center was generating analogs of the cyclohexanedione herbicide sethoxydim, an inhibitor or acetyl-coenzyme-A carboxylase. Some of the second-generation herbicidal derivatives with a dimedone backbone caused bleaching symptoms similar to that from leptospermone. Combination of the syncarpic

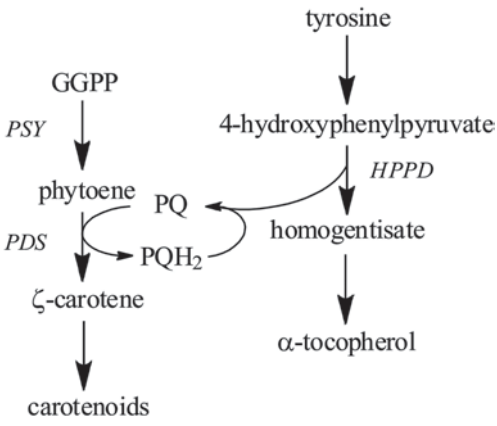


Fig. 5.10 Role of HPPD in carotenoid synthesis. *PSY* phytoene synthase, *PDS* phytoene desaturase *HPPD p*-hydroxyphenylpyruvate dioxygenase, *PQ* plastoquinone

acid of leptospermone to this chemistry ultimately served as the basis for the development of the triketone synthetic herbicides (Fig. 5.9) [44].

β-Triketones (e.g., leptospermone, flavesone, agglomerone, tasmanone, papuanone, and grandiflorone) are common in many Australasian woody plant genera (e.g., *Leptospermum*, *Eucalyptus*, *Callistemon*, *Xanthostemon*, *Backhousia*, *Calytrix*, *Baeckea*, *Melaceuca*, and *Corymbia*) [42, 45, 46]. On average, steam-distilled manuka oil accounts for 0.3 % of the dry weight of *L. scoparium* [47]. However, the amount of β-triketone present in these oils varies widely across New Zealand. Some chemotypes contain as little as 0.1 % triketone while others can accumulate up to 33 % [47]. More than 200 individual manuka plants from 87 sites throughout New Zealand were analyzed and the triketone-rich chemotypes were almost exclusively limited to the East Cape region [47]. Cluster analysis of the composition of these samples identified 11 geographical chemotypes distinguished by different levels of monoterpenes and sesquiterpenes, methyl cinnamate, and triketones. The reason for this chemotaxonomic geographical distribution is not well understood.

Little is known about the chemotypes outside of New Zealand, though clearly the *Callistemon* samples studied by Gray in 1977 contained sufficient amounts of leptospermone for isolation and purification. Interestingly, this adds to the serendipity of the discovery of its herbicidal activity since the analysis of a number of *Callistemon* species either did not report the presence of detectable amounts of triketones [42] or only trace amounts [48]. The primary constituent of the essential oil of this genus is the monoterpene 1,8-cineole [49].

Synthetic β-triketone herbicides (e.g., sulcotrione and mesotrione) cause bleaching of newly emerging tissues [50]. This symptom was traditionally associated with inhibitors of phytoene desaturase, but triketone herbicides do not inhibit this enzyme. It was later found that these herbicides inhibit *p*-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme involved in the biosynthesis of prenyl quinones and tocopherols [50]. Plastoquinone (a prenylquinone) is an essential cofactor for phytoene desaturase [51] (Fig. 5.10). In the absence of plastoquinone, phytoene desaturase activity is reduced which results in the bleaching of young foliage and accumulation

of phytoene customarily associated with phytoene desaturase inhibitors [52]. Chlorophyll levels are also affected because the photosynthetic apparatus is no longer protected from reactive oxygen species generated under high light intensity.

Gray observed that leptospermone caused bleaching of plant tissues [43]. Work with the bioactive components of manuka oil demonstrated that some natural β-triketones also inhibit plant HPPD [53]. Most of the activity of manuka oil was attributed to leptospermone because it was the most abundant triketone in the examined oil. However, grandiflorone, a minor constituent that has a more lipophilic side chain, was a much more potent inhibitor of HPPD. Conversely, the short methyl side chain of flavesone nullified the activity of this triketone. The important role of the lipophilicity of the side chain was confirmed by a structure–activity study using a series of natural and synthetic leptospermone analogs [54].

Greenhouse experiments using agricultural soils showed that manuka oil was active both when applied to the foliage and to the soil surface. While most essential oils have little to no soil activity, preemergence application of manuka oil controlled the growth of large crabgrass at a rate of 3 L/ha. The soil activity of manuka oil is due in part to the relatively slow dissipation of leptospermone, which remained active in soil for at least 2 weeks [55].

Triketones and other phytotoxic natural products are often produced and stored in specialized structures, which may serve in part as a mechanism to prevent autotoxic effects [56, 57]. In the leaves of members of the Myrtaceae family, which encompasses most of the known herbicidal triketone-producing species, specialized secretory glands consisting of roughly spherical secretion-filled spaces lined with specialized glandular cells are found (Fig. 5.11). In the genus *Leptospermum*, the gland is typically covered by two to four cells, which have thin, straight walls and are generally of the same approximate size. These cells are encircled by 5–14 unspecialized epidermal cells in a spiral orientation. Although there has been a debate on the method of glandular cavity production, evidence suggests that in the Myrtaceae this formation occurs schizogenously. Schizogenous formation proceeds by the division of single cells within the epidermis or mesophyll layer with the oil cavity forming as an intracellular space [58]. The schizogenous cavity is lined with a single layer of four to six epithelial cells that are thought to be responsible for the production of the volatile oils stored within the cavity [59, 60]. In *Melaleuca* species, the cells lining the immature gland cavity were shown to be metabolically active by osmophilic staining, supporting their role in oil synthesis. In this species, the mature glands contain highly vacuolated epithelial cells lining the gland cavity that are unlikely to lead to continued oil synthesis and accumulation [60]. It has also been demonstrated that essential oil has the potential for release through the modified epidermal cells covering the gland, although the physiological and ecological aspects of this phenomena remain to be investigated.

The *in vitro* chemical synthesis of leptospermone and many other triketones has been well studied [61], but much work remains to unravel the *in vivo* biosynthesis of these compounds. Although an *in planta* biosynthetic route has yet to be established, a hypothetical pathway can be proposed based on the structure of the final compounds (Fig. 5.11). In a series of conversions analogous to the well-examined

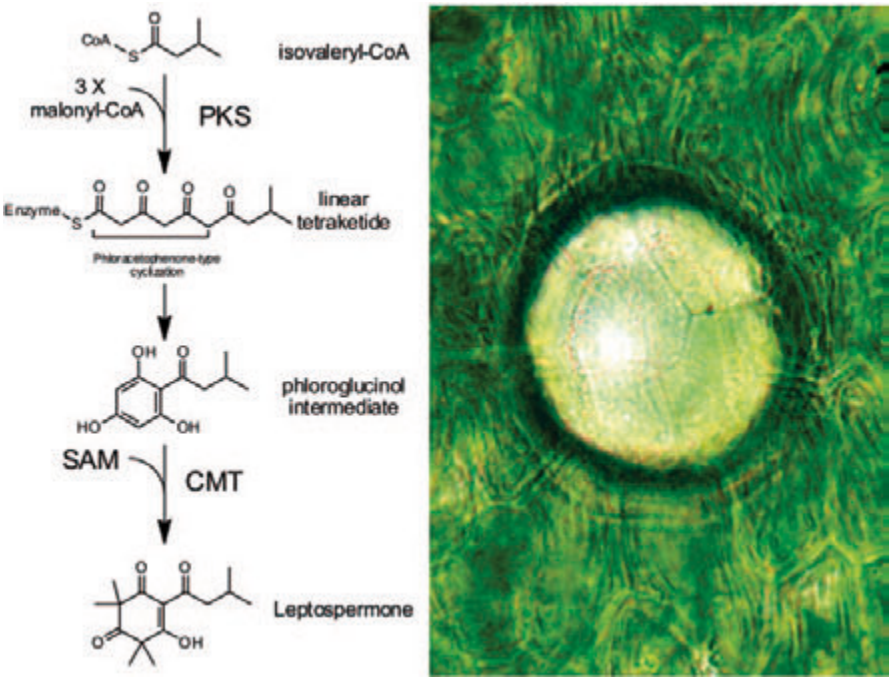


Fig. 5.11 Micrograph of a representative *Leptospermum scoparium* (manuka) schizogenous gland, and a proposed biosynthetic pathway to leptospermone

chalcone synthase enzyme [62], a type III polyketide synthase (PKS) sequentially condenses three malonyl CoA molecules into a polyketide chain extending from an isovaleryl CoA starter molecule. The enzyme subsequently cyclizes the linear tetraketide intermediate via a Claisen-type condensation to generate a phloroglucinol intermediate. A PKS enzyme, valeropenone synthase (VPS), with this activity has been purified to homogeneity and biochemically characterized from *Humulus lupulus* L. (hops) cone glandular hairs [63]. VPS is thought to be involved in the production of the beer flavoring iso-acids of hops which have been shown to contain a β,β-triketone moiety [64]. Subsequently, a gene for this enzyme has been identified and characterized [65]. Efforts are currently underway to isolate and characterize enzymes homologous to VPS from *Leptospermum scoparium* as an initial effort to characterize the leptospermone biosynthetic pathway.

After the production of the phloroglucinol intermediate, the compound would be proposed to undergo spontaneous keto–enol tautomerization, and subsequently to undergo methylation by an as-of-yet unidentified C-methyltransferase (CMT). Early work with methionine-methyl-C^{14} labeled adult *Dryopteris marginalis* ferns demonstrated that the C- and O-methyl substituents of isolated phloroglucinols were derived from methionine [66]. If these findings are consistent with leptospermone, the biosynthetic methyltransferases are likely to be similar to S-adenosylmethionine using CMTs identified in other species.

Table 5.1 Bioassay evaluation results of 9,10-anthraquinone and the analog anthraquinone-59 against the cyanobacterium *Planktothrix perornata* and the green alga *Selenastrum capricornutum*.

Compound	LOEC[a]	LCIC[b]	IC50[c]
P. perornata			
9,10-Anthraquinone	100	100	nd
Anthraquinone-59	10	100	6.3
S. capricornutum			
9,10-Anthraquinone	>100,000	>100,000	nd
Anthraquinone-59	10,000	100,000	5,623

nd not determined
[a] Lowest-observed-effect concentration (nM)
[b] Lowest-complete-inhibition concentration (nM)
[c] 50% inhibition concentration (nM)

5.5.2 Algicides

Some blue-green algae (cyanobacteria) synthesize secondary compounds that can impart unsavory flavors to pond-cultured fish. Currently, aquaculturists are using copper sulfate and chelates, as well as diuron, a synthetic herbicide, to kill cyanobacteria. However, these products generally kill all algae, including beneficial eukaryotic species that are better oxygenators of the water and are not associated with off-flavor compound production. Our laboratory has had a research program to discover natural product-based compounds that are selective for killing cyanobacteria. Among thousands of plant crude extracts and pure compounds tested in the laboratory, 9,10-anthraquinone was one of the most promising compounds [67], with about a thousand times greater activity against a noxious cyanobacterium than on a green alga (Table 5.1). However, the physicochemical properties of this compound were not suitable for use in aquaculture ponds, so the molecule was modified to impart water solubility, while retaining its biological activity [68]. The best modification was the analog anthraquinone-59, which has been patented to control cyanobacteria (Fig. 5.12) [69].

5.6 Transgenic Approaches to Phytotochemical-Based Pest Resistance

All phytochemicals are produced by enzymes encoded by plant genes. With transgenic approaches, we can impart production of new pest management compounds into crops or manipulate the production of such compounds that already exist in crops. Our laboratory has been interested in using these methods to alter the production of sorgoleone in *Sorghum* spp. and other crop species such as rice.

Sorgoleone, a major component of the hydrophobic root exudate of sorghum [*Sorghum bicolor* (L.) Moench], represents one of the most extensively studied

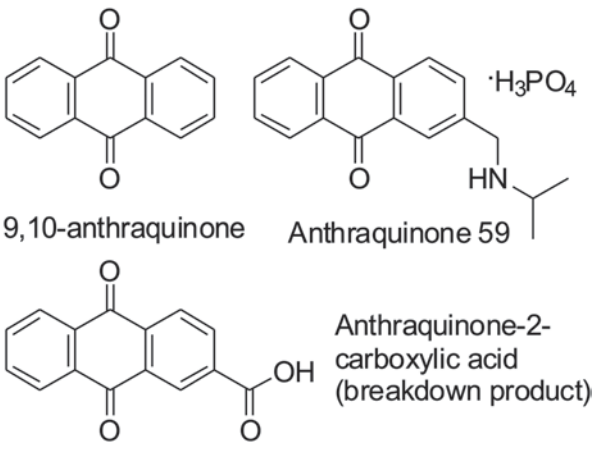


Fig. 5.12 Structures of 9,10-anthraquinone, patented water-soluble analog anthraquinone-59, and the major breakdown product of anthraquinone-59

allelochemicals. In contrast to phenolic lipid-type compounds found in other plants, sorgoleone is an uncommon lipid benzoquinone possessing significant herbicidal activity and is produced exclusively by *Sorghum* spp. [70]. Sorgoleone suppresses the growth of a large number of plant species and appears to be most active on small-seeded plants [71–76]. Additionally, sorgoleone has a relatively long half-life in soil and has been reported to inhibit multiple cellular targets, including plastoquinone/photosystem II, *p*-hydroxyphenylpyruvate dioxygenase, and mitochondrial respiration [77–83]. Thus, evolution of resistance to sorgoleone would presumably be less likely in comparison with a phytotoxin possessing a less-complex mode of action. For the above-mentioned reasons, significant interest has been generated in the potential development of sorgoleone as a natural product-based herbicide.

The sorgoleone biosynthetic pathway appears to exclusively or predominantly reside in root hair cells, with the end product sorgoleone comprising a major portion of the hydrophobic exudate material released into the rhizosphere [80, 84–87]. The biosynthetic pathway of sorgoleone has been previously investigated, and these studies have shown that the aromatic moiety within sorgoleone's structure is synthesized via iterative condensation reactions catalyzed by alkylresorcinol synthase (ARS) enzymes utilizing a C16:3 fatty acyl-CoA precursor [88, 89]. The resulting 5-pentadecatrienyl resorcinol intermediate (produced by ARS) is next methylated by a root hair-specific *O*-methyltransferase [90] and subsequently dihydroxylated by a P450 monooxygenase to yield sorgoleone (Fig. 5.13).

A strategy for the cloning and functional characterization of genes and enzymes involved in sorgoleone biosynthesis has been pursued, involving the screening of candidate gene sequences derived from a root hair-specific *S. bicolor* expressed sequence tag (EST) database [90]. Using this approach, two root hair-specific fatty acid destaurase enzymes were identified, designated SbDES2 and SbDES3, which are likely responsible for the generation of the C16:3 fatty acyl-CoA precursor by consecutive desaturation reactions, starting with palmitoleoyl-CoA (Fig. 5.13) [86]. Heterologous co-expression of *SbDES2* and *SbDES3* in yeast cells resulted in the

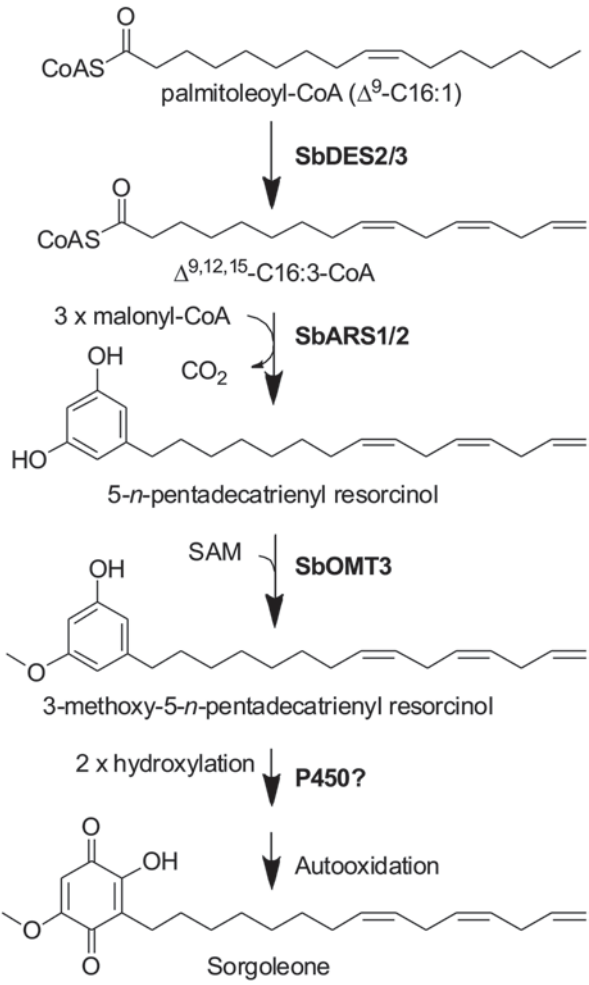


Fig. 5.13 Sorgoleone biosynthetic pathway

production of hexadecatrienoic acid (16:3$\Delta^{9,12,15}$; Fig. 5.13). Co-expression of the two enzymes was required, given that SbDES2 was found to convert endogenous palmitoleic acid (16:1Δ^{9}) to hexadecadienoic acid (16:2$\Delta^{9,12}$), thus providing a substrate that recombinant SbDES3 was capable of converting into hexadecatrienoic acid in vivo [86].

Two root hair-specific ARSs (designated SbARS1 and SbARS2), representing the first such enzymes described from higher plants, have also been characterized and linked to the biosynthesis of sorgoleone [87]. The recombinant SbARS1 and SbARS2 enzymes have both been shown to accept a variety of fatty acyl-CoA starter units using *in vitro* enzymatic assays, including the physiological substrate hexadecatrienyl-CoA (C16:3$\Delta^{9,12,15}$-CoA; Fig. 5.13). The 5-pentadecatrienyl resorcinol intermediate produced by SbARS1 and SbARS2 *in planta* is likely next methylated

by a root hair-specific *O*-methyltransferase designated SbOMT3 [90]. Recombinant SbOMT3 was found by *in vitro* enzymatic assays to exhibit a marked preference for alkylresorcinolic substrates among a panel of phenolic compounds tested [90]. As mentioned, the final steps in the sorgoleone biosynthetic pathway involve the dihydroxylation of the 3-methoxy-5-*n*-pentadecatrienyl resorcinol intermediate (Fig. 5.13), and work on several candidate root hair-specific P450 monooxygenase sequences identified within the root hair ESTs is ongoing (Z. Pan, unpublished).

Initial efforts to alter sorgoleone levels in transgenic *S. bicolor* events have been successfully performed with RNA interference (RNAi), using SbARS1/2 3′ coding and contiguous untranslated region (UTR) sequences incorporated within hairpin RNA (hpRNA)-forming binary transformation vectors [87]. For these experiments, segregating T_1 populations representing multiple independent transgenics were first analyzed by quantitative real time polymerase chain reaction (qRT-PCR) for the presence of the transgene-derived hpRNA and individual seedlings were scored as hpRNA "+" or "–". Pooled samples from hpRNA "+" or "–" individuals were analyzed by gas chromatography–mass spectrometry (GC-MS), and sorgoleone levels were found to be reduced to unquantifiable levels in the hpRNA-expressing transformants (Fig. 5.14; see also [87]). The work performed to date on sorgoleone biosynthesis, involving heterologous expression experiments and RNAi in transgenic *S. bicolor*, has provided compelling evidence that SbDES2, SbDES3, SbOMT3, SbARS1, and SbARS2 participate in sorgoleone biosynthesis *in vivo*. These sequences should provide a powerful new toolbox for the manipulation of sorgoleone biosynthesis in *S. bicolor* and the potential transfer of this trait to other crop species.

