Chemogenetische Ansätze zur Identifizierung des molekularen Wirkmechanismus der Reaktiven Schwefelspezies Allicin

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Diese Doktorarbeit entstand unter der Anleitung von Prof. Dr. Claus Jacob am Institut für Bioorganische Chemie der Universität des Saarlandes in Zusammenarbeit mit Prof. Alan Slusarenko, PhD am Institut für Biologie III der RWTH Aachen im Zeitraum von Februar 2015 bis April 2018.

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In diese kumulative Dissertation sind folgende drei Publikationen eingeflossen:

An Optimized Facile Procedure to Synthesize and Purify Allicin

Albrecht, F.*, **Leontiev, R.***, Jacob, C., Slusarenko, A.J.

*equal contribution

Molecules 2017, 22(5), 770

Allicin is a reactive sulfur species (RSS) and defence substance from garlic (*Allium sativum* L.). The compound is a broad-spectrum antibiotic that is also effective against multiple drug resistant (MDR) strains. A detailed protocol for allicin synthesis based on diallyl-disulfide (DADS) oxidation by H_2O_2 using acetic acid as a catalyst was published in 2001 by Lawson and Wang. Here we report on improvements to this basic method, clarify the mechanism of the reaction and show that it is zero-order with respect to DADS and first-order with respect to the concentration of H_2O_2. The progress of allicin synthesis and the reaction mechanism were analyzed by high-performance liquid chromatography (HPLC) and the identity and purity of the products was verified with LC-MS and ^{1}H-NMR. We were able to obtain allicin of high purity (>98%) and >91% yield, with standard equipment available in any reasonable biological laboratory. This protocol will enable researchers to prepare and work with easily and cheaply prepared allicin of high quality.

Yap1p, the central regulator of the *S. cerevisiae* oxidative stress response, is activated by allicin, a natural oxidant and defence substance of garlic

Gruhlke, M.C.H., Schlembach, I., **Leontiev, R.**, Uebachs, A., Gollwitzer, P., Weiss, A., Delaunay, A., Toledano, M., Slusarenko, A.J.

Free Radical Biology and Medicine 2017, 108, 793-802

Allicin is a thiol-reactive sulfur-containing natural product from garlic with a broad range of antimicrobial effects against prokaryotes and eukaryotes. Previous work showed that the *S. cerevisiae* OSI1 gene is highly induced by allicin and other thiol-reactive compounds, and *in silico* analysis revealed multiple Yap1p binding motifs in the OSI1 promoter sequence. An OSI1-promoter::luciferase reporter construct expressed in Wt and Δyap1 cells showed absolute Yap1p-dependence for allicin-induced OSI1-expression.

A GFP:Yap1p fusion protein accumulated in the nucleus within 10 min of allicin treatment and a Δyap1 mutant was highly sensitive to allicin. Yap1p regulates glutathione (GSH) metabolism genes, and Δgsh1, Δgsh2 and Δglr1 mutants showed increased sensitivity to allicin. Allicin activated the OSI1-promoter::luciferase reporter construct in Δgpx3 and Δybp1 cells, indicating that allicin activates Yap1p directly rather than via H_2O_2 production. A systematic series of C-to-A Yap1p exchange mutants showed that the C-term C598 and C620 residues were necessary for allicin activation. These data suggest that Yap1p is an important transcriptional regulator for the resistance of yeast cells to allicin, and that activation occurs by direct modification of C-term cysteines as shown for other electrophiles.

A Comparison of the Antibacterial and Antifungal Activities of Thiosulfinate Analogues of Allicin

Leontiev, R., Hohaus, N., Jacob, C., Gruhlke, M.C.H., Slusarenko, A.J.

Scientific Reports 2018, 8(1), 6763

Allicin (diallylthiosulfinate) is a defence molecule from garlic (*Allium sativum* L.) with broad antimicrobial activities in the low µM range against Gram-positive and -negative bacteria, including antibiotic resistant strains, and fungi. Allicin reacts with thiol groups and can inactivate essential enzymes. However, allicin is unstable at room temperature and antimicrobial activity is lost within minutes upon heating to >80 °C. Allicin's antimicrobial activity is due to the thiosulfinate group, so we synthesized a series of allicin analogues and tested their antimicrobial properties and thermal stability. Dimethyl-, diethyl-, diallyl-, dipropyl- and dibenzyl-thiosulfinates were synthesized and tested *in vitro* against bacteria and the model fungus *Saccharomyces cerevisiae*, human and plant cells in culture and *Arabidopsis* root growth. The more volatile compounds showed significant antimicrobial properties via the gas phase. A chemogenetic screen with selected yeast mutants showed that the mode of action of the analogues was similar to that of allicin and that the glutathione pool and glutathione metabolism were of central importance for resistance against them. Thiosulfinates differed in their effectivity against specific organisms and some were thermally more stable than allicin. These analogues could be suitable for applications in medicine and agriculture either singly or in combination with other antimicrobials.

Stellungnahme über die geleisteten Beiträge des Autors

Der Autor möchte zu den Publikationen in der Dissertation Stellung beziehen:

An Optimized Facile Procedure to Synthesize and Purify Allicin

Synthese, Aufreinigung und Charakterisierung des Allicins sowie der Nebenprodukte. Durchführung der Experimente sowie Auswertung der HPLC, LC-MS und NMR Daten. Konzeption und (Mit-) Verfassung des Manuskripts.

Die Arbeiten wurden in gleichen Anteilen von Herrn Dr. Frank Albrecht und dem Autor durchgeführt.

Yap1p, the central regulator of the *S. cerevisiae* oxidative stress response, is activated by allicin, a natural oxidant and defence substance of garlic

Transformation verschiedener Plasmide in *Escherichia coli* und *Saccharomyces cerevisiae*. Untersuchungen von Hefestämmen bezüglich ihres Verhalten gegenüber Reaktiven Sauerstoff und Schwefel Spezies. Interpretation der Ergebnisse sowie (Mit-) Verfassung des Manuskripts.

A Comparison of the Antibacterial and Antifungal Activities of Thiosulfinate Analogues of Allicin

Synthese, Aufreinigung und Charakterisierung der verschiedenen genutzten Thiosulfinate. Planung und Durchführung des chemogenetischen Screenings sowie der biologischen Aktivitätsassays. Interpretation der Ergebnisse sowie (Mit-) Verfassung des Manuskripts.

Weitere Publikationen des Autors, die nicht Bestandteil dieser Dissertation sind**:**

Inorganic Reactive Sulfur-Nitrogen Species: Intricate Release Mechanisms or Cacophony in Yellow, Blue and Red?

Grman, M., Nasim, M.J., **Leontiev, R.**, Misak, A., Jakusova, V., Ondrias, K., Jacob, C.

Antioxidants 2017, 6(1), 14

Finding the Starting Point for Mode-of-Action Studies of Novel Selenium Compounds: Yeast as a Genetic Toolkit

Leontiev, R., Slusarenko, A.J.

Current Organic Synthesis 2017, 14(8)

The Small Matter of a Red Ox, a Particularly Sensitive Pink Cat, and the Quest for the Yellow Stone of Wisdom

Nasim, M.J., Denezhkin, P., Sarfraz, M., **Leontiev, R.**, Ney, Y., Kharma, A., Griffin, S., Masood, M.I., Jacob, C.

Current Pharmacology Reports 2018, 4(5), 380-396

Interspecies comparison of the bacterial response to 1 allicin reveals species-specific defense strategies

Wüllner, D., Haupt, A., Prochnow, P., **Leontiev, R**., Slusarenko, A.J., Bandow, J.E.

Proteomics 2019

Kurzzusammenfassung

In der vorliegenden Dissertation werden verschiedene neue Erkenntnisse im Hinblick auf die Wirkungsweise des Naturstoffes Allicin beschrieben.

Eine neu entwickelte Methode zur Synthese des Allicins wird beschrieben und ihre Vorteile werden diskutiert. Der Wirkmechanismus von Allicin wird am Modellorganismus Bierhefe (*Saccharomyces cerevisiae*) untersucht. Dabei zeigt sich, dass die Konzentration von Glutathion in der Zelle mit dem Wachstum unter Allicin-Stress korreliert. Dies ist damit zu erklären, dass die antioxidative Antwort von Hefe gegenüber Allicin vom Transkriptionsfaktor Yap1p abhängig ist. Dessen Oxidation führt zur Expression von Genen, die wiederum die Bildung von antioxidativen Enzymen und Molekülen bewirkt.

Des Weiteren wird die Wirkung von strukturell dem Allicin ähnlichen Thiosulfinaten auf verschiedene Modellorganismen untersucht. Es wird gezeigt, dass die verschiedenen Organismen gegenüber unterschiedlichen Thiosulfinaten anfällig sind, und dass manche Thiosulfinate aufgrund ihrer hohen Volatilität sogar über die Gasphase wirken können. Die Daten legen nahe, dass der Wirkungsmechanismus der untersuchten Thiosulfinate dem des Allicins ähnlich ist.

Insgesamt ist Allicin ein sehr vielversprechendes Molekül mit starken antimikrobiellen Eigenschaften. Es zeigt ein sehr breites Wirkungsspektrum und eine hohe Reaktionsbereitschaft. Somit ist Allicin ein sehr interessanter Naturstoff.

Short summary

The thesis at hand gives an overview over the various gains in scientific knowledge about the sulfur containing natural product allicin.

A novel method for the synthesis of allicin is described and the advantages thereof discussed. The mode of action of allicin is investigated in the model organism brewer's yeast (*Saccharomyces cerevisiae*). We could show that the concentration of glutathione inside the cell correlates with the cell growth under conditions of allicin stress. This phenomenon is caused by the fact, that the oxidative stress response of yeast against allicin is dependent on the transcription factor YAP1p. The oxidation of this protein leads to an expression of specific genes, which again leads to an enhanced production of antioxidative enzymes and molecules.

Furthermore, we studied the activity of thiosulfinates with an allicin resembling structure against various model organisms. The results show that different organisms are hypersusceptible towards different thiosulfinates. Due to their high volatility some tested compounds even show antimicrobial activity through the gas phase. The data suggest that the mode of action of the tested thiosulfinates is similar to the one of allicin.

All things considered is allicin a promising molecule with strong antimicrobial properties. It has a broad range of efficacy and highly reactive. Therefore, Allicin must be considered a fascinating natural product.

Inhalt

Abbildungsverzeichnis

1. Abkürzungsverzeichnis

ABC	ATP binding cassette
ATP	Adenosintriphosphat
DADS	Diallyldisulfid
DNA	Deoxyribonucleic acid
GPX	Glutathionperoxidasen
GR	Glutathion Reduktase
GSH	Glutathion
GSSG	oxidiertes Glutathion Dimer
GST	Glutathion-S-Transferasen
LDL	Low Density Lipoprotein
MRSA	Methicillin-resistenter *Staphylococcus aureus*
NADPH	Nicotinamidadenindinukleotidphosphat
OS	Oxidativer Stress
PDI	Protein Disulfid Isomerasen
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSS	Reactive Sulfur Species
SOD	Superoxid Dismutasen
TR	Thioredoxin Reduktase
TRX	Thioredoxin
UV	Ultraviolett
YAP1	Yeast AP-1
YBP1	Yap1-Binding Protein

2. Einleitung

2.1. Oxidativer Stress

Der Begriff Oxidativer Stress (OS) wurde von Helmut Sies im Jahr 1985 [1] geprägt und beschreibt die Auslenkung des Gleichgewichts von Pro- und Antioxidantien hin auf die Seite der Prooxidantien. Hauptsächlich wird der Begriff OS genutzt, um einen Überschuss an Reaktiven Sauerstoff Spezies (Reactive Oxygen Species – ROS) in einem biologischen System zu beschreiben. ROS entstehen hauptsächlich durch endogene Quellen, wie zum Beispiel durch die mitochondriale Atmungskette. Neben den endogenen Quellen spielen aber auch exogene Quellen eine entscheidende Rolle. Dazu gehören primär UV-Strahlung und Radioaktivität und die Aufnahme verschiedenster Faktoren aus der Umwelt, wie zum Beispiel durch Luftverschmutzung oder Pestizide, Metalle und Arzneimittelrückstände in der Nahrung [2].

Zu den wichtigsten ROS zählen das Superoxid Radikal ($O_2^{\bullet -}$) und das nicht-radikalische Wasserstoffperoxid (H_2O_2), sowie Hydroxyl-Radikale ($HO^{\bullet}$), Ozon (O_3) und Singulett Sauerstoff ($^1O_2$). Diese Verbindungen kommen in biologischen Systemen in sehr geringen Konzentrationen vor und haben meist eine sehr geringe Lebensdauer, allerdings sind sie, wie der Name schon vermuten lässt, äußerst reaktiv und in der Lage, weitere Verbindungen zu oxidieren. Dies ist physiologisch von großer Bedeutung, wenn es sich bei der oxidierten Verbindung um ein Biomolekül, wie beispielsweise ein Protein oder die DNA handelt [3].

Viele Krebszellen zeichnen sich durch eine erhöhte Zellteilung aus und sind metabolisch aktiver als gesunde Zellen. In diesem Zusammenhang herrscht in diesen Zellen eine erhöhte intrazelluläre Konzentration an ROS [4, 5, 6]. Dieser Umstand wird in der Chemotherapie ausgenutzt. Viele Krebszellen, die ohnehin schon einen erhöhten Anteil an ROS enthalten, sind beispielsweise gegenüber ROS-generierenden Verbindungen empfindlicher als normale Zellen. Durch eine weitere Induktion von ROS in den Zellen wird die Krebszelle somit eher über den jeweiligen physiologisch kritischen Grenzwert gehoben als normale Zellen. Dadurch kann die kranke Zelle nicht mehr proliferieren und wird zur Apoptose oder Nekrose gezwungen [7, 8]. Bei gesunden Zellen würden diese Verbindungen zwar genauso zu einer

Erhöhung der Konzentration oxidativer Spezies führen, allerdings würde in diesen Zellen der kritische Grenzwert nicht erreicht werden. Hier würde die antioxidative Antwort der Zelle dem OS entgegenwirken können (Abb. 1).

OS wird aber nicht nur negativ gesehen. So kann in geringen Dosen induzierter OS zum Beispiel die Toleranz gegenüber Ischämie erhöhen und das Voranschreiten der Parkinson-Krankheit verzögern [9, 10].

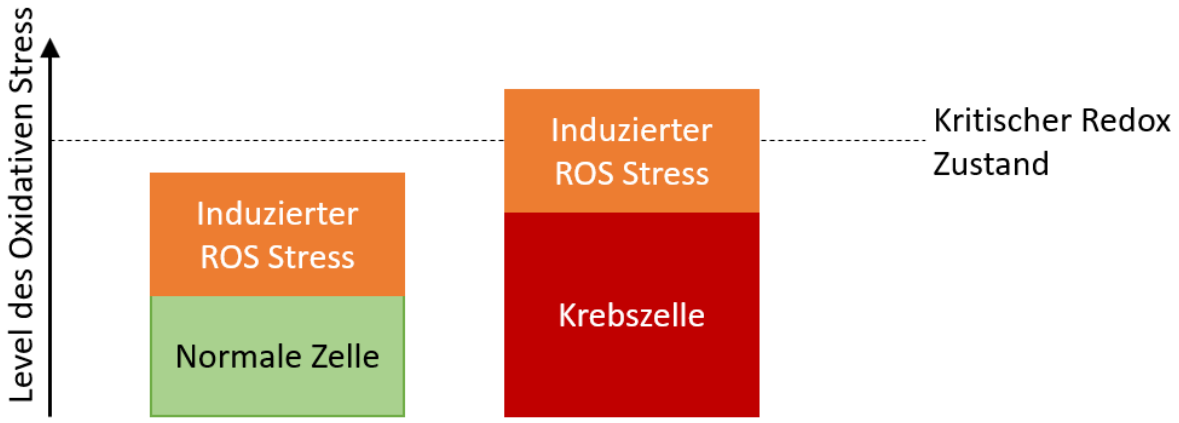

Abbildung 1: Vereinfachte Darstellung des postulierten Modells zur selektiven Zytotoxizität von ROS induzierenden Substanzen nach Jamier (2010).

2.2. Redox Modifikation und Regulation

Die Möglichkeit zur Oxidation und Reduktion von Molekülen ist eine Notwendigkeit für das Leben und eine zentrale Säule der Biochemie [11]. Viele Biomoleküle können oxidiert bzw. reduziert werden und Proteine ändern teilweise sogar ihre Funktion in Abhängigkeit von ihrem Redoxzustand [12]. So reagieren z.B. die Thioredoxin Proteine (TRX) bereitwillig mit einem Disulfid. Infolgedessen wird die Disulfidbrücke reduziert und es entstehen zwei Thiole und das Thioredoxin selbst wird oxidiert. Bis es erneut seine Funktion als Antioxidans erfüllen kann, muss es selbst wiederum von einem anderen Enzym, der Thioredoxin Reduktase (TR) reduziert werden, wobei NADPH hier

als Elektronendonor dient [13]. Die TR von Menschen und höheren Eukaryoten, die sich in ihrem Wirkmechanismus von bakterieller TR unterscheiden, enthalten dabei ein Selenocystein im aktiven Zentrum [14].

In der Tat finden biochemische Redox-Reaktionen an Thiolen statt. Thiole sind Schwefelgruppen, in denen das Schwefelatom maximal reduziert vorliegt, was formell der Oxidationsstufe -2 entspricht. Die Signifikanz des Schwefels in biologischen Systemen beruht auf den vielen Oxidationsstufen, die von -2 bis +6 reichen können, den diversen Schwefel enthaltenden Molekülen sowie auf der Verbreitung und der hohen Konzentration von Thiolgruppen in Zellen [15]. Abbildung 2, welche aus Gruhlke und Slusarenko (2012) entnommen wurde, zeigt dabei die möglichen Reaktionen eines Thiols.

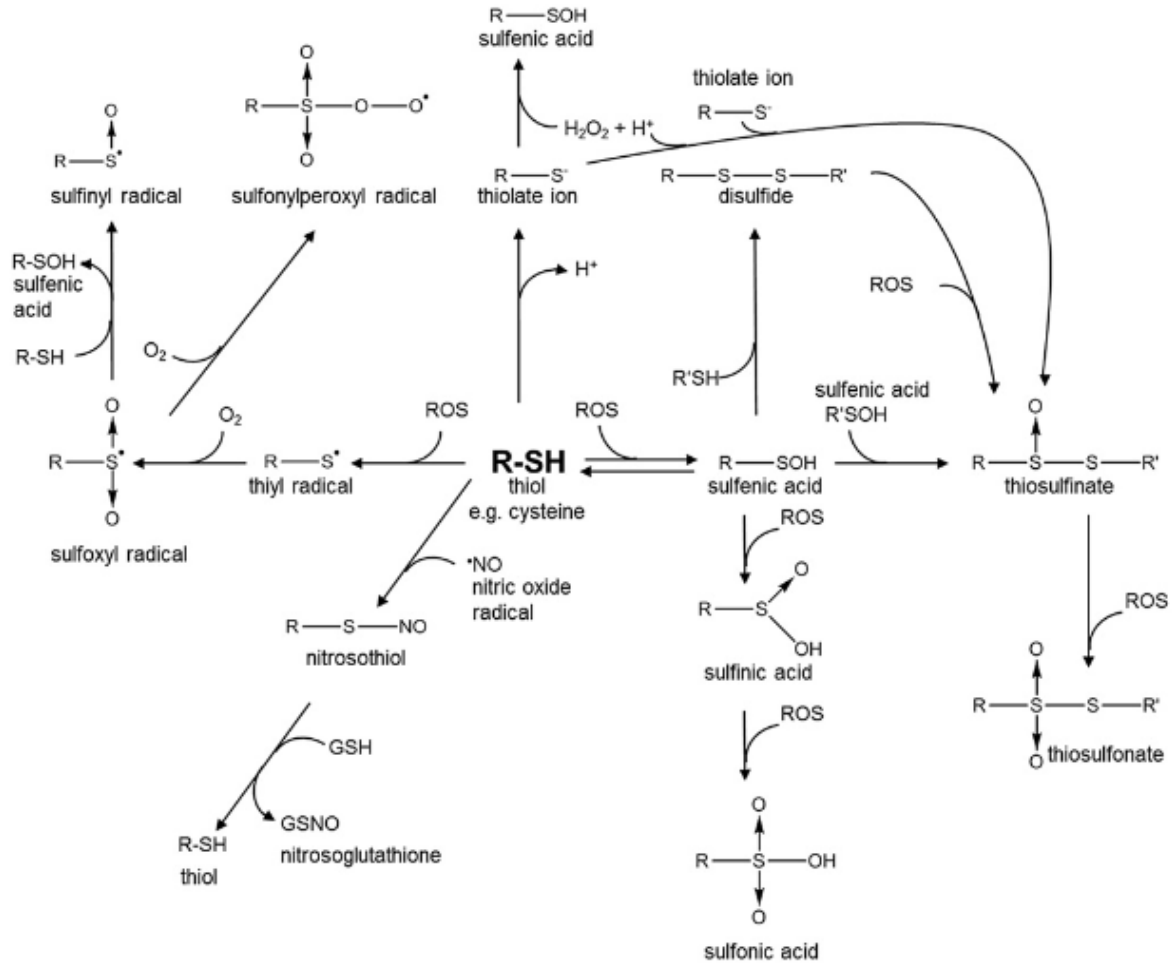

Abbildung 2: Mögliche Reaktionen eines Thiols nach Gruhlke und Slusarenko 2012 [13].

Die häufigste Oxidation eines Thiols in einer Zelle geschieht unter Ausbildung von Disulfid-Brücken. Dabei laufen grundsätzlich mehrere Mechanismen ab, was zu verschieden Produkten führt.

2.2.1. Bildung intermolekularer Disulfide

Hier werden zwei Proteine, welche jeweils mindestens einmal die Aminosäure Cystein enthalten, oxidiert. Dadurch entsteht zwischen den beiden Schwefelatomen der Cysteine eine Schwefel - Schwefel Brücke, auch S-S Disulfid-Bindung genannt [16].