5.7 Summary

We have provided examples of natural product-based pesticides that are now commercially successful, as well as a few examples of the many natural compounds that we have studied which are active against pests. Our group's research is but a small sampling of the extensive, international effort to discover natural product-based pest management products. Some have argued that we have reached diminishing returns with this approach, but an analysis of the number of new phytochemicals being discovered yearly and the relatively few bioassays for potential pesticidal activity that most of the known phytochemicals have been subjected to, indicates that this is not the case. Furthermore, chemical modification of these compounds and their use to discover new modes of action greatly expands the scope for future work.

It is even clearer that we have only scratched the surface of the possibilities of using transgene technology to protect crops from pests by imparting production or manipulation of production of pest management phytochemicals. We are especially interested in using this method for crop/weed allelopathy [91, 92], as success in this effort would greatly decrease the most used form of synthetic pesticides, herbicides. Furthermore, these efforts provide much-needed experimental verification of the plant/plant allelopathy role of putative allelochemicals. For example, strong support

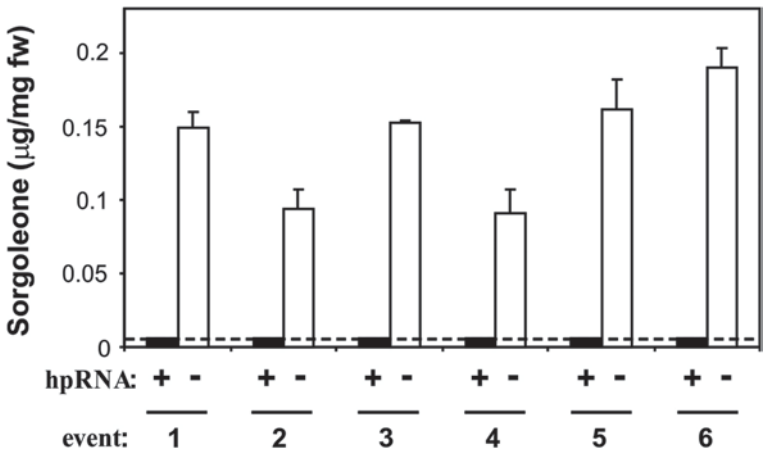


Fig. 5.14 Sorgoleone levels in different *S. bicolor* RNAi transgenic events. Sorgoleone levels were determined by GC-MS analysis of root exudates prepared from pooled hpRNA "+" and hpRNAi "–" seedlings. Data are expressed as mean ± SD

for the allelopathic role of momilactones in rice was recently generated by using gene knockouts to reduce expression of two genes encoding enzymes in the momilactone pathway [93]. These early efforts are promising and should stimulate further research along these lines. Lastly, there can be unexpected spin-off from such research. For example, members of our group have used an *O*-methyltransferase gene of the sorgoleone pathway [90] with a peanut stilbene synthase gene to impart the production of pterostilbene in plants [94]. Pterostilbene is a phytochemical fungicide [e.g., 95], so this technology could be used to improve fungal pathogen resistance in crops. Additional potential benefits of such a transgenic crop are the health-promoting properties of pterostilbene [e.g., 96–98]. Such creative use of the genetics of phytochemical production bodes well for the future of meshing phytochemistry with transgene technology.

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Chapter 6
Sulfhydryl-Reactive Phytochemicals as Dual Activators of Transcription Factors NRF2 and HSF1

Ying Zhang, Sharadha Dayalan Naidu, Rumen V. Kostov, Ashley Pheely, Vittorio Calabrese and Albena T. Dinkova-Kostova

Abstract Two central regulators, nuclear factor-erythroid 2 p45-related factor 2 (NRF2) and heat shock factor 1 (HSF1), control the KEAP1/NRF2/ARE pathway and the heat shock response, two essential cellular defense mechanisms. Both systems are highly inducible under conditions of stress. Many small molecules, including certain phytochemicals, such as isothiocyanates and phenylpropanoids, and/or their metabolites, have the capacity to induce the KEAP1/NRF2/ARE pathway. Recent results suggest that a common signal that is sensed through cysteine modification(s) within Kelch-like ECH-associated protein 1 (KEAP1) and HSF1, or possibly within a negative regulator of HSF1, is responsible for triggering both pathways. Celastrol, withaferin A, gedunin, curcumin, and sulforaphane are examples of structurally diverse phytochemicals with a common chemical signature: reactivity with sulfhydryl groups. This reactivity underlies their biological activities as multitarget agents for which protective effects have been documented in numerous animal models of human disease and which include induction of large networks of transcriptional programs regulated by transcription factors NRF2 and HSF1.

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6.1 Introduction

The KEAP1/NRF2/ARE pathway and the heat shock response represent two essential cellular defense mechanisms that are controlled by two central regulators, nuclear factor-erythroid 2 p45-related factor 2 (NRF2) and heat shock factor 1 (HSF1), respectively. Both systems do not normally operate at their maximal capacity, but are highly inducible under conditions of stress. Induction leads to the transcriptional upregulation of networks of proteins that protect against the potentially devastating consequences of thermal, oxidative, and electrophilic stress, and chronic inflammation. Nearly 25 years ago, Paul Talalay and his colleagues discovered that many small molecules, including certain phytochemicals, such as isothiocyanates and phenylpropanoids, and/or their metabolites, have the capacity to induce the KEAP1/NRF2/ARE pathway. Although structurally very diverse, excluding the possibility of ligand–receptor interactions as the underlying mechanism, all inducers possess a common chemical signature: reactivity with sulfhydryl groups [1]. Based on this finding, it was predicted that there exists in the cell a protein sensor for inducers that is endowed with highly reactive cysteine residues [2], which was later identified by Masayuki Yamamoto and his colleagues as Kelch-like ECH-associated protein 1 (KEAP1) [3], the main negative regulator of transcription factor NRF2 [4]. More recently, reactivity with sulfhydryl groups has emerged as also being important for the activation of HSF1 by various small molecules [5–9]. Thus, it appears that a common signal that is sensed through cysteine modification(s) within KEAP1 and HSF1, or possibly within a negative regulator of HSF1, is responsible for triggering both pathways.

6.2 The KEAP1/NRF2/ARE Pathway

The KEAP1/NRF2/ARE pathway is at the forefront of the cellular defense. In numerous experimental systems, induction of this pathway has been shown to be protective against various conditions of stress. Conversely, failure to upregulate the pathway (such as under conditions of NRF2 deficiency) leads to increased sensitization and accelerated disease pathogenesis. Under basal conditions, transcription factor NRF2 is continuously targeted for ubiquitination and proteasomal degradation by the repressor protein KEAP1, which serves as a substrate adaptor for Cullin 3 (Cul3)-based E3 ubiquitin ligase (Fig. 6.1) [10–12]. In addition to KEAP1, the levels of NRF2 within the cell are also controlled by the action of glycogen synthase kinase-3β (GSK3β) and β-transducin repeat-containing protein (β-TrCP) which serves as a substrate adaptor for Cullin 1 (Cul1)-based ubiquitin ligase [13]. The precise mechanistic details of regulation of the KEAP1/NRF2/ARE pathway are not completely understood and several different models have been proposed [14]. It is clear, however, that many inducers of the pathway chemically modify specific cysteine residues within KEAP1, leading to loss of its ability to target NRF2

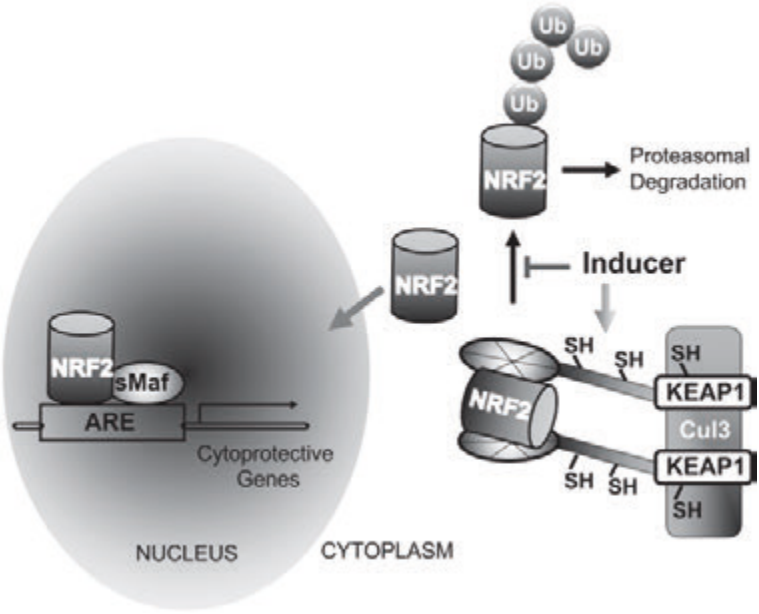


Fig. 6.1 The KEAP1/NRF2/ARE pathway. Under basal conditions, NRF2 is targeted for ubiquitination and proteasomal degradation by its repressor KEAP1, which serves as a substrate adaptor for Cullin 3 (Cul3)-based ubiquitin ligase. Inducers chemically react with cysteine residues of KEAP1, rendering it unable to target NRF2 for degradation. As a result, NRF2 accumulates and undergoes nuclear translocation, where it binds to antioxidant response elements (AREs) as a heterodimer with a small Maf protein, driving the expression of cytoprotective genes

for ubiquitination and proteasomal degradation. Subsequently, NRF2 accumulates, enters the nucleus, binds as a heterodimer with a small Maf transcription factor to antioxidant response elements (AREs, specific sequences that are present in the promoter regions of NRF2-target genes), and activates transcription [15–17].

NRF2-dependent genes encode a large network of cytoprotective proteins, including those that are involved in the metabolism and transport of a wide array of endo- and xenobiotics, proteins that have antioxidant functions, as well as those that participate in the synthesis, utilization, and regeneration of glutathione and NADPH. The number of genes that are under the transcriptional control of NRF2 is fascinatingly large: a recent study integrating chromatin-immunoprecipitation with parallel sequencing (ChIP-Seq) and global transcription profiling identified 645 basal and 654 inducible direct targets of NRF2, with 244 genes at the intersection [18]. Moreover, the functional diversity of the NRF2-dependent cytoprotective proteins is extraordinary and provides the cell with multiple layers of protection. Examples of NRF2-dependent proteins include: (1) antioxidant enzymes (e.g., heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), and thioredoxin reductase); (2) conjugating enzymes (e.g., glutathione *S*-transferases (GSTs) and uridine 5′-diphospho (UDP)-glucuronosyltransferases); (3) proteins

that facilitate the export of xenobiotics and/or their metabolites (e.g., solute carriers and adenosine triphosphate (ATP)-binding cassette transporters); (4) anti-inflammatory enzymes (e.g., leukotriene B_4 dehydrogenase); (5) enzymes that participate in the synthesis and regeneration of glutathione (e.g., χ-CT, the core subunit of the cystine/glutamate membrane transporter, γ-glutamate cysteine ligase catalytic (GCLC) and modulatory (GCLM) subunits, glutathione reductase); (6) enzymes that are responsible for the synthesis of reducing equivalents (e.g., glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme); (7) proteins that protect against metal overload (e.g., ferritin and metallothioneins); and (8) proteins that participate in the repair and removal of damaged proteins (e.g., subunits of the 26S proteasome).

6.3 The Heat Shock Response

The heat shock response is another critical inducible defense mechanism which is essential in protecting the cell under conditions of acute and chronic proteotoxic stress affecting the integrity of the proteome. The heat shock response is controlled by a family of heat shock (transcription) factors, among which HSF1 plays the major role [19, 20]. Under homeostatic conditions, HSF1 is an inactive monomeric phosphoprotein bound to Hsp90 (Fig. 6.2). Following stimulation, HSF1 dissociates from the Hsp90 complex, trimerizes, and binds to heat shock elements (HSEs) of its target genes, thereby driving their expression [19–23]. In addition, a number of posttranslational modifications, such as phosphorylation, sumoylation, and acetylation, are involved in regulating the transcriptional activity of HSF1, and there is also negative feedback regulation by heat shock proteins, such as Hsp70 and Hsp40. Several different ways of activation of HSF1 have been proposed and the experimental evidence for each one of them was recently reviewed [23]. Displacement of HSF1 from its negative regulator Hsp90 is one major mechanism: indeed, pharmacological inhibition of Hsp90 or its antibody-mediated depletion is sufficient to induce trimerization and DNA binding of HSF1 [24, 25].

Similarly to the KEAP1/NRF2/ARE pathway, the number of genes that are regulated by the heat shock response is strikingly large: various studies employing differential display, transcriptional profiling, or proteomic approaches have shown that, depending on the organism, approximately 50–200 genes are induced [26]. According to their functions, the proteins encoded by these genes have been grouped into seven distinct classes: (1) molecular chaperones that prevent unspecific aggregation of nonnative or partially misfolded proteins (e.g., Hsp70, Hsp40); (2) proteolytic proteins that participate in the removal of irreversibly damaged proteins (e.g., BAG3 (BCL2-associated protein), APG5 L (protein involved in autophagy), the cysteine protease, caspase 1 (CASP1), neural precursor cell-expressed developmentally downregulated 4 like (NEDD4 L), and ubiquitin-protein ligase)); (3) RNA- and DNA-modifying enzymes, which are necessary to repair DNA damage (e.g., the bacterial DNA glycosylase MutM); (4) metabolic enzymes that are needed

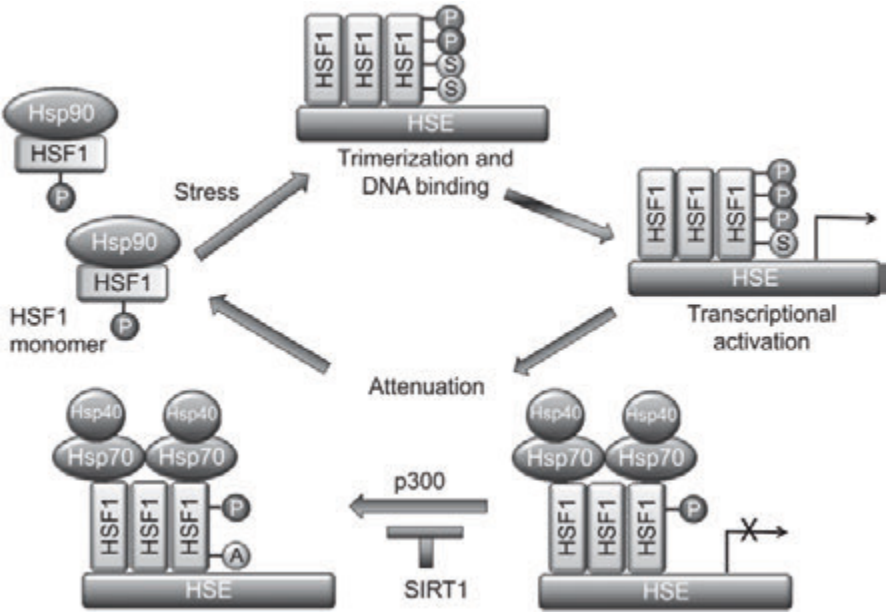


Fig. 6.2 The heat shock response. Under basal conditions, HSF1 is an inactive monomeric phosphoprotein bound to Hsp90. Following stimulation, HSF1 dissociates from Hsp90, trimerizes, and binds to heat shock elements (HSEs) of its target genes, thereby driving their expression. In addition, multiple posttranslational modifications regulate the transcriptional activity of HSF1, such as phosphorylation (P), sumoylation (S), and acetylation (A). HSF1 is also negatively feedback-regulated by heat shock proteins. (Adapted from [19])

to reorganize and maintain the energy supply of the cell (e.g., ACAT2 (acetyl-CoA acetyltransferase), ALAS1 (aminolevulinate synthase), ChGn (chondroitin α-1,4-*N*-acetylgalactosaminyltransferase)); (5) transcription factors, kinases, or phosphatases that further activate other stress response pathways (e.g., RHOH (Ras homolog), PTPG1 (tyrosine phosphatase), RGS2 (regulator of G-protein signaling), and IER5 (regulator of immediate early response)); (6) proteins involved in sustaining cellular structures such as the cytoskeleton and membranes (e.g., tight junction-associated protein (TJP4) and signal-induced proliferation-associated 1-like protein 3 (SIPA1L3)); and (7) proteins involved in transport and detoxification (e.g., the amino acid transporter SLC38A2).

It should be emphasized that the KEAP1/NRF2/ARE pathway and the heat shock response are two distinct defense mechanisms. Thus, induction of the KEAP1/NRF2/ARE pathway occurs in the absence of HSF1; likewise, induction of the heat shock response is independent of NRF2 [9]. Nevertheless, there is some functional overlap between the two pathways which is perhaps best exemplified by HO-1, also known as Hsp32. Indeed, the gene encoding HO-1 is one of the most highly inducible genes (in terms of both kinetics and magnitude of induction) in response to both heat shock as well as inducers of the KEAP1/NRF2/ARE pathway.

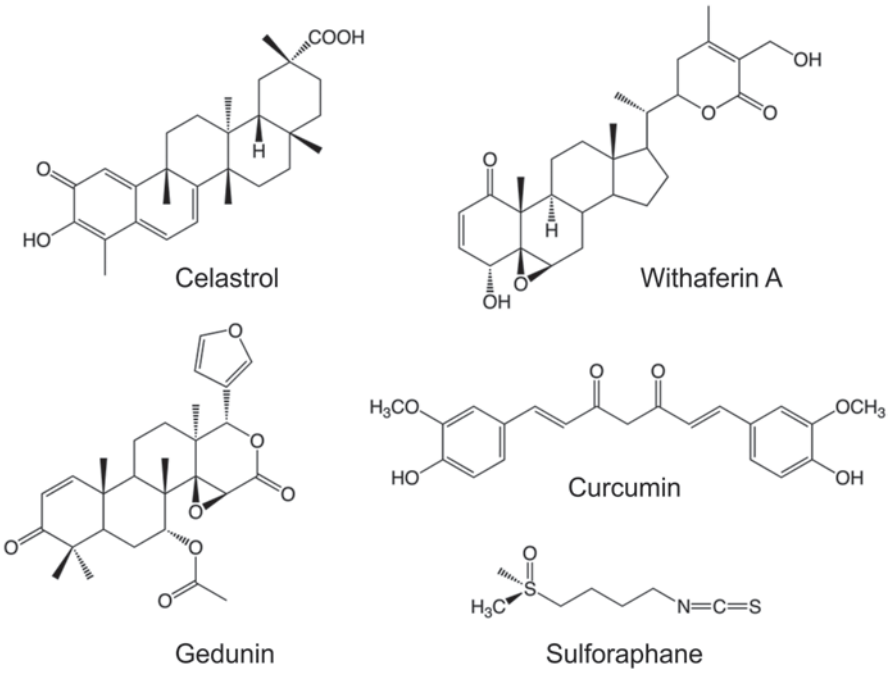


Fig. 6.3 Examples of sulfhydryl-reactive phytochemicals—dual activators of transcription factors NRF2 and HSF1

6.4 Phytochemicals that Activate Transcription Factors, NRF2 and HSF1, and the Consequences of Activation

6.4.1 Celastrol

The quinone methide triterpenoid celastrol (Fig. 6.3) was isolated from the Chinese plant *Tripterygium wilfordii*. Celastrol was reported to induce the heat shock response following a screen of bioactive small molecules in the human HeLa cell line hsp70.1pr-luc [27]. This cell line is stably transfected with a luciferase-encoding construct under the transcriptional control of the *hsp70* promoter. The same study showed that celastrol activates the *hsp70* promoter reporter in several different cell types (i.e., in the breast cancer cell lines MCF7 and BT474, the non-small-cell lung carcinoma cell line H157, and the neuroblastoma cell line SH-SY5Y) to levels comparable with those induced by heat shock (42 °C). Treatment with celastrol led to hyperphosphorylation of HSF1, enhanced binding of HSF1 to the heat shock element in the Hsp70 promoter, and transcriptional activation of endogenous heat shock genes.

Expression profiling of RNA isolated from the androgen-dependent prostate cancer cell line LNCaP that had been treated with celastrol was performed in order to

obtain a gene expression signature for celastrol activity [28]. A collection of gene expression profiles of drug-treated cell lines, termed the Connectivity Map [29], was then used to identify known drugs with similar effects on gene expression. Strikingly, the celastrol gene expression signature was found to be very similar to those of four known inhibitors of Hsp90, strongly suggesting that celastrol functions as an inhibitor of Hsp90. This conclusion was further supported by the ability of celastrol to: (1) decrease the levels of the Hsp90 client proteins AR, FLT3, EGFR, BCL-ABL1, AKT, and HER-2 in a concentration-dependent manner in several different cell lines [28, 30], (2) inhibit the ATP-binding activity of Hsp90 [28], and (3) reduce the interaction of Hsp90 with the co-chaperone p23 [28, 31].

Treatment with celastrol was found to disrupt the interaction of Hsp90 and cell division cycle protein 37 (Cdc37), a co-chaperone which is essential for the association of client proteins to Hsp90 [32]. As a result, the Hsp90 client proteins Cdk4 and AKT were destabilized and degraded via the ubiquitin proteasome, and apoptosis was initiated in the pancreatic cancer cell line Panc-1. Further mechanistic studies revealed that celastrol reacts with the C-terminus of Hsp90 and inhibits the ATPase activity of the chaperone without affecting the ATP binding pocket [33]. To identify target proteins of celastrol, stable isotope labeling with amino acids in cell culture (SILAC) approach was used by Hansen et al. [34] in cultured human lymphoblastoid cells that had been exposed to celastrol for 24 hours. It was found that 158 of the ~1,800 proteins with robust quantitation had at least a 1.5-fold change in their levels, with 112 being upregulated and 46 being downregulated. Upregulated proteins include those involved in cellular homeostatic processes, stress responses, cell death, and intracellular transport. A prominent group is that involved in protein quality control, such as the endoplasmic reticulum molecular chaperones GRP78 (HspA5), Grp94 (Hsp90B1), calnexin (CANX), calreticulin (CALR), ERp29 (ERP29), multiple protein disulfide isomerases, glucosidases, and glycosyltransferases. A second group of celastrol-induced proteins comprises those involved in the cellular defense against oxidative stress, such as peroxiredoxins, thioredoxins, and HO-1. These findings are in agreement with an earlier study by Trott et al. [5] in *Saccharomyces cerevisiae* in which transcriptional profiling showed that treatment with celastrol induced heat shock genes as well as antioxidant genes. Celastrol caused hyperphosphorylation of the yeast HSF1 and upregulation of heat shock proteins. In addition, transcription factor Yap1, which is activated in response to oxidants and electrophiles and triggers the transcription of cytoprotective genes, was also activated by celastrol treatment, via the carboxy-terminal redox center of the transcription factor. Similar to its effects in yeast, celastrol also induces antioxidant response genes (e.g., GCLM, χ-CT, and NQO1), in parallel with heat shock target genes (e.g., Hsp70) in RKO human colorectal carcinoma cells [5], Hepa1c1c7 mouse hepatoma cells, and mouse embryonic fibroblasts [9]. Induction of Hsp70 requires functional HSF1, but is independent of NRF2, whereas upregulation of NQO1 occurs in the absence of HSF1, but the presence of NRF2 is essential [9].

The ability of celastrol to upregulate the KEAP1/NRF2/ARE pathway and the heat shock response suggests that celastrol could have cytoprotective effects, a conclusion that has received experimental support in a number of different studies.

One protective effect of celastrol is its ability to inhibit endogenous peroxynitrite formation and to prevent endothelial barrier dysfunction [35]. Celastrol was also shown to protect against aminoglycoside-induced hair cell death and to reduce hearing loss in mice receiving systemic aminoglycoside treatment [36]. Exposure to celastrol was protective against lethal heat stress in both HeLa cells and SH-SY5Y cells, and to a similar extent as a 42° C heat shock [37]. In cells ectopically expressing a mutant polyglutamine (Q57-YFP) protein, celastrol treatment prevented the aggregation of the fusion protein and the associated cytotoxicity [37]. In rodent models for Alzheimer's disease, celastrol reduced the amyloid-α-associated pathology [38] and improved memory, learning, and psychomotor activity [39]. In mice, celastrol protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- and 3-nitropropionic acid-induced neurotoxicity [40]. In a transgenic mouse model of amyotrophic lateral sclerosis (ALS), celastrol prevented neuronal cell death, improved weight loss and motor performance, and delayed disease onset [41].

In addition to its cytoprotective effects, celastrol has also been shown to inhibit the proliferation of a number of cancer cell lines and to suppress tumor development and metastasis in various animal models of carcinogenesis. Thus, treatment of the oral leukoplakia cell line MSK-Leuk1 with celastrol inhibited the activation of AhR-dependent transcription of *CYP1A1* and *CYP1B1*, which encode proteins that are responsible for the conversion of polycyclic aromatic hydrocarbons to genotoxic metabolites [42]. Consequently, the formation of benzo(a)pyrene [B(a)P]-induced DNA adducts was reduced. The antitumor activity of celastrol has also been demonstrated using a panel of human breast cancer cell lines with selectivity toward those overexpressing the receptor tyrosine kinase ErbB2, an Hsp90 client protein [43]. Furthermore, celastrol inhibited tumor growth of ErbB2-overexpressing human breast cancer cells in a mouse xenograft model [43]. Celastrol downregulated the expression of CXCR4, a chemokine receptor that is closely linked with tumor metastasis, in breast cancer, colon cancer, squamous cell carcinoma, and pancreatic cancer cells, and inhibited invasion [44]. Reduction of hypoxia-induced angiogenesis and metastasis by celastrol has also been demonstrated and shown to be partly mediated by inhibition of Hsp90 [45]. Hsp90 inhibition was also implicated in the ability of celastrol to increase the sensitivity of the NCI-H460 lung cancer cell line to radiation [46]. In the RIP1-Tag2 transgenic mouse model of pancreatic islet carcinoma, tumor metastasis was suppressed by more than 80 % when celastrol was administered at a dose of 3 mg/kg body weight, once every 3 days, for 4 weeks [32].

6.4.2 Gedunin

Gedunin (Fig. 6.3) is a tetranortriterpenoid isolated from the Indian neem tree *Azadirachta indica* which has antimalarial, insecticidal, and anticancer activity. Using the Connectivity Map [29], it was found that, similar to celastrol-, gedunin-

induced genes were enriched in the profile of known Hsp90 inhibitors; likewise, gedunin-repressed genes were repressed by known Hsp90 inhibitors [28]. Although less potent, gedunin appears to share with celastrol some of the same mechanisms by which it inhibits Hsp90 and activates NRF2. Thus, gedunin treatment caused a decrease in ATP binding to Hsp90 and a reduction of the protein levels of the Hsp90 client proteins AR, FLT3, EGFR, and BCR-ABL1 in a concentration-dependent manner in three different cell lines: LNCaP, K562, and Ba/F3 [28]. Similarly, when MCF-7 cells were incubated with gedunin for 24 h, there occurred a dose-dependent degradation of the Hsp90 client proteins HER-2 and AKT, strongly suggesting that Hsp90 is the cellular target for this compound [30]. Indeed, gedunin has been shown to disrupt the interaction between Hsp90 and its co-chaperone, Cdc37 [30, 47].

Recently, gedunin was identified as an NRF2-dependent inducer of cytoprotective enzymes in a high-throughput screen of the Spectrum library comprising 2,000 biologically active compounds [48]. This screening assay used a Neh2-luciferase reporter system in which the Neh2 domain of NRF2, through which the transcription factor binds to its negative regulator KEAP1, was fused to firefly luciferase, thus allowing the direct monitoring of induction based on the time course of reporter activation. The same study showed that gedunin protected neurons against oxidative stress in an astrocyte-dependent manner, and via an NRF2-dependent mechanism. Thus, when primary cultured astrocytes were pretreated with gedunin for 24 h followed by the addition of neurons in the presence of the glutathione-depleting compound homocysteic acid, significant neuroprotection was observed. Protection by gedunin was accompanied by an increase in the levels of GSH and HO-1, and was abrogated by silencing of NRF2.

Gedunin was also shown to induce apoptosis and inhibit cell growth in Caco-2 (colon cancer), MCF-7 (breast adenocarcinoma), NCI-H460 (non-small-cell lung cancer), and A375-C5 (melanoma) cells [49, 50]. In the SKOV3, OVCAR4, and OVCAR8 ovarian cancer cell lines, treatment with gedunin decreased cell proliferation by 80% [51]. When a bioinformatics analysis was performed, integrating gedunin sensitivity and gene expression data from 54 cancer cell lines, 52 genes were found to be associated with gedunin sensitivity, 49 of which had decreasing expression with increasing gedunin sensitivity [51]. Pathway analysis revealed significant alterations in signaling pathways controlled by the aryl hydrocarbon receptor, phosphatidylinositol 3-kinase (PI3K)/AKT, nitric oxide, neuregulin, and extracellular signal-regulated kinase/mitogen-activated protein kinase. In agreement with the protective effects of gedunin, three closely related compounds, deoxygedunin, deacetoxy-7-oxogedunin, and deacetylgedunin, were shown to activate HSF1 and induce Hsp70 [52]. Moreover, in an MG-132-induced protein misfolding neuronal cell culture model, the compounds protected against cell death, and RNAi knockdown of HSF1 significantly reversed the cytoprotective effect. In HeLa cells that had been transiently transfected with a polyglutamine-expanded toxic isoform (Q103) of huntingtin, a model for Huntington's disease, all three gedunin derivatives improved cell survival.

6.4.3 Withaferin A

Withaferin A (Fig. 6.3) is a withanolide found in the Indian medicinal plant *Withania somnifera*. More than 30 withanolides have been reported to induce the NRF2-dependent enzyme NQO1 [53, 54]. Withaferin A was among the active compounds which were recently identified as inducers of the HSF1-dependent heat shock response when a library of more than 80,000 natural and synthetic compounds were evaluated using a reporter cell line optimized for high-throughput screening [8]. This reporter cell line expresses enhanced green fluorescent protein (EGFP) under the transcriptional control of a minimal consensus HSE-containing promoter. Withaferin A was also shown to bind to the C-terminus of Hsp90, disrupt the Hsp90–Cdc37 complex, inhibit the activity of the chaperone, and promote the degradation of the Hsp90 client proteins AKT, Cdk4, and the glucocorticoid receptor [55]. Molecular docking analyses of withaferin A into the active Hsp90–Cdc37 complex support the hypothesis that this withanolide has the potential to inhibit the association of the chaperone with its co-chaperone by disrupting the stability of attachment of Hsp90 to Cdc37 [56, 57].

The anticancer effects of withaferin A have been demonstrated in various cell culture and xenograft models. In MCF-7 cells, withaferin A downregulated estrogen receptor alpha (ERα), and caused apoptosis and cell growth inhibition [58]. Withaferin A induced apoptosis and inhibited cell proliferation in the pancreatic cancer cell lines Panc-1, MiaPaCa2, and BxPc3 [55] and in the glioma cell lines LN428, LN827, U87, and BT70, irrespective of their p53 status [8]. Furthermore, in xenograft models of pancreatic Panc-1 cells, tumor growth was reduced by withaferin A in a dose-dependent manner [55]. Tumor growth inhibition by withaferin A was also demonstrated in an orthotopic xenograft model in mice which involved intracranial implantation of BT70 glioma progenitor cells [8]. In this model, evaluation of the mRNA levels for HO-1 within the intracranial tumor mass showed a dramatic (7.7-fold) increase in the withanolide A-treated animals.

6.4.4 Curcumin

Curcumin (1,7-bis(4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3,5-dione, diferuloylmethane; Fig. 6.3) is a component of turmeric, a yellow spice that is obtained from the rhizomes of the East Indian plant *Curcuma longa* L. (Zingiberaceae). We have described the ability of curcumin and other related phenolic Michael acceptors to induce the NRF2-dependent enzyme NQO1 in Hepa1c1c7 cells [59, 60]. Curcumin has also been shown to increase the expression of HO-1 in rat neurons and astrocytes [61], renal epithelial cells [62], and human cardiac myoblasts, hepatocytes, monocytes, and endothelial cells [63–66]. In the human proximal tubule cell line HK-2, curcumin increased the expression of Hsp70 and protected against shiga toxin-induced apoptosis and necrosis [67]. Curcumin treatment increased the levels of HO-1 and Hsp70, and improved the functional recovery of pancreatic islets

following cryopreservation [68]. In cultured *Xenopus laevis* A6 kidney epithelial cells, curcumin led to induction of Hsp30 and Hsp70 and was protective against a subsequent cytotoxic thermal challenge [69]. In the chronic myelogenous leukemia cell line K562, exposure to curcumin disrupted the binding the Hsp90–p23 complex to its client protein p210 BCR/ABL, and thus decreased the levels of this oncogenic tyrosine kinase [70]. Hsp27, Hsp70, and Hsp40 were induced when lung adenocarcinoma (CL1–5) cells were treated with curcumin [71].

The hepatic enzyme activities of catalase and superoxide dismutase as well as the levels of Hsp70 were increased when curcumin was administered intravenously to Sprague-Dawley rats [72]. This treatment protected the liver against the damaging effects of ischemia/reperfusion, including lipid peroxidation, activation of inducible nitric oxide synthase and myeloperoxidase, and apoptosis, and improved survival. Pretreatment with curcumin enhanced induction of heat shock proteins Hsp70, Hsp27, and alpha B crystalline in liver and adrenal gland of rats that had been subjected to heat stress [73]. Numerous studies have demonstrated the protective effects of curcumin in animal models of neurodegeneration, cardiovascular disease, diabetes, and cancer; these have been recently reviewed [74–76]. In humans, curcumin is well tolerated at doses up to 12 g per day [77]. As of October 2013, curcumin has been or currently is in 85 different clinical trials targeting various disease conditions, such as psoriasis, radiation dermatitis, atopic asthma, mild cognitive impairment, Alzheimer's disease, ulcerative colitis, multiple myeloma, pancreatic cancer, colorectal cancer, and myelodysplastic syndrome (www.clinicaltrials.gov).

6.4.5 Sulforaphane

Sulforaphane (1-isothiocyanato-(4*R*)-(methylsulfinyl)butane; Fig. 6.3) is an isothiocyanate that is formed upon plant tissue injury from a glucosinolate precursor (glucoraphanin), a phytochemical present in cruciferous plants. Sulforaphane was isolated from extracts of broccoli (*Brassica oleracea*) as the principal inducer of the NRF2-dependent enzyme NQO1 using a highly quantitative bioassay in murine hepatoma Hepa1c1c7 cells [78]. Over the past 20 years, voluminous experimental evidence that is beyond the scope of this *Perspective* has convincingly shown the ability of sulforaphane to induce NRF2-dependent genes in cells and animals, and to protect against chronic degenerative conditions, such as cancer, cardiovascular disease, and neurodegenerative diseases (reviewed in [79–81]). Induction of HSF-1 dependent genes by this isothiocyanate is a more recent discovery. Global gene expression profiling of liver tissue isolated from C57BL/6J mice that had been treated with a single dose of sulforaphane (90 mg/kg, *p.o.*) revealed that, in addition to the classical NRF2-dependent genes, there was a prominent induction of heat shock proteins, including alpha B crystallin, Hsp40, and Hsp70 [82]. In HL60 and K562 cells, two human leukemic cell lines that have a chromosome abnormality known as the Philadelphia chromosome, sulforaphane treatment was reported to cause nuclear accumulation of both NRF2 and HSF1, and to increase the expression

of Hsp70; interestingly, these effects were enhanced following hGSTA1-1 overexpression [83]. Activation of HSF1 and the heat shock response was also shown in human HeLa and monkey COS-1 cells, with increased expression in Hsp27 being implicated in upregulation of the proteasomal activity [84].

In a pancreatic cancer xenograft model, co-treatment with sulforaphane enhanced the antitumor effect of the 17-allylamino 17-demethoxygeldanamycin (17-AAG), an Hsp90 inhibitor, resulting in inhibition of tumor growth by more than 70% [85]. The isothiocyanate disrupted the Hsp90–Cdc37 interaction and, synergistically with 17-AAG, downregulated several Hsp90 client proteins, including mutant p53, Raf-1, and Cdk4 [85]. Thus, inhibition of Hsp90 may underlie the ability of sulforaphane to activate HSF1 and the heat shock response. In addition to disrupting the association of Hsp90 with its co-chaperone, another potential mechanism of sulforaphane inhibiting the activity of Hsp90 is through altering the acetylation of the chaperone. Sulforaphane was discovered to downregulate the activity of histone deacetylase (HDAC) in a number of human cell lines established from colon, prostate, pancreatic, and breast cancer, as well as in leukemic cells [86–88]. Incorporation of sulforaphane in the diet was also shown to downregulate HDAC and to increase global histone acetylation, with specific increase at the *bax* and the *p21* promoter regions, in polyp tissue isolated from Apc^{min} mice and in PC-3 xenografts [89, 90]. Based on the structural similarities between the HDAC inhibitor trichostatin A and the sulforaphane metabolites sulforaphane-cysteine and sulforaphane-*N*-acetlcysteine revealed by molecular modeling, it was suggested, and then confirmed experimentally, that the metabolites were the actual inhibitors [86, 91]. Interestingly, targeted inhibition or knockdown of HDAC6 leads to acetylation of Hsp90 and disruption of its chaperone function, resulting in polyubiquitylation and depletion of Hsp90 client proteins, including BCR-ABL [92] and the androgen receptor [93]. Treatment with sulforaphane downregulates HDAC6's deacetylase activity, resulting in hyperacetylation of Hsp90 and inhibition of its association with the androgen receptor. Consequently, the proteasomal degradation of the androgen receptor is accelerated, leading to attenuation of androgen receptor-mediated signaling [93]. Thus, inhibition of Hsp90 activity by either disrupting its association with a co-chaperone or promoting its acetylation is a potential mechanism for HSF1 activation by sulforaphane.