2.2.2. Bildung intramolekularer Disulfide

Dabei muss ein Protein oder Enzym mindestens zwei Cysteine enthalten, deren Schwefelatome bei einer Oxidation über eine Disulfidbrücke verbunden werden. Durch diese Oxidation ändert sich die Faltung des Proteins und dadurch bedingt auch dessen Funktion und Beweglichkeit. Ein Beispiel hierfür ist das YAP1 Protein, welches als Transkriptionsregulator eine wichtige Rolle in der antioxidativen Antwort von Hefezellen aus *Saccharomyces cerevisiae* gegenüber von OS spielt. Das Prooxidants H_2O_2 oxidiert mit Hilfe der Enzyme YBP1 und GPX3 zwei bzw. vier Thiole des YAP1 Proteins zu einer bzw. zwei Disulfidbrücken. Durch die damit einhergehende Konformationsänderung lässt sich das YAP1 Protein somit nicht mehr aus dem Zellkern exportieren und reichert sich in seiner oxidierten Form an. Solange YAP1 in einer oxidierten Form im Überschuss vorliegt, reguliert es die Transkription von Genen hoch, die antioxidative Enzyme codieren. Ein Export aus dem Zellkern findet erst wieder statt, wenn Thioredoxine, deren Expression ebenfalls durch YAP1 reguliert wird, die Disulfidbrücken reduzieren. Somit handelt es sich um eine gekoppelte Autoregulation [17].

2.2.3. Oxidation von Cysteinresten mit niedermolekularen Thiolen

Oft werden die Cysteinreste von Proteinen auch durch die Reaktion mit niedermolekularen Verbindungen zu Disulfidbrücken oxidiert. Dabei findet ein sogenannter Thiol-Disulfid Austausch statt. Thiol und Disulfid bilden ein Redoxpaar. Zwei Thiole werden oxidiert und geben formell jeweils ein Elektron ab, während das Disulfid reduziert wird und die beiden Elektronen aufnimmt. Es ist zu beachten, dass, obwohl freien Thiolen formell die Oxidationszahl -2 zuzuordnen ist und Schwefel in Disulfiden formell die Oxidationszahl -1 besitzt, aus mechanistischer Sicht keine freien Elektronen übertragen werden.

Genau wie die Bildung von intramolekularen Disulfidbrücken kann die Oxidation einzelner Cysteinreste zu einer Änderung der Konfirmation und einer Veränderung der Funktion oder Aktivität des Proteins führen [18].

2.2.4. Oxidation von niedermolekularen Thiolen

In allen eukaryotischen und vielen prokaryotischen Zellen liegt eine relativ hohe Konzentration des niedermolekularen Thiols Glutathion (*γ*-L-Glutamyl-L-cysteinylglycin, GSH) vor. Glutathion ist ein Tripeptid und besteht aus den Aminosäuren Glutaminsäure, Cystein und Glycin und wird oft als intrazellulärer Redox-Puffer bezeichnet, da es in seiner reduzierten Form vielen Enzymen als Substrat dient. Dabei wird das Glutathion zum Glutathion-Disulfid (GSSG) oxidiert, während das entsprechende Enzym reduziert wird. Viele Enzyme, die GSH als Substrat nutzen, sind relevant für den intrazellulären Redox-Status. Dazu gehören zum Beispiel die Glutaredoxine (GRX) und die Glutathione Peroxidasen (GPX) [19].

Der von Claus Jacob im Jahr 2010 geprägte Begriff „Zellulärer Thiolstat“ beschreibt das Modell eines intrazellulären Redox-Netzwerkes in dem, ähnlich einem Thermostaten, Signale gesendet und durch Rückkopplung gesteuert werden, sodass das System gesteuert wird. In dem Fall handelt es sich aber um lebende Zellen, die auf intern und extern bedingte Änderungen des Redoxzustandes angemessen reagieren. Ermöglicht wird dies durch die komplexe Redoxchemie der Aminosäure

Cystein, welche in vielen Proteinen und Enzymen und dem Glutathion enthalten ist und an vielen Redoxprozessen beteiligt ist [20, 21].

Mit Hilfe eins thermodynamischen Modells und der Nernst Gleichung lässt sich das elektrochemische Reduktionspotenzial der Zelle berechnen. Da Glutathion in der Zelle in hohen Konzentrationen vorkommt und sämtliche Redoxpaare der Zelle im stetigen Austausch zwischen und miteinander stehen, ist das Verhältnis von oxidiertem und reduziertem Glutathion der zentrale Bestandteil der Berechnungen.

Da eine Erhöhung des Oxidationszustandes zu einem größeren Anteil an GSSG führt bzw. ein größerer Anteil an GSSG einen höheren Oxidationsgrad der Zelle beschreibt, lässt sich das Reduktionspotential als ein Maß für den Redoxstatus der Zelle und somit ihres physiologischen Zustandes nutzen [22, 23].

Eine Zelle, deren Glutathionpool sich im reduzierten Zustand befindet, proliferiert. Eine Erhöhung des GSSG Anteils erhöht das elektrochemische Reduktionspotential und die Zelle befindet sich im Zustand der Differenzierung. Dabei exportiert der ABC Transporter Ycf1 (yeast-cadmium factor 1) GSSG aus dem Cytosol in die Vakuole, um einen reduzierten Zustand im Cytosol aufrecht zu erhalten [24]. Eine weitere Erhöhung des GSSG Anteils in der Zelle führt schließlich zur Apoptose und Nekrose der Zelle (Abb. 3) [25]. GSSG wird durch das Enzym Glutathion-Reduktase unter Oxidation von NADPH zu $NADP^+$ wieder zum reduzierten Glutathion reduziert [26]. Neue Studien von Horn *et al.* [27] zeigen, dass die Glutathion-Reduktase (GR) auch in der Lage ist, gemischte Disulfide aus Glutathion und Reaktiven Schwefel Spezies wieder zu reduzieren.

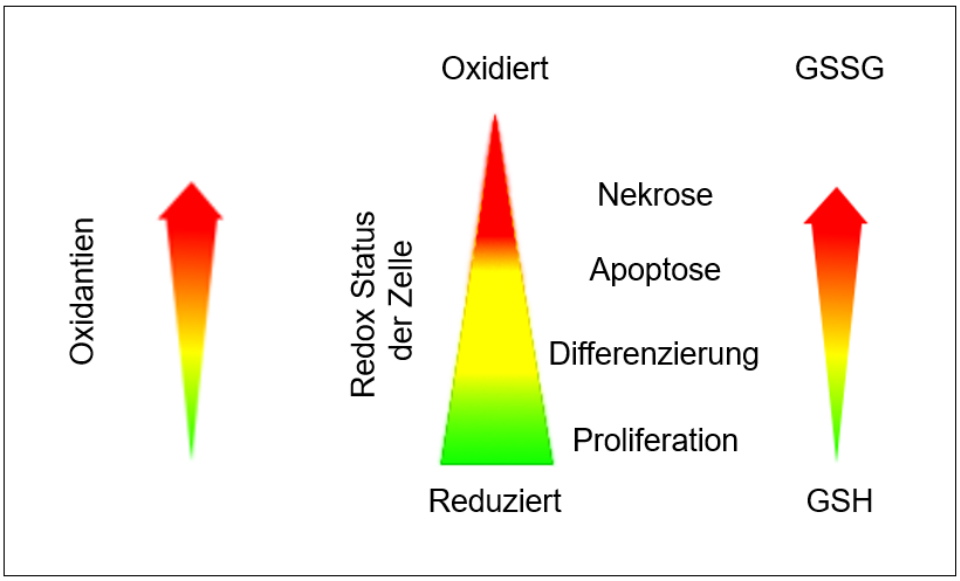

Abbildung 3: Einfluss von Oxidantien auf den Redoxzustand der Zelle sowie auf das Verhältnis von oxidiertem und reduziertem Glutathion, angepasst nach Gruhlke et al. (2011) [25]

2.3. Antioxidantien

Antioxidantien sind definiert als Substanzen, die Oxidationsprozesse verlangsamen oder unterdrücken. Neben dem Glutathion und dem Cystein gibt es noch weitere Antioxidantien, die eine wichtige Rolle in eukaryotischen Zellen spielen. Diese lassen sich in niedermolekulare Verbindungen und Enzyme unterteilen.

2.3.1. Niedermolekulare Verbindungen

Eine Vielzahl niedermolekularer Verbindungen kann in der Zelle als Antioxidans fungieren. Dazu gehören das schon erwähnte Glutathion sowie weitere Verbindungen, wie Harnsäure und α-Liponsäure. Zu den prominentesten in unserer Nahrung vorkommenden Antioxidantien gehören die bekannten Verbindungen Ascorbinsäure, auch Vitamin C genannt, Vitamin E und Carotinoide sowie viele Polyphenole wie Phenolcarbonsäuren, Flavonoide und Proanthocyanidine (Abb. 4) [28].

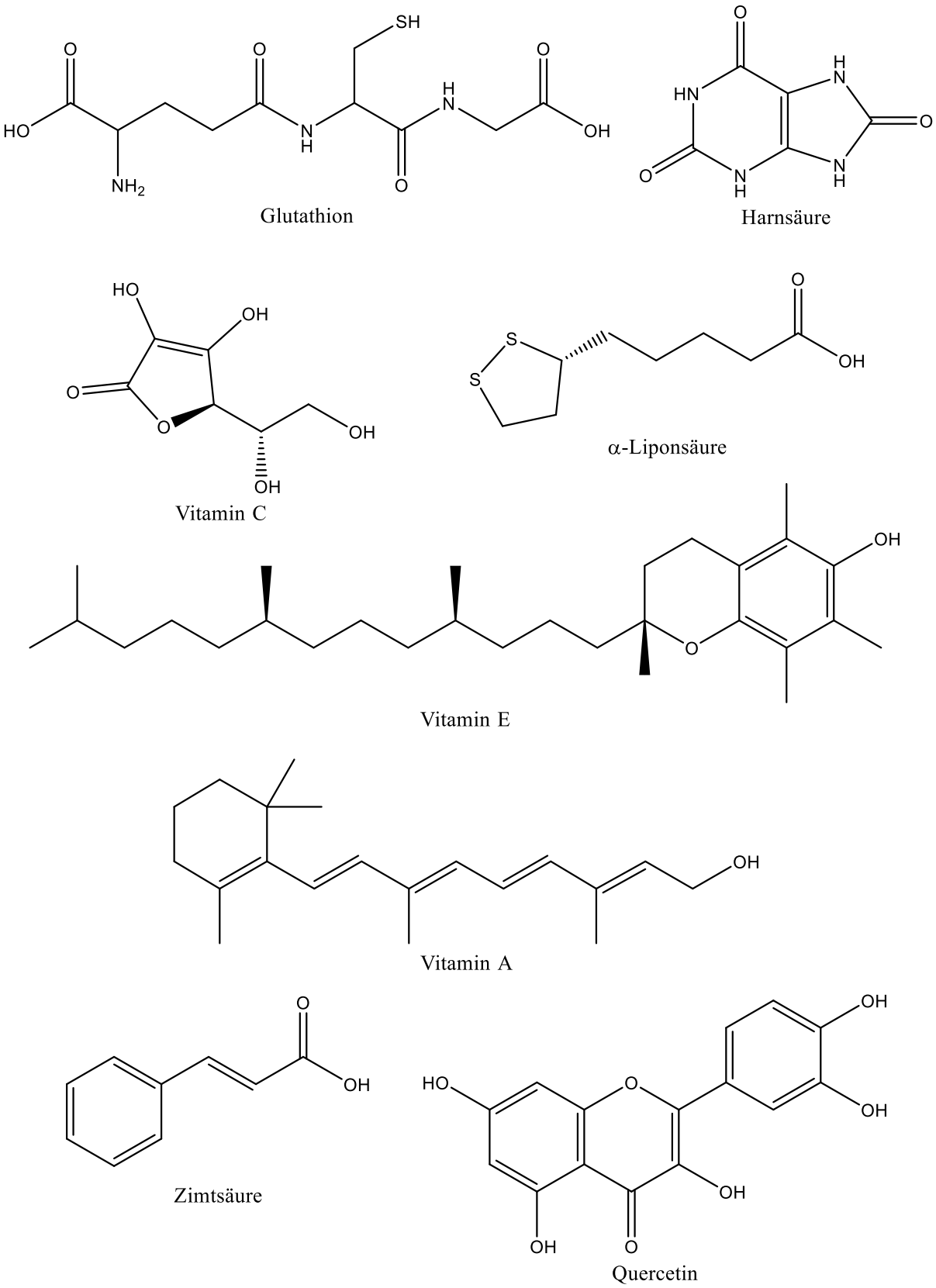

Abbildung 4: Chemische Struktur einiger ausgewählter Antioxidantien.

2.3.2. Enzyme

In Zellen stellen enzymatische Antioxidantien eine wichtige Säule im Redoxsystem dar [29]. Viele davon sind schon vorhanden, wenn die Zelle gesund und ungestresst vorliegt, und halten den physiologisch optimalen Redoxzustand aufrecht. Im Falle von OS werden sie jedoch vermehrt von der Zelle exprimiert. Zu den wichtigsten Enzymen mit antioxidativer Aktivität gehören:

2.3.2.1. Superoxid Dismutasen (SOD)

Wie der Name „Superoxid Dismutase“ schon impliziert, wandeln diese Enzyme das sehr reaktive $O_2^{\bullet-}$ in H_2O_2 und O_2 um. SOD können anhand der Proteinfaltung und der beteiligten Metallatome in drei unterschiedliche Klassen eingeteilt werden: die Kupfer- und Zink-SOD, welche hauptsächlich von Eukaryoten genutzt wird, die Eisen- oder Mangan-SOD, welche entweder eins der beiden oder beide Metallatome als Cofaktor nutzen und sowohl von Prokaryoten als auch von Eukaryoten genutzt werden und die Nickel-SOD, welche ausschließlich von Prokaryoten genutzt werden. Bei Menschen und Säugetieren kommen drei verschiedene SOD vor, SOD1 im Cytoplasma, SOD2 in den Mitochondrien und SOD3, welche in der Zelle lokalisiert ist [30].

2.3.2.2. Peroxidasen

Peroxidasen sind in der Lage, H_2O_2 und aliphatische Peroxide zu reduzieren. Die dabei benötigten Elektronen werden aus verschiedenen Quellen, wie z.B. Cytochrom *c*, Iodid, GSH oder Mangan (II) entnommen. Einen besonderen Stellenwert haben hier die Katalasen, die H_2O_2 sowohl als Elektronendonor als auch als Elektronenakzeptor nutzen, um es als Teil einer Redox Disproportionierung zu H_2O und O_2 umzuwandeln [31, 32].

2.3.2.3. Glutathionperoxidasen (GPX)

Die Glutathionperoxidasen sind spezielle Peroxidasen, die ebenfalls Wasserstoffperoxid und organische Peroxide zu Wasser und Alkoholen reduzieren, allerdings oxidieren sie dabei GSH zum GSSG, indem GSH als Elektronendonor genutzt wird. Bei Menschen konnten bisher acht GPX identifiziert werden. Im aktiven Zentrum dieser Enzyme steht dabei ein Selenocystein, das oxidiert wird, um H_2O_2 zu reduzieren [33].

2.3.2.4. Glutaredoxine (GRX)

Glutaredoxine bilden eine Gruppe von redoxaktiven Enzymen, deren katalytisches Zentrum zwei Cysteine enthält, die im Falle einer Reduktion des Substrates und einer Oxidation des Enzymes zu einer Disulfid-Brücke oxidiert werden können. Glutaredoxine dienen als Elektronendonatoren für Ribonukleotid-Reduktasen und als Teil der antioxidativen Abwehr, indem sie Dehydroascorbinsäure und Peroxiredoxine reduzieren. Glutaredoxine selbst werden wiederum durch Glutathion reduziert [34].

2.3.2.5. Glutathion Reduktasen (GR)

Glutathion wird von vielen Enzymen als Elektronendonator genutzt, um die reduzierte Form des Enzymes wiederherzustellen. Es kann allerdings auch direkt durch die Reaktion mit einer anderen niedermolekularen Verbindung oxidiert werden. Die Glutathion Reduktasen (GR) reduzieren das oxidierte Glutathion Disulfid GSSG zurück zu GSH unter Verwendung des Reduktionsäquivalentes NADPH. Aktuell konnten Horn *et al.* am Beispiel von *S*-Allylmercaptoglutathion, einem Reaktionsprodukt von Allicin und GSH, zeigen, dass im Modellsystem Hefe Glutathion Reduktasen auch andere Verbindungen oxidierten Glutathions, wie z.B. *S* – Allylmercaptoglutathion, wieder reduzieren können [26, 27].

2.3.2.6. Thioredoxine (TRX)

Die Gruppe der Thioredoxine ist in der Lage, oxidierte Cysteinreste anderer Enzyme und Proteine wieder zu reduzieren. Dabei werden die Cysteine am katalytischen Zentrum oxidiert und es bildet sich eine intramolekulare Disulfid-Bindung aus [35, 36].

2.3.2.7. Thioredoxin Reduktase (TR)

Die Thioredoxin-Reduktase hat eine ganz ähnliche Funktion wie die Glutathion-Reduktase: Unter Verwendung des Reduktionsäquivalents NADPH ist die Thioredoxin-Reduktase in der Lage, die Disulfidbrücke eines oxidierten Thioredoxins durch Reduktion zu öffnen und somit ein antioxidatives Enzym wieder in seinen aktiven Zustand zu überführen. Somit sind die Cysteinreste des Thioredoxins wieder reduziert und können erneut ihrer Funktion nachgehen [36].

2.3.2.8. Protein Disulfid Isomerasen (PDI)

Protein Disulfid Isomerasen (PDI) sind Enzyme, die eine wichtige Rolle bei der Proteinfaltung spielen, indem sie die passenden Cysteinreste zu Disulfidbrücken verbinden. Im aktiven Zentrum dieser Enzyme stehen zwei Disulfidbrücken, welche als Elektronenakzeptor fungieren [37].

2.4. Reaktive Schwefel Spezies

Früher ging man davon aus, dass Oxidativer Stress (OS) hauptsächlich durch Reaktive Sauerstoff- und Stickstoff Spezies (ROS, RNS) ausgelöst wird. Schwefel hingegen wurde als Teil der antioxidativen Antwort des Systems betrachtet, in dem es eine passive Rolle annimmt und oxidiert werden kann. Erst 2002 wurde von Giles und Jacob das Konzept der Reaktiven Schwefel Spezies eingeführt, nachdem sie erkannten, dass Schwefel-Verbindungen existieren, die ähnlich hohe Reaktivität wie die ROS besitzen und in der Lage sind, Proteine und Enzyme mit Thiolen zu oxidieren. Per

Definition sind Reaktive Schwefel Spezies (RSS) Schwefelverbindungen, die in biologischen Systemen Redoxaktivität besitzen. Schwefel kann in diesen Verbindungen Oxidationszustände von -2 bis +6 annehmen. Daher sind RSS in der Lage, sowohl oxidierend als auch reduzierend zu wirken. Ein Beispiel für ein physiologisch oxidierendes RSS ist das Allicin (Prop-2-en-1-thiosulfinsäure-S-allylester) [38].

2.5. Knoblauch, Allicin und Thiosulfinate

Die Aufzeichnungen, in denen Knoblauch (*Allium sativum*) als Gewürz- und Heilpflanze genutzt wurde, gehen tausende Jahre zurück [39]. Bereits im antiken Ägypten nutzten die Menschen Knoblauch. Es wurde nicht nur als Gewürz genutzt, sondern auch, um Salben herzustellen und um Sklaven gesund zu halten. Es sind Belege für seine Nutzung von Griechenland hin bis nach Japan vorhanden [40]. Erst kürzlich wurde ein Rezept in England für eine Augensalbe aus dem 10. Jahrhundert im Bald‘s Leechbook gefunden [41], welches ein erstaunlich potentes Arzneimittel hervorbringt und Knoblauch enthält. Den Grund bzw. das Molekül, das für die biologische Aktivität des Knoblauchs verantwortlich ist, konnten Cavallito und Bailey 1944 identifizieren und isolieren: das Allicin [42].

Allicin ist ein Thiosulfinat (RS(O)SR‘; R, R‘≠H) bei dem zwei Allylgruppen die Reste bilden und in dem beiden Schwefelatomen unterschiedliche Oxidationszahlen zuzuordnen sind (+1 und -1). Die volatile organische Schwefelverbindung bildet sich erst, sobald Knoblauchgewebe verletzt wird. Dabei befinden sich die Vorläufer-substanz, die Aminosäure Alliin (*S*-Allyl-L-cystein-*S*-oxid) in intakten Zellen im Zytoplasma und die Alliinase, das Enzym, welches die Umwandlung des Alliins zum Allicin katalysiert und zur Klasse der C-S-Lyasen (Carbon-Schwefel-Lyasen) gehört, in den Vakuolen. Diese räumliche Trennung des „Zwei-Komponenten Wirkstoffs“ ist auch daran zu erkennen, dass unbeschädigter Knoblauch nicht den charakteristischen Knoblauchgeruch absondert. Sobald die Zelle verletzt wird, verbinden sich Vorläufersubstanz und Enzym und aus Alliin entsteht unter Abspaltung von Brenztraubensäure (2-Oxopropansäure, $C_3H_4O_3$) und Ammoniak (NH_3) die

Allylsulfensäure. Diese ist reaktiv und kondensiert mit einem weiteren Molekül Allylsulfensäure zum Allicin (Abb. 5).

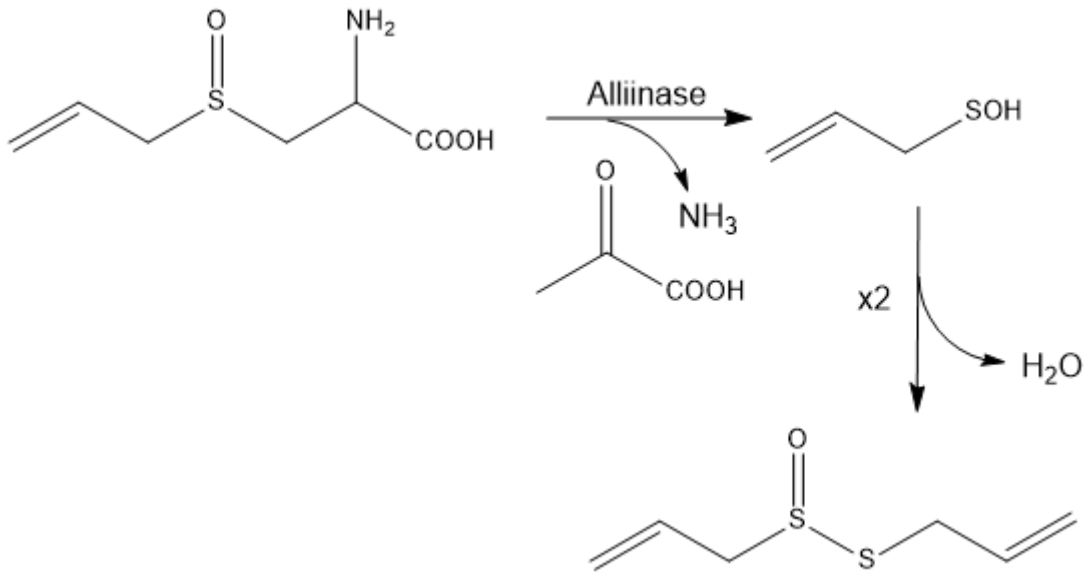

Abbildung 5: Biosynthese des Allicins aus der Vorläufersubstanz Alliin in Gegenwart der C-S-Lyase Alliinase.