6.5 The Importance of Reactivity with Sulfhydryl Groups for Activation of the KEAP1/NRF2/ARE Pathway and the Heat Shock Response

Reactivity with sulfhydryl groups is the only common feature of the phytochemicals discussed in this *Perspective*. Furthermore, the presence of this "chemical signature" is essential for activation of transcription factors NRF2 and HSF1. Thus, the central carbon of the isothiocyanate (—N=C=S) group of sulforaphane is highly

electrophilic and reacts avidly with sulfhydryl groups. In addition, the isothiocyanates participate in transthiocarbamoylation reactions in which they are readily transferred from one (e.g., glutathione) conjugate to another sufhydryl group-containing molecule [94, 95]. Indeed, this reactivity with sulfhydryl groups underlies the cellular uptake and metabolism of sulforaphane, and the binding to its protein targets, including KEAP1 [96]. Similarly, the electrophilicity of the α,β-unsaturated carbonyl functionality within the chemical structures of celastrol, gedunin, withaferin A, and curcumin makes them highly reactive with sulfhydryl groups, although the epoxide moiety within the structures of gedunin and withaferin A may also contribute to this reactivity. In a high-throughput screen for inducers of the HSF1-dependent heat shock response that used a library of more than 80,000 compounds, the presence of the α,β-unsaturated carbonyl moiety was found to be required for both activation of the heat-shock response as well as for inhibition of glioma tumor cell growth [8]. In a recent large-scale study of approximately 1 million small molecules, electrophilicity was a prominent feature in several of the major clusters of more than 200 activators of the heat shock response that were identified [97]. The importance of sulfhydryl reactivity of the phytochemicals discussed here for activation of the KEAP1/NRF2/ARE pathway and the heat shock response is also supported by studies which have used these agents in combination with classical nucleophiles [5, 97, 98]. Thus, both induction of NRF2-dependent genes and of HSF1 target genes by celastrol are prevented by incubation with dithiothreitol (DTT) [5]. The ability of celastrol to increase the levels of Hsp70, and to decrease the levels of the Hsp90–Cdc37 complex can be reversed by *N*-acetylcysteine or glutathione, but not vitamin C, again implying that sulfhydryl reactivity is critical for these biological effects of celastrol [98]. Similarly, the celastrol-mediated degradation of the Hsp90 client protein ErbB2 is abolished by pretreatment of celastrol with DTT [43]. By use of one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy and application of density functional theory, it was recently shown that nucleophiles (e.g., cysteine and glutathione) add to celastrol regioselectively and stereospecifically to form Michael adducts, such that nucleophilic attack is favored exclusively at the α-face with the nucleophile approach *syn* to the α-C9 methyl [99]. Taken together, these findings imply that cysteine reactivity plays a critical role in triggering both the KEAP1/NRF2/ARE pathway and the heat shock response.

It is now widely recognized that cysteine residues within KEAP1 are the sensors for NRF2 activators. KEAP1 is a multidomain homodimeric protein which has five distinct domains (Fig. 6.4): NTR, N-terminal region (amino acids 1−60), broad complex, tramtrack, bric-á-brac (BTB; amino acids 61−179) which is a dimerization domain, IVR, intervening region (amino acids 180−314) which is a particularly cysteine-rich region containing eight cysteine residues among its 134 amino acids, Kelch domain (amino acids 315−598), through which KEAP1 binds to NRF2, and CTR, C-terminal region (amino acids 599−624). Murine KEAP1 contains 25 cysteine residues among its 624 amino acids (its human homolog has 27 cysteines), nine of which (i.e., C23, C38, C151, C241, C273, C288, C297, C319, and C613) are flanked by basic amino acids. The presence of neighboring basic amino acids is

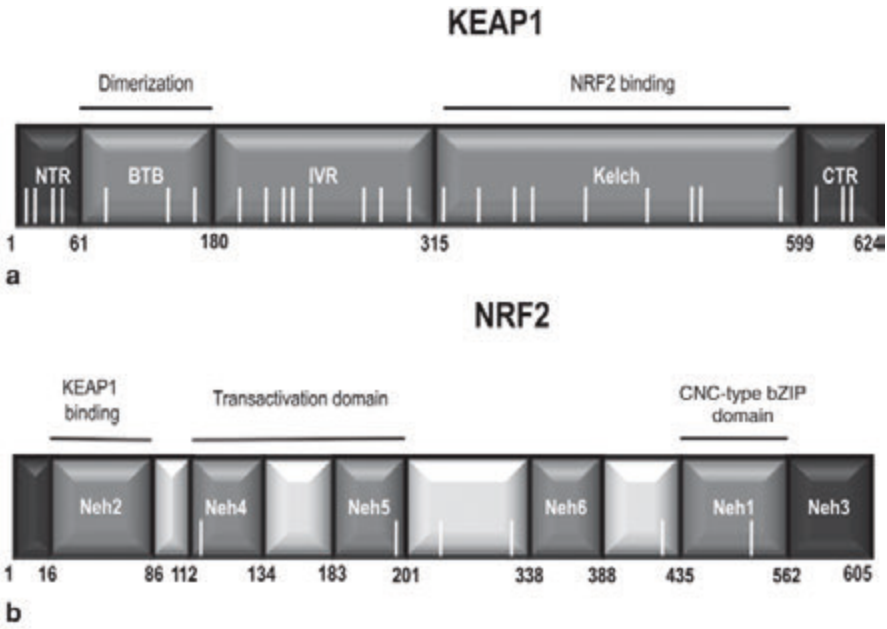


Fig. 6.4 Domain structure of KEAP1 (**a**) and NRF2 (**b**). In KEAP1, the BTB domain is the homodimerization domain and the site of interaction with Cullin 3. The Kelch domain is the NRF2-binding domain. In NRF2, the Neh2 domain is responsible for binding to KEAP1. Neh4 and Neh5 form the transactivation domain, and the Neh1 and Neh3 domains comprise the DNA-binding site of the transcription factor. The white bars indicate the distribution of the cysteine residues within the two proteins

known to lower the pK_a value of cysteine, favoring the formation of the thiolate anion at neutral pH, and thus increasing the cysteine reactivity [100]. Modifications of specific cysteine residues of KEAP1 by sulforaphane, or its sulfoxythiocarbamate derivative STCA, have been detected using both purified recombinant protein as well as lysates of cells that are expressing ectopically KEAP1 following exposure to these compounds [101–104]. By use of mass spectroscopy and mutagenesis analysis, it was established that C151 in the BTB domain, and C273 and C288 in the IVR domain are of particular importance for the repressor function of KEAP1, although depending on the reaction conditions and the experimental system, other cysteine residues can also be modified by sulforaphane, such as C38 in the N-terminal domain, C368, C489, and C583 in the Kelch domain, and C624 in the CTR [101, 104]. Mutation of C151 rendered KEAP1 a constitutive repressor of NRF2, which was unresponsive to induction by sulforaphane [10, 105]. In contrast, substitution of C273 or C288 with either serine or alanine led to complete loss of repressor activity and KEAP1 was unable to repress NRF2 even under basal conditions [105–107]. The increased activity of NRF2 in the presence of C273S/A or C288S/A mutant KEAP1 was caused by reduced ubiquitination of NRF2, resulting in stabilization of the transcription factor [10, 11]. Experiments using transgenic mice expressing

either C273A or C288A KEAP1 mutants confirmed that these residues are required for repression of NRF2 under basal conditions [108].

Two studies—one conducted in zebrafish and another in cultured mammalian cells—have established that KEAP1 contains multiple inducer sensors. The study in zebrafish categorized a series of activators of NRF2 into two groups: those which react with C151 of KEAP1 (e.g., sulforaphane), and those which react with C273 (e.g., 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) [109]. When murine KEAP1 was ectopically expressed in mammalian cells, exposure of these cells to inducers of different types revealed that C151 and C288 each form discrete sensors, and there also exists a third sensor which is formed by H225, C226, and C613 [103]. Molecular modeling showed that C151 is in close spatial proximity with four positively charged amino acids, i.e., K131, R135, K150, and H154, an environment that most likely contributes to the increased reactivity of C151 by decreasing its pK_a and favoring the thiolate formation at physiological pH [103, 110]. Indeed, mutant KEAP1 in which K131, R135, and K150, were substituted with methionine residues had much lower sensitivity to sulforaphane [103]. Another model proposes that binding of inducers to C151 leads to a steric clash with residues in the adjacent α-helix, which may alter the interaction between KEAP1 and Cul3 [111]. It was recently suggested that gedunin may react directly with KEAP1 and impair its ability to target NRF2 for ubiquitination and proteasomal degradation by disrupting the association of KEAP1 with NRF2, although the cysteine reactivity of this phytochemical was not implicated in this model [48].

The importance of cysteine modifications for triggering the heat shock response is also apparent with regard to both HSF1 as well as its negative regulator Hsp90. Human and murine HSF1 contain five conserved cysteine residues. Based on the amino acid sequence of human HSF1, C153 and C373 might be predicted to be particularly reactive with electrophiles; they are in close proximity to basic amino acids (K148, K150, and K157 nearby C153; K372 directly adjacent to C373). An intermolecular disulfide bond formation between C36 and C103 within the human HSF1 has been reported to cause trimerization and DNA binding, whereas formation of an intramolecular disulfide bond (in which C153, C373, and C378 participate) inhibits trimerization and binding to heat shock elements in the promoter regions of heat shock genes [112]. Activation of murine HSF1 by H_2O_2 is dependent on C35 and C105, and the redox regulation of HSF1 is essential for induction of heat shock genes and for protection against thermal and oxidative stress [113]. C35 and C105 are localized within the DNA-binding domain of HSF1, and are required for disulfide bond formation in response to stress.

The reactivity of the cysteine residues of Hsp90 has also been investigated. C521 and C589/590 the rat Hsp90β, corresponding to C529 and C597/C598 in Hsp90α, have been predicted to be highly reactive [114]. Human Hsp90 is a dimeric multidomain protein that contains an N-terminal domain (amino acids 1–210) where ATP binds, a flexible linker region (amino acids 210–272) which affects the function, co-chaperone interaction, and conformation of Hsp90, a middle domain (amino acids 272–629) where many of its client proteins bind, and a C-terminal domain (amino acids 629–732) which contains a dimerization motif (Fig. 6.5) [115, 116].

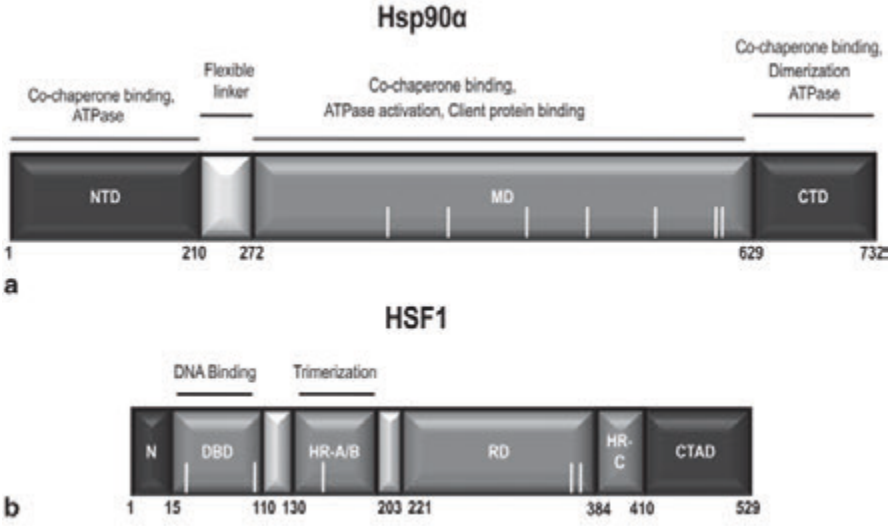


Fig. 6.5 Domain structure of human Hsp90α and HSF1. **a** In Hsp90α, the N-terminal domain (NTD) is the site of binding of ATP and some co-chaperones. The middle domain (MD) is where many of the Hsp90 client proteins and co-chaperones interact, and the C-terminal domain (CTD) contains a dimerization motif and a second ATP-binding site. **b** In HSF1, the DNA-binding domain (DBD) is at the N-terminus of the protein. Trimerization of the transcription factor is through the heptad repeat (HR-A/B) region and is negatively regulated by the HR-C domain. The transactivation domain (CTAD) is at the C-terminus of the protein. The regulatory domain (RD) has a negative regulatory function over the transactivation domain. The *white bars* indicate the distribution of the cysteine residues within the two proteins

In addition, the C-terminal domain has a conserved MEEVD amino acid sequence implicated in binding to co-chaperones with tetratricopeptide repeat (TRP) domains [117, 118], and can also bind to ATP when the N-terminal domain contains one ATP molecule. The binding of ATP causes the N-terminal domains to dimerize and become compacted, allowing them to function as a molecular clamp. Following ATP hydrolysis, the N-terminal domains dissociate. The ATPase cycle of Hsp90 is regulated at multiple levels. It can be stimulated by the co-chaperone Aha1 [119–121] or inhibited by Hop/Sti1 [122–124] and p23/Sba1 [125–129]. Posttranslational modifications of Hsp90 such as acetylation [130], phosphorylation [132, 132], and *S*-nitrosylation [133–137], represent another level of regulation. It has been shown that in human Hsp90α, *S*-nitrosylation at C597 inhibits the ATPase activity of the chaperone [137]. Further work by Retzlaff et al. [138] reported that substituting C597 in human Hsp90α with *S*-nitrosylation-mimicking residues, such as asparagine and aspartic acid, shifts the conformational equilibrium of the protein toward the open conformation, thus decreasing its chaperone activity. This conclusion is also supported by *in silico* studies indicating that C597 is involved in regulating the conformation in Hsp90 [139]. Whereas the identity of the individual cysteine residues of Hsp90 that could be modified by the phytochemicals discussed in this

Perspective is presently unknown, it is notable that C572, adjacent to a lysine residue, has been found to be modified by the electrophilic lipid peroxidation product 4-hydroxy-2-nonenal [140], whereas C521 was identified as the site of thiocarbamoylation when recombinant Hsp90α was incubated with 6-methylsulfinylhexyl isothiocyanate [95]. In agreement, by use of proteomics and click chemistry approaches, Hsp90 was identified as being modified when HEK293 cells were exposed to the sulfoxythiocarbamate derivative of sulforaphane, STCA [9, 102]. Because all cysteine residues reside in the middle client protein-binding domain of Hsp90, it is possible that their modification may lead to disruption of the Hsp90–client protein interactions.

Another possible target for sulfhydryl-reactive phytochemicals is the Hsp90 co-chaperone Cdc37. By use of heteronuclear single quantum coherence (HSQC) NMR spectroscopy, the interaction between the $^{1}H,^{15}N$-labeled N-terminal domain of Hsp90 with unlabeled full-length Cdc37 was investigated in the absence or presence of celastrol [141]. This approach, in combination with mutagenesis analysis and chemical modification (with *N*-ethylmaleimide) of the nine cysteine residues of Cdc37, revealed that celastrol is able to react with the three cysteine residues within the N-terminal domain of Cdc37. Reaction with celastrol leads to large conformational changes both in the N-terminal and in the middle Hsp90-binding domains of Cdc37, ultimately disrupting the Cdc37–Hsp90 association. As Cdc37 is an essential component of the Hsp90 complex machinery, its displacement from the complex will undoubtedly affect the function of the chaperone. In sum, the exact protein targets of the phytochemicals discussed here, which lead to activation of the heat shock response, are incompletely understood, likely to be multiple, and represent a subject of intense investigations by many laboratories. However, it is clear that the reactivity with cysteine residues within those protein targets is critical for the underlying mechanism(s).

It is noteworthy that activation of NRF2 generally occurs at lower inducer concentrations than those that activate HSF1. The most likely reason for this observation could be the relative nucleophilicity of the cysteine residues and their accessibility within the structure of the protein targets for reaction with the electrophilic inducers. Whereas the identification of the precise reason(s) requires further investigation, based on the available experimental evidence it could be proposed that, at low concentrations (Fig. 6.6a), inducers react first with KEAP1, activating the KEAP1/NRF2/ARE pathway. At higher inducer concentrations (Fig. 6.6b), a second target, such as Hsp90, or a co-chaperone within the Hsp90 complex machinery, is also affected, leading to induction of the heat shock response.

6.6 Conclusions

Celastrol, withaferin A, gedunin, curcumin, and sulforaphane are examples of structurally diverse phytochemicals with a common chemical signature: reactivity with sulfhydryl groups. This reactivity underlies their biological activities as

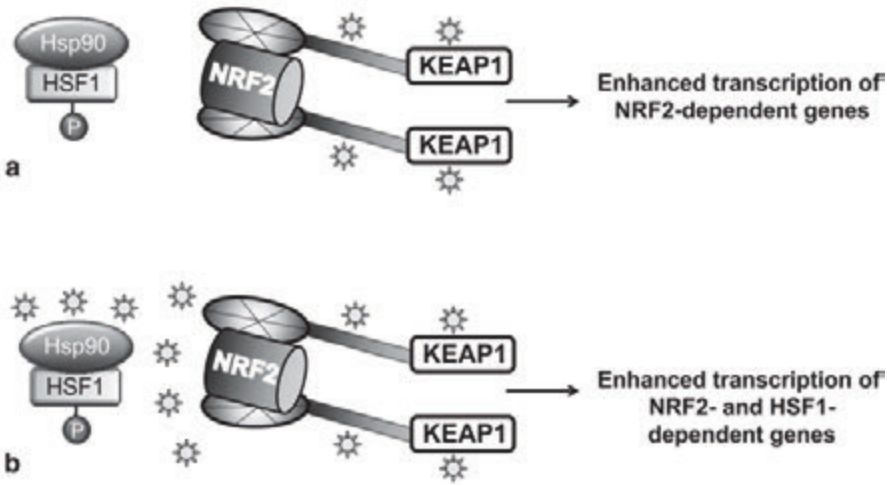


Fig. 6.6 Proposed model for concentration-dependent activation of the KEAP1/NRF2/ARE pathway and the heat shock response by sulfhydryl-reactive phytochemicals. **a** At low concentrations, inducers (depicted with solar symbols) react first with cysteine residues of KEAP1, activating transcription of NRF2-dependent genes. **b** At higher concentrations of inducers, a second target, such as Hsp90, HSF1, or a co-chaperone within the Hsp90 complex machinery, is also affected, leading to enhanced transcription of NRF2- and HSF1-dependent genes

multitarget agents for which protective effects have been documented in numerous animal models of human disease. The effects of such phytochemicals in biological systems are long lasting and comprehensive as they are due to induction of large networks of transcriptional programs regulated by transcription factors NRF2 and HSF1. The resulting upregulation of cytoprotective proteins provides the cell with multiple layers of protection against electrophiles, oxidants, and chronic inflammation, which are the major causes for the development of chronic degenerative conditions, such as cancer, cardiovascular disease, and neurodegeneration. Notably, lower concentrations of phytochemicals are required for induction of NRF2-dependent genes than those which induce HSF1-dependent responses, suggesting that activation of NRF2 precedes that of HSF1. It can be hypothesized that the KEAP1/NRF2/ARE pathway functions to defend against imminent danger. It is then followed by the heat shock response to protect against a potentially devastating damage and preserve the proteome. Collectively, the two pathways ensure adaptation and survival.

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Chapter 7
Chemical and Biological Mechanisms of Phytochemical Activation of NRF2 and Importance in Disease Prevention

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Abstract Plants are an incredibly rich source of compounds that activate the Nrf2 transcription factor, leading to upregulation of a battery of cytoprotective genes. This perspective surveys established and proposed molecular mechanisms of Nrf2 activation by phytochemicals with a special emphasis on a common chemical property of Nrf2 activators: the ability as "soft" electrophiles to modify cellular thiols, either directly or as oxidized biotransformants. In addition, the role of reactive oxygen/nitrogen species as secondary messengers in Nrf2 activation is discussed. While the uniquely reactive C151 of Keap1, an Nrf2 repressor protein, is highlighted as a key target of cytoprotective phytochemicals, also reviewed are other stress-responsive proteins, including kinases, which play nonredundant roles in the activation of Nrf2 by plant-derived agents. Finally, the Perspective presents two key factors accounting for the enhanced therapeutic windows of effective phytochemical activators of the Keap1–Nrf2 axis: enhanced selectivity toward sensor cysteines and reversibility of addition to thiolate molecules.