Allicin ist ein chirales Molekül, allerdings kommt es in der Natur nur als Racemat vor, auch wenn die Vorläufersubstanz Alliin selbst in enantiomer reiner Form vorliegt. Dies liegt daran, dass die bei der Umwandlung entstehende Sulfensäure kein Stereozentrum besitzt und die chirale Information dadurch verloren geht. Somit wird bei der Kondensation Allicin als Racemat gebildet. Der Biosyntheseweg, der zur Bildung des Alliins führt, ist bis heute allerdings immer noch nicht ausreichend geklärt.

Da Allicin thermisch nicht stabil ist und sich auch schon bei Raumtemperatur durch Reaktionen mit sich selbst zersetzt [43], ist die Möglichkeit, Allicin auch auf chemischem Wege herzustellen, von großem Interesse. Dabei wird Diallyldisulfid durch H_2O_2 unter Verwendung eines Katalysators- meistens Essigsäure- zum Allicin oxidiert [44]. Als Oxidationsmittel werden aber auch häufig Monoperoxyphthalate und Chlorperbenzoesäure genutzt [45, 46, 47]. Ein genaues Protokoll zur Herstellung von reinem Allicin wurde im Jahr 2001 von Lawson und Wang veröffentlich. Albrecht *et al.* haben davon ausgehend im Jahr 2017 eine verbesserte Methode veröffentlicht und den chemischen Reaktionsmechanismus aufgeklärt [48].

Die Reaktion besteht aus zwei Reaktionsschritten. Im ersten Reaktionsschritt oxidiert H_2O_2 die organische Säure zur korrespondierenden Persäure (RCOOOH). Im zweiten Reaktionsschritt oxidiert die Persäure das Diallyldisulfid (DADS) und wird dabei zur ursprünglichen organischen Säure reduziert, die dadurch als Katalysator fungiert. Aus der Literatur ist bekannt, dass solche Persäuren wie Perameisen (HCOOOH)- und Peressigsäure (CH_3COOOH) in organischen Lösemitteln löslich sind, wohingegen das DADS mit der wässrigen H_2O_2- Lösung nicht mischbar ist und die Reaktion daher in einem Zwei-Phasen-System stattfindet.

2.5.1. Biologische Aktivität von Allicin

Allicin und damit auch Knoblauch besitzt eine große Brandbreite an biologischer Aktivität. Es konnte gezeigt werden, dass Allicin in der Lage ist, LDL (Low Density Lipoprotein) -Cholesterin, welches verantwortlich für die Entstehung von Arteriosklerose und anderer gefäßeinengender Herzerkrankungen ist, zu senken. Nachdem Mäuse 12 Wochen lang einer täglichen Diät mit 20 mg Allicin pro kg Körpergewicht unterzogen wurden, betrug der LDL-Cholesterin-Gehalt im Serum nur noch 50% der Kontrollgruppe [49]. Schon in geringen Konzentrationen ($IC_{50} < 10\mu M$) inhibiert Allicin die Fettsäuresynthese [50]. Weitere positive Effekte sind zum Beispiel die Senkung des Blutdruckes [51] und damit einhergehend die allgemeine Verringerung von Herz-Kreislauf-Erkrankungen. Auch wurde gezeigt, dass Allicin eine cytostatische Aktivität besitzt und somit als ein potenzieller Kandidat für den Einsatz in der Krebstherapie fungieren könnte. So liegen z.B. die IC_{50} Werte gegenüber der Brustkrebs-Zelllinie MCF-7 und der Darmkrebs-Zelllinie HT-29 zwischen 10 und 25 µM [52].

Allicin wirkt nicht nur auf menschliche Zellen, sondern ist ebenfalls in anderen Lebewesen aktiv. Es besitzt ausgeprägte antibakterielle, fungizide und auch viruzide Eigenschaften. Interessanterweise sind sogar Methicillin-resistente *Staphylococcus aureus* (MRSA) Bakterien gegen Allicin empfindlich [53].

Auch die sehr starke fungizide Wirkung des Allicins ist von großem wirtschaftlichem und ökologischem Interesse. So sind viele phytopathogene Pilze, die einen großen Schaden in der landwirtschaftlichen Produktion von Obst und Gemüse verursachen,

schon durch geringste Konzentrationen von Allicin am Wachstum gehindert. Da es sich bei Allicin um ein Naturprodukt handelt, ist auch der Einsatz in der ökologischen Landwirtschaft möglich [54].

Die Tatsache, dass Diallyldisulfid keine mit Allicin vergleichbare Aktivität besitzt, obwohl der Unterschied zwischen den beiden nur die Oxidation eines Schwefelatoms zum „Sulfoxid“ beträgt, zeigt, dass die Thiosulfinat-Gruppe für die Aktivität von Allicin verantwortlich ist. Andererseits zeigen sowohl das Diallyldisulfid als auch die Vorläufersubstanz Alliin andere, ebenfalls interessante biologische Aktivitäten [55].

Sorlanzanao-Puerto und seine Kollegen konnten zeigen, dass nicht nur die Thiosulfinate, sondern auch die Thiosulfonate ($RS(O)_2SR'$; R, R‘ ≠ H; Oxidationsstufe des Schwefels: +3 und -1), bei denen ein Schwefelatom durch zwei Sauerstoffatome oxidiert ist, eine bemerkenswerte Aktivität gegen verschiedene Gram-positive und Gram-negative Bakterien besitzen [56]. Diese Gruppe von Verbindungen zeigt ebenso wie die der Thiosulfinate ein sehr breites antibakterielles Spektrum und wirkt gegen eine Vielzahl von multiresistenten Bakterien, die von Patienten isoliert werden konnten. Solche Thiosulfonate können chemisch hergestellt werden, kommen aber auch natürlich vor, wie z.B. in der südostasiatischen Frucht *Scorodocarpus borneensis* und dem afrikanischen Baum *Scorodophloeus zenkeri* [57].

Allerdings wurde auch gezeigt, dass Knoblauchextrakt eine höhere antibakterielle Aktivität besitzt als Allicin der gleichen Konzentration. Dies ist auf den ersten Blick überraschend, aber damit zu erklären, dass in Knoblauch eben nicht nur das Diallylthiosulfinat gebildet wird, sondern auch andere Thiosulfinate wie beispielsweise Dimethylthiosulfinat und Dipropylthiosulfinat vorkommen [58].

2.6. Resistenzen gegenüber klassischen Antibiotika

Durch die Vielzahl der identifizierten, isolierten, synthetisierten und modifizierten Antibiotika sollten die durch Bakterien hervorgerufenen Krankheiten heutzutage keine bedeutende Rolle mehr spielen. Allerdings sind Bakterien lebende Organismen und stellen sich schnell auf neue Gegebenheiten ein. Bei Anwendung eines Antibiotikums bedeutet dies, dass die Bakterien Resistenzmechanismen entwickeln. Nur wenige Jahre nach Einführung eines neuen Antibiotikums werden meist die ersten resistenten

Bakterienstämme isoliert. Diese schnelle Anpassung von Bakterien ist in der ebenso schnellen Reproduktionsrate und der hohen Anzahl an Bakterien begründet. Sie wird unterstützt durch eine große genetische Vielfalt und der Möglichkeit des Transfers von Resistenzgenen von verschiedenen Organismen (Pflanzen, Pilze, andere Bakterien). Sobald nur eine einzige Zelle von mehreren Millionen eine Mutation aufweist, die zu einer Resistenz führt, wird sich diese Resistenz unter Selektionsdruck durchsetzen, weil die anderen Zellen durch das Antibiotikum abgetötet werden [59].

Baym *et al*. haben vor kurzem eine große mediale Aufmerksamkeit mit einem wissenschaftlichen Artikel erfahren, in dem sie auf einer übergroßen Petrischale die Evolution von resistenten Bakterien zeitaufgelöst gezeigt haben. Dabei sind 12 Tage ausreichend, um Bakterien entstehen zu lassen, die selbst das 3000-fache der ursprünglichen minimal inhibierenden Konzentration nicht mehr von der Proliferation abhalten [60].

Bakterien und Pilze nutzen im Prinzip häufig vier verschiedene Möglichkeiten, um eine Resistenz gegenüber verschiedenen Wirkstoffklassen zu erreichen:

- Entwicklung einer wirkstoffundurchlässigen Membran
- Export des Wirkstoffes aus der Zelle
- Metabolisierung des Wirkstoffes
- Modifizierung des Zielmoleküls

2.6.1. Entwicklung einer wirkstoffundurchlässigen Membran

Ein Mechanismus, den Bakterien entwickeln, um sich gegen antimikrobielle Wirkstoffe zu schützen, ist die Modifizierung der Zellmembran. Indem die Membran keine Wirkstoffe mehr durchlässt, wirkt sie wie ein Schutzschild für die Bakterien. Um dies zu erreichen, werden Transmembranproteine, sogenannte Porine, welche ähnlich einem Kanal den Stoffaustausch von Zellen ermöglichen, entweder in geringerer Anzahl gebildet oder so modifiziert, dass sie den betreffenden Wirkstoff nicht mehr durchlassen können (Abb. 6). Dadurch wird entweder die Möglichkeit zum Eintritt in die Zelle bzw. die Eintrittsrate in die Zelle herabgesetzt und keine wirksame

Wirkstoffkonzentration in der Zelle mehr erreicht. Somit können die Zellen bei erhöhten äußeren Wirkstoffkonzentrationen proliferieren und eine Resistenz ist gegeben [61].

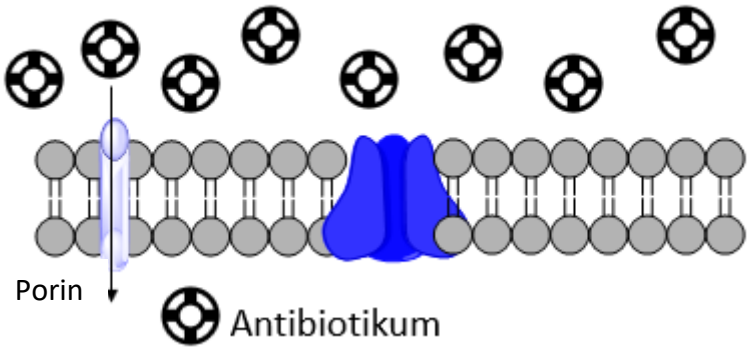

Abbildung 6: Bei geringerer Anzahl an Porinen mit geringerer Durchlässigkeit für Wirkstoffe ergibt sich eine geringere Wirkstoffkonzentration innerhalb der Zelle.

2.6.2. Auspumpen des Wirkstoffes aus der Zelle

Einige Organismen produzieren Membranproteine, sogenannte ABC Transporter (ATP binding cassette), welche in der Lage sind, Wirkstoffe aktiv aus der Zelle zu pumpen. Bei einem resistenten Organismus sind diese Proteine entweder modifiziert, um die Wirkstoffe schneller oder effektiver aus der Zelle zu pumpen, oder aber es werden mehr dieser Efflux-Proteine produziert. In beiden Fällen hat dies eine geringere Wirkstoffkonzentration im Inneren der Zelle zur Folge (Abb. 7). Diese Konzentration ist dann nicht mehr ausreichend, um die betroffenen Zellen zu töten mit dem Resultat einer Proliferation der resistenten Zellen [62].

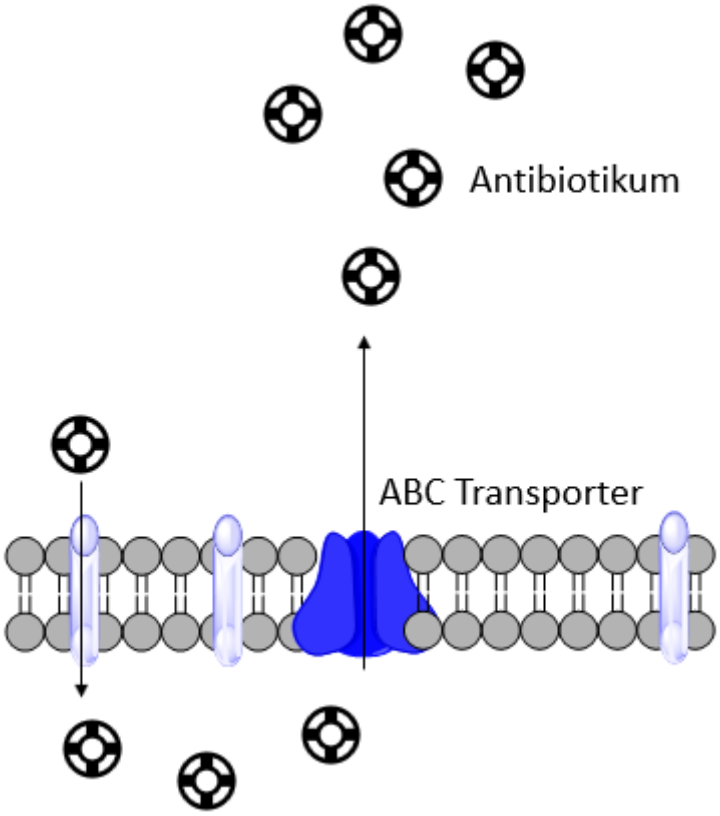

Abbildung 7: ABC Transporter entfernen Antibiotika aus der Zelle.

2.6.3. Metabolisierung des Wirkstoffes

Eine weitere Möglichkeit, für Organismen Resistenzen gegenüber antimikrobiellen Wirkstoffen zu entwickeln, stellt die Metabolisierung des Wirkstoffmoleküls dar. Dabei werden zwei verschiedene Strategien beobachtet. In dem einen Fall hat das resistente Bakterium spezielle Enzyme entwickelt, um die Wirkstoffe zu deaktivieren. Ein Beispiel hierfür sind *β*-Lactamasen. Diese Enzyme greifen mit der OH-Gruppe eines Serins im aktiven Zentrum der *β*-Lactamase den Lactam-Vierring von *β*-Lactam Antibiotika an. Der Vierring öffnet sich unter Hydrolyse und das Enzym spaltet sich ab, um weitere *β*-Lactame zu desaktivieren. In der Regel decarboxyliert das entstandene Zwischenprodukt und die Deaktivierung ergibt sich daher als irreversibel [63].

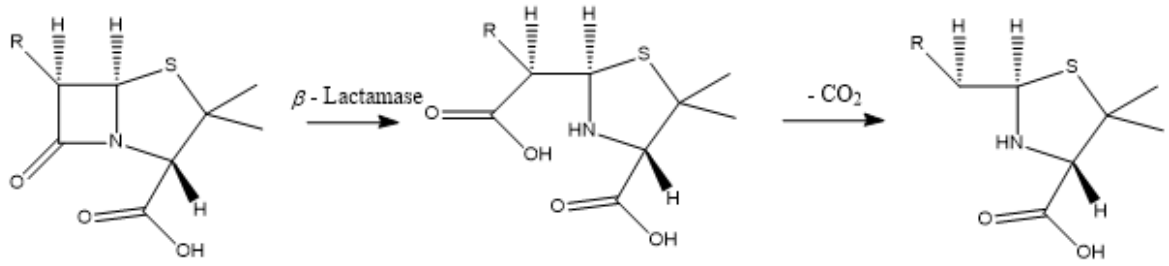

Abbildung 8: Öffnung des β-*Lactamrings durch die* β-*Lactamase mit anschließender Decarboxylierung.*

Eine weitere Möglichkeit, den Wirkstoff zu desaktivieren, besteht darin, ihn sterisch zu modifizieren. Somit ist er nicht mehr in der Lage, an sein Ziel zu binden. Dies kann enzymatisch oder Säure/ Base-katalysiert ablaufen. Dabei werden meistens eine oder mehrerer Seitenketten acetyliert, phosphoryliert oder es wird eine Adenosinmonophosphat Gruppe hinzugefügt [64]. Zum Beispiel werden Aminoglycoside Antibiotika wie Kanamycine durch Phosphotransferase phosphoryliert und somit desaktiviert.

2.6.4. Modifizierung des Zielmoleküls

Eine gewissermaßen umgekehrte Strategie verfolgen die Bakterien, die in der Lage sind, bei höheren Wirkstoff-Konzentrationen zu proliferieren. Diese Bakterien haben dabei einen Weg gefunden anstelle des Wirkstoffes selbst, das Zielmolekül der Antibiotika so zu modifizieren, dass es zu keiner Reaktion mit dem Wirkstoff mehr kommt. Oft geschieht dies über die geringfügige Änderung an einem Protein, wodurch der Wirkstoff nicht mehr bzw. nicht mehr richtig an das Protein binden kann und somit unwirksam wird. Das prominenteste Beispiel dafür ist wahrscheinlich die Resistenz beim Erreger MRSA (Methicillin-resistenter *Staphylococcus aureus*). Normalerweise reagieren Methicillin und andere *b*-Lactam Antibiotika mit Penicillin bindenden Proteinen (PBP), die für den Aufbau der Zellwand benötigt werden. Dabei binden die *b*-Lactame an das Zentrum des Proteins, welches für die Transpeptidase-Aktivität und somit für die Quervernetzung der Zellwand Peptide verantwortlich ist und inhibieren die PBP dadurch. Die seit den 60er Jahren bekannten MRSA Staphylokokken-Arten bilden ein modifiziertes Penicillin Bindungsprotein (PBP2a) aus, welche eine geringere

Affinität zu β-Lactamen hat. Dieses ist auch in Anwesenheit von Antibiotika funktionsfähig und führt die Transpeptidation und somit die Ausbildung einer intakten Zellwand fort [65].

2.7. Post – Antibiotika - Ära

Der durch diese Resistenzmechanismen zunehmend hervorgerufene Wirkungsverlust von Antibiotika stellt eine globale Bedrohung dar. Schon einfache Infektionen, die relativ schnell durch effektive Wirkstoffe bekämpft werden konnten, können dadurch zukünftig zu einer Gefahr werden. Die bestehenden Antibiotika-Klassen wurden fast ausschließlich im letzten Jahrhundert entdeckt. Daher ist es nun unerlässlich neue Antibiotika zu finden und die gegenwärtige – teils sorglose - Nutzung von Antibiotika im Übermaß zu hinterfragen [66].

2.8. Verwendete biologische Modellorganismen

2.8.1. Backhefe *Saccharomyces cerevisiae*

Die heutige Bierhefe oder auch Bäckerhefe genannt hat ihren Ursprung in den obergärigen Bierhefen. Dies geht auch aus ihrem lateinischen Namen *Saccharomyces cerevisiae* hervor, *cerevisiae* bedeutet „des Bieres“ und *Saccharomyces* bedeutet „Zuckerpilz“.

Die Zellen von *Saccharomyces cerevisiae* besitzen eine ovale Form, sind in etwa 10 µm lang und vermehren sich durch Knospung.

Bierhefe ist in der Lage, Energie sowohl durch Zellatmung als auch durch Gärung zu gewinnen. Dabei werden hauptsächlich Mono- und Disaccharide metabolisiert. Bei aerober Atmung ist das Endprodukt dementsprechend Kohlenstoffdioxid (CO_2) und im Falle der anaeroben Gärung Ethanol (CH_3CH_2OH) [67].

Bierhefen werden als Zusatz beim Backen verwendet. Ebenso werden die Hefen bei der Gärung von Bieren und Weinen und zunehmend bei der Herstellung von Bioethanol als Kraftstoff verwendet. Hefeextrakte werden in der Biochemie als Zusatz

zu Nährmedien verwendet und durch gentechnische Veränderungen lassen sich Hefen so programmieren, dass sie Aminosäuren und Proteine wie z.B. Insulin für den Menschen produzieren [68].

Hefen sind, genau wie Tiere und Pflanzen, Eukaryoten. Das bedeutet, dass ein Zellkern und weitere intrazelluläre Organellen, wie Mitochondrien, Vakuolen und das endoplasmatische Retikulum, einzeln innerhalb einer Zelle vorliegen und autarke Funktionen, besitzen. Prokaryotische Organismen, wie zum Beispiel Bakterien, hingegen besitzen keine einzelnen, Membran-gebundenen Organellen und können daher auch die biochemischen und metabolischen Prozesse nicht auf einzelne Bereiche aufgliedern [69].

Bierhefezellen und menschliche Zellen sind sich auf genetischer und biochemischer Ebene überraschend ähnlich. Beispielsweise reagieren Hefezellen auf viele Chemikalien ähnlich wie auch menschliche Zellen und daher eignet sich *Saccharomyces cerevisiae* hervorragend als Modellorganismus für die Forschung. Zum Beispiel kann die Wirkungsweise bestimmter potenzieller Wirkstoffe und Substanzklassen vorab an Bierhefe experimentell bestimmt werden und somit erste Anhaltspunkte für Versuche mit Säugetieren liefern. So hat die Bierhefe in der jüngeren Vergangenheit auch als Modellorganismus in der Erforschung einiger weit verbreiteter menschlicher Krankheiten wie Krebs, Fettleibigkeit und von neurodegenerativen Erkrankungen gedient [70, 71, 72]. Es wurden aber auch seltenere Krankheiten wie das Werner-Syndrom, bei dem die Betroffenen vorzeitig altern, untersucht. In dem Fall liegt ein Defekt im humanen WRN-Gen vor, dessen Struktur der des SGS1 Gens der Hefe gleicht [73].

Bäckerhefe war der erste eukaryotische Organismus, dessen Genom in Jahr 1996 vollständig sequenziert und veröffentlicht wurde. 13 Millionen Basenpaare codieren über 6000 Gene, die auf 16 Chromosomen angeordnet sind. Im Vergleich dazu enthält das menschliche Genom ungefähr 20000 Gene und ist somit dreimal so groß [74, 75]. Und trotz den so offensichtlich enormen Unterschieden zwischen einem Menschen und einer Hefezelle sind viele Gene ähnlich und besitzen ähnliche Eigenschaften. Einige Gene sind sogar insoweit homolog, dass sie zwischen den Organismen ausgetauscht werden können und eine funktionale Komplementation herstellen können. Das bedeutet, dass ein Gen aus einer Hefe in einer menschlichen Zelle die Funktion des entsprechenden menschlichen Gens ersetzen kann [76].

Durch diese Eigenschaften und die relativ einfache Kultivierung im Labor ist Bierhefe hervorragend als Testorganismus geeignet. Durch den Einsatz in sogenannten Mutanten-Bibliotheken, bei denen einzelne Gene der Hefe entfernt wurden, und dem chemogenetischen Profiling lassen sich Rückschlüsse auf die Art der Wirkungsweise einer chemischen Substanz und auf ihre zellulären Angriffsziele ziehen. Für die Charakterisierung neuer Wirkstoffe sind solche Studien unerlässlich [77].

2.8.2. Escherichia coli

Escherichia coli Bakterien wurden erstmals im Jahr 1886 vom Kinderarzt Theodor Escherich (1857-1911) beschrieben und sind nach diesem benannt worden. Dabei handelt es sich um ein fakultativ anaerobes, stäbchenförmiges Bakterium (Abb. 9). Es ist der Familie der *Enterobacteriaceae* zugeordnet und als Gram-negativ klassifiziert [78]. Damit besitzen *Escherichia coli* Bakterien nur eine dünne Zellwand, die allerdings sowohl von innen als auch von außen durch eine Lipidmembran umschlossen wird. Normalerweise leben *E. coli* Bakterien als Teil der Darmflora von Menschen und Säugetieren und spielen eine entscheidende Rolle beim Metabolismus und der Produktion von Vitaminen (insbesondere Vitamin K_2) [79]. Da *E. coli* Bakterien eine hohe Lebensdauer auch außerhalb des Darms haben, werden sie oftmals als Indikator für die Qualität von Trinkwasser und Lebensmittelhygiene genutzt. *E. coli* Bakterien sind leicht durch biochemische und analytische Methoden nachzuweisen und deuten auf fäkale Rückstände und unhygienische Verarbeitung hin. Viele *E. coli* Stämme besitzen darüber hinaus pathogene Eigenschaften und können zu einer Vielzahl von verschiedenen Krankheitsbildern, wie Blasenentzündungen und Diarrhöe, führen [80].

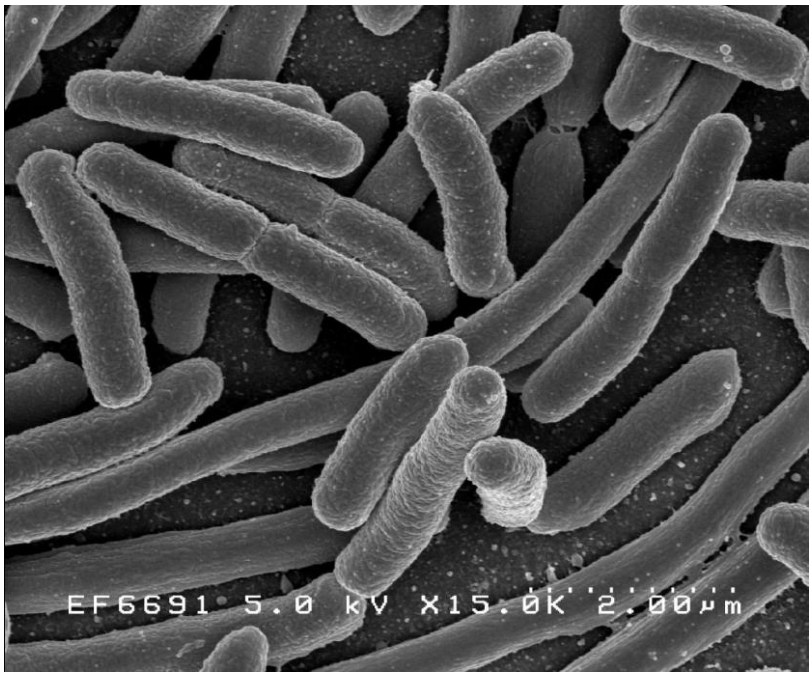

Abbildung 9: Rasterelektronenmikroskopische Aufnahmen von Escherichia coli *Bakterien. Die Stäbchenform ist gut zu erkennen. Credit: Rocky Mountain Laboratories, NIAID, NIH.*

Durch die relativ einfache Kultivierung unter Laborbedingungen sind nicht-pathogene Varianten von *E. coli* Bakterien zu einem der am häufigsten genutzten Forschungsorganismen geworden. Zum einen werden *E. coli* Bakterien als Modellorganismus für physiologische Tests eingesetzt [81], zum anderen werden die Bakterien aufgrund ihrer guten Handhabbarkeit und ihres schnellen Wachstums als beliebtes Werkzeug für biochemische und genetische Arbeiten genutzt [82].

2.8.3. Pseudomonaden

Bakterien der Gattung *Pseudomonas* wurden erstmals 1894 von Walter Migula (1863-1938) beschrieben. Ebenso wie *E. coli* sind Pseudomonaden Gram-negative Bakterien und besitzen eine stäbchenartige Form. Pseudomonaden sind allesamt aerobe, nicht sporenbildende Bakterien. Man findet sie praktisch überall, d.h. im Boden, in Gewässern, auf Pflanzen und auf Tieren [83].

Die meisten Pseudomonaden-Spezies besitzen eine Resistenz gegenüber verschiedenen Antibiotika, die dadurch entsteht, dass die Antibiotika aus der Zelle gepumpt werden. Dank ihrer robusten Zellwand proliferieren Bakterien der Spezies *Pseudomonas* in relativ widrigen Umgebungen. Dazu sind sie in der Lage, einen

Biofilm auszubilden und sich somit gegen weiße Blutzellen oder das Eindringen von Antibiotika zu schützen. So wird zum Beispiel das Bakterium *Pseudomonas aeruginosa* ein immer größer werdendes Problem im medizinischen Bereich, da es ein opportunistischer Erreger ist, der bereits geschwächte Patienten angreift und aufgrund seiner geringen Anfälligkeit gegenüber Antibiotika schwer zu behandeln ist [84]. Da sowohl Pflanzen- als auch humanpathogene Pseudomonaden-Spezies existieren, sind Pseudomonaden ein interessanter Modellorganismus für antibakterielle Wirkstoffe.

2.8.3.1. Pseudomonas fluorescens

Pseudomonas fluorescens hat seinen Namen aufgrund der fluoreszierenden Siderophore – der Pyoverdine, die von diesen Bakterien ausgeschieden werden, erhalten [85]. In der Medizin ist *Pseudomonas fluorescens* von geringerer Bedeutung, spielt aber eine große Rolle bei verderblichen Lebensmitteln und in der Bodenbiologie. Im Boden sind *Pseudomonas fluorescens* Bakterien von Vorteil, da sie einige Wurzeln vor Pflanzenpathogenen schützen. Die Hintergründe sind noch nicht zweifelsfrei geklärt, allerdings geht man davon aus, dass entweder die betreffenden Pflanzen durch die Pseudomonaden eine induzierte Resistenz aufbauen oder aber die Pseudomaden in direkter Konkurrenz zu pflanzenpathogenen Mikroben stehen und der Boden somit keinen Platz für andere Mikroben mehr bietet [86, 87].

2.8.3.2. Pseudomonas syringae

Pseudomonas syringae haben ihren Namen vom Gewöhnlichen Fliederbaum (*Syringa vulgaris*) erhalten, da sie auf ihm zum ersten Mal isoliert worden sind. Sie gehören zu den eisbildenden Mikroorganismen. Gelegentlich werden *Pseudomonas syringae* Bakterien durch den Wind fortgetragen und gelangen in höhere Luftschichten, wo sie die Bildung von Eiskristallen initiieren. Die Eiskristalle fallen als Niederschlag in Form von Hagel, Schnee oder Regen zurück zu Boden. Dies erklärt auch, dass *Pseudomonas syringae* im Prinzip überall auf der Erde zu finden ist. *Pseudomonas syringae* Bakterien sind in der Lage, eine Reihe verschiedenster Baum- und

Pflanzenarten anzugreifen. Dabei kann es unter anderem zu Baumkrebs kommen, einer pflanzlichen Reaktion auf die Infektion, bei der das produzierte Wundgewebe zu einer massiven Wucherung führt, die optisch an einen Tumor erinnert. Weiterhin ist auch die Katalyse der Kristallisationskeimbildung ein Faktor, der für die pathogenen Eigenschaften von *Pseudomonas syringae* verantwortlich ist. Ein Protein (InaZ) an der Zellwand ordnet Wasser in einem Eis-ähnlichen Raster an und begünstigt dadurch die Kristallisation. Somit kann es zu einer Vereisung der Pflanzen bei relativ hohen Temperaturen von über -5°C kommen und durch die entstehenden Gewebeschäden in den benetzten Flächen sind die Pflanzen leichter für die Bakterien zugänglich [88, 89].

Einige *Pseudomonas syringae* Stämme wurden relativ früh vollständig genetisch sequenziert und sind in der Lage, die ebenfalls gut charakterisierten Pflanzen *Arabidopsis thaliana* und *Nicotiana benthamiana* zu infizieren. Dadurch werden sie bevorzugt für die Studien von Wechselwirkungen zwischen Pflanzen und Pathogenen herangezogen und wurden bereits beispielsweise genutzt, um den Zusammenhang zwischen pathogenen Genen und der Inhibition pflanzlicher Abwehrsysteme nachzuweisen [90].

2.8.4. Micrococcus luteus

Micrococcus luteus ist ein Gram-positives, aerobes Bakterium und kann im Erdboden, Staub, aber auch in Gewässern oder in der Luft isoliert werden. Die Form des Bakteriums ist rund, allerdings verläuft die Zellteilung nicht vollständig, und die Zellen sind über die Zellwand miteinander verbunden. Dadurch bilden sich sogenannte Tetraden aus. *Micrococcus luteus* Kolonien sind gelblich gefärbt, woher auch der Name stammt. Die gelbe Färbung kommt durch die Produktion des Stoffes Sarcinaxanthin (Abb. 10) zustande. Sarcinaxanthin ist ein Carotinoid und damit ein Antioxidants. Der Einsatz des Sarcinaxanthin als Nahrungsergänzungsmittel oder Lebensmittelfarbstoff oder aber als Zusatzstoff für Sonnencremes wird zurzeit erforscht [91, 92].

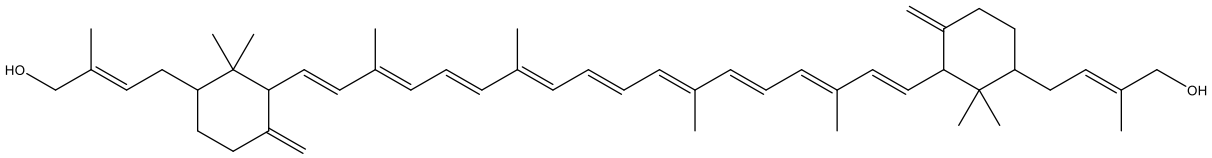

Abbildung 10: Chemische Struktur von Sarcinaxanthin.

Im Normfall weist *Micrococcus luteus* keine Pathogenität gegenüber Menschen auf, allerdings wurden in seltenen Fällen bei Patienten mit geschwächtem Immunsystem Infektionen der Herzinnenhaut festgestellt [93].

Micrococcus luteus spielt eine große Rolle beim Verderb von Lebensmitteln, da es unter aeroben Bedingungen proliferiert und sehr weit verbreitet ist. Dabei werden Proteine im Lebensmittel abgebaut und es kommt zum Verderb des Produktes ohne die Folge einer Lebensmittelvergiftung [94].

In der Forschung wird *Micrococcus luteus* hauptsächlich als Modellorganismus genutzt, um die Wirksamkeit von antibakteriellen Wirkstoffen gegen Gram-positive Bakterien festzustellen.

2.8.5. Arabidopsis thaliana

Die Ackerschmalwand *Arabidopsis thaliana* ist eine Pflanze aus der Familie der Brassicaceaen. Oft auch als Unkraut gesehen, ist *Arabidopsis thaliana* vor allem auf der Nordhalbkugel verbreitet.

Arabidopsis thaliana wurde zum ersten Mal im Jahr 1577 vom deutschen Botaniker Johannes Thal (1542-1583) beschrieben und nach mehreren Umbenennungen schließlich auch nach ihm benannt [95].

Schon früh wurde *Arabidopsis thaliana* als Modellpflanze in der Forschung eigesetzt, da sie relativ wenig Pflege benötigt, geringe Ansprüche an Platz stellt und mit nur sechs Wochen einen sehr kurzen Generationszyklus durchläuft [96]. *Arabidopsis thaliana* kann auch deshalb als Modellorganismus so oft verwendet werden, weil sie ein relativ kleines Genom von 125 Millionen Basenpaaren besitzt, welche auf insgesamt fünf Chromosomenpaaren über 25000 Gene codieren. Das Genom war

dementsprechend von großem wissenschaftlichem Interesse und wurde schon im Jahr 2000 durch internationale Kooperation im Rahmen der Arabidopsis Genom Initiative vollständig sequenziert [97].

Arabidopsis thaliana wird zunehmend in der Erforschung und genetischen Analyse von Wirt-Pathogen-Beziehungen eingesetzt.

3. Ziele der Arbeit

Die Forschung, die der vorliegenden Arbeit zugrunde liegt, beschäftigt sich mit Allicin, einer Reaktiven Schwefelspezies (RSS) aus der Gruppe der Thiosulfinate und seinen Derivaten. Das Hauptaugenmerk dieser Dissertation richtet sich dabei auf folgende Punkte:

- Verbesserung der Synthese und Aufreinigung des Allicins
- Identifizierung der molekularen Wirkmechanismen von Allicin und seiner zellulären Ziele
- Vergleich der Wirksamkeit und Wirkungsweise verschiedener Thiosulfinate in unterschiedlichen Modellsystemen (u.a. Hefezellen, Bakterien, humanen und pflanzlichen Zellen)

4. Ergebnisse

Im Folgenden sind die Publikationen, auf denen diese kumulative Dissertation aufbaut, aufgeführt:

4.1. Publikation I

An Optimized Facile Procedure to Synthesize and Purify Allicin

Albrecht, F.*, **Leontiev, R.***, Jacob, C., Slusarenko, A.J.

*gleicher Beitrag zu Publikation

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An Optimized Facile Procedure to Synthesize and Purify Allicin

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Abstract: Allicin is a reactive sulfur species (RSS) and defence substance from garlic (*Allium sativum* L.). The compound is a broad-spectrum antibiotic that is also effective against multiple drug resistant (MDR) strains. A detailed protocol for allicin synthesis based on diallyl-disulfide (DADS) oxidation by H_2O_2 using acetic acid as a catalyst was published in 2001 by Lawson and Wang. Here we report on improvements to this basic method, clarify the mechanism of the reaction and show that it is zero-order with respect to DADS and first-order with respect to the concentration of H_2O_2. The progress of allicin synthesis and the reaction mechanism were analyzsd by high-performance liquid chromatography (HPLC) and the identity and purity of the products was verified with LC-MS and ^{1}H-NMR. We were able to obtain allicin of high purity (>98%) and >91% yield, with standard equipment available in any reasonable biological laboratory. This protocol will enable researchers to prepare and work with easily and cheaply prepared allicin of high quality.

Keywords: allicin; *Allium sativum*; diallyl-disulfide; catalytic oxidation; reactive sulfur species; dipropyl-disulfide; thiosulfinate

1. Introduction

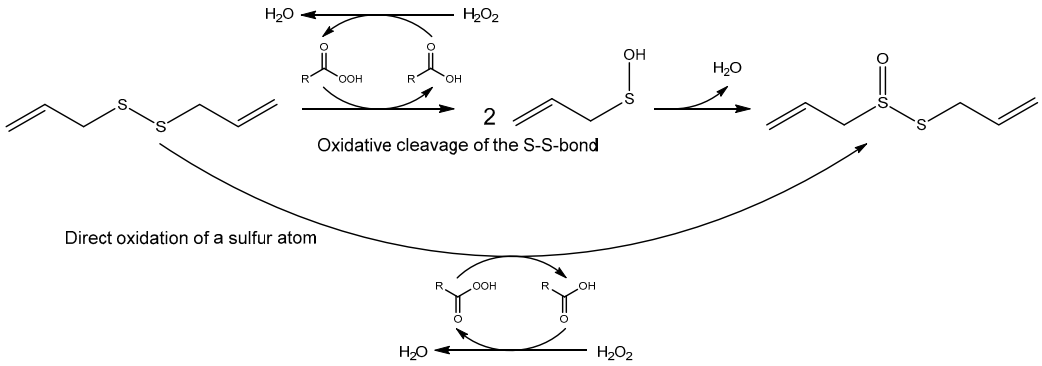
The sulfur-containing compound allicin (2-Propene-1-sulfinothioic acid *S*-2-propenyl ester, or diallyl-thiosulfinate, DATS) is produced in damaged tissue of garlic (*Allium sativum*), ramsons (*Allium ursinum*), and hooker chives (*Allium hookeri*) and gives these plants their typical odours [1]. Garlic is highly valued in the cuisines of many nations because of its excellent flavour and its pungent smell. Additionally, it has long been believed that allicin, or at least garlic consumption, is beneficial to health [2]. In 1944 Cavallito and Bailey demonstrated that allicin inhibited the growth of *Staphylococcus aureus* and other bacteria in liquid culture [3]. Furthermore, allicin was shown to induce apoptosis, often selectively, in mammalian cancer cells cultured in vitro [4,5], in intact tissues in vivo [6], and in cells of yeast (*Saccharomyces cerevisiae*), a model fungal eucaryote [7]. These properties turn allicin into a highly interesting compound for clinical investigations. Stoll and Seebeck first reported the synthesis of allicin in 1947, but without specifying experimental details [8]. Their chemical synthesis of allicin was based on the oxidation of diallyl-disulfide (DADS) by peracetic acid as a mild oxidizing agent. A more detailed protocol of this basic method was published by Lawson and Koch in 1994 and Lawson and Wang in 2001 [9,10]. Other methods to synthesize allicin utilizing magnesium monoperoxyphthalate [11] or chloroperbenzoic acid have also been reported [12,13]. Nevertheless, it is still challenging to obtain pure allicin in acceptable yields.

In the original protocol, DADS was stirred into a mixture of acetic acid and H_2O_2 and incubated at room temperature (RT) for 4 h with constant stirring. The reaction was stopped by adding five volumes of water and extracted with dichloromethane (DCM) to retrieve allicin along with unreacted DADS, some acetic acid, and DCM-soluble reaction byproducts. The lipophilic undissociated acid catalyst in the DCM phase was neutralized with aqueous sodium carbonate solution which facilitated partitioning of the hydrophilic sodium acetate generated into the aqueous phase. DCM was removed by rotary evaporation at RT at reduced pressure to yield an oily residue of allicin, unreacted DADS, and byproducts. Further purification of allicin was based on the differential partitioning of the constituents of the oily residue between *n*-hexane and an aqueous phase (two washes). Unreacted DADS and some allicin accumulated in the *n*-hexane phase, but allicin, which is more polar than DADS, concentrated to some extent in the aqueous phase. The separation method was inefficient, however, and allicin losses occurred at this stage. Finally, the allicin-containing aqueous phase was partitioned against DCM to isolate allicin and dried over anhydrous $CaSO_4$. Allicin was obtained as an oily residue after evaporation of the DCM under reduced pressure at RT.

This synthesis consists of at least two reaction steps. Firstly, the organic peracid is formed by oxidation of the organic acid by H_2O_2. Secondly, DADS is oxidized by the peracid, thus regenerating the parent organic acid. It has been reported that peracids, such as performic and peracetic acids, are adequately soluble in the organic phase [14], but DADS is immiscible with the aqueous H_2O_2 solution and the reactions therefore take place in a two phase system.

In the optimized method described in this paper we used a formic acid catalyst instead of acetic acid, which enabled us to carry out the reaction at 0 °C under more controlled conditions and we systematically varied the concentrations of the reactants, while following the progress of the reaction using HPLC. Furthermore, we developed a silica gel column chromatography protocol for allicin purification which avoided the losses associated with the original solvent partitioning procedure.

A reaction mechanism for Stoll and Seebeck's synthesis was postulated by Nikolic et al. [15] proposing oxidative cleavage of the S–S bond in DADS by hydroxyl-radicals generated from the acidic H_2O_2 to give allyl-sulfenic acid which condenses to yield allicin (Scheme 1). In contrast, an alternate mechanism, namely direct oxidation of one of the S-atoms in DADS without oxidative cleavage of the S–S bond, is also plausible (Scheme 1). Here we provide data supporting an oxidative cleavage mechanism and condensation of two sulfenic acid molecules to yield allicin, but without a need for hydroxyl-radicals.



Scheme 1. Allicin synthesis from diallyl-disulfide (DADS) through oxidation by a peracid generated with H_2O_2 in the reaction mixture. The organic acid serves as an intermediate catalyst.

2. Results and Discussion

2.1. Comparison of DADS Oxidation Catalyzed by Acetic Acid or Formic Acid

Preliminary experiments substituting formic acid for acetic acid at RT resulted in rapid overheating of the reaction mixture accompanied by massive byproduct formation, therefore, we carried out the

formic acid catalyzed allicin syntheses at 0 °C. The progress of the oxidation reactions was followed by HPLC analysis; i.e., disappearance of the DADS peak and appearance of the allicin peak. In a first attempt, we withdrew small samples of the proceeding reaction, diluted them with methanol and measured the amount of allicin and DADS. This approach was not reliable, however, due to the fact that the reaction mixture was an emulsion. It was, therefore, difficult, despite thorough mixing, to guarantee the same distribution of content in withdrawn samples and the remainder of the reaction mix in the flask. Measurements confirmed these concerns and showed unrealistic kinetics (data not shown). Therefore, in a second approach, the reaction was carried out in several parallel aliquots on a micro-scale and each aliquot was diluted with methanol as a whole to give a single data point. Thus, every time point presented in Fig. 1 shows an independent parallel reaction run. Reaction progress was followed by calculating the percentage ratio of actual allicin yield divided by the theoretical maximum yield (100% of DADS converted to allicin) to indicate the percent conversion during the course of the reaction. Despite the lower reaction temperature, allicin was formed more rapidly and to a greater yield (78% conversion by 4 h) with formic acid as catalyst than with acetic acid (58% conversion at 4 h) (Figure 1).