Abbreviations

Nrf2	NF-E2-related factor 2
ARE	antioxidant response element
ROS/RNS	reactive oxygen/nitrogen species
QR1	NAD(P)H: quinone oxidoreductase 1
wt	wild-type
GSH	glutathione
NAC	*N*-acetylcysteine
EGCG	epigallocatechin gallate

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tBHQ	*tert*-butylhydroquinone
DAS	diallylsulfide
CYP2E1	cytochrome P450 2E1
DASO	diallyl sulfoxide
$DASO_2$	diallyl sulfone
I3C	indole-3-carbinol
DIM	3,3′-diindolylmethane
3MI	3-methylindole
CYP	cytochrome P450
AhR	aryl hydrocarbon receptor
β-NF	β-naphthoflavone
ERβ	estrogen receptor β
QR2	quinone reductase 2
SOD	superoxide dismutase
HO-1	heme oxygenase 1
BAECs	bovine aortic endothelial cells
GSTs	glutathione *S*-transferases
MRPs	multidrug resistance-associated proteins
PI3K	phosphatidylinositol 3-kinase
MAPKs	mitogen-activated protein kinases
PEITC	phenethyl isothiocyanate
PKC	protein kinase C

7.1 Introduction

Numerous phytochemicals have shown great promise for prevention and treatment of various human diseases. For example, ClinicalTrials.gov lists 25 different intervention trials investigating the effects of standardized preparations of broccoli sprouts. These trials include the amelioration of symptoms of diseases as diverse as autism,[1] cystic fibrosis, influenza, asthma, and chronic obstructive pulmonary disease. The trials also encompass studies on the prevention of breast, lung, and prostate cancers, carcinogenesis from aflatoxin exposure, and cardiovascular disease. The purpose of this Perspective is to (1) review a key molecular mechanism of phytochemicals in the prevention and amelioration of diseases: the activation of the transcription factor NF-E2-related factor 2 (Nrf2); (2) outline common chemical features of potent Nrf2 activators; and (3) provide perspectives for harnessing these features for more effective disease prevention or treatment.

[1] ClinicalTrials.gov identifiers, in order as listed above—amelioration studies: NCT01474993, NCT01315665, NCT01269723, NCT01183923, and NCT00994604; prevention studies: NCT00982319, NCT00255775, NCT00607932, NCT01437501, and NCT00252018.

7.2 Upregulation of Cytoprotective Genes by Nrf2

Oxidative stress and associated inflammation contribute to the progression of many chronic degenerative diseases in humans [1, 2]. Exposure to oxidant and electrophilic agents from air, water, food, and other environmental sources has also been implicated in a large (70–90 %) component of cancer and cardiovascular disease risks [3]. The Nrf2 transcription factor has emerged as a key player in protecting cells against various intrinsic and extrinsic assaults. Nrf2 regulates more than 600 genes, including over 100 that encode cytoprotective proteins [4], named for their ability to protect cells against oxidative stress, reactive electrophilic species, and other types of stress (reviewed in [5]). In brief, these proteins include antioxidant enzymes, NADPH regeneration enzymes, glutathione (GSH) biosynthesis enzymes, heat shock proteins, enzymes that facilitate the elimination of xenobiotic toxicants such as detoxification enzymes and drug-efflux pumps, as well as subunits of the 26S proteasome. Collectively, these Nrf2-regulated genes share at least one copy of the antioxidant response element (ARE, $5'\text{-}^{A}/_{G}TGA^{C}/_{G}{}^{T}/_{A}{}^{C}/_{N}{}^{A}/_{N}GC^{A}/_{T}\text{-}3'$) in their promoter region [4]. Nrf2 binds to the ARE as a heterodimer with one of several small Maf transcription factors, leading to upregulation of gene transcription.

Upregulation of this battery of cytoprotective proteins through Nrf2 activation is a critical component of an organism's ability to cope with intrinsic and extrinsic stress factors, including inflammation, reactive oxygen/nitrogen species (ROS/RNS), shear stressors such as endothelial stressors, and environmental toxins. Studies on *Nrf2*$^{-/-}$ mice, which have low and largely noninducible levels of many cytoprotective proteins [4], serve as striking and comprehensive examples of the importance of these proteins in maintaining health and preventing disease. Nrf2-deficient mice are prone to develop disorders that are caused by ROS and inflammation, including macular degeneration [6], neurodegeneration in a murine model of Parkinson's disease [7], cardiac disorders [8, 9], and chemically induced tumorigenesis [10–12]. Furthermore, *Nrf2*$^{-/-}$ mice are more susceptible to damage of the blood–brain barrier following brain injury [13], formation of carcinogen–DNA adducts in the lung exposed to diesel exhaust [14], acute pulmonary injury induced by butylated hydroxytoluene [15], and hepatic damage by acetaminophen [16]. In addition, they are deficient in their intrinsic capacity for skin wound healing [17]. Since loss of Nrf2 increases pathological cell and tissue damage in response to intrinsic and extrinsic factors, it is believed that upregulation of Nrf2 serves both cytoprotective and preventive roles in diverse pathophysiological situations.

In support of the importance of Nrf2 in preventing human diseases, inherited DNA polymorphisms that reduce the abundance of Nrf2 are associated with various pathologies, including chronic gastritis, ulcerative colitis, skin pathologies such as skin vitiligo, and adult respiratory distress syndrome (reviewed in [18]). In fact, a number of clinical trials that assess the effects of broccoli sprouts include direct measurements of Nrf2 activation. For example, Nrf2 levels, along with markers of oxidative stress, will be assessed after administration of macerated broccoli sprouts

in both healthy volunteers and those with cystic fibrosis,[2] in nasal epithelial cells obtained by curettage, as well as in alveolar macrophages and bronchial epithelial cells of patients with chronic obstructive pulmonary disease.[3]

7.3 Phytochemical Activation of Nrf2

7.3.1 Overview

Plants have been an incredibly rich source for the identification of compounds that activate cytoprotective genes. The development of a simple microtiter-plate-based assay [19] to assess induction of the cytoprotective enzyme NAD(P)H: quinone oxidoreductase 1 (QR1) in mouse Hepa1c1c7 cells has greatly facilitated the ability to screen for and identify many cytoprotective phytochemicals. For example, a collective effort of colleagues at the University of Illinois at Chicago and Purdue University has identified 66 compounds from 18 plant species that are active in the QR1 assay [20]. Representative phytochemicals that have been shown to activate cytoprotective genes are listed in Table 7.1.

Several plant families important for human diets are particularly rich in ARE inducers. For example, many organosulfur activators have been isolated from garlic and onion, edible members of the *Allium* family. The *Cruciferae* family of vegetables, including broccoli, cabbage, Brussels sprouts, horseradish, mustard, and watercress, produces a particularly large and functionally diverse number of potent ARE inducers (Table 7.1). These plants contain glucosinolates, the thioglucoside conjugates of the ARE-activating species. Altogether, over 120 glucosinolates have been identified from various plants [21]. These are enzymatically converted to the ARE-inducing forms either by myrosinase, a thioglucosidase that is localized in a separated cellular compartment and is released upon maceration or chewing, or by intestinal microflora after ingestion [22]. Three general classes of ARE inducers produced by myrosinase-catalyzed hydrolysis of glucosinolates are isothiocyanates, indoles, and epithionitriles (Table 7.1). The enzymatic hydrolysis mechanisms involved in their release are reviewed elsewhere [23, 24]. Finally, phenolic compounds, another important class of Nrf2-activating agents, have been isolated from diverse plant families, including grapes (*Vitaceae*) and teas (*Theaseae*), which are rich sources of flavonoids and related catechins.

Importantly, *Nrf2*$^{-/-}$ mice experiments highlight the key role of Nrf2 in mediating the cytoprotective effects of the phytochemicals discussed above. Thus, sulforaphane, one of the active components of broccoli sprout extracts, has been reported to inhibit skin [12] and forestomach [10] carcinogenesis in wild-type (wt) mice, but its ability to do so is significantly attenuated in *Nrf2*$^{-/-}$ animals. In addition,

[2] Clinicaltrials.gov identifier NCT01315665.

[3] Clinicaltrials.gov identifier NCT01335971.

Table 7.1 Structures of phytochemicals that upregulate cytoprotective enyzmes through Nrf2

Structural/functional classes	Representatives	Plant sources
Chalcone, MA, phenol	Isoliquiritigenin	Licorice, shallot, tonka bean
Chalcone, MA, phenol	Xanthohumol	Hops
Cinnamate, 1,2-diphenol	Caffeic acid	Lignin-containing plants
Cinnamate, 1,2-diphenol	Ferulic acid	Apples, cabbages, plums
Diarylheptanioid, MA, 1,2-diphenol	Curcumin	Turmeric
Diarylheptanioid, MA, 1,2-diphenol	Yakuchinone B	*Alpinia oxyphylla* Miquel
Masked 1,2-diphenol	Capsaicin	Spicy peppers
Terpenoid 1,2-diphenol	Carnosol	Rosemary
Flavanol, 1,2,3-triphenol	EGCG	Green tea
1,2-Diphenol	Ellagic acid	Berries
Flavonoid mixture	Silymarin (mixture)	Milk thistle
Flavanone, masked 1,2-diphenol	Silibin (single compound)	Milk thistle
Masked 1,2-diphenol, MA	10-Shogaol	Ginger
Flavone, phenol	Apigenin	Chamomile, thyme
Flavone, phenol	Luteolin	Celery, thyme
Isoflavone, phenol	Genistein	Soy
Isoflavone, phenol	Biochanin A	Red clover
Flavonol, 1,2,6-triphenol	Quercetin	Apples, capers
Flavonol, 1,6-diphenol	Kaempferol	Apples, broccoli, tea
Flavonol, 1,2,6-triphenol	Fistein	Acacia, mangos
Flavonol, phenol	Galangin	*Alpinia officinarum*
Trans-stilbene, phenol	Resveratrol	Grape skins, berries
Trans-stilbene, 1,2-diphenol	Piceatannol	Grape skins, berries
Withanolide, MA	18-Hydroxywithanolide D	Tomatillo
Withanolide, MA	Withaphysacarpin	Tomatillo
Norwithanolide, MA	Subtrifloralactone A	*Deprea subtriflora*
Coumarin, MA (?)	Coumarin	Leguminosae spp.
Butenolide, MA (?)	β-Angelica lactone	*Archangelica officinalis*
Coumarinoid, MA (?)	Auraptene	Citrus
Terpenoid, furan	Cafestol	Green coffee beans
Terpenoid, furan	Kahweol	Green coffee beans
Terpenoid, MA	Zerumbone	Tropical ginger
Terpenoid, MA	Citral (geranial)	Lemongrass
Terpenoid, MA	Celastrol	Thunder of God vine (*T. wilfordii*)
Terpenoid (carotene)	Lycopene	Tomatoes
Phytosterol, MA	*E*-Guggulsterone	*Commiphora mukul*
Alkaloid, MA	Cryptolepinone	Sida acuta
Organosulfide	Allicin	Garlic
Organosulfide	Diallyl disulfide	Garlic
Organosulfide	Diallyl trisulfide	Garlic
Organosulfide	Dilallyl sulfide	Garlic
Organosulfide	S-allyl cysteine	Garlic
Isothiocyanate	Sulforaphane	Broccoli
Isothiocyanate	Phenethyl ITC	Turnips, watercress
Isothiocyanate	6-Methylsulfinylhexyl ITC	Wasabi
Indoles	Indole-3-carbinol > DIM[a]	Brussels sprouts, cabbages
Indoles	Brassinin	Chinese cabbage
Epithionitrile	1-Cyano-2,3-epithiopropane	Cabbages
Epithionitrile	1-Cyano-3,3-epithiobutane	Cabbages

MA Michael acceptor, *ITC* isothiocyanate

[a] As described in the text, indole-3-carbinol is a precursor for diindolylmethane (DIM), which is considerably less toxic

sulforaphane was able to protect the blood–brain barrier post injury only in wt Nrf2 mice [13]. Similarly, resveratrol, a flavonoid-like molecule produced by many plants, protected against high-fat-diet-induced oxidative stress in aortas of wt but not *Nrf2*$^{-/-}$ mice [9].

7.3.2 The Chemistry Required for Phytochemicals to Activate Nrf2

7.3.2.1 Reactivity with Thiolates

Despite the high level of overall structural diversity among ARE inducers, many cytoprotective phytochemicals from different plant sources share common thiol-reactive chemical motifs (collectively shown in Fig. 7.1 as red moieties). For example, the presence of an α,β-unsaturated carbonyl group, a potential Michael acceptor, is a particularly common feature in ARE inducers, including withanolides (e.g., withaphysacarpin), chalcones (e.g., isoliquritigenin), butenolides (e.g., β-angelica lactone), oxidized terpenoids (e.g., zerumbone, *E*-guggulsterone, and citrals), and curcuminoids (e.g., curcumin). Other inducer classes are similarly electrophilic epithionitriles (e.g., 1-cyano-2,3-epithiopropane), isothiocyanates (e.g., sulforaphane), and organopolysulfides (e.g., allicin). Talalay and colleagues in 1988 first recognized that the diverse structures share the ability to react with thiolate groups [25]. They hypothesized that gene induction takes place by virtue of an intracellular sensor that contains one or more reactive cysteine residues, modification of which by inducing agents would lead to target gene activation. This seminal hypothesis is supported by numerous studies that followed, which directly linked the presence of particular functional groups to Nrf2–ARE induction, as shown with zerumbone [26], chalcones [27], flavonoids [28], and withanolides [20, 29]. Furthermore, a strong correlation has been identified between inducer potencies and chemical reactivities toward thiolates [30].

Finally, in 1999 a key repressor of Nrf2 was discovered, the Keap1 protein, which was found to possess an unusually large number of cysteines (25 and 27 in the mouse and human proteins, respectively) [31]. As described in detail in Sect. 7.3.4.1, a subset of these cysteines, C151 in particular, has been found to be important for Nrf2 activation by phytochemicals.

7.3.2.2 Phytochemicals that Do Not Have the Ability to React with Thiolates

While the critical feature of an Nrf2 activator appears to be the ability to react with thiolates, a large number of phytochemicals, including phenols, monosulfides, furans, and indoles, would need to acquire this property through metabolic and/or chemical processing. Studies on the biotransformations of many such molecules are surprisingly limited, despite the current interest in the health benefits of phy-

tochemicals. Herein, we summarize the available experimental evidence from the literature implicating mechanisms of converting phytochemicals to thiol-reactive species. We also discuss two classes for which transformation to a thiolate-reactive species is considerably more difficult to ascertain, carotenoids and 1,3-polyphenols.

Electron-rich phenolic compounds featuring "additive" distribution of electron-donating groups are one relatively well-characterized chemical class of phytochemicals that require electrophilic conversion for inducer activity (Fig. 7.1, blue structural fragments). These are compounds that contain phenolic hydroxyl groups in a conjugated system with an even number of carbons separating them. Representative examples of this class include 1,2-diphenols (e.g., epigallocatechin gallate (EGCG), caffeic acid, and piceatannol) and vinylogous 1,6-diphenols, with an alkene spacer acting as the "electron conduit" (e.g., quercetin and kaempferol). The 1,2- 1,4-, and 1,6-diphenols, but not the mono- or 1,3-dihydroxy variants, can be readily oxidized in vivo by a variety of mechanisms (Fig. 7.2) to the corresponding quinoids and will be referred to herein as quinoid-forming phenols. In fact, such phenols have been associated with induction of carcinogen-detoxifying enzymes by one of the earliest observations in the field [32]. The resulting Michael acceptors can then readily react with cysteine thiolates (Fig. 7.2). For example, the well-characterized flavonoid quercetin is expected to be a very weak electrophile intrinsically due to the electron-donating effect of the 3-hydroxyl group and both aromatic rings (see Fig. 7.3a for numbering scheme). However, in human blood plasma, quercetin is readily oxidized to a significantly more electrophilic quinone methide [33] (Fig. 7.3a). Importantly, the oxidized species is much more reactive toward thiolates, and its conjugation products have been detected with GSH, N-acetylcysteine (NAC), cysteine [34, 35], and protein cysteine residues [36]. Similarly, EGCG contains several aromatic 1,2-dihydroxy units, and thus can form quinones that react with isolated and protein thiolates, as demonstrated in both biochemical experiments and cells [37, 38]. EGCG has also been found in mouse urine as the S-cysteinyl–EGCG conjugate after a high oral dose [39]. Furthermore, the oxidative conversion of phenolic compounds to Michael acceptors has been shown to correlate strongly with cytoprotective enzyme induction in studies evaluating *tert*-butylhydroquinone (tBHQ) [40] and EGCG analogs [38], as well as a broad series of phenols [41]. As further evidence that the oxidation of phenols is a prerequisite to ARE induction, Cu^{2+}or other oxidized transition metal cations in the media strongly stimulated the ARE induction potential of *para*- and *ortho*-hydroquinones [42]. These metal ions act as catalysts in the oxidation of phenols to Michael reaction acceptors (Fig. 7.2) under aerobic conditions. Importantly, transition metal salts had no effect on inducer activity of the corresponding quinones or sulforaphane [43].

The organosulfur compounds from garlic and onions were shown by the Wattenberg group in 1988 to have interesting structural requirements for inducer activity [44]. Garlic organopolysulfides and derivative thiosulfinates, such as allicin (Fig. 7.1), were able to induce the cytoprotective enzyme glutathione S-transferase (GST), as might be expected from their ability to modify cellular thiols. However, the monosulfide diallylsulfide (DAS) is also an inducer, and the mechanism by which it activates Nrf2 is yet to be delineated. In addition, the diallyl forms of the

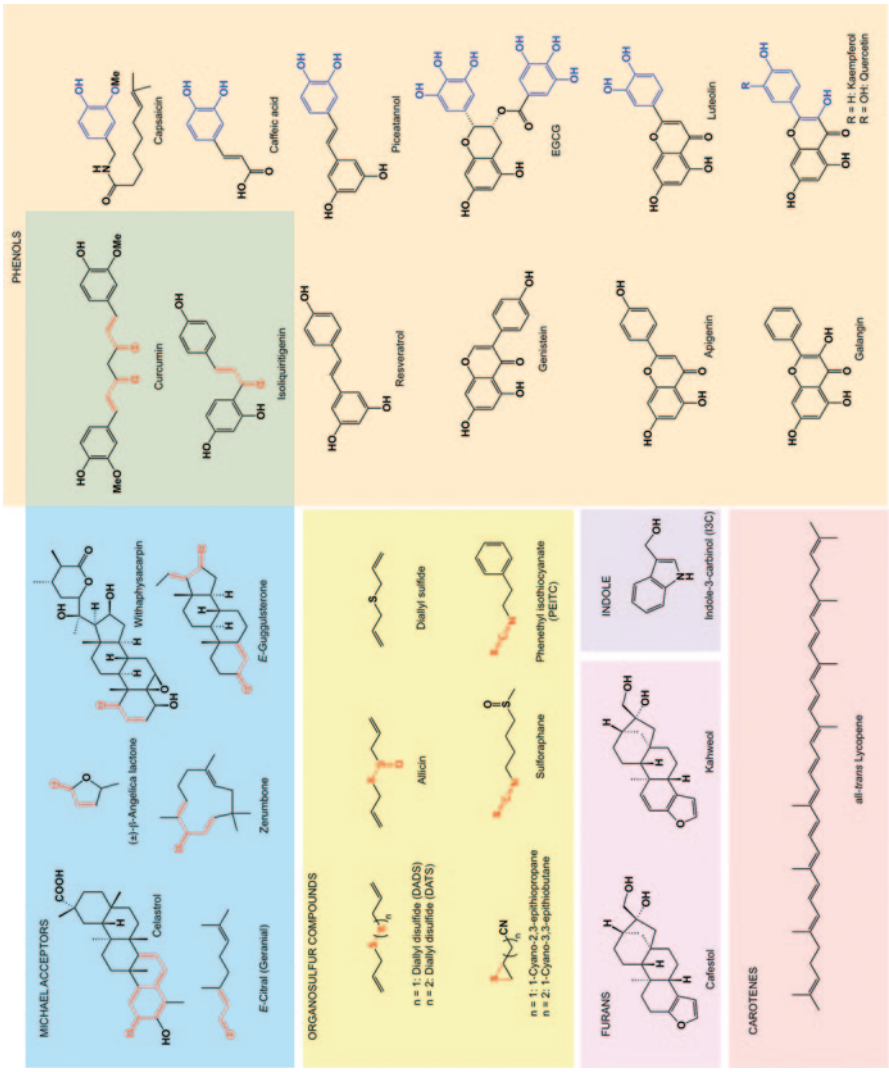


Fig. 7.1 Structures of phytochemicals arranged by the chemistry that leads to Nrf2 activation. All compounds shown activate Nrf2. Thiol-reactive chemical motifs are shown in *red*, and "additive" distribution of electron-donating groups, which can be oxidized to quinoids, are shown in *blue*

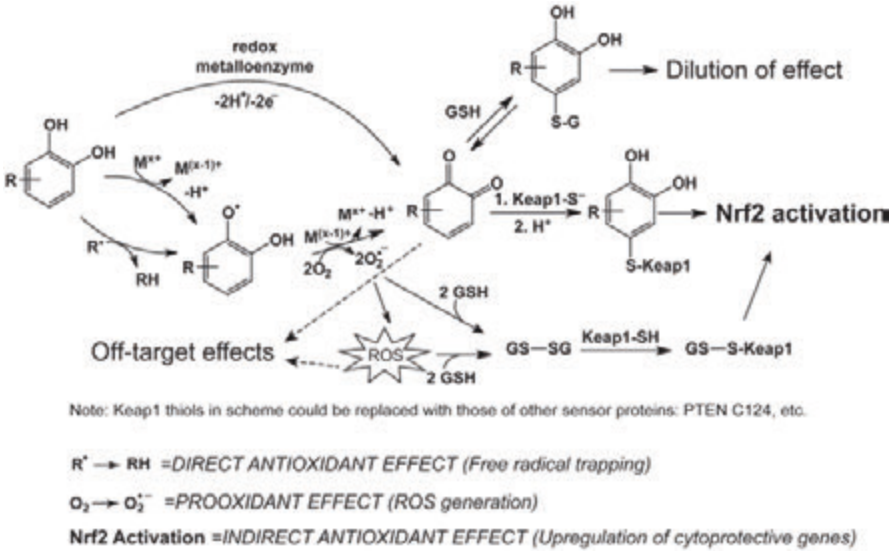


Fig. 7.2 The three roles a quinoid-forming polyphenol (represented by the R-catechol) can play: prooxidant, direct antioxidant, and indirect antioxidant. As a *direct antioxidant*, in the presence of a high concentration of free radical species, a polyphenol can trap the radical, forming a relatively stable radical species. As a *prooxidant*, in the presence of catalytic amounts of a transition metal, a polyphenol can promote the formation of superoxide and other ROS, en route to formation of a Michael acceptor. An alternate path to oxidation of the polyphenol is catalyzed by a metalloenzyme and occurs without the production of ROS. Once the quinoid group is formed, the Michael acceptor group can react with a thiolate molecule. There is evidence that a quinone reacts with a key Keap1 sensor cysteine, C151, leading to Nrf2 activation, as described in the text. Upon activation, Nrf2 upregulates a battery of antioxidant enzymes and other cytoprotective enzymes, known as the *indirect antioxidant* effect. Reaction of the quinoid with GSH and subsequent elimination from the cell will lead to dilution of the effect. Alternative mechanisms of Nrf2 activation by radicals or ROS not depicted are oxidation of sensor cysteines, or formation of disulfides among sensor cysteines

di- and tri-sulfides were much more potent than the propyl version. In surmising why the diallyl sulfides might have higher potency, it is interesting to note that cytochrome P450 2E1 (CYP2E1) is implicated in sequential conversion of the sulfide to the corresponding sulfoxide (DASO) and sulfone ($DASO_2$) derivatives [45]. Further oxidation of the sulfone metabolite generated an electrophile that was shown to act as a suicide inhibitor of CYP2E1, as well as other unidentified cytochromes implicated in bioactivation of various cytotoxins [45]. While the exact mechanism of this inhibition remains to be established, the irreversible nature of the antagonism implies generation of a reactive intermediate capable of covalent modification of the enzyme involving, in all likelihood, an active-site cysteine [46]. In addition, liquid chromatography–mass spectrometry (LC–MS/MS) analysis of bile fluids from rats treated with DAS, DASO, and $DASO_2$ identified several GSH conjugates, implicating epoxidation of the allylic group as an important metabolic activation step for all three compounds [47]. Unlike the epoxides of DAS and DASO, the $DASO_2$-

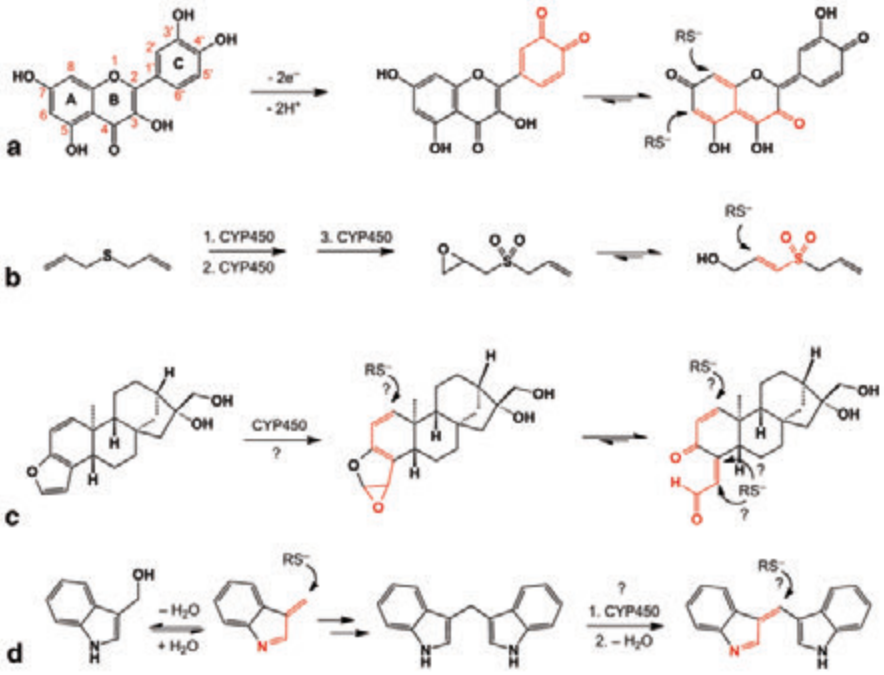


Fig. 7.3 Biotransformations implicated in conversions of quercetin (**a**), DAS (**b**), coffee triterpenoids (kahweol shown here) (**c**), and I3C and DIM (**d**) into thiol-reactive conjugated electrophiles: quinoids (quercetin), sulfone (DAS), epoxide or γ-ketoenal (kahweol), and indolenines (I3C and DIM). The established or proposed reactive groups are highlighted in *red*

derived epoxide provides a unique entry into a sulfone-activated Michael acceptor containing a sulfonylprop-2-en-1-ol group (see Fig. 7.3b for the proposed mechanism). This so-called "soft" electrophile, with highly distributed charge density, is much more likely to react with a thiolate, a similarly polarizable "soft" nucleophile, rather than the "hard" epoxide (see Sect. 7.3.3 for a brief discussion of hard and soft reagents and other factors affecting electrophile chemoselectivity). Therefore, oxidation of the sulfide, in combination with epoxidation of the allylic group, could be responsible for the inactivation of CYP2E1 and, possibly, induction of Nrf2 by modifying sensor cysteines.

Furan-containing compounds, such as the diterpenes cafestol and kahweol, are known to act as the principal Nrf2 activators in coffee despite lacking a thiol-reactive functional group [48, 49]. Although the exact mechanism of induction is yet to be established, a recent mass spectrometric study of bile fluids from mice injected with cafestol was interpreted by the authors to suggest that epoxidation is the key step in converting coffee furans into thiol-reactive species [50]. However, the study did not distinguish between direct addition to the epoxide and addition at a remote double bond conjugated to the epoxide or Michael addition to a γ-ketoenal, arising from a ring-opening reaction of the oxidized furan (depicted in Fig. 7.3c).

The distinction is important because furan epoxides and corresponding dicarbonyl derivatives have long been associated with severe cytotoxicity of furan-containing compounds [51], due to both high reactivity and a tendency to react with oxygen and nitrogen nucleophiles, in addition to thiolates. This, however, is inconsistent with the generally cytoprotective properties of the coffee furans. The particular structural environment of electrophiles derived from these furans may account for the observed shift in the balance of toxic and protective effects (Fig. 7.3c). Thus, the kahweol epoxide presents a doubly conjugated epoxide (epoxydiene) requiring a soft thiolate nucleophile to attack the soft electrophilic center next to a highly sterically congested quaternary carbon. The corresponding ketoenal is also deactivated toward nonspecific additions by both cross-conjugation of the dienone functionality and β,β-dialkyl substitution of the unsaturated aldehyde [52]. Alternatively, participation of less reactive metabolites, such as conjugated lactones, for example, which have been detected as metabolic derivatives of furan-containing compounds [53], may account for the low toxicity of these phytochemicals. Further studies will be required for understanding the unique properties of these furans.

Indole-3-carbinol (I3C), a glucosinolate breakdown product, and its digestive product 3,3′-diindolylmethane (DIM) are ARE inducers [54] and have many other cancer chemopreventive effects [55]. However, while DIM has been described as an effective inducer of the ARE with little associated long-term toxicity [56], I3C has a weak level of Nrf2 activation and is associated with a complex interplay of pro- and anticarcinogenic effects (for a review see [56]). Although no specific mechanism accounting for the ARE induction ability of DIM has been established, reaction schemes for producing electrophilic species capable of covalent modification of thiols could be inferred from known metabolic and chemical transformations of I3C, DIM, and 3-methylindole (3MI), a by-product of tryptophan metabolism by intestinal microflora [57]. In the case of the last compound, a highly electrophilic thiolate-reactive derivative, 3-methyleneindolenine, an α,β-unsaturated imine, is produced via cytochrome P450 (CYP)-mediated dehydrogenation [58]. This species is postulated to be responsible for the cytotoxicity of this substituted indole [58, 59]. The reactivity of the α,β-unsaturated imine toward nucleophiles is promoted by a concomitant rearomatization of the indole nucleus, which makes it a rather nondiscriminating and thus toxic agent, capable of modifying a variety of functionalities found in a cellular environment. 3-Methyleneindolenine also serves as an intermediate in the dehydration–conjugate addition–retro-aldol cascade in the acid-catalyzed conversion of I3C to DIM [60] (Fig. 7.3d). Therefore, the low level of Nrf2 activation and pro- and anticarcinogenic effects associated with I3C could be explained by the formation of both the toxic electrophile and DIM, respectively, in the course of the same chemical process. On the basis of established biotransformations of DIM [61], it is tempting to propose that an oxidation event similar to that seen for 3MI can lead to the extended conjugation-stabilized Michael acceptor, 3-(3-indolylmethylene)-indolenine (Fig. 7.3d). We must note that no thiol conjugates of DIM metabolites have been isolated, perhaps, due to reversibility of such additions to the conjugation-stabilized electrophile (see Sect. 7.3.3 for further discussion of the importance of reversibility). Significantly, a sulfate-conjugated

hydration product of the proposed species has been isolated as one of the major biotransformants from cultured cancer cells [61]. The Michael acceptor produced by both I3C and 3MI may be the principal agent responsible for associated toxicities. Unlike 3MI, however, the biotransformation of I3C can also lead to DIM, a more stable indole derivative [62] that is unlikely to produce a nondiscriminating electrophile. This example underscores the fact that the level of reactivity of phytochemically derived electrophiles could be the key determinant in a sensitive balance of cytotoxic and cytoprotective effects.

Carotenoids, lycopene in particular, have been shown to activate the ARE via Nrf2 [63]. The carotenes are unsaturated hydrocarbons and thus contain no thiol-reactive species. However, the authors point out that the ethanolic extract of the lycopene preparation, containing unidentified hydrophilic lycopene derivatives, activated the ARE with a similar potency as lycopene. Therefore, it seems likely that oxidation of the polyene, leading to formation of an electrophilic species (e.g., citral [64]), is a prerequisite for Nrf2 activation.

In addition, there are a handful of phenols (non-quinoid forming) that have no electrophilic moieties, or facile nonenzymatic autoxidative paths to obtaining these moieties, which have been shown to activate cytoprotective enzymes, such as resveratrol [65] and galangin [28]. The Nrf2-dependence of ARE activation by resveratrol has been particularly well established in studies in *Nrf2*$^{-/-}$ mice [9] and normal human small airway epithelial cells (SAECs) [66]. Resveratrol and other related phenols can be hydroxylated to quinoid-forming species by a member of the cytochrome P450 (CYP) superfamily of monooxygenases, such as pro-carcinogen activating CYP1A1, CYP1A2, and CYP1B1 [67, 68]. These enzymes are transcriptionally controlled by the aryl hydrocarbon receptor (AhR). So-called bifunctional ARE inducers, such as β-naphthoflavone (β-NF), activate Nrf2 by first binding to and activating AhR, which in turn leads to upregulation of CYP enzymes [69]. However, resveratrol is a known inhibitor of AhR [70, 71], and therefore in uninduced normal cells resveratrol may not be hydroxylated by CYPs prior to Nrf2 activation. Resveratrol activation of QR1 through the estrogen receptor β (ERβ) in breast cancer cells has been explored as another mechanistic possibility. Various studies support a model in which binding of phytoestrogens, including resveratrol, to ERβ causes ERβ to bind and activate the QR1 ARE [72–75]. However, this mechanism appears to be restricted to cancer cells overexpressing ERβ [75]. A third mechanism has been suggested based on the observation that resveratrol binds to and inhibits quinone reductase 2 (QR2) with low nanomolar affinity [76]. QR2's function is not clearly elucidated, but it is known to catalyze the reduction of quinones, among several other classes of electron-deficient compounds. The authors suggested that resveratrol may activate Nrf2 by inhibiting QR2, resulting in the accumulation of endogenous quinones that can then induce electrophilic stress by modifying cellular thiols [76]. A similar indirect induction mechanism could apply to other phytochemical inhibitors of QR2, including quercetin, kampferol, apigenin, or genistein, that inhibit this reductase at physiologically relevant concentrations [76, 77]. This hypothesis warrants further investigation. Finally, a fourth mechanism accounting for activation of Nrf2 by non-quinoid-forming phenols by means of kinase activation is explored for genistein in Sect. 7.3.4.3.

7.3.2.3 Role of ROS in Nrf2 Activation by Phenolic Compounds

One important area of consideration is to what extent ROS are involved in the activation of Nrf2 by phytochemical inducers. Phenolic ARE inducers can play multiple roles in the redox status of a cell (Fig. 7.2). They are able to act as both direct antioxidants, scavenging free radicals, and, if converted to thiolate-reactive electrophiles, as indirect antioxidants by upregulating antioxidant genes [78]. Depending on the experimental conditions, they can also act as pro-oxidants [79]. As shown in Fig. 7.2, the nonenzymatic autoxidation of a polyphenol can lead to ROS formation. This generation of ROS may play a role in Nrf2 activation, as well as have potential off-target effects such as toxicity. The ability of ROS to upregulate Nrf2 is well established, for example by treatment of cells with H_2O_2 [80]. One mechanism by which ROS may activate Nrf2 is depicted in Fig. 7.2. In this scenario, the generation of superoxide and other ROS can lead to oxidation of GSH to GSSG [81]. GSSG then could modify sensor thiolates such as Keap1 cysteines [82, 83], thereby activating Nrf2 (see also Sect. 7.3.4.1). In addition, sensor thiolates can be directly oxidized by ROS (reviewed in [84] and [85]).

There are indications that ROS mediate signaling for some ARE inducers. For example, the generation of ROS in cells [86, 87] and cell media [88] by EGCG has been well established. Importantly, ROS scavengers NAC, GSH, superoxide dismutase (SOD), and catalase all inhibited the induction of heme oxygenase 1 (HO-1) by EGCG, as shown in bovine aortic endothelial cells (BAECs) [89]. Thus, an EGCG-induced ROS, rather than an EGCG-derived electrophile, mediated EGCG-induced HO-1 expression under these conditions. A role for ROS as a secondary messenger in Nrf2 activation, specifically H_2O_2, has also been shown for a different ARE inducer class, dithiolethiones, in Hepa 1c1c7 cells [80]. In addition, a role for ROS in Nrf2 activation by 21 flavonoids (including fisetin, kaempferol, and quercetin) was explored [90]. A high level of correlation was observed between the flavonoids' ability to activate the ARE and their computed energy levels of the highest occupied molecular orbitals (E_{HOMO}), representing the tendency of the flavonoid to donate electrons in redox processes, for example. Thus, more oxidizable flavonoids possessing less negative E_{HOMO}values were generally more potent inducers of ARE-mediated gene expression. Therefore, ROS may be important secondary messengers for flavonoids.

Interestingly, we note that there are three outliers in the flavonoid correlation analysis [90] with much greater abilities to activate the ARE than predicted by their E_{HOMO} values (and hence their tendency to donate electrons in the formation of ROS). This boost in induction potency could be associated with a particular set of structural features. Thus, only these three flavonoids (quercetin, morin, and myricetin) out of 21 tested can be predicted to form extended conjugation-stabilized quinone methides involving the C ring, as products of a two-electron oxidation sequence (Fig. 7.3a). We hypothesize that while generation of ROS by flavonoids may contribute to activation of Nrf2, the ability to form a conjugation-stabilized electrophile, which can react with thiolates such as quinoid, produces a much greater extent of ARE activation due to its ability to react directly with sensor thiolates,

such as Keap1 cysteines. Importantly, the quinone methide derived from quercetin has a remarkably high stabilization effect of the extended conjugation, as shown by its ability to form reversible thiol adducts [34]. Finally, in support of this hypothesis, out of a series of five structurally similar flavonoids, only the quinoid-forming ones (kaempferol, quercetin, and luteolin) were able to react with a thiol (GSH) to form *mono*- and *bis*-GSH conjugates, without forming radicals and ROS [91, 92]. In contrast, the other two flavonoids (apigenin and naringenin), which are not able to form conjugation-stabilized electrophiles, generated radicals and ROS and could not form conjugates with GSH.