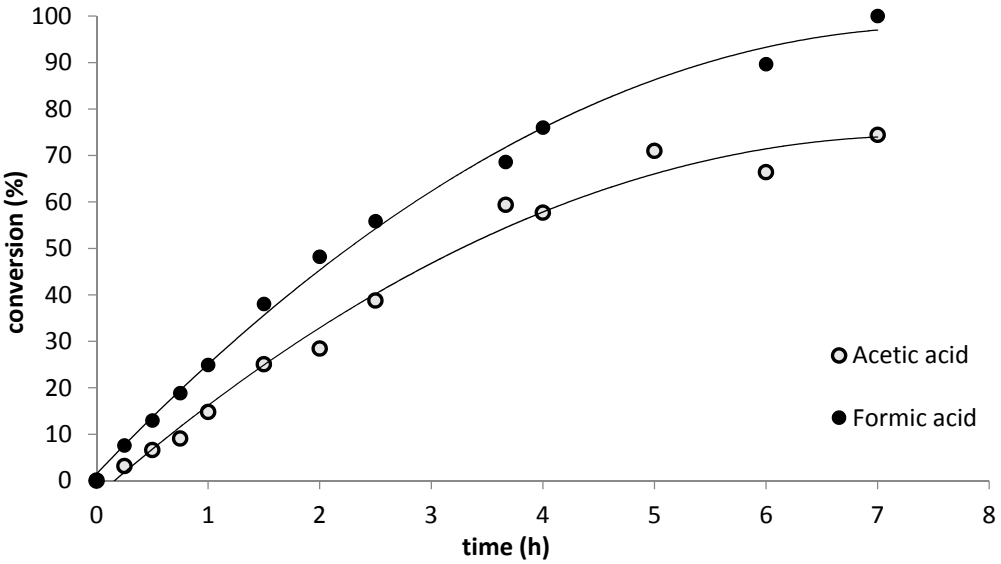


Figure 1. Kinetics of allicin synthesis. The oxidation of DADS to allicin was catalyzed by either acetic acid at 20 °C or formic acid at 0 °C. All reactions took place in sealed 2 mL reaction tubes in temperature-controlled rotary shakers and via continual shaking at 1200 rpm to ensure optimal mixing. The products were separated by HPLC and quantified with a UV detector at 254 nm. Reaction progress was followed by calculating the percentage ratio of actual allicin yield divided by the theoretical maximum yield to indicate the percentage of conversion during the course of the reaction.

Byproducts detectable by HPLC and presumably arising via decomposition, were observed increasingly with incubation times longer than 4 h. Quantitatively slightly lower amounts of byproducts were observed at 0 °C with formic acid as a catalyst than with acetic acid at 20 °C (Figure 2).

Lesser byproduct formation using formic acid at 0 °C as shown in Figure 2 may be explained by allicin's increased reactivity and inherent instability at higher temperatures. The instability of allicin at higher temperatures was reported to be increased by hydrophobic solvents such as any residual DADS [16]. For those reasons, the reaction should be stopped at the latest after 4 h, by adding five volumes of H_2O, even though conversion is incomplete. Furthermore, if not tempered to 20 °C different RTs will lead to different kinetics for the reaction and the need for new calibrations. Therefore,

we propose that it is advantageous for reasons of increased yield and reaction consistency to use formic acid as a catalyst and to carry out the reaction on ice at 0 °C.

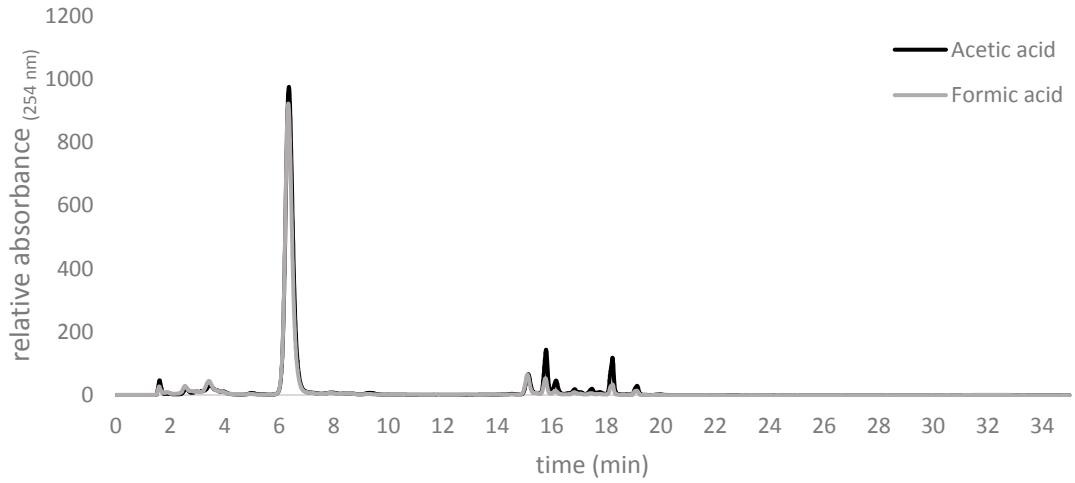


Figure 2. Comparison of representative end point traces of the products of routine allicin syntheses with either acetic or formic acids.

2.2. Reaction Order with Respect to Individual Reactants

The kinetics shown in Figure 1 not only reveal a faster reaction when formic acid is used as a catalyst, but also give information about the reaction order. Thus, after 2 h—48%, after 4 h—76% and after 6 h—90% of the DADS was converted to allicin. This is an approximate halving of the amount of DADS every 2 h indicating that the overall reaction followed first order kinetics. We investigated the reaction kinetics in more detail and showed that the shaking conditions for the two phase reaction were a limiting factor for the reaction speed (Figure 3). The reaction rate can be seen to increase proportionally up to 1200 rpm, which was thus chosen as the routine shaking velocity for micro-scale synthesis reactions.

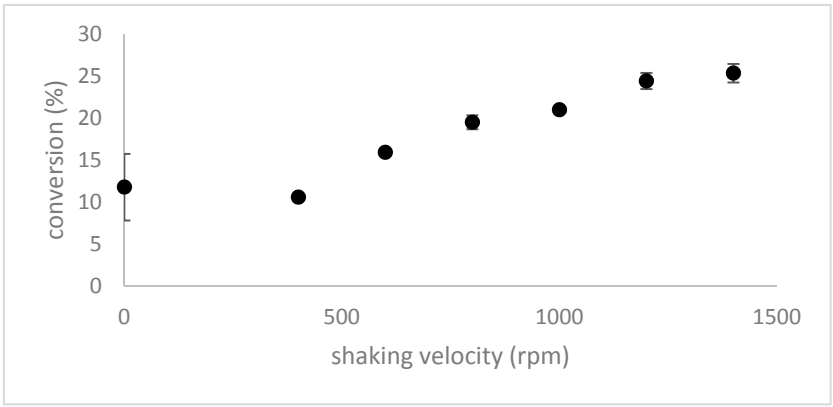


Figure 3. The effect of shaking velocity on the reaction rate. Micro-scale reactions using a pre-incubated mixture of H_2O_2 and formic acid (the reagent mix was stored two weeks at 4 °C) and shaken at various speeds at 0 °C to mix the reactants. Reactions were stopped after 5 min. The maximum reaction rate was achieved by 1200 rpm and statistical analysis according to the Holm-Sidak method showed no significant difference between the rates at 1200 rpm and 1400 rpm.

Since the oxidation of DADS occurs in the organic DADS phase by peracid dissolved in it, varying the amount of DADS in the reaction mix does not actually affect its concentration relative

to the peracid. Therefore, the reaction follows a pseudo-zero-order kinetic with respect to DADS. Furthermore, it was observed that with pre-incubation of H_2O_2 and formic acid a higher rate of DADS conversion to allicin was achieved than when all reactants were mixed at once, suggesting that peracid formation was a rate-limiting step. This aspect will be investigated in the next section.

In contrast, varying the concentration of H_2O_2 affected the rate of product formation. As shown in Figure 4 there was a linear relationship between the concentration of H_2O_2 and product formation. Therefore, the reaction follows first-order kinetics with respect to the concentration of H_2O_2.

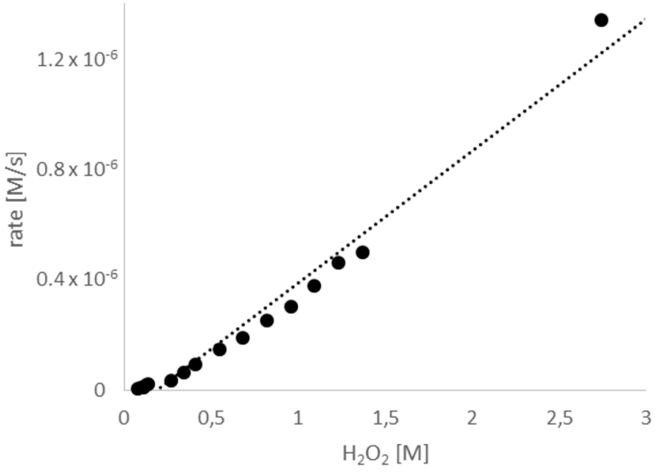


Figure 4. The rate of allicin formation in relation to the H_2O_2 concentration. Micro-scale reactions were performed with varying H_2O_2 concentrations. The reactions were stopped after 30 min, when turnover of H_2O_2 was between 14% (start conc. 2.74 M) and 0.9% (start conc. 0.082 M), the linear progression indicates that neither H_2O_2 nor DADS were limiting in the reaction.

2.3. Preformation of Performic Acid

We observed that when H_2O_2 and formic acid were mixed 3 h before the addition of DADS, a 37% conversion of DADS to allicin was observed within seconds and that the conversion was >80% complete after 120 min (Figure 5).

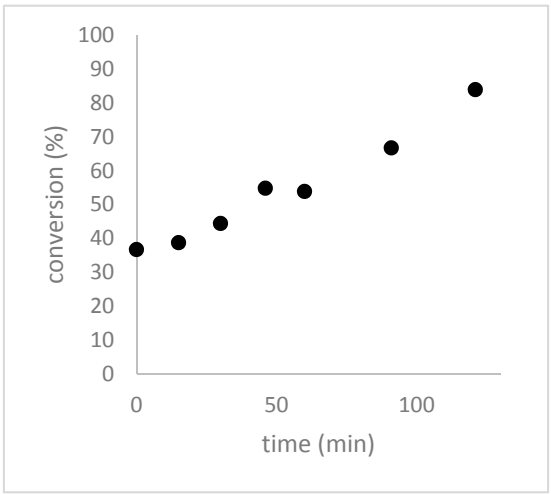


Figure 5. Effect of allowing 3 h at 0 °C for the preformation of performic acid on the rate of conversion of DADS to allicin. H_2O_2 and formic acid were mixed according to the micro-scale reaction procedure and incubated on ice for 3 h before DADS was added. The reactions were stopped with methanol, separated by HPLC and quantified with a UV detector at 254 nm.

Without preformation of the performic acid the reaction needs ~1.5 h to reach >35% conversion and showed >80% conversion only after ~4 h (Figure 1). This illustrates clearly that the formation of performic acid is rate limiting for allicin synthesis, therefore, we decided to investigate systematically the effect of the pre-incubation time of H_2O_2 and formic acid on the conversion rate of DADS to allicin in order to optimize this step in the protocol (Figure 6).

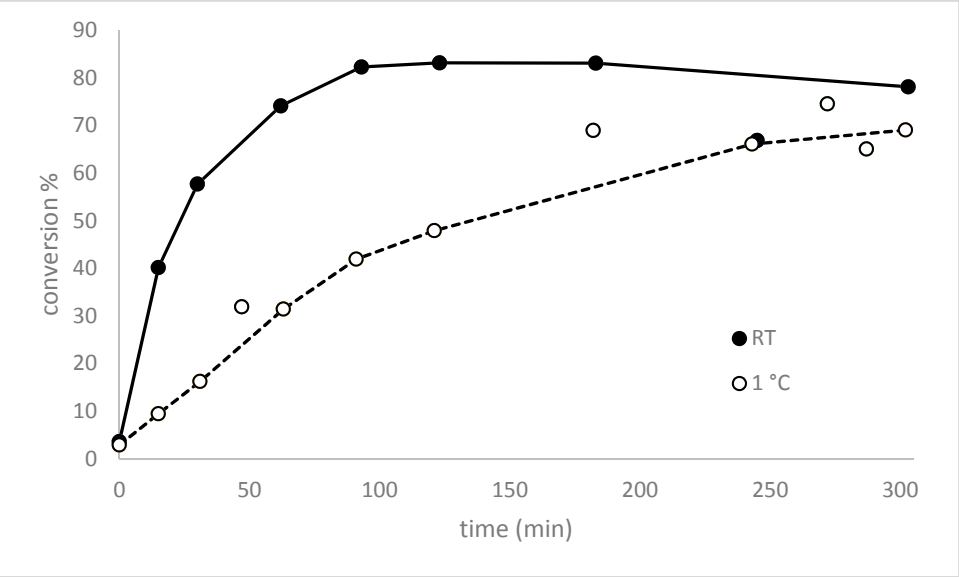


Figure 6. The effect on the pre-incubation time for performic acid formation on the rate of DADS conversion to allicin. H_2O_2 and formic acid were mixed according to the micro-scale reaction procedure and incubated at room temperature or 1 °C for the indicated times before DADS was added. The reactions were stopped after 5 min by addition of methanol, separated by HPLC and quantified with a UV detector at 254 nm. A maximal turnover was reached after 100 min at RT, indicating that maximum performic acid formation was achieved after that time.

We investigated the turnover of DADS to allicin depending on the pre-incubation time of the standard amounts of H_2O_2 and formic acid at 0 °C and at RT. The maximum turnover was reached between 100 and 180 min, followed by decrease of the turnover. Our observations are in accordance with those of Filippis et al. [17] who showed, that the formation of performic acid was a slow temperature dependent mechanism. In their experimental setup the maximum turnover of 25% of the H_2O_2 was reached after 100 min at 30 °C; thereafter the concentration started to decrease due to performic acid decomposition. Thus, in our optimized allicin synthesis protocol we recommend a 100 min pre-incubation step at RT to pre-form the performic acid.

2.4. Influence of Formic Acid Concentration and Amount of H_2O_2 on the Conversion of DADS to Allicin

Having established that preformation of performic acid greatly enhanced the conversion of DADS to allicin, we analysed the procedure with respect to formic acid and H_2O_2 concentrations. In micro-scale reactions higher amounts of acid and H_2O_2 increased the rate of the reaction (Figure 7), but when the reaction volume was scaled up this effect was less pronounced. See Section 2.5.

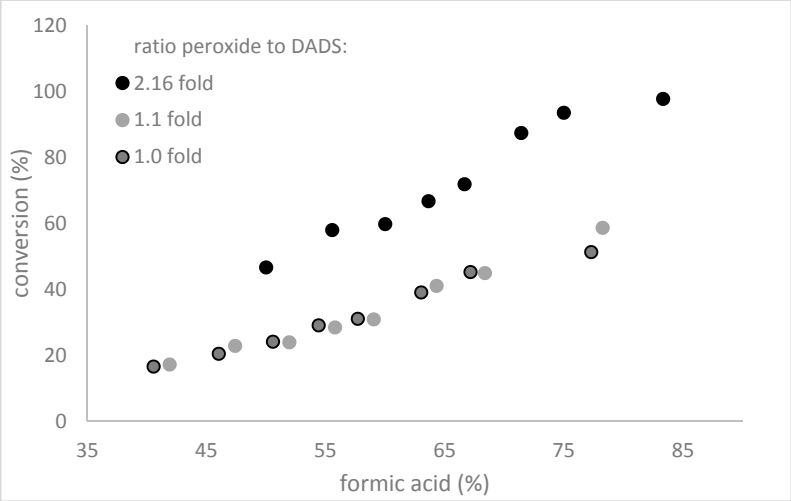


Figure 7. The influence of formic acid concentration and amount of H_2O_2 on the conversion of DADS to allicin. In a series of microscale reactions, the amount of H_2O_2 was varied three times from 1 mol H_2O_2 per mol DADS to 2.16 mol H_2O_2 per mol DADS, indicated with different data point styles. The concentration of formic acid was also varied between 40% and 85% in the reaction. All H_2O_2-formic acid mixtures were pre-incubated for 3 h on ice. The lower black data points (50% formic acid; 47% conversion) are conform to standard micro-scale reaction conditions. The figure shows that higher concentrations of acid and peroxide enhance the speed of the reaction.

2.5. Accelerated Allicin Synthesis

Not all of the advantages observed by altering parameters in the micro-scale reactions were completely transferable to scaled up reactions. The reaction speed was not as high as on the microscale and the formation of byproducts became more prevalent (data not shown). The latter are problems which could be due lesser mixing inefficiency and emulsion formation on the larger scale. In order to avoid inadequate mixing, we used methanol to combine the two phases and prevent emulsion formation (Section 3.2.3). In this way, a conversion of >98.46 ± 0.45% in just 15 min was achieved (Figure 8).

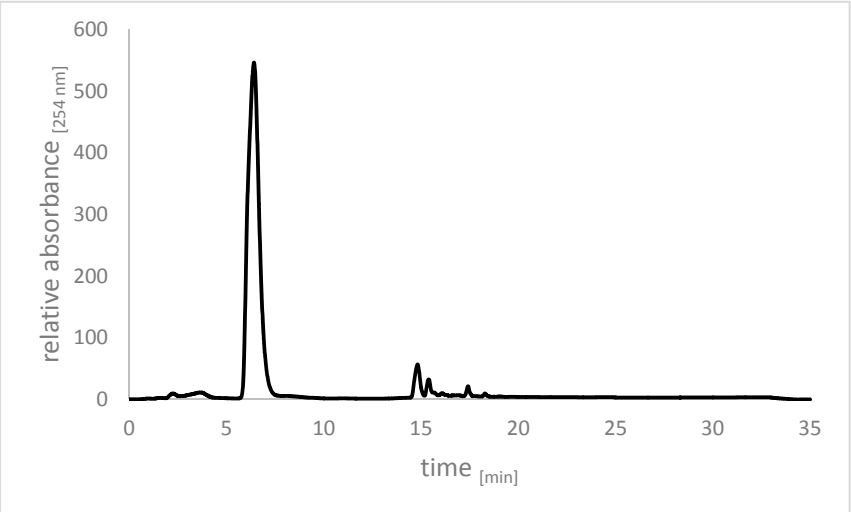


Figure 8. Chromatogram of crude allicin after synthesis using pre-formed performic acid. The reaction was extracted with dichloromethane (DCM) and the solvent was removed by rotary evaporation. The crude product already showed good purity.

2.6. Purification of Allicin

After quenching the reaction by addition of H_2O, the reaction mixture consists of allicin, DADS, formic acid, H_2O_2, and byproducts. The organic compounds were extracted by partitioning against either dichloromethane (DCM) or diethyl-ether. In the Lawson method remaining acetic acid was removed by washing the organic phase with Na_2CO_3 solution or extracting several times with water. This, however, leads to a loss of allicin, some of which partitions into the aqueous phase. A further advantage of using formic acid as a catalyst becomes apparent here. Formic acid is more volatile than acetic acid and, therefore, more easily removable under reduced pressure at room temperature, thus switching to evaporation instead of washing and, hence, avoiding the Na_2CO_3 washing step.

After rotary evaporation, separating allicin, DADS and byproducts is challenging, due to the similar physical properties of these compounds. The Lawson method partitioned repeatedly between *n*-hexane and water to accumulate allicin in the aqueous phase. The calculated log*P* values (clog*P*) of allicin (1.35), DADS (2.95), and probable byproducts such as vinyl-dithiine (2.69) and ajoene (1.97) (Chemdraw, see Section 3) indicate that allicin is the least hydrophobic molecule. Nonetheless, repeated extractions lead to further losses of allicin. To circumvent this we used silica gel chromatography to separate allicin from the other compounds (Figure 9). The structure of the final product was confirmed and the purity was determined by ^{13}C-NMR and ^{1}H-NMR, respectively.

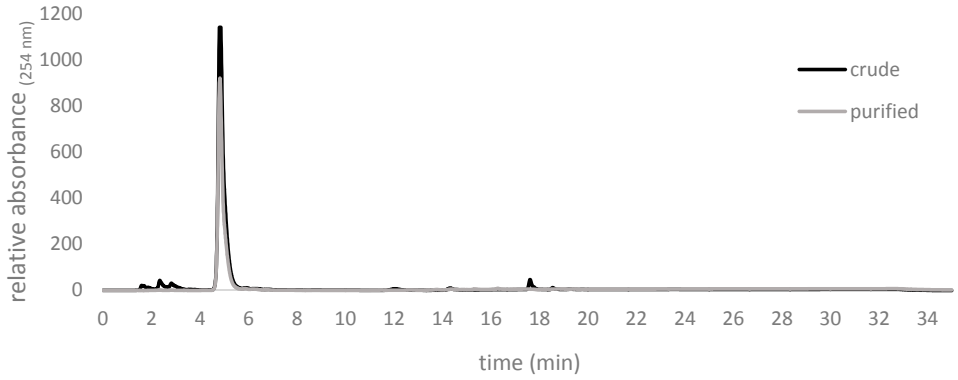
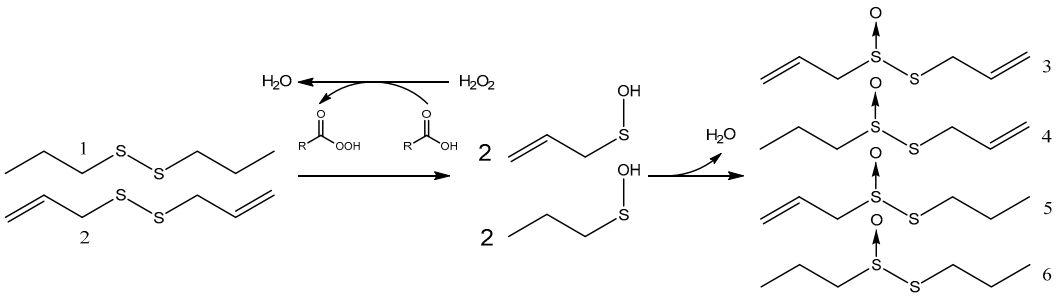


Figure 9. Chromatograms of crude allicin after synthesis using formic acid as a catalyst and rotary evaporation and afterwards purification by silica gel chromatography. The crude product contained impurities with retention times of about 2 min and 18 min, respectively, whilst the purified product showed >98% purity. Respresentative traces of our repeated routine syntheses are shown.

2.7. Reaction Mechanism

We reasoned that if allicin synthesis proceeded by direct oxidation of DADS (1) without oxidative cleavage of the S–S bond, then a mixture of DADS and dipropyl-disulfide (DPDS, 2) would yield allicin (DATS, 3) and dipropyl-thiosufinate (propicin, DPTS, 6) only. In contrast, if oxidative cleavage of the S–S bond occurred, then mixed allyl-propyl thiosulfinates should be further products because of random condensation of the respective sulfenic acids (Scheme 2). Thus, formation of *S*-allyl-propane-1-sulfinothioate (4) and *S*-propyl-prop-2-ene-1-sulfinothioate (5) would be predicted.

A mixture of DADS (1) and DPDS (2) was oxidized by performic acid, as described in Section 3.2.4. After the reaction was quenched products were extracted with DCM. LC-MS analysis of the crude extracts showed, in addition to single peaks at 6 min and 11 min, which were identified as allicin (3) and propicin (6), respectively, a double peak at 8 min from the mixed thiosulfinates (4,5). Data in Figure 10 are a combination of the UV absorption chromatogram detected by HPLC and the mass signals detected with LC-MS. These data indicate that oxidation of alkyl disulfides to thiosulfinates by peracids proceeds via oxidative cleavage of the S–S bond, but does not formally rule out parallel direct *S*-atom oxidation without S–S bond cleavage. Therefore, we suggest the reaction mechanism shown in Scheme 3.