In considering whether generation of ROS is a secondary messenger in Nrf2 activation by flavonoids and other phytochemicals, it is important to note that the two-electron oxidation of phenols need not necessarily lead to ROS formation. If a polyphenol can be recognized as a substrate of an oxidizing metalloenzyme, it can be converted to the Michael acceptor without production of ROS (Fig. 7.2). To our knowledge, this theory remains to be tested. Dosage is likely critical as to whether useful or harmful levels of ROS are produced by treatment with ARE inducers [93]. Much work remains to determine the role(s) that ROS play in Nrf2 activation by phytochemicals, including whether the ROS generated by therapeutic and physiologically relevant concentrations of phytochemicals have deleterious off-target effects, or are relatively harmless and perhaps relevant for participating in numerous signaling mechanisms, which are beyond the scope of this Perspective (reviewed in [85, 94, 95]). The dosage amount is likely very important, as illustrated through the example of the synthetic oleanane triterpenoid CDDO-Im. While at low concentrations ($\leq$100 nM) CDDO-Im is a potent Nrf2 inducer with undetectable adverse effects, above 300 nM ROS-mediated toxicity is observed [96]).

7.3.3 Basis of Phytochemicals as Therapeutic, Rather than Toxic, Agents

The cytoprotective roles of phytochemicals discussed thus far could be considered surprising, given that most if not all were produced for biodefense against insects, bacterial parasites, and other animals, including humans, consuming plant parts. The question arises then as to how these molecules have a cumulatively cytoprotective effect. One critical factor is dosage, as at high concentrations most of these molecules have been shown to display at least some level of toxicity. At low concentrations then, the residual "toxicity" maintains cells in a state of adaptive stress, providing them with tools for counteracting a variety of adverse conditions [97]. The ability of a phytochemical to induce such a state selectively plays a critical role in the cytotoxicity/cytoprotection balance. This balance appears to be rather sensitive to subtle changes in chemical structure. Two considerations that appear to be significant are selectivity for stress-sensing cysteines and reversibility of thiol modification.

There are various factors that contribute to the preference of an electrophile for stress-sensing cysteines. First, cytoprotective compounds (or their bioactivated derivatives) contain "soft" electrophilic centers, which generally favor an attack by a corresponding soft nucleophile, best represented in a cellular environment by a thiolate anion. In phytochemicals, soft electrophilicity is associated with a carbon center conjugated through a network of π-bonds to a reactive functional group, such as a carbonyl (Fig. 7.3a), sulfone (Fig. 7.3b), epoxide (Fig. 7.3c), or imine (Fig. 7.3d), or with an electrophilic center activated directly by large, polarizable atoms (e.g., epithionitriles, isothiocyanates, etc.). The molecular orbital effects at the root of these preferences are important but are beyond the scope of this Perspective (see [98] for relevant discussion). Soft electrophilicity, however, is not sufficient for shifting the therapeutic window toward cytoprotection, as highly reactive albeit soft agents will modify unintended thiols (i.e., hemoglobin, human serum albumin, GSH, etc.) [3] and even non-thiol targets (i.e., nucleic acids).

Second, stress-sensing cysteines are likely maintained by their immediate environment in a highly reactive state. This could include pK_a reduction [99] and the presence of Brønsted acids to orient and activate the incoming electrophiles through hydrogen bonding [52] (see Fig. 7.4a). It is highly likely that one or several Keap1 cysteines are in fact presented in such an environment (see Sect. 7.3.4.1).

Third, the structural context of presenting the electrophilic functionalities is likely to play an essential role in an electrophile's ability to react selectively. Thus, another contributing factor noted in enhancing reactivity with sulfur nucleophiles is the presence of a neighboring group, such as a hydroxyl functionality, in the vicinity of a reactive β-carbon of cinnamates, chalcones, curcuminoids, bis(2-hydroxybenzylidene) derivatives [30], and some withanolides [20]. A neighboring hydroxyl can guide an incoming thiolate anion via a transient charge–dipole interaction (hydrogen bond) resulting in a highly selective process that enhances the effectiveness of such agents (Fig. 7.4b).

In addition, a sterically hindered electrophile may exercise a superior degree of selectivity toward uniquely reactive thiolates (e.g., Keap1 cysteines). For example, both the high effectiveness and low toxicity profile of synthetic oleanane triterpenoids have been ascribed to a special combination of the high intrinsic electrophilicity of the Michael acceptor activated by both keto and cyano groups (Fig. 7.4c) and sterics, specifically the presence of a Michael acceptor functionality directly adjacent to a highly congested quaternary carbon center [100] (Fig. 7.4d). Congestion in the vicinity of the reactive β-carbon reduces the reactivity of the Michael acceptor by crowding the transition state, where trigonal planar geometry is converted into a more sterically demanding tetrahedral state. This should allow only the most reactive thiolates to overcome the steric barrier and form covalent adducts. It must be noted, however, that mere reduction of reactivity without adjustment of other factors is expected to result in a situation where the enhanced selectivity and reduced toxicity come at the expense of potency. Thus, of two stereoisomers of guggulsterone, a terpenoid that lacks any known toxicity, only the *E*-form displays moderate ARE induction [101], due to, presumably, a poor accessibility by nucleophiles of the trisubstituted α,β-unsaturated ketone furnished with a quater-

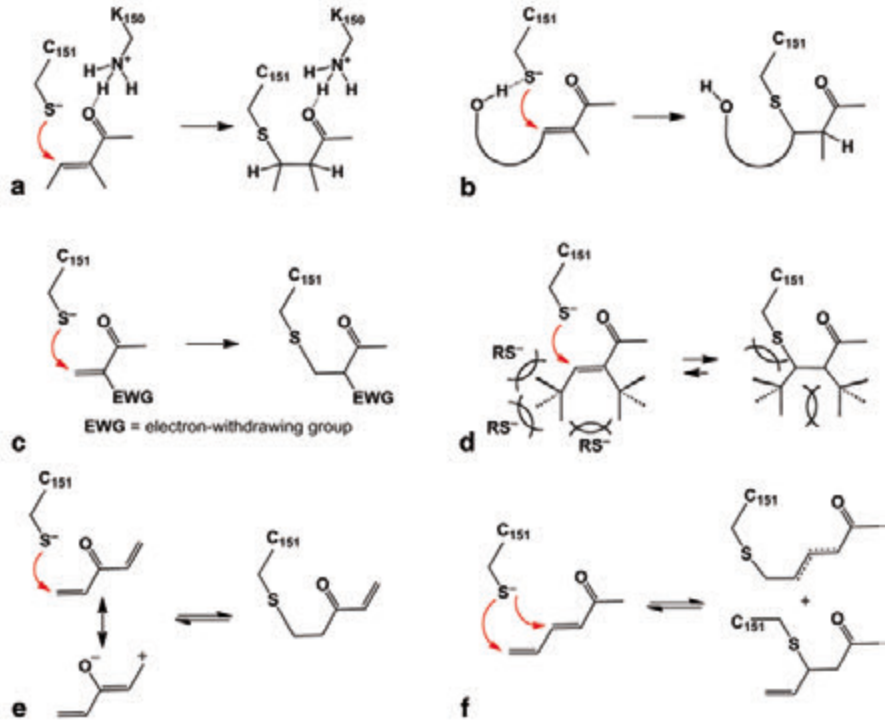


Fig. 7.4 Structural features of Michael acceptors affecting the rates of forward and reverse reactions with thiols. Keap1 C151 is shown as an example thiol. **a** Brønsted acid catalysis by a neighboring residue (Keap1 K150). **b** Neighboring group (proximity) catalysis through hydrogen bonding with a hydroxyl adjacent to the β-carbon. **c** Alkene polyactivation, favoring addition. **d** Steric congestion, preventing additions by less reactive nucleophiles via transition state crowding. **e** Cross-conjugation and **f** extended conjugation stabilize an electrophile by diluting the partial positive charges at the electrophilic centers and thereby reducing its reactivity and promoting reversibility

nary center as the α-carbon substituent. The relatively low toxicity of coffee furans, as compared to other furans, can also be explained by steric factors, if the operative electrophile is a conjugated epoxydiene formed directly from kahweol (Fig. 7.3c) or after oxidative processing of cafestol.

The reversibility of addition, the fourth factor on our list, is emerging as a crucial characteristic of highly effective inducers with low associated toxicity [102]. Clearly, the entropically favored reversal of electrophilic additions is expected to (1) alleviate many potentially adverse outcomes associated with unintended alkylation events, (2) activate multiple stress sensors at a greatly reduced concentration, and (3) exhibit catalytic turnover (recycling) in maintaining the state of adaptive stress. ARE inducers must compete with prominent blood proteins—hemoglobin and serum albumin—and GSH, among many other potential nucleophiles. GSH, the most available cellular thiol responsible for detoxification, can be conjugated with electrophiles in an uncatalyzed manner, or the conjugation can be catalyzed by

GSTs (reviewed in [103]). Once formed, if sufficiently stable, these conjugates are excreted from the cell by transmembrane multidrug resistance-associated proteins (MRPs), leading to dilution of the ARE inducer and the signaling event (Fig. 7.2). Thus, highly reversible ARE inducers may escape elimination from the cell by deconjugating from GSH prior to being transported by an MRP, maintaining their cellular concentration more effectively than ARE inducers that form much more stable conjugates. For "reversible" ARE inducers, conjugation with GST has been hypothesized to be an intermediate step [104], leading via continuous deconjugations to activation of less represented but highly reactive "sensory" thiolates, responsible for antioxidant effects (Fig. 7.2).

For example, the reversible addition of sulforaphane to thiols has long been suspected to be responsible for the pleiotropic nature of its cellular activity [78, 105]. In addition, the dramatically enhanced Nrf2-induction potential of CDDO and its variants over traditional phytochemical inducers may be due in large part to the facile reversibility of their thiol conjugates [100]. While a systematic analysis of chemical features responsible for the reversibility of thiol conjugations would benefit the field, certain generalizations about these features can be made herein. Thus, unusual stabilization of the electrophile through cross-conjugation (Fig. 7.4e) or extension of conjugation (Fig. 7.4f) could be invoked in a few cases documenting the favorability of addition reversal [34, 52, 100]. Both effects should lead to the enhanced delocalization of the partial positive charge induced by the associated carbonyl, reducing the electrophilicity of β- (δ-, etc.) carbon centers. For example, in celastrol, a quinone methide triterpene that has submicromolar potency for ARE activation, extended conjugation can account for the observed reversibility of its reaction with GSH [106]. Similarly, reversibility due to enhanced stabilization of the electrophile could explain the reduced toxicity and unique effectiveness of quercetin, when compared to structurally related flavonoids and flavonols in particular. Thus, the glutathionyl adducts of the quercetin-derived quinone methide have been shown to be reversible on the order of minutes to hours [34]. In addition, we suggest that the reversibility of addition may play an important role in shifting the effectiveness-to-toxicity balance in yet-to-be established cases. For example, the divergent toxicity profiles of I3C and DIM (see Sect. 7.3.2.2) could be explained by the reversibility of the DIM-derived thiol conjugate due to conjugation extension by a second aromatic group.

Alternatively, the destabilization of an adduct through steric congestion at positions adjacent to the nascent C–S bond may also facilitate elimination, as carbon hybridization changes from trigonal planar to tetrahedral. Thus, we hypothesize that the unusually low toxicity of CDDO [100] is likely the result of addition reversibility due to steric congestion in the corresponding adduct (Fig. 7.4d). In contrast, ARE inducers that do not form relatively reversible adducts, such as the EGCG *o*-quinone, for example, may be eliminated from the cell rather quickly through sequestration by GSH and subsequent elimination by an MRP. For these compounds, generation of superoxide and ROS as signaling intermediates may represent a more effective pathway for Nrf2 activation (Fig. 7.2).

7.3.4 Mechanisms of Nrf2 Activation

7.3.4.1 Modification of Keap1 Cysteines: Identification of Key Sensors for ARE Inducers

A key mechanism by which phytochemicals activate Nrf2 is revealed by their common ability to react with cysteine residues. As mentioned in Sect. 7.3.2.1, the Nrf2-repressor protein Keap1 is particularly cysteine-rich. Keap1 directly binds to Nrf2 [31] and represses the transcription factor in at least two ways. First, Keap1 contains a Crm1-dependent nuclear export signal sequence that appears to be important for maintaining Nrf2 primarily in the cytoplasm under basal conditions [107–109]. In addition, and likely most important for phytochemical signaling, Keap1 serves as a bridge between Nrf2 and the Cul3-E3-ubiquitin ligase complex, leading to the ubiquitination of Nrf2 and subsequent degradation by the 26S proteasome (reviewed in [110]). There are two separate sites in Nrf2 that bind to Keap1, termed the ETGE and DLG motifs [111]. Keap1 forms a homodimer through its N-terminal BTB domain, and it is the C-terminal Kelch domain of Keap1 that binds to the Nrf2 ETGE and DLG motifs. The seven lysines of Nrf2 that are targets for Cul3-mediated ubiquitination are located between the ETGE and DLG motifs [112]. Binding of the two Kelch domains of a homodimer of Keap1 to the ETGE and DLG sites of an Nrf2 protein molecule is believed to be critical for presentation of those lysines to an E2 ligase for ubiquitination [113]. Ubiquitination-directed degradation maintains the Nrf2 protein at a low level under normal conditions. Upon exposure to stress or compounds that modify cysteines, Nrf2 escapes Keap1 repression, and thereby is no longer ubiquitinated and degraded. This in turn leads to Nrf2 accumulation, resulting in activation of ARE-regulated genes.

Two primary methods have been used to illustrate which of the Keap1 cysteines (27 in human Keap1) are implicated in sensing ARE inducers: overexpression of Keap1 containing cysteine mutated to serine or alanine in mammalian cell lines or zebrafish, and mapping of cysteine modification in the purified protein by peptidic digestion and mass spectrometry. These complementary methods have revealed that a “cysteine code” may exist, in which particular types of ARE inducers react most readily with particular Keap1 cysteines. Cysteines 226 and 613 were shown to sense heavy metals [114], while C273 and C288 have been implicated in sensing alkenals, some cyclopentenone prostaglandins, and nitro-fatty acids [114–116]. Keap1 C151 has been shown to be required for sensing many electrophilic ARE inducers [115, 117–121]. For example, a potent imidazole derivative of CDDO (CDDO-Im), tBHQ, a quinone-forming phenol, and sulforaphane are all highly dependent on C151 to upregulate the ARE [107, 115, 120]. Finally, the importance of Keap1 C151 in sensing ARE inducers is illustrated by the Keap1 C151S transgenic mouse [122]. This mouse is not only less responsive to tBHQ for upregulation of cytoprotective enzymes but the basal levels of these enzymes are also repressed [122]. Thus, Keap1 C151 emerges as the key sensor for both endogenous agents as well as phytochemicals. The role of this cysteine as a sensor has been proposed to have

evolved as a means of sensing nitric oxide [114]. Interestingly, mutation of C226 and C623 to serine has no effect on ARE activation by sulforaphane or tBHQ [114]. Therefore, C151 is currently the only known Keap1 sensor cysteine for a phytochemical, sulforaphane, and a synthetic quinoid-forming polyphenol, tBHQ.

The role of Keap1 C151 as a principal sensor for phytochemicals is further supported by peptide-mapping studies. Keap1 C151 is the only cysteine consistently and highly modified by all phytochemicals tested thus far: isoliquiritigenin, 10-shogaol, xanthohumol [123], and sulforaphane [124] (Fig. 7.5). There have been discrepant results for modification of Keap1 cysteines, in particular C151, by sulforaphane. Initially, peptide-mapping studies of human Keap1 cysteines modified by sulforaphane found C151 to be one of the least readily modified cysteines (Fig. 7.5) [125]. Based on the high dependence of sulforaphane ARE activation on C151, as well as the known labile, reversible nature of dithiocarbamates [126], i.e., the products of sulforaphane reaction with thiols, further experiments were conducted using a streamlined method to limit the reverse reaction after labeling Keap1 cysteines with sulforaphane [124]. Under these conditions, C151 emerged as one of the four most readily modified cysteines of Keap1. The adduction of Keap1 C151 by sulforaphane was also observed indirectly for Keap1 overexpressed in Cos1 cells using a biotin-switch technique, where C151 appeared to be modified to a greater extent than other cysteines [114]. The entire pattern of modification of Keap1 by sulforaphane generally shows large variability depending on the method used (Fig. 7.5 and [124]), in particular as to whether C151 is detected as modified, illustrating the importance of considering the reversible nature of electrophile–cysteine adducts. Interestingly, several cysteines including C77, C226, C368, and C489 (Fig. 7.5) were detected as readily modified regardless of the method used, indicating that certain dithiocarbamate–cysteine adducts are much more stable than others. The C151–dithiocarbamate adduct, on the other hand, is both one of the most reactive and reversible. Modeling of the BTB domain of Keap1 containing C151 [114, 120] depicts various residues nearby, including K131, R135, K150, and H154, that could participate both in lowering the pK_a of C151 and in providing acid/base catalysis for promoting both the forward (addition) and the reverse (elimination) reactions. As a confirmation, a single mutation of K150 to threonine significantly reduces the response to C151-dependent ARE inducers [115].

While the modification pattern of quinoid-forming polyphenol phytochemicals has not yet been assessed, the oxidized form of tBHQ was found to react with Keap1 C151, shown directly for purified Keap1 [127] (Fig. 7.5) and indirectly for Keap1 overexpressed in Cos1 cells [114]. Interestingly, C151 is not found to be readily modified by the oxidized form of GSH, GSSG [82] (Fig. 7.5). Phytochemicals for which ROS production is a key means of activating Nrf2 (Sect. 7.3.2.3) likely activate Nrf2 by first generating GSSG (Fig. 7.2). Thus, their Nrf2 activation would presumably be much less dependent on Keap1 C151.