Scheme 2. The basic reaction for mixed thiosulfinate synthesis from DADS and DPDS by oxidation by peracid generated with H_2O_2 in the reaction mixture.

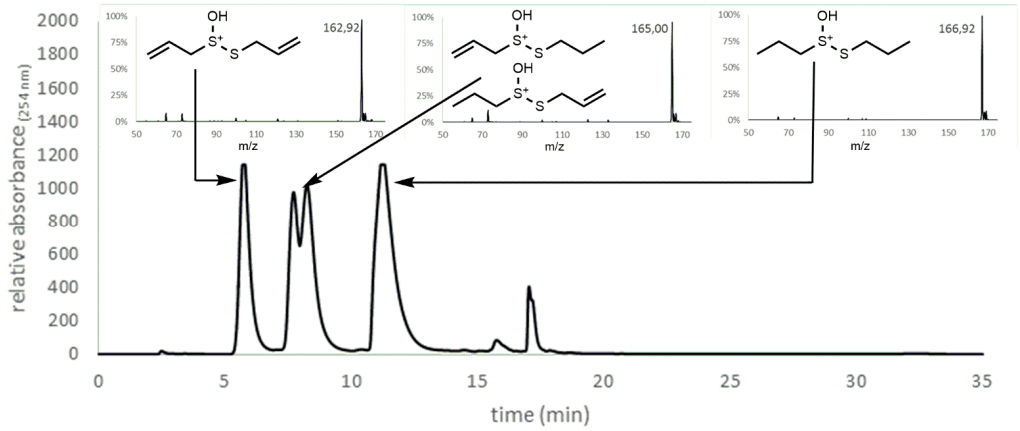
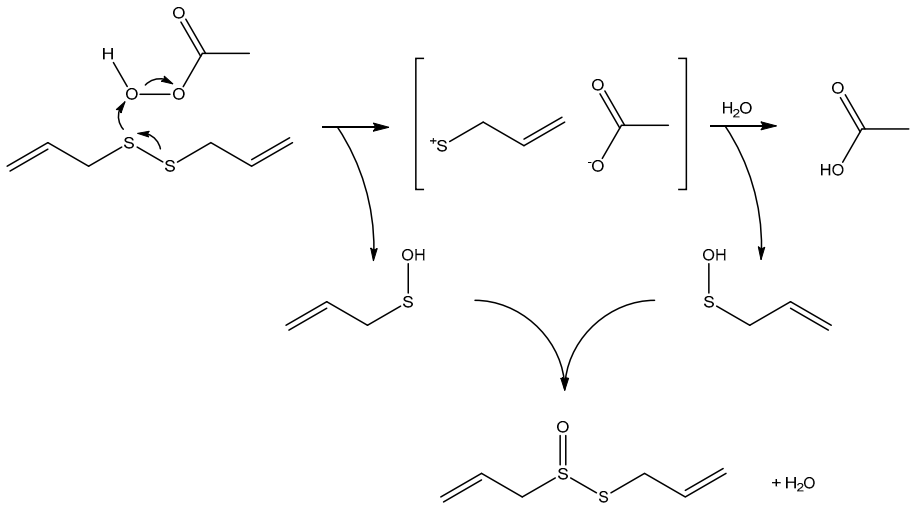


Figure 10. Synthesis of mixed thiosulfinates. According to Scheme 2 a reaction mechanism via the formation of sulfenic acid would be expected to produce mixed thiosulfinates from a mixture of reacting alkyl disulfides. The chromatogram was obtained by HPLC and quantified with a UV detector at 254 nm. Masses were identified in a separate LC-MS analysis using the same column and gradient. Insets show the *m*/*z* ratios in MS-traces and the respective structures of the major ions. The masses obtained fit to the expected molecules: 6 min, 162.92 Da—allicin (3); 8 min, 165.00 Da—*S*-allyl-propane-1-sulfinothioate (4) and *S*-propyl-prop-2-ene-1-sulfinothioate (5); 11 min, 166.92 Da—propicin (6).



Scheme 3. The oxidation of disulfides proceeds via the formation of an allyl cation and an allylsulfenic acid in the first step. In a second step the allyl cation reacts with water to form a second allylsulfenic acid. The resulting sulfenic acids condense in a third step to form allicin.

3. Materials and Methods

3.1. Materials

DADS (≥80%) was purchased from Sigma Aldrich (Munich, Germany). DPDS (98%) was purchased from Sigma Aldrich. Formic acid (≥98%, p.a.) was purchased from Carl Roth (Karlsruhe, Germany). H_2O_2 (30%) was purchased from Merck (Darmstadt, Germany). Acetic acid (100%, p.a.) was purchased from Carl Roth. TLC was performed using Merck TLC Silica gel 60 F_{254} with concentration zone. Solvent A (*n*-hexane ≥99% p.s.) was purchased from Carl Roth. Solvent B (ethyl acetate ≥99.5% p.s.) was purchased from Carl Roth. Liquid chromatography was performed using silica gel 60 (0.04–0.063 mm (230–400 mesh)) purchased from Carl Roth. HPLC was performed using a Bischoff Chromatography Hyperchrome HPLC column 150 mm× 4.6 mm packed with Prontosil Kromaplus 100-5-C18 5.0 µm (Leonberg, Germany) in a Jasco System composed of: a Jasco DG-2080-53 3-Line-Degasser, a Jasco LG-980-02 ternary gradient unit, a Jasco PU-980 intelligent HPLC pump, a Jasco CO-2060Plus Intelligent column thermostat, a Jasco AS-1555 intelligent sampler, a Jasco UV-2077 multi-wavelength UV-VIS detector, and a Jasco LC-Net II/ADC. Jasco ChromPass Chromatography Data System Version 1.8.6.1 was used for control and analysis (Groß-Umstadt, Germany). Solvent A (H_2O) was obtained using a Satorius Stedim Biotech Arium® Pro VF (Goettingen, Germany). Solvent B (methanol (ultra) Gradient HPLC Grade) was purchased from J.T. Baker. (Center Valley, PA, USA). LC-MS was performed using a Bischoff Chromatography Hyperchrome HPLC Column 150 mm × 4.6 mm packed with Prontosil Kromaplus 100-5-C18 5.0 µm in an Agilent 1200 System (Santa Clara, CA, USA). To solvent A 0.1% formic acid (≥98%, p.a.; Carl Roth (Karlsruhe, Germany)) was added. Shaking of the micro-scale reactions was performed using an Eppendorf Thermomixer comfort (Hamburg, Germany) to define 20 °C and a Hettich Benelux MKR 23 (Geldermalsen, The Netherlands) to define 0 °C. Calculation of log*P* values was performed using ChemDraw Professional 16.0.0.82 (PerkinElmer, Waltham, MA, USA).

3.2. Methods

3.2.1. Distillation of DADS

DADS is commercially only available at 80% purity. For further purification we used distillation under reduced pressure. To enhance the efficacy of distillation a Vigreux column (600 mm) was used. The crude DADS was stirred and tempered in an oil bath. The pressure was reduced to approximately 50 mbar. At an oil bath temperature of 120 °C the DADS fraction evaporated. The boiling point under these conditions was 80.5 °C. A purity of 98% was determined by HPLC.

3.2.2. Synthesis of Allicin without Pre-Formed Performic Acid

Distilled diallyl disulfide (DADS; 2 g, 13.7 mmol) was mixed in 5 mL formic acid and stirred for 5 min at 0 °C. H_2O_2 (30%; 3 mL, 29.6 mmol) was added slowly to the mixture. The reaction was stopped after approximately 4 h by addition of 25 mL distilled water and the mixture was extracted three times with DCM. The solvent was removed under reduced pressure and the product was dissolved in the eluent, a mixture of *n*-hexane and ethyl-acetate (2:1).

Separation was performed via liquid chromatography using 150 mm silica gel 60 in a column with a diameter of 30 mm. Fractions were collected into tubes cooled in an ice bath and TLC was used to identify fractions containing solely allicin. Those fractions were combined, dried over amorphous anhydrous sulfate (e.g., $MgSO_4$ or $CaSO_4$) and filtered. The solvents were removed under reduced pressure at RT to yield a clear, oily substance that smells like garlic. Yield: 1.64 g, 10.1 mmol, 74%.

^{1}H-NMR (500 MHz, $CDCl_3$): δ3.70–3.75 (m, 4H); 5.14–5.42 (m, 4H); 5.68–5.88 (m, 2H); ^{13}C-NMR: (125 MHz, $CDCl_3$) δ35.08, 59.82, 119.11, 124.10, 125.78, 132.8.

3.2.3. Synthesis of Allicin Using Pre-Formed Performic Acid

Distilled diallyl-disulfide (DADS; 0.5 g, 3.5 mmol) was mixed in 2.5 mL methanol and stirred for 5 min at 0 °C. Performic acid solution (2.0 mL) (as described in Section 3.2.6.) was added slowly to the mixture. The reaction was quenched after 15 min by addition of 25 mL distilled water and the mixture was extracted three times with DCM. The solvent was removed under reduced pressure and the product was dissolved in a mixture of *n*-hexane and ethyl-acetate (2:1).

Separation was performed via liquid chromatography using 150 mm silica gel 60 in a column with a diameter of 30 mm and *n*-hexane and ethyl acetate (2:1) as eluent. Fractions were collected into tubes cooled in an ice bath and TLC was used to identify fractions solely containing allicin. Those fractions were combined, dried over an anhydrous sulfate, and filtered. The solvents were removed under reduced pressure at RT to yield a clear, oily substance that smells like garlic. Yield: 0.52 g, 3.204 mmol, 92%.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.70–3.75 (m, 4H); 5.14–5.42 (m, 4H); 5.68–5.88 (m, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ35.08, 59.82, 119.11, 124.10, 125.78, 132.8.

3.2.4. Synthesis of Mixed Thiosulfinates

Diallyl disulfide (DADS; 1 g, 6.84 mmol) and dipropyl disulfide (DPDS; 1g, 6.65 mmol) were mixed in 5 mL formic acid and stirred for 5 min at 0 °C. H_2O_2 (30%; 3 mL, 29.6 mmol) was added slowly to the mixture. The reaction was quenched after approximately 4 h by addition of 25 mL distilled water and the mixture was extracted three times with DCM. The solvent was removed under reduced pressure and the crude products were analysed by HPLC and HPLC-MS.

3.2.5. Micro-Scale Reaction

DADS (10 mg, 68.4 µmol) was mixed in 25 µL of either formic or acetic acid in a 2.0 mL reaction tube on ice. The formic acid-containing tubes were placed in a cooling shaker at 0 °C, the acetic acid-containing tubes were placed in a shaker at 20 °C. Then H_2O_2 solution (30%, 15 µL, 148 µmol) was added to the mixture and the reaction was initiated by shaking at 1200 rpm. For sample collection, single tubes were removed and the reaction was quenched by diluting the mixture to 2 mL with methanol. The samples were stored at −20 °C prior to HPLC analysis.

3.2.6. Performic acid Pre-Formation

If not explained differently, H_2O_2 and formic acid were mixed (in a ratio of 3:5) and incubated at RT for 90 min. In micro-scale reactions, for instance, 40 µL of that mixture was used instead of adding 25 µL formic acid and 15 µL H_2O_2.

3.2.7. High-Performance Liquid Chromatography (HPLC) Analysis

Reaction mixtures were analyzed by loading each 20 µL sample onto the HPLC. Separation was performed using H_2O as mobile phase A and methanol as mobile phase B with the following gradient: 56% A (pre-run); 53% A (10 min); 7% A (15 min); 7%A (30 min); 56% A (31 min); 56% A (35 min) at a flow rate of 1 mL/min and a column thermostat temperature of 25 °C. Under these conditions retention times were 4.8 min for allicin and 18.2 min for DADS. Byproducts appearing at 14.9 min and 17.7 min due to their calculated log*P* values are assumed to be forms of ajoene and vinyldithiine, but were not investigated further at this stage. To quantify allicin and DADS, external standards were used.

3.2.8. Liquid Chromatography-Mass Spectrometry (LC-MS)

The LC-MS protocol used the same gradient and column as the HPLC protocol, except for the use of 0.1% formic acid, which was used instead of pure water. The following source conditions were employed: heater—350 °C; sheath gas flow rate machine settings (without units)—30; auxiliary

gas flow rate—5; sweep gas flow rate—0; ion spray voltage—400 kV; capillary temperature—250 °C; capillary voltage—82,5 V; tube lens—120 V in a ThermoFischer LTQ XL (Waltham, MA, USA).

3.2.9. Thin Layer Chromatography (TLC)

Approximately 2 µL of the reaction mixture was loaded on a silica plate. After drying, the substances were separated using *n*-hexane/ethyl-acetate mixture (in a ratio of 2:1) as mobile phase. Under these conditions spots were visible under UV light (254 nm). Allicin's R_f value was 0.70 and DADS's R_f value was 0.95.

4. Conclusions

Our data provide evidence that the reaction mechanism underlying the conversion of DADS to allicin in the presence of formic acid and H_2O_2 is similar to that already proposed by Nikolic, but without the need for hydroxyl radicals. The unpaired electrons in such radicals might delocalize and would surely result in a number of additional side products for which we see no evidence. As the four possible products from the mixture of disulfides (DPDS and DADS) were formed in approximately equal amounts, we surmise that direct oxidation of the disulfides without chain cleavage is probably not quantitatively significant and, thus, we suggest an oxidative cleavage mechanism for the reaction as shown in Scheme 3.

We also show that the optimized method we describe here to synthesize allicin is an improvement on the previously-published procedures based on the one of Lawson [10]. Not only does the utilization of formic acid as a catalyst lead to a purer product, since the formation of by-products is decreased, the reaction also occurs faster and is easier to perform under standard conditions. Formic acid offers another advantage during the purification of the product allicin because it is more volatile than acetic acid and therefore easily removed under reduced pressure. Other peroxy-acids such as magnesium monoperoxyphthalate, or chloroperbenzoic acid have also been used [11–13]. In light of economical reasoning, however, the price of formic acid compared to aromatic peroxy-acids is just another argument, which points to formic acid as the catalyst of choice for the synthesis of allicin. Additionally, formic acid, as a naturally-occurring organic molecule produced, for example, by red ants and stinging nettles, is more eco-friendly than most alternatives (with the possible exception of acetic acid) and certainly 'greener' than the aromatic alternatives. The use of silica gel chromatography offers the advantage whereby a separation of the product and byproducts can be achieved without further diluting the allicin excessively. Therefore, it is possible to continue the reaction until a maximal turnover is reached, purify the crude product, and obtain pure allicin rather easily. A suggested optimized protocol for the synthesis of allicin, taking into account the various individual improvements we describe here are, therefore, as follows:

1. Use redistilled DADS and add methanol to combine the aqueous and organic phases (see Section 3.2.3.) or keep the final reaction volume small to promote efficient mixing and achieve a high conversion rate.
2. Use formic acid as the acid catalyst and pre-form performic acid as described in Section 3.2.6.
3. Cool the reagents and carry out the reaction on ice.
4. Slowly add performic acid solution.
5. Continually stir the reactants as efficiently as possible and carry out the reaction at 0 °C for just 15 min.
6. Quench the reaction with water.

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Author Contributions: F.A. and R.L. contributed equally to the practical work and writing the manuscript. A.J.S. provided supervision and wrote and edited the manuscript. C.J. edited the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Sample Availability: Samples of the compounds are not available from the authors.

4.1. Publikation II

Yap1p, the central regulator of the *S. cerevisiae* oxidative stress response, is activated by allicin, a natural oxidant and defence substance of garlic

Gruhlke, M.C.H., Schlembach, I., **Leontiev, R.**, Uebachs, A., Gollwitzer, P., Weiss, A., Delaunay, A., Toledano, M., Slusarenko, A.J.

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Yap1p, the central regulator of the *S. cerevisiae* oxidative stress response, is activated by allicin, a natural oxidant and defence substance of garlic

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Gpx3, yeast

ABSTRACT

Allicin is a thiol-reactive sulfur-containing natural product from garlic with a broad range of antimicrobial effects against prokaryotes and eukaryotes. Previous work showed that the *S. cerevisiae OSI1 gene* is highly induced by allicin and other thiol-reactive compounds, and *in silico* analysis revealed multiple Yap1p binding motifs in the *OSI1* promoter sequence. An *OSI1*-promoter::luciferase reporter construct expressed in Wt and Δ*yap1* cells showed absolute Yap1p-dependence for allicin-induced *OSI1*-expression. A GFP::Yap1p fusion protein accumulated in the nucleus within 10 min of allicin treatment and a Δ*yap1* mutant was highly sensitive to allicin. Yap1p regulates glutathione (GSH) metabolism genes, and Δ*gsh1,* Δ*gsh2 and* Δ*glr1* mutants showed increased sensitivity to allicin. Allicin activated the *OSI1*-promoter::luciferase reporter construct in Δ*gpx3* and Δ*ybp1* cells, indicating that allicin activates Yap1p directly rather than via H_2O_2 production. A systematic series of C-to-A Yap1p exchange mutants showed that the C-term C598 and C620 residues were necessary for allicin activation.

These data suggest that Yap1p is an important transcriptional regulator for the resistance of yeast cells to allicin, and that activation occurs by direct modification of C-term cysteines as shown for other electrophiles.

1. Introduction

Allicin (diallylthiosulfinate) is a sulfur-containing, redox-active compound that is produced in garlic tissue upon injury and is the molecule that gives freshly cut garlic its characteristic odour. A single clove of garlic can produce up to 5 mg of allicin [1]. The precursor alliin (S-allyl cysteinesulfoxide) is separated from the enzyme alliinase by cellular sub-compartmentation. However, mixing of these two components after cell damage leads to allylsulfenic acid production, the condensation of which yields allicin. Allicin is a volatile reactive

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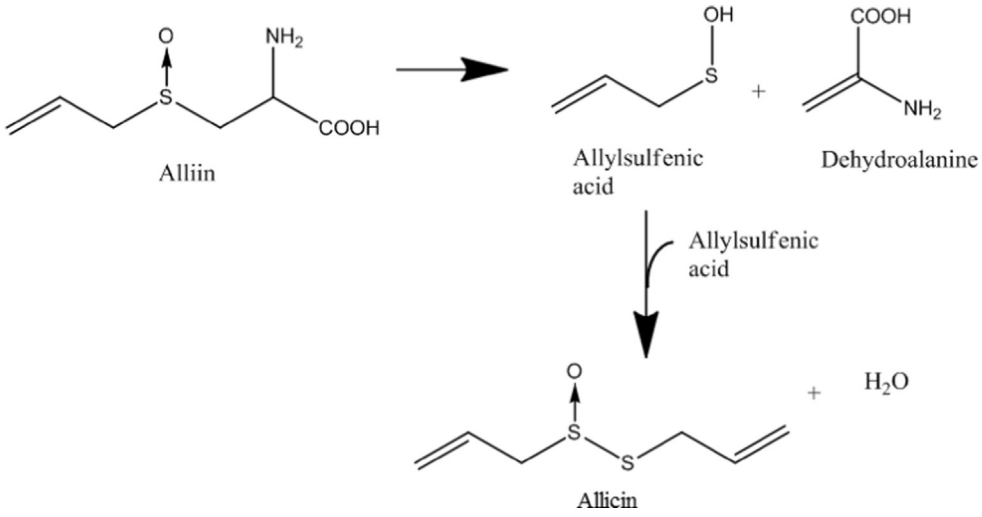

sulfur species (RSS) and reacts with itself upon heating and ageing to produce a cascade of downstream sulfur-containing molecules [2,3]. Allicin has a protective role against bacteria, fungi and insects [4]. Allicin is not only a dose-dependent biocide, but also shows a broad range of physiological effects with pharmaceutical impact for humans. Thus, effects on lipid metabolism [5–7], and on blood-pressure [8,9] have been reported. Furthermore, allicin has been shown to be a potent inducer of apoptosis in a variety of cancer cell lines [10,11,12–15] and in yeast, where it was shown to act as a redox toxin, oxidizing and reducing the size of the glutathione pool and shifting cellular redox to be less negative [16].

Allicin shows strong antimicrobial activity against human-pathogens like *Candida albicans*, *Helicobacter pylori*, methicillin resistant *Staphylococcus aureus* (MRSA), and several bacterial and fungal plant pathogens [3,4,17–19]. The potential to develop allicin-containing preparations for applications in medicine and organic agriculture and horticulture has been recognized [1,20,21].

Cavallito and Bailey [22] first identified allicin as the antimicrobial principle in garlic and determined its structure. They also first suggested that thiol-reactivity might be the basis of allicin's antimicrobial activity [22]. Allicin is an electrophile and undergoes nucleophilic substitution with thiols to produce mixed disulfides. Thus, chemically, allicin readily oxidizes cellular thiols by a variant thiol-disulfide exchange reaction, which leads to S-allyl formation on accessible thiols of cellular proteins [23–25]. We previously carried out OxICAT-based redox proteomic studies in *E. coli* and identified several allicin protein targets [26]. We have also shown that allicin depletes and oxidizes the cellular glutathione pool [16]. The inhibition of certain thiol-containing enzymes by allicin was first demonstrated by Wills [27]. The antimicrobial activity of allicin was long assumed to be due to direct inactivation of essential thiol-containing enzymes. It was later shown that allicin has broader effects on cellular thiol-redox systems, directly impacting the glutathione pool, and thus potentially influencing redox-sensitive proteins [16]. Furthermore, it was demonstrated that allicin can permeabilize biological and artificial lipid membranes independently of its chemical reactivity [28]. This latter activity may be the reason for allicin's reported synergistic effects with membrane-active antibiotics like amphotericin-B and polymixin-B [29,30].

Yu et al. characterized the yeast transcriptomic response to sub-lethal doses of allicin and reported that 292 genes were differentially expressed by 90 min after allicin treatment [31]. In particular, transcripts of the *YKL071w* ORF showed the highest accumulation (~27 fold), a gene also reported to be strongly induced by a variety of oxidative and thiol-reactive substances, for example patulin [32], celastrol [33] and furfural [34]. Because of these and our own observations, we gave to *YKL071w* the name Oxidative Stress Induced 1 (*OSI1*). The function of the Osi1p protein is unknown, but homology searches indicate similarities to short chain alcohol dehydrogenases. Interestingly, our *in silico* analysis of the *OSI1* promoter revealed multiple overlapping putative binding sites for the Yap1p transcription factor, which motivated investigating the role of Yap1p in the yeast response to allicin.

The redox-regulated transcription factor Yap1p is the central regulator of the *S. cerevisiae* oxidative stress response (OSR) [35]. Yap1p regulates the expression of antioxidant, glutathione metabolism and detoxifying genes such as ABC transporters [36,37]. For example, Yap1p controls the expression of *GSH1* and *GSH2* of the glutathione biosynthetic pathway, glutathione reductase, glutaredoxins, thioredoxins and thioredoxin reductases [38,39].

Yap1p activity is regulated by a redox-dependent control of its subcellular location [40,41]. Yap1p shuttles between the nucleus and cytoplasm by virtue of active nuclear import and export. In unstressed cells, however, Yap1p is predominantly cytoplasmic as a result of a more proficient nuclear export, relative to its import. In response to H_2O_2, Yap1p becomes oxidized, forming two disulfide bonds between N- and C-terminal cysteine (Cys) residues, which by concealing the export receptor-Crm1 cognate nuclear export signal (NES), causes Yap1p to accumulate in the nucleus and to activate gene expression [42]. Oxidation of Yap1p by H_2O_2 is not direct, involving the thiol-peroxidase Gpx3p, also named Orp1p for Oxidant Receptor Peroxidase, which acts as the upstream sensor of the pathway [43]. Oxidation of Yap1p by Gpx3 also requires Ybp1p, a probable scaffold protein the function of which is not well understood. Electrophilic compounds, such as 4-hydroxynonenal, iodoacetamide and the metal cadmium activate Yap1p in a manner independent of Gpx3, which involves the direct modification of Yap1p C-terminal Cys residues and leads also to the concealment of the C-terminal NES motif [41,44,45,46].

We show here that the natural product allicin, produced in large amounts by the common foodstuff garlic, induces expression of the *OSI1* gene in a Yap1p-dependent manner. We investigate the role of Yap1p in coordinating the resistance response of yeast to allicin through the genes encoding activity of the glutathione pathway. We also show that, as for synthetic electrophilic compounds, allicin activates Yap1p in a manner independent of Gpx3, which involves the probable modification of C-terminal Cys residues. This is the first report characterizing the interaction of a natural electrophile from a common foodstuff with the Yap1p transcription factor.

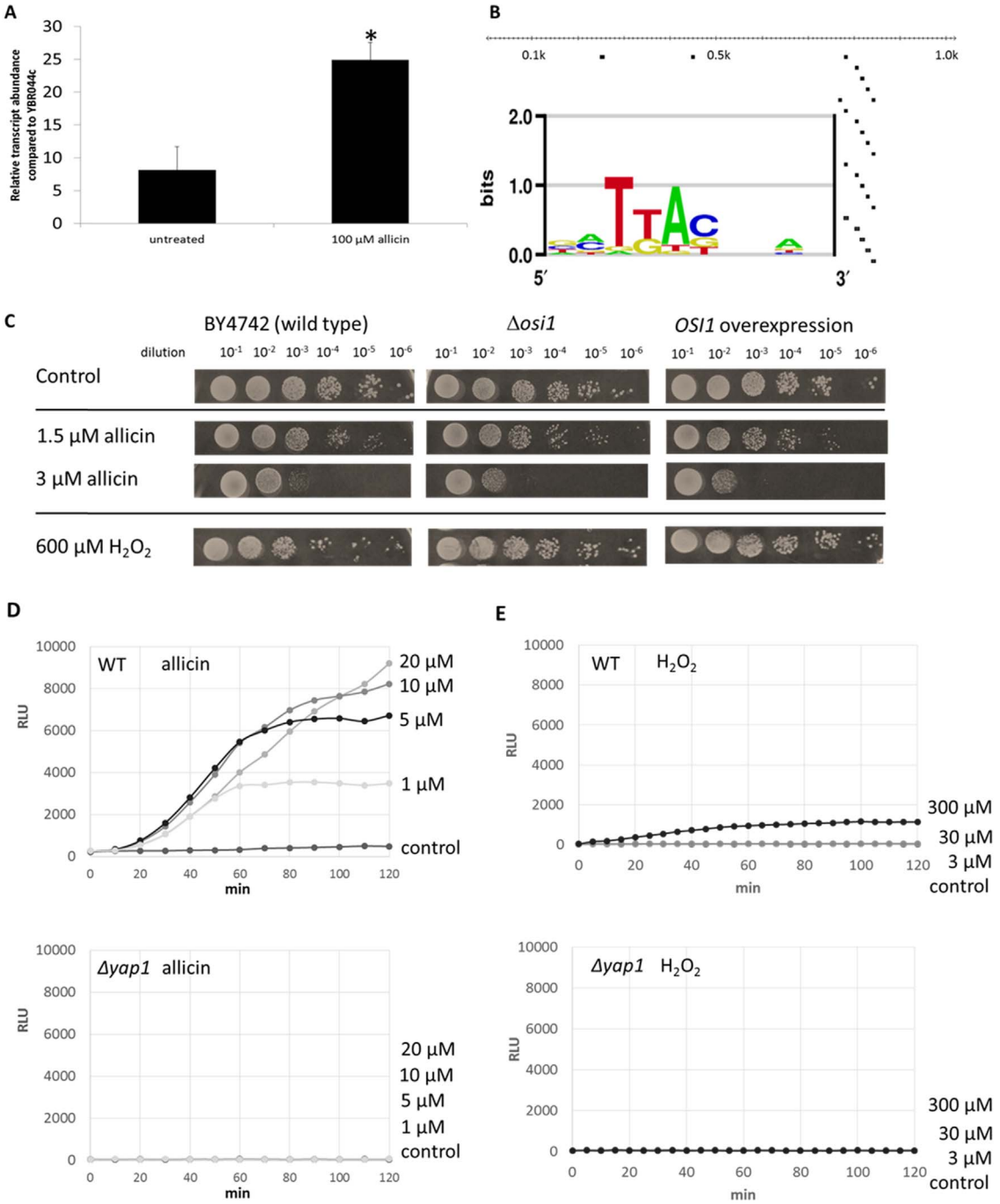

Fig. 1. *OSI1* (YKL071w) characteristics. Allicin-induced accumulation of *OSI1* (YKL071w) transcripts in BY4742 cells was determined by qPCR 1 h after treatment and shows the relative transcript abundance compared to the reference gene YBR044c (n = 4, $p < 0.05$ Student's *t*-test) (A). Multiple overlapping Yap1p binding sites (consensus shown in the inset) in the + and – strands of the 1 kb sequence upstream of the *OSI1* (*YKL071w*) ATG start codon. The diagram was prepared using The Yeast Transcription Factor Specificity Compendium, Version: 1.02 (http://yetfasco.ccbr.utoronto.ca) and shows the position of consensus Yap1p binding sequences #327T(G/T)A(C/G)(T/A)AA, #329T(G/T)ACTAA, #1150 AAGCTTTTT and #2186 (G/C/T)(A/C)TTA(C/G/T)NN(A/T) (B). BY4742, Δ*osi1* and an *OSI1* overexpression plasmid [34] transformed into BY4742 cells were grown to stationary phase and serial dilutions plated onto CSM medium containing allicin or H_2O_2 at the concentrations indicated. Results are shown after two days incubation at 28 °C (C). An *OSI1*-promoter::luciferase reporter construct was transformed into Wt and Δ*yap1* deletion mutant backgrounds and showed dose-dependent expression after allicin treatment in the Wt background but was completely inactive in the Δ*yap1* background (D). The quantitatively much weaker response of the *OSI1*::luciferase reporter construct to H_2O_2 in Wt cells and absence of a response in the Δ*yap1* deletion mutant background is shown for comparison in (E). (RLU = relative luminescence units).

2. Results

2.1. Allicin induces expression of the OSI1 gene

In an *S. cerevisiae* microarray study, Yu et al. [31] reported that *YKL071w* (hereafter referred to as *OSI1*) transcript abundance had the highest increase among the many genes induced by allicin. We confirmed here that in BY4742 cells, allicin potently induces *OSI1* gene-transcript accumulation (Fig. 1A).

2.2. Osi1p is similar to short-chain alcohol dehydrogenases and its gene promoter carries multiple Yap1p binding sites

In silico analysis of the OSI1 promoter identified multiple overlapping

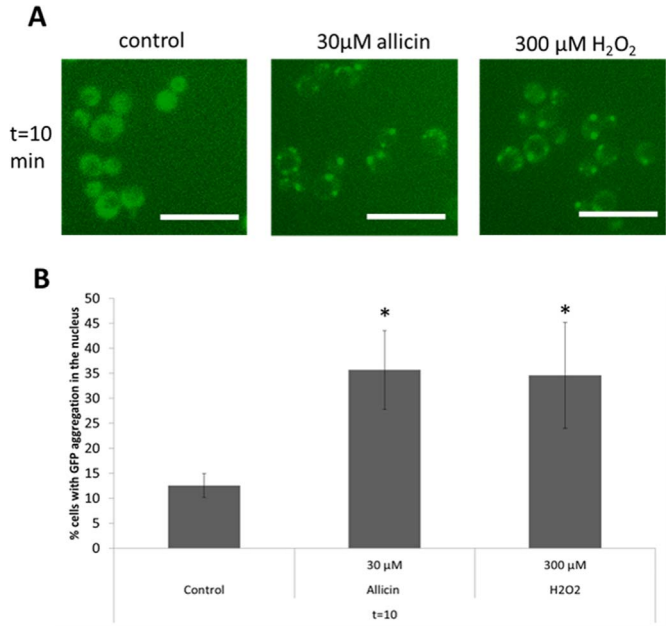

Fig. 2. Allicin-induced Yap1 nuclear localization. (A) Within 10 min of exposure to 30 μM allicin or 300 μM H_2O_2 a Yap1-GFP fusion protein can be seen to concentrate in the nuclei. (B) Quantitative representation of the data shown in A after counting ~900 cells. Student's *T*-test (* = $p < 0.05$).

putative Yap1p binding sites, particularly in the region from −234 to −156 upstream of the ATG translational start codon (Fig. 1B). A Blast-P search revealed that the Osi1p amino acid sequence has high similarity to yeast proteins with oxidoreductase and dehydrogenase activities. The highest similarity was to members of the short-chain alcohol dehydrogenase/reductase family (SDR), a heterogeneous family of proteins with a conserved NAD(P)H binding motif (a so-called 'Rossmann fold'). Irrespective of the function of its gene product, however, and despite potent *OSI1* induction by allicin, cells with an *OSI1* null deletion (Δ*osi1*), or those overexpressing this gene from a high copy number plasmid, showed the same allicin sensitivity relative to their Wt counterpart (Fig. 1C).

2.3. Yap1p-dependence of OSI1 expression

The multiple overlapping Yap1p binding sites in the *OSI1* promoter (Fig. 1B) suggested that expression of the *OSI1* gene is dependent on the Yap1p transcription factor. To test this hypothesis we prepared an *OSI1*-promoter::luciferase reporter construct spanning the portion between −860 up to the translation start site. This gene fusion was transformed into Wt and a Δ*yap1* deletion mutant, and its induction by H_2O_2 and allicin was comparatively measured. In Wt cells the *OSI1*-promoter::luciferase reporter responded both to H_2O_2 and to allicin. Furthermore, the dose-dependent response showed that sublethal concentrations of allicin as low as 1 μM (0.16 μg mL^{-1}) were active in inducing the gene fusion (Fig. 1D, E). At this concentration, allicin-dependent gene induction reached a value of ~3500 relative luminescence units

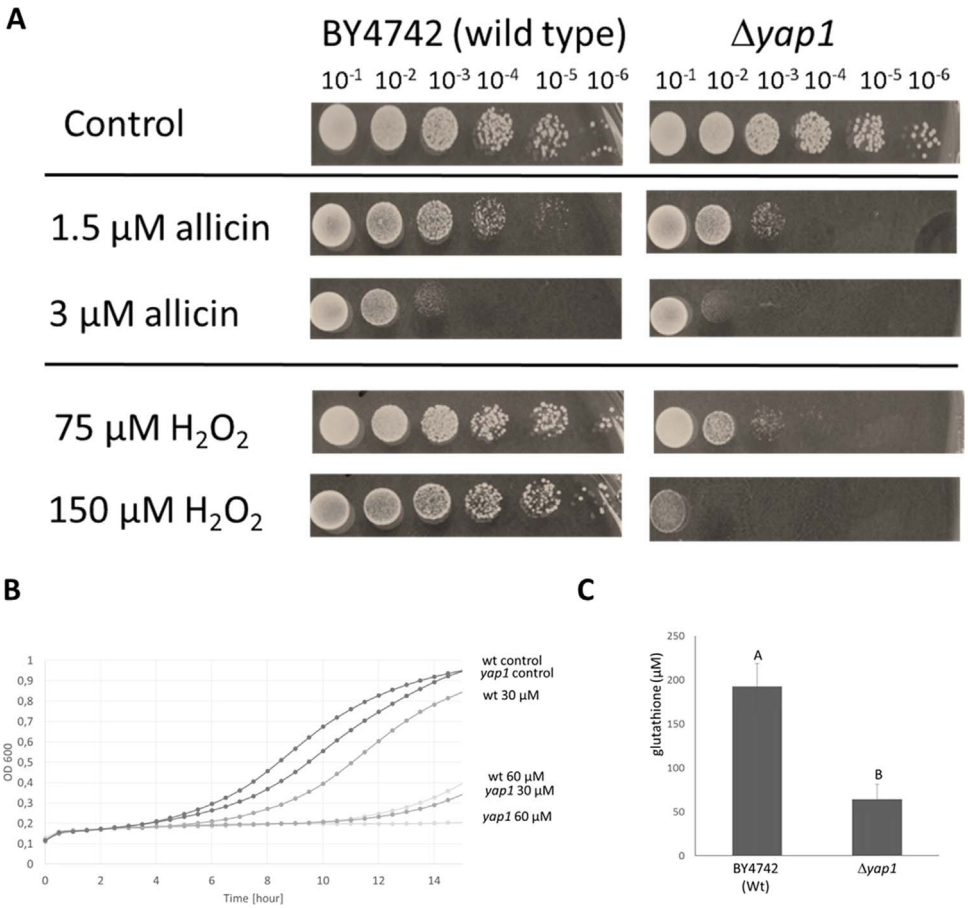

Fig. 3. A Δ*yap1* mutant is hypersensitive to allicin and H_2O_2 and has a smaller relative GSH pool than Wt BY4742 and Δ*osi1* cells. (A) BY4742 yeast cells or Δ*yap1* cells were grown to stationary phase and serial dilutions plated onto CSM medium containing allicin or H_2O_2 at the concentrations indicated. After two days incubation at 28 °C the hypersensitivity of Δ*yap1* cells to allicin and H_2O_2 is visible. (B) Growth kinetics of BY4742 or Δ*yap1* cells in CSM medium in the presence of various allicin concentrations. (C) Total cellular glutathione was released from overnight cultures of cells by vortexing with glass beads and measured in a glutathione reductase recycling assay as described in Section 4. n = 3, error bars show standard deviation. Columns with the same letter were not significantly different from one another ($p > 0.05$) in a one way ANOVA test.

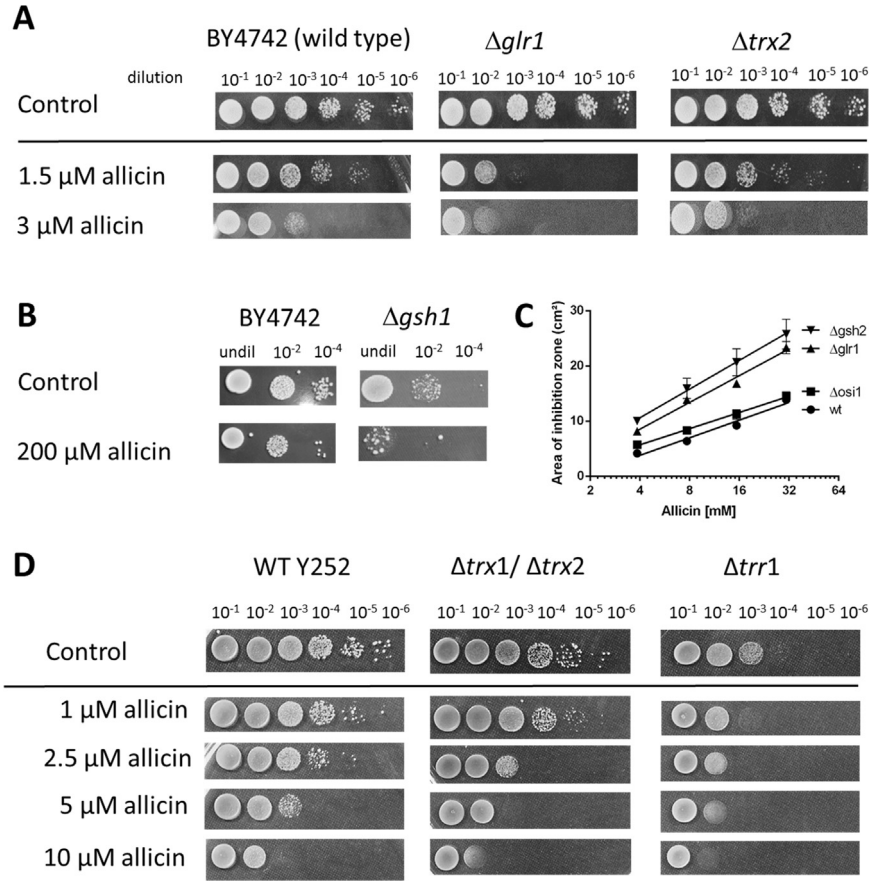

Fig. 4. Sensitivity of mutants to allicin. (A) BY4742 yeast cells, Δ*glr1* or Δ*trx2* cells were grown to stationary phase and serial dilutions plated onto CSM medium containing allicin at the concentrations indicated. Results are shown after two days incubation at 28 °C. (B) Because the Δ*gsh1* mutant is non-viable in the absence of GSH, Wt BY4742 and Δ*gsh1* cells were grown in pre-culture in CSM broth containing 50 µM GSH and for 16 h in shake culture either with or without allicin in the same medium during the experiment and 10 µL of the dilution series plated onto CSM agar containing 50 µM GSH but without allicin. The comparatively high concentration of allicin was necessary in this experiment because the effective concentration is reduced by reaction with GSH. (C) Allicin solution (25 µL) was pipetted into wells cut out of yeast-seeded CSM agar in Petri plates and the inhibition zones measured after incubation at 28 °C. BY4742 Wt, Δ*osi1*, Δ*glr1*, and Δ*gsh2* mutants are shown. Bars show standard deviation of four replicates. (D) WT Y252 Wt cells and the corresponding Δ*trx1*/Δ*trx2* and Δ*trr1* cells were grown to stationary phase and serial dilutions plated onto CSM medium containing allicin at the concentrations indicated. Results are shown after two days incubation at 28 °C.

(RLU), which was higher than the response elicited by 300 µM H_2O_2 (~1100 RLU), known to elicit the maximal Yap1p response to H_2O_2 [42]. In contrast, no luciferase activity could be recorded in Δ*yap1*, thus demonstrating the absolute Yap1p dependence of allicin-induced *OSI1* promoter activation (Fig. 1D, E). Another indication of the activation of Yap1 by allicin was that it induced the nuclear accumulation of a Yap1p-GFP fusion (Fig. 2A, B).

2.4. Yap1p is required for tolerance to allicin

Yap1p is the central regulator of the OSR in yeast, regulating, among other things, activities of the glutathione pathway. Because allicin modifies thiols by oxidation, we predicted that Δ*yap1* might be sensitive to allicin. The Δ*yap1* strain was indeed highly sensitive to allicin, relative to Wt, on both solid media and liquid culture growth assays (Fig. 3A, B). Tolerance to H_2O_2 served here as a control. We also measured total cellular levels of glutathione in these cells, which showed that Δ*yap1* cells had approximately 30% of the Wt amount of GSH (Fig. 3C). In both Wt and Δ*yap1* cells the proportion of GSSG in the total glutathione pool in unstressed cells was less than 1%.

2.5. Glutathione is important in the tolerance to allicin

As Yap1p controls the activities of the glutathione pathways [35], and as Δ*yap1* is hypersensitive to allicin, we next tested the importance of these genes in allicin tolerance, specifically *GSH1*, which encodes gamma-glutamyl cysteine synthase, the limiting enzyme in glutathione biosynthesis, *GSH2* encoding glutathione synthase which couples gamma-glutamyl cysteine to glycine to make GSH, and *GLR1*, which encodes glutathione reductase. Deletion of either *GSH1* (Δ*gsh1*), *GSH2* (Δ*gsh2*) or *GLR1* (Δ*glr1*) made cells hypersensitive to allicin (Fig. 4A, B, C). Because glutathione is essential for growth [47], allicin tolerance of the Δ*gsh1* strain was tested in plates containing 50 µM glutathione, a concentration that is sufficient to fully rescue growth at levels of the Wt [48]. Hence, the observed defective growth of these cells in the presence of allicin indicates that glutathione is important to prevent allicin toxicity. In comparison, deletion of *TRX2*, which encodes the major yeast cytoplasmic thioredoxin, barely increased sensitivity to allicin, relative to Wt. Taken together these results indicate the importance of the Yap1p-dependent glutathione response to sustain resistance to allicin toxicity. In a standard agar-diffusion inhibition zone test with yeast cells embedded in the agar and 25 µL of allicin solution pipetted into wells cut in the agar, it can be seen that the inhibition zones for Wt and Δ*osi1* cells are very similar over the concentration range 2–32 mM whereas Δ*glr1* and Δ*gsh2* mutants show much larger inhibition zones over the same concentration range (Fig. 4C).