While C151 is the cysteine most readily modified by phytochemicals based on current knowledge, other cysteines are sites of phytochemical addition in purified Keap1 protein (Fig. 7.5). For example, C319 is readily modified by all four phytochemicals shown in Fig. 7.5 as well as oxidized GSH. To our knowledge, this

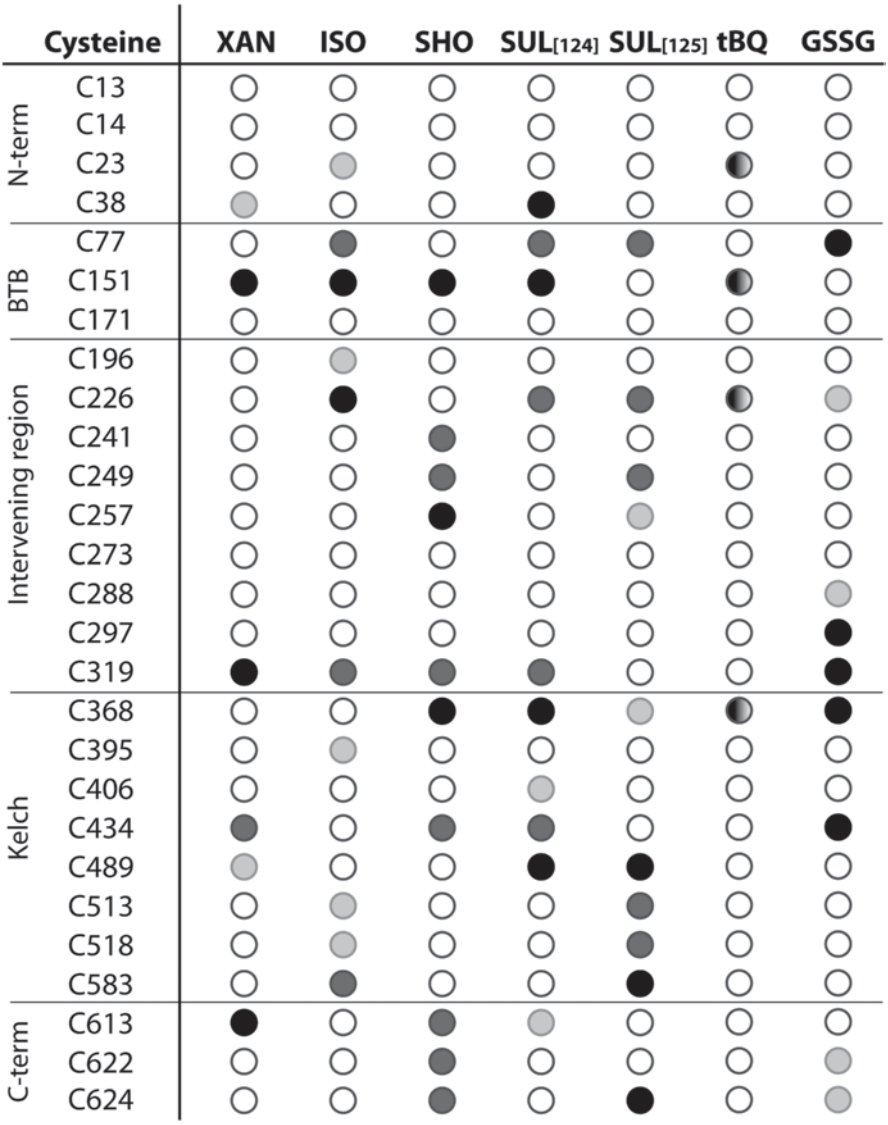


Fig. 7.5 Keap1 cysteines readily modified by phytochemicals, glutathione, and a quinone-forming polyphenol. Abbreviated name, chemical name and reference: XAN, xanthohumol [123]; ISO, isoliquiritigenin [123]; SHO, 10-shogaol [123]; SUL, sulforaphane [124, 125]; tBQ, tert-butylquinone [127]; and GSSG, oxidized glutathione [82]. Cysteines are ranked in order of most readily modified for all but tBQ, as determined by increasing concentrations of the electrophile, with the *darkest circles* being the most readily modified, and the *empty circle* indicating weakly modified or not modified cysteines. For tBQ, the cysteines are not ranked

cysteine has not yet been evaluated as a sensor for phytochemicals, and it has been shown not to be required to sense the soft cations, such as As^{3+} and Se^{4+} , but there is an indication that it may play a role in sensing the Zn^{2+} ion [114].

Notably, none of the phytochemicals evaluated in Fig. 7.5 modified C273 or C288 appreciably, although GSH modified C288 to some extent. These cysteines are considered to be potential sensors, as their mutation to serine renders Keap1 unable to repress Nrf2 [107, 122]. As these cysteines have been implicated in sensing more reactive Michael acceptors—alkenals, prostaglandins, and nitro-fatty acids [114–116]—they may serve to detect primarily these endogenous signaling agents, rather than more stable phytochemical electrophiles. Keap1 C226 and C613, which sense heavy metal salts and other soft cations [114], are readily modified by phytochemicals, with isoliquiritigenin and sulforaphane targeting the former and xanthohumol, 10-shogaol, and, to some extent, sulforaphane targeting the latter. However, as described above, mutation of C226 or C613 to serine had no effect on the ability of Keap1 to sense sulforaphane [114]. It is likely that the C151 residue present in these mutant proteins could be modified by sulforaphane, leading to a loss of Keap1 repression of Nrf2. Further work is required to ascertain whether modification of Keap1 cysteines other than C151 contributes to Nrf2 activation by phytochemicals.

7.3.4.2 Mechanism of Nrf2 Activation upon Modification of Keap1 Cysteines

The means by which Nrf2 escapes Keap1–Cul3-directed ubiquitination and degradation is still under active investigation. Disruption of the Keap1–Nrf2 interaction upon modification of Keap1 cysteines, allowing Nrf2 to escape Keap1, was originally proposed on the basis of experiments using recombinant proteins [126]. However, further investigations of a similar nature showed that the overall affinity of the Keap1–Nrf2 complex is maintained after modification of Keap1 at sensor cysteines, including C151 and C288, by ARE inducers including sulforaphane and isoliquiritigenin [128]. While a reduced affinity of Keap1 for Nrf2 after treatment of cells with tBHQ has also been observed by pull-down assays [129], other studies have reported that Keap1 and Nrf2 remain associated after treatment of cells with tBHQ [107, 130], sulforaphane [107], a synthetic triterpenoid derivative, dh404 [131], or quercetin [132]. Pull-down assays are a useful but imprecise means of determining relative affinities of protein–protein interactions, making it difficult to ascertain whether the observed perturbation of the Keap1–Nrf2 complex occurs in response to inducers in a cellular context. It is noteworthy that preventing Nrf2 degradation is sufficient to promote Nrf2 nuclear accumulation and ARE activation [133]. It has been hypothesized that the affinity of the weaker Nrf2 DLG site is decreased after modification of Keap1 cysteines including C151, C273 and/or C288, leading to decreased ubiquitination, without affecting substantially the overall stability of the complex [122].

Significantly, the stability of the Keap1–Cul3 interaction, also essential for ubiquitination, is reduced upon treatment with ARE inducers as observed by pull-down assays [112, 134]. These studies show that the reduced affinity is dependent on Keap1 C151, as mutation of C151 to serine largely abolished the effect. Modification of the C151 residue has clearly been shown to disrupt Keap1-mediated ubiquitination of Nrf2, as mutation of C151 to tryptophan had the same effect as electrophile treatment of cells [120]. The Keap1 C151W mutant was largely unable to mediate Nrf2 ubiquitination, leading to stabilization of Nrf2 with concurrent activation of ARE-regulated genes, and the mutant had reduced affinity for Cul3, as assessed by the pull-down assay. Remarkably, Nrf2 binding to Keap1 in this assay was unaffected by the tryptophan mutation. While other Keap1 cysteines do very likely participate as well in Nrf2 activation, the results with Keap1 C151W in cells, as well as in a zebrafish model [115], show that modification of Keap1 C151 alone is sufficient for signaling for Nrf2 activation.

It has been proposed that the negative charge of the C151 thiolate anion is essential for Nrf2 turnover, and that modification of C151 by an electrophile neutralizes the negative charge, leading to the signaling event [114]. However, a series of 13 mutations at position 151 showed that the size of the residues was the key determinant, rather than hydrophobicity or charge, with the largest residue, tryptophan, showing the largest effect [120]. In particular, asparagine, with a relatively small partial molar volume (i.e., size) and no capacity to carry a negative charge, rendered the corresponding Keap1 mutant effective in suppressing Nrf2. Therefore, the size of the residue at position 151 appears to be the major determining factor for Keap1's ability to repress Nrf2. Since all phytochemicals that were found to react with the C151 thiolate have partial molar volumes larger than that of tryptophan, they would all be large enough to trigger the signaling mechanism.

Alternatively, formation of disulfide bonds between Keap1 cysteines has been proposed to mediate loss of Nrf2 repression. Disulfide-linked Keap1 dimers have been observed in extracts of cells treated with the chalcone derivative *bis*(2-hydroxybenzylidene)acetone, which is a phenolic Michael acceptor [135]. In addition, treatment of cells with oxidizing agents (e.g., H_2O_2, nitric oxide, hypochlorous acid, or *S*-nitrosocysteine) leads to a Keap1 dimer linked through C151 [136]. Nonenzymatic oxidation of phenolic compounds will generate phenoxyl radicals of variable stability (Fig. 7.2), and it has been proposed that these reactive intermediates could mediate the formation of thiyl radicals, promoting the formation of a disulfide bond or bonds between Keap1 cysteines [78]. Furthermore, both ROS and GSSG could also participate in Keap1 disulfide bond formation (Fig. 7.2). In addition, a Keap1 dimer that was stable under reducing conditions was induced by treatment of cells with a quinoid-forming polyphenol, tBHQ [107]. Formation of this non-disulfide-linked dimer was dependent on C151. Radical-mediated hydrogen abstraction from surface tryptophans or tyrosines by subsequent cross-linking may account for the formation of the non-reducible dimer [137]. Further studies are required to determine, on a structural/molecular level, how direct modification of C151 and other Keap1 residues by ARE inducers or GSSG, or Keap1 dimer formation, leads to the loss of their ability to repress Nrf2.

7.3.4.3 Role of Protein Kinases in Nrf2 Activation by Phytochemicals

In addition to the direct Keap1-repression–inactivation mechanism discussed above, a number of kinases have been implicated in Nrf2 activation. The kinase pathways that currently appear to be the most important for Nrf2 activation by phytochemicals are the ones mediated by phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs). The PI3K/Akt pathway mediates carnosol-induced expression of the cytoprotective enzyme HO-1 in rat pheochromocytoma PC12 cells [138]. In addition, EGCG, guggulsterone, and piceatannol activate expression of cytoprotective enzymes including HO-1 in the human mammary epithelial (MCF10A) cells in a PI3K/Akt-dependent manner [101, 139, 140]. EGCG activation of Nrf2 in BAECs is also PI3K/Akt dependent [89]. The Nrf2 activation by CDDO-Im is dependent upon PI3K/Akt activity in ARPE-19 retinal epithelial cells [141].

The mechanism by which Nrf2 is regulated by PI3K/Akt has been investigated in some detail. The GSK-3β kinase, which is inactivated upon phosphorylation by Akt1, phosphorylates and negatively regulates Nrf2 via two distinct mechanisms [142, 143]. The phosphorylation of Nrf2 by GSK-3β leads to both cytoplasmic localization of Nrf2 [142] and Keap1-independent ubiquitination by the SCF/β-TrCP/Cul1 complex, leading to Nrf2 degradation [143]. A key cysteine residue, C124 in phosphatase and tensin homolog (PTEN), a negative regulator of the PI3K/Akt axis [144], is directly modified by biotinylated CDDO in ARPE-19 cells [141], and the same cysteine is required for guggulsterone-induced Nrf2 accumulation in MCF10A cells [101]. Remaining to be demonstrated is whether the modification of PTEN C124 by electrophilic agents also inactivates GSK-3β, and whether this in turn leads to Nrf2 accumulation and Nrf2 nuclear localization.

While the inhibition of GSK-3β is implicated in the initial response to Nrf2 activators, the re-activation of GSK-3β has been proposed to occur several hours later, leading to eventual downregulation of the response by increasing Nrf2 nuclear export [145]. In this proposed mechanism, H_2O_2 treatment of cells activates GSK-3β, leading to subsequent activation of Fyn/Src kinases, which then phosphorylate Nrf2 Y568. Phosphorylation of Y568 enhances the interaction of Nrf2 with the Crm1 nuclear export protein. This mechanism has not yet been evaluated for phytochemical ARE inducers. However, these investigations were prompted by the observation that genistein, an ARE inducer, is a tyrosine kinase inhibitor. Whether inhibition of Nrf2 Y568 phosphorylation is in part or wholly responsible for the ARE induction by genistein or other natural phenols is unknown.

The role of MAPKs in activation of Nrf2 is somewhat controversial. A large number of studies with various phytochemicals, including sulforaphane, phenethyl isothiocyanate (PEITC), curcumin, quercetin, and EGCG, have implicated MAPKs in Nrf2 activation (reviewed in [146]). In order to assess the importance of this kinase family in Nrf2 signaling, MAPKs were overexpressed in HEK293T cells, and potential sites of MAPK-dependent phosphorylation on Nrf2 were identified by mass spectrometry. As a result, five phosphorylated residues were identified [146]. An Nrf2 mutant with all five sites mutated to alanine showed a moderate decrease

in the transcriptional activity of Nrf2, concomitant with a slight reduction in its nuclear accumulation. However, the stability of the Nrf2 protein, which is primarily controlled by Keap1, was not affected. The general conclusion drawn from this work is that direct phosphorylation of Nrf2 by MAPKs has limited contribution to modulating Nrf2 activity [110, 146]. However, the Nrf2 protein with five mutated residues was only evaluated for blocked phosphorylation by overexpression of c-Jun N-terminal kinase (JNK2), rather than each MAPK relevant for Nrf2 activation [146]. Therefore, an additional site on Nrf2 beyond those five identified by mass spectrometry may in fact be a relevant target. Regardless of whether direct Nrf2 phosphorylation is the means by which MAPKs activate Nrf2, the sheer number of studies that implicate this pathway in Nrf2 activation by phytochemicals calls for further mechanistic investigation.

Activation of Nrf2 is also mediated by other kinases such as PERK [147, 148] and casein kinase 2 [149, 150]. However, to our knowledge, these pathways have not been evaluated for phytochemical activation of Nrf2. In addition, protein kinase C (PKC) isoforms are involved in the upregulation of cytoprotective genes by several phytochemicals. PKC-dependent ARE gene upregulation was observed for curcumin in HUH7 hepatoma cells and human monocytes [151, 152], piceatannol in BAECs [153], and epigallocatechin in human monocytic cells [154]. Nrf2 nuclear accumulation and ARE activation are also PKC-dependent for tBHQ in HepG2 and rat H4IIEC3 hepatoma cell lines [129, 155, 156], as well as nontumorigenic human keratinocytes (HaCaT cells) [157]. However, ARE activation by quercetin or kaempferol was not dependent on PKC in Hepa1c1c7 hepatoma cells [90], nor was ARE activation by diallyl trisulfide dependent on PKC in HepG2 cells [158]. Further investigations are required to ascertain under what circumstances a phytochemical might activate Nrf2 through PKC isoforms.

7.4 Summary and Future Directions

Nrf2 is emerging as a master control of cytoprotective mechanisms important for defense against environmental challenges and stress factors. The phytochemical activation of Nrf2 promises to be an important mechanism in the prevention and amelioration of a wide variety of human diseases. In addition, Nrf2 is under investigation as a drug target [159], and there is much to be learned from investigations of phytochemical ARE inducers with regard to what makes an effective agent with minimal toxicity. A large effort has been put toward understanding the mechanisms by which Nrf2 is activated, although phytochemicals are still relatively understudied compared to other compounds [160].

In general, a common trait of agents that activate Nrf2 effectively is the ability to react with thiols either directly or upon bioactivation (spontaneous or enzyme-catalyzed). Alternative mechanisms of Nrf2 de-repression involving induction of oxidative stress, for example, have also been discussed. The electrophilic and/or oxidative nature of Nrf2 activators means that a balance between toxic and protec-

tive effects is necessary for safe administration of these agents. This balance is described in conventional drug development as a "therapeutic window" or effective dosage range. Within this range, beneficial effects are exhibited at optimal strength without being compromised by adverse processes. Our primary recommendation for future studies in the phytochemical activation of Nrf2 is that, as with conventional drugs, efforts are put toward understanding how this dosage range is widened. This will enable identification or development of more effective agents for disease prevention and strategies for administering those agents.

Widening this dosage range does not appear to be a trivial exercise. The challenge lies in the fact that a strategy for increasing potency that merely relies on higher reactivity of these electrophiles is likely to enhance the extent of deleterious events. These may range from effect dilution through GSH conjugation followed by efflux to severe cytotoxicity associated with covalent modification of unintended targets. At the same time, reduction of toxicity through attenuation of electrophilicity would be expected to compromise potency. However, nature provides some important examples of how an effective therapeutic window can be achieved, which involves both partners in the modification reaction: sensor cysteines and naturally occurring cytoprotective agents.

The chemistry of the sensor cysteines plays an important role in ensuring advantageous biological effects of phytochemicals. The unique reactivity of soft thiolates of the sensor cysteines implicated in ARE induction, including Keap1 C151, ensures a high degree of "chemoselectivity" through the intrinsic preference for π-conjugated and thereby polarizable (soft) electrophilic centers, such as those found in effective phytochemical activators of Nrf2 (Fig. 7.1 and Sect. 7.3.3). Chemoselectivity is further ensured by the high reactivity of the sensor cysteines implicated in ARE induction, including Keap1 C151. What remains to be explored is whether yet further preference toward particular sensor cysteines could be provided by the remaining structural features of the covalent modifiers, such as overall size or presence of particular functionalities that could interact favorably with the protein environment surrounding the thiol.

Certain phytochemical ARE inducers, either relatively potent ones or those with low or no observable toxicity, also provide clues as to the features that widen the therapeutic window. Thus, preference for sensor thiolates can be enhanced by neighboring hydroxyl groups guiding a thiolate toward an attack on an electrophile. Alternatively, since sensor thiolates are so reactive, poor Michael acceptors could conjugate to them preferentially and, thereby, act as viable ARE inducers, while evading less reactive "off-target" nucleophiles. Examples of phytochemicals that are expected to be relatively unreactive Michael acceptors include those deactivated by alkene polysubstitution, bulky neighbors, and/or extended conjugation (Sect. 7.3.3). Remarkably, these deactivating factors are also expected to facilitate the reverse elimination process either by enhancing the stability of the electrophile, or by destabilizing the adduct. The reversibility of conjugates is emerging as an important feature for high potency and low toxicity. As discussed in Sect. 7.3.3, continuous deconjugation may simultaneously contribute to both toxicity reduction due to the instability of off-target conjugates and poten-

cy enhancement through efflux prevention. In addition, there is much to learn as to how modification of Keap1 cysteines leads to Nrf2 de-repression, for example, whether the size of modifiers influences the extent of the response, as suggested by a mutagenesis study [120].

Another factor important in the therapeutic window for phytochemicals is the ability to induce Nrf2 by a variety of pathways. Key sensor cysteines of phytochemical ARE inducers are being identified—Keap1 C151 and C124 of PTEN kinase—and other cysteines will likely emerge as important in sensing ARE inducers. Depending on the nature of the chemical and biological events associated with a given phytochemical in a given condition, different signaling "nodes" can be activated, all leading to Nrf2–ARE regulation. For example, the ultimate messenger molecule producing the Nrf2-dependent signal may not only be the phytochemical itself and/or its metabolite, but a concomitantly formed ROS/RNS or GSSG, as the GSH:GSSG ratio drops. These secondary signaling messengers target not only Keap1 and PTEN, but also numerous other kinases, including those involved in Nrf2 regulation. For example, various flavonoids including quercetin and EGCG can associate noncovalently with and modulate a number of protein kinases with high affinity (reviewed in [161]). There appears to be an intricate network of regulatory proteins that modulate Nrf2 activity, and multiple sensing steps can lead to the ability of Nrf2 upregulation to be tuned as is appropriate. Importantly, the presence of such a network implies that, if multiple nodes are activated by a single phytochemical, the convergence of the responses may lead to a more pronounced effect. This rationale also provides the basis for the unexpected effectiveness exhibited by mixtures of Nrf2 activators. Indeed, impressive synergism has been observed in a few studies conducted thus far, including for glucosinolate breakdown products [54] and a commercially available phytochemical supplement mixture [162]. Nrf2 activation, while clearly important, is certainly not the only mediator of the cytoprotective and disease-preventive attributes of phytochemicals (reviewed in [163]). Thus, phytochemicals that modulate a number of targets in disease prevention within their "therapeutic window" will likely be most effective in humans.

In summary, despite the multitude of cellular targets affected by phytochemicals, upregulation of Nrf2 is emerging as a key factor in their cytoprotective properties. In addition, while it is unlikely that a single phytochemical or even a plant source will emerge as a magic bullet for disease prevention or amelioration, we expect that unraveling the chemical and biological aspects of the action of phytochemicals may lead to unprecedented opportunities. These prospects could range from dietary/supplement recommendations to phytochemical "cocktails" specially formulated for synergistic effects and to nature-inspired synthetic molecules harnessing the most effective features of the plant-derived prototypes.

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