Because under some circumstances *TRX1* can complement *TRX2*, we also tested a Δ*trx1*/Δ*trx2* double mutant and a thioredoxin reductase mutant (Δ*trr1*) (Fig. 4D). The Δ*trx1*/Δ*trx2* double mutant is, similarly to the Δ*trx2* single mutant, only slightly more susceptible to allicin than the Wt, which contrasts with the high susceptibility shown by the Δ*glr1*

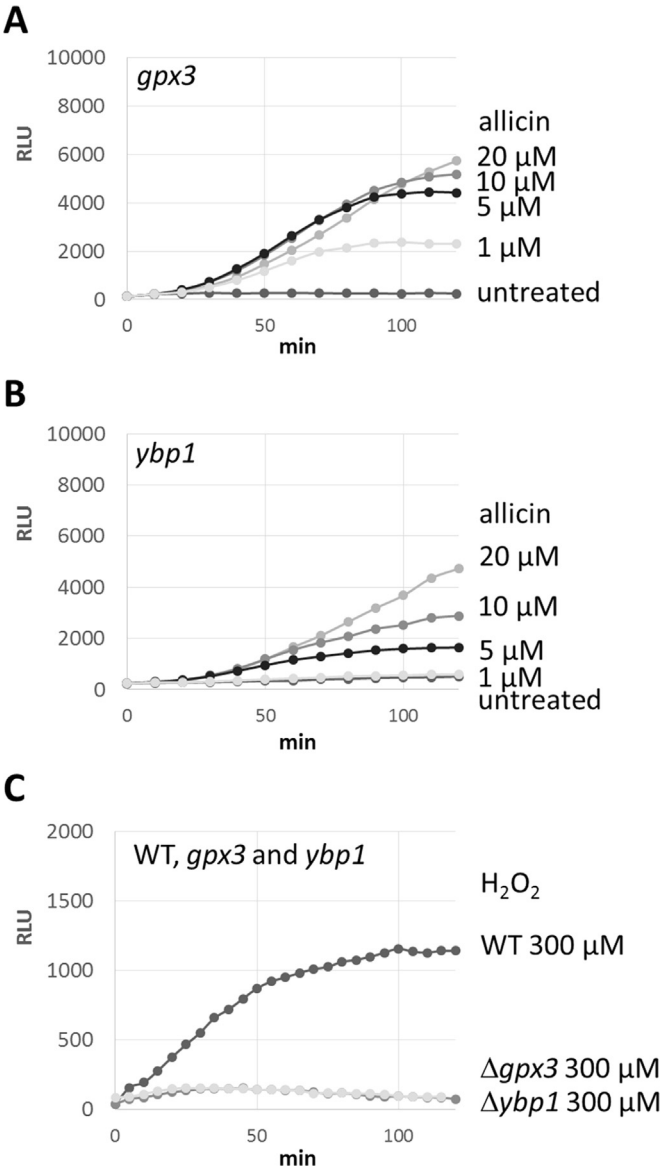

Fig. 5. Activation of Yap1p by allicin is independent of H_2O_2 production. (A) Dose dependent response kinetic to allicin of the *OSI1* promoter::luciferase reporter in the Δ*gpx3* background. (B) Dose dependent response kinetic to allicin of the *OSI1* promoter::luciferase reporter in the Δ*ybp1* background. (C) Activation kinetic of the *OSI1* promoter::luciferase reporter by H_2O_2 in the Wt BY4742 background but not in Δ*gpx3* or Δ*ybp1* backgrounds. (RLU = relative luminescence units).

mutant, even at very low allicin concentrations. The Δ*trr1* mutant has an intrinsic poor growth but is not obviously hypersensitive to allicin (Fig. 4D).

2.6. Activation of Yap1p by allicin occurs independently of H_2O_2 production

As allicin modifies Cys residues by S-thioallylation, it could activate Yap1p through modification of regulatory Cys residues, as shown for synthetic electrophilic compounds [44]. However, its impact on thiol-redox control pathways, and especially on glutathione also suggest that allicin could also activate Yap1p through H_2O_2 production. We thus monitored the activation of Yap1p by allicin in cells lacking Gpx3p, in which the Yap1p response to H_2O_2, but not to electrophilic compounds, is totally blunted [43,44]. In Δ*gpx3* the *OSI1*-promoter::luciferase reporter was still induced by allicin, although not as potently as in Wt (Figs. 1D and 5A). Ybp1p is a protein essential for the function of the

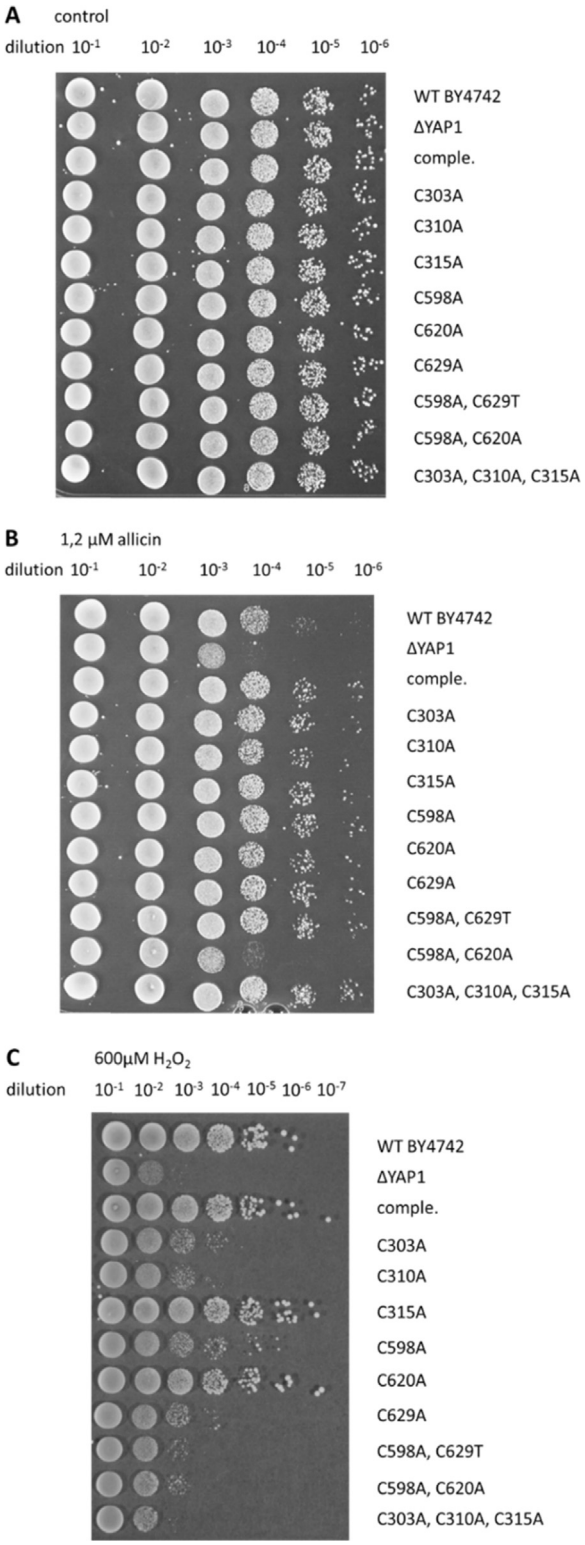

Fig. 6. Drop test showing the relative sensitivity of cells expressing various cysteine exchange mutant constructs in the Δ*yap1* background. (A) All transformants grew as the Wt in the absence of oxidative stress on CSM. (B) Cells were plated onto CSM containing 1.2 µM allicin. The Δ*yap1* mutant was hypersusceptible to allicin stress and this could be complemented by transforming with the Wt *Yap1* sequence (comple.). Allicin stressed C→A exchange mutants all grew as the Wt with the exception of the C598A, C620A transformant which showed the background Δ*yap1* mutant phenotype. (C) Cells were plated onto CSM containing 600 µM H_2O_2. The Δ*yap1* mutant was hypersusceptible to H_2O_2 stress and this could be complemented by transforming with the Wt *Yap1* sequence (comple.). The C315A and the C620A mutants were as resistant to H_2O_2 as the Wt cells but all other transformants were hypersusceptible to H_2O_2.

Gpx3p-Yap1p redox relay [49]. In Δ*ybp1* cells also, the *OSI1*-reporter luciferase fusion still responded to allicin, but here again not as potently as in Wt (Figs. 1D and 5B). In contrast, in both Δ*gpx3* and Δ*ybp1* the *OSI1*::luciferase reporter was not at all induced by H_2O_2 (Fig. 5C), as previously shown [43]. The persistence of the Yap1p response to allicin in Δ*gpx3* and Δ*ybp1* thus clearly indicates that this natural substance activates Yap1p in a manner independent of cellular H_2O_2 production. However, the slightly lower amplitude of this response, relative to Wt cells, could also suggest that it is partially elicited through H_2O_2 (Figs. 1D and 5A, B).

To confirm the mechanism by which allicin activates Yap1p, we monitored allicin tolerance of cells that carried mutant versions of Yap1p with Ala substitution of Cys regulatory residues. Yap1p carries six Cys residues clustered in N-terminal and C-terminal domains (see Fig. 7). Activation of Yap1p by H_2O_2 leads to formation of at least two disulfides, one between Cys303 and Cys598 and the other between Cys310 and Cys629 [42,50,51], and Ala substitution of any of these four Cys residues abrogate the Yap1p response to H_2O_2.

Yap1p cysteine substitution mutants were expressed in Δ*yap1* cells to assess which cysteines are important for allicin activation. All transformants had a Wt growth in the absence of stressor (Fig. 6A). As already shown, the Δ*yap1* mutant was hypersensitive to allicin (Fig. 6B), and this allicin phenotype was rescued by *YAP1* (Fig. 6B), and by all Yap1p Cys-to-Ala substitution mutants, except for Yap1pC598A and Yap1pC620A, which remained allicin hypersensitive (Fig. 6B). As expected, the H_2O_2 control experiment revealed that mutants with Ala substitution of Cys303, Cys310, Cys598 and Cys629 were hypersensitive to H_2O_2 (Fig. 6C). Thus, allicin-induced Yap1p activation in cells lacking Gpx3p or Ybp1p suggested that the mechanism by which it activates the transcription factor is by direct modification of Yap1p Cys residues. The results of the sensitivity assay confirm these results, now indicating that Yap1p Cys598 and Cys620 are the important targets of allicin Cys residues modification.

3. Discussion

Many studies of the cellular effects of electrophilic compounds have utilized synthetic compounds, such as acrolein, diamide, diethylmaleimide, or menadione and have emphasized the overlapping and distinct sub-sets of genes induced by these different stressors e.g [52]; e.g [53]. However, organisms are only likely to come into contact with these substances in the laboratory, and in this regard, it is interesting and novel to study the effects of the natural electrophilic oxidant and defence substance allicin on cells, a potent thiol-reactive agent and dose-dependent biocide [3]. Because garlic is a common foodstuff and an ingredient in many dishes, it is particularly relevant to understand allicin's effects on cells at sublethal doses. We report here on the regulation of the cellular response to allicin in yeast cells via its effect on the Yap1p transcription factor.

In a microarray study with the diploid yeast strain L1190 Yu et al. [31] reported that *YKL071w* (*OSI1*) transcripts accumulated to the highest degree of all yeast genes after allicin treatment. We confirmed here that *OSI1* transcripts accumulated after allicin treatment of the haploid yeast strain (BY4742) (Fig. 1A). However, Δ*osi1* mutants and *OSI1*-overexpression showed no greater susceptibility or resistance to allicin in drop tests than the wild type (Fig. 1C). Thus, Osi1p activity may not be required for allicin resistance, or the loss of Osi1p activity is compensated for by similar enzyme activities, or possibly also the quantitative effect of Osi1p in a multicomponent resistance response is not measurable. However, our *in silico* analysis of the *OSI1* gene revealed multiple binding motifs for the redox-regulated Yap1p transcription factor (Fig. 1B). Yu et al. [31] also reported a 1.5 fold allicin-induced accumulation of YAP1 transcripts. We show here the Yap1p-dependence of *OSI1* expression using an *OSI1* promoter::luciferase reporter in Wt and Δ*yap1* cells (Fig. 1D, E).

Yap1p regulates the expression of antioxidant genes, including genes involved in glutathione synthesis and metabolism, and thioredoxins [35]. Glutathione is the major low M_r thiol in eukaryotic cells and acts as a major cellular thiol-redox buffer [16,54,55]. We previously showed that allicin targets the GSH pool in yeast cells, shifts the cellular redox to a more oxidized state and elicits apoptosis or necrosis in a concentration dependent manner [55]. In keeping with these results we found that the size of the GSH pool in Δ*yap1* cells is approximately 30% of those of Wt (Fig. 3C), which should contribute to the hypersensitivity of these cells to allicin toxicity (Fig. 3A, B). If the relative efficacy of allicin and H_2O_2 are compared, both the Wt BY4742 and the Δ*yap1* mutant are sensitive to much lower concentrations of allicin than of H_2O_2 (Fig. 2A), attesting to the potency of allicin as an antimicrobial agent. Furthermore, comparing the response of the *OSI1*-promoter::luciferase reporter to allicin and H_2O_2, shown in Fig. 1D and E respectively, it can be seen that the *OSI1* promoter responds to allicin more sensitively than to H_2O_2. As little as 1 μM allicin elicits a response of ~3500 RLU whereas 300 μM H_2O_2 gave a response of ~1100 RLU.

Significantly, the GSH content of Δ*yap1* cells is much reduced compared to Wt cells (Fig. 3C). The natural product allicin is a potent thiol reagent and reacts with glutathione as well as cysteine-containing proteins [3,16,24]. In this regard it behaves similarly to the synthetic substance and electrophile dipyridyl disulfide (DPS), for which it was demonstrated that the cytoplasmic GSH redox status determined the survival of yeast cells [45]. Thus, it seemed likely that yeast mutants compromised in the regulation of the glutathione pool (e.g. gamma-glutamyl cysteine synthase Δ*gsh1*, glutathione synthase Δ*gsh2*, glutathione reductase Δ*glr1*) might also be affected in resistance to allicin and this was indeed shown to be the case (Fig. 4A, B, C). Thus, the Δ*yap1* mutant is doubly compromised in its resistance to allicin; on the one hand because of its reduced GSH pool and redox-buffering capacity, and on the other hand because of its inability to mount an effective OSR. In this regard, we tested the resistance of a Δ*trx2* mutant, a Yap1p-dependent OSR gene and the major cytoplasmic NADPH-dependent thioredoxin, and showed that it too was more susceptible to allicin than the wild type, although the effect was not as pronounced as with GSH mutants (Fig. 4A, B). In addition, because under some circumstances

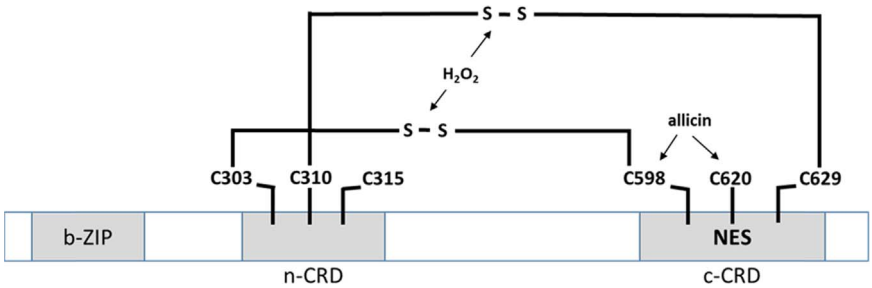

Fig. 7. Schematic representation of the cysteine targets in Yap1p for allicin and H_2O_2 respectively. As previously reported C303/C598 and C310/C629 are the sites of H_2O_2–induced cystine bridge formation in Yap1p and in this work the C-term C598 and C620 as a combined site of allicin action are shown. b-ZIP = basic leucine zipper domain, n-CRD = N-term cysteine rich domain, c-CRD = C-term cysteine rich domain, NES = nuclear export sequence.

Δ*trx1* can complement for Δ*trx2* [56], we tested a double Δ*trx1*/Δ*trx2* mutant and found that it behaved similarly to the Δ*trx2* single mutant and was not as sensitive to allicin as the Δ*glr1* mutant (Fig. 4D). In a further test of the role of thioredoxins in resistance to allicin we investigated a thiordoxin reductase deletion mutant (Δ*trr1*) for allicin sensitivity. The Δ*trr1* mutant grew much less well than the Wt even in the absence of allicin but its differential susceptibility to allicin in comparison to the Wt did not seem as great as for the Δ*glr1* mutant. The data for theΔ*trx1*/Δ*trx2* and the Δ*trr1* mutants strengthens our conclusion that GSH and GSH metabolism play a major role in resistance to allicin.

Activation of Yap1 depends upon its oxidation and accumulation in the nucleus. As a further confirmation of the involvement of Yap1p in the cell's response to allicin, we used a *YAP1-GFP* fusion construct and showed that Yap1p concentrates in the nucleus within 10 min of allicin treatment (Fig. 2A, B), further demonstrating the activation of Yap1p by allicin.

Allicin reacts with accessible thiol groups and can S-thioallylate proteins directly [25,26] and thus it seemed likely that allicin was activating Yap1p by directly oxidizing cysteines in the C-term CRD of Yap1p as do other electrophiles [41,44–46]. However, it was also possible that secondarily generated reactive oxygen species (ROS) e.g. H_2O_2 might mediate the activation of Yap1 by allicin. In order to test this possibility, the *OSI1* promoter::luciferase reporter construct was transformed into Δ*gpx3* and Δ*ybp1* mutant backgrounds because these genes had previously been shown to be crucial for H_2O_2 activation of Yap1p [43,49]. The activation of the *OSI1* promoter by allicin in these mutant backgrounds (Fig. 5C, D) showed that allicin can activate Yap1p directly but it does not rule out that allicin might also lead to H_2O_2 accumulation in stressed cells.

The target cysteines for allicin in Yap1p were identified using a series of cys replacement constructs transformed into the Δ*yap1* mutant background (Fig. 6). Only the C598A, C620A double mutant was hypersensitive to allicin, indicating that either one of these cysteines in the C-term CRD must be present for allicin-dependent Yap1p activation but when both are lacking, allicin cannot activate Yap1p. This result is consistent with observations that electrophilic thiol reagents need the presence of the C-term CRD cysteines to activate Yap1p [41,44–46]. However, C629 was not needed for activation of Yap1p by allicin (Fig. 6). The NES encompasses C620 and it was shown that the L619 residue was essential for its function [51,57]. Our results suggest that the S-thioallylation of both C598 and C620 are needed to prevent the binding of the export receptor Crm1 to Yap1p [35] (Fig. 7). Furthermore, replacement of the of the N-term cysteines C303 or C310, necessary for H_2O_2 activation of Yap1p, did not affect the allicin phenotypic response (Fig. 6B) but did as expected render the cells hypersensitive to H_2O_2 (Fig. 6C), which further indicates that allicin does not activate Yap1p through the intermediacy of H_2O_2, but is mainly due to the direct modifications of C-terminal cysteines C598 and C620.

Taken together our results show that the cellular response in yeast against the natural product allicin from garlic is dependent upon the Yap1p transcription factor and on the integrity of the GSH pool.

4. Materials and methods

4.1. Yeast strains and cultivation

Yeast strains used were based on the BY4742 wildtype strain (Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0). The mutants Δ*osi1* (YKL071w), Δ*yap1* (YML007w), Δ*ybp1* (YBR216C), Δ*gpx3* (YIR037W) Δ*glr1* (YPL091w), Δ*trx2* (YGR209c) and Δ*gsh1* (YJL101c) were obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). According to the SGD gene nomenclature rules (http://genome.www.stanford.edu/Saccharomyces/) these deletion mutants, which are all gene replacement mutants, are correctly written: *osi1Δ::KanMX*, *yap1Δ::KanMX*, *ybp1Δ::KanMX*, *gpx3Δ::KanMX*, *glr1Δ::KanMX*, *trx2Δ::KanMX* and *gsh1Δ::KanMX*, respectively. However, for the sake of simplicity a simple Δ has been written in front of the lower case gene symbol throughout (e.g. Δ*yap1*). The Δ*trx1*/Δ*trx2* and Δ*trr1* mutants were based on the Y252 Wt strain and are from the culture collection of Laboratoire Stress Oxydants et Cancer, SBMS.

Yeast cells were cultivated on CSM-Medium (7 g L^{-1} Yeast Nitrogen Base containing $(NH_4)_2SO_4$ without amino acids (ForMedium Ltd., Norwich, UK); 40 g L^{-1} Glucose (Carl Roth, Karlsruhe, Germany); 0,8 g L^{-1} CSM complete dropout (ForMedium Ltd., Norwich, UK). For solid media, 15 g L^{-1} agar (Carl Roth, Karlsruhe, Germany) were added. Yeast cells were cultivated at 28 °C and liquid culture was shaken at 220 rpm.

4.2. RNA-preparation, cDNA synthesis and q-PCR

Yeast cells were cultivated as mentioned above. 20 mL yeast freshly grown cell culture (OD = 0.5) were treated with allicin (16 μg mL^{-1} = 10 μM) for one hour while incubated under standard conditions (after [31]). After one hour, the cells were centrifuged, decanted and resuspended in 200 μL extraction buffer (10 mM TRIS, pH 7.5; 10 mM EDTA; 0.5% sodium dodecyl sulfate (SDS)). 200 μL phenol-chloroform-isoamyl alcohol (PCI; 25:24:1) and 0.4g glass beads were added and vortexed for 3 × 1 min (the samples were cooled on ice in between). 300 μL PCI and 300 μL extraction buffer were added and the mixture was vortexed for one additional minute, before the tubes were centrifuged at 4 °C for 5 min at 5800 × *g*. The upper (= aqueous) phase was transferred to a new reaction tube and again extracted with an equal volume of PCI. After centrifugation (5 min, 4 °C, 5800 × *g*), RNA was precipitated by adding a two-fold volume of 95% ethanol (DEPC-treated) and cooling for 1 h at −70 °C. The tube was than centrifuged at 4 °C for 20 min at 5800 × *g*. After decanting, the pellet was washed twice with 95% DEPC-treated ethanol and dried. The pellet was resuspended in 100 μM water. Residual gDNA was removed by DNase digestion according to manufacturer's instructions (Fermentas, St. Leon Roth, Germany). Quality of RNA was checked by denaturating gel electrophoresis. cDNA was synthesized after thermal denaturation (5 min at 65 °C) using a Fermentas RevertAid cDNA first strand cDNA synthesis kit (Fermentas, St. Leon Roth, Germany).

qPCR was conducted in a ABI7300 Real time PCR system using the following conditions: 2 min 50 °C, 10 min 95 °C, 40 cycles 15 s (95 °C, 1 min 60 °C) and Cyber ® GreenER (Invitrogen GmbH, Karlsruhe, Germany) as master mix. Expression of *OSI1* was compared with *TCM62* (YBR044c) using the following qPCR-primer: TCC AAC AAA GGC CCT GGC ACA (YBR044c, forward); CCT CTG GCT CCG TAT CGC CG (YBR044c; reverse); ACC AGA AGA AAG TGC CTC TGC GCT (*OSI1*, forward); GAC GCC TTC GCT GCC GTC AT (*OSI1*, reverse). The 'RefGenes application' of the GENEVESTIGATOR software package (https://genevestigator.com/gv/) suggested YBR044c as a reference gene choosing a microarray dataset investigating oxidative stress [58] as targeted condition and YKL071w as targeted gene.

4.3. OSI1-promoter::luciferase reporter construct

The *OSI1*-Promoter was amplified by PCR using the following primers TGG GCT CTT ATT GAA TCG CTT CCG (forward) and TCC ACG GCT ACC ACC GAT GA (reverse) and blunt end ligated into the pJET1.2 plasmid (Thermo Fisher Scientific GmbH) resulting in the pJET OSIp plasmid. The *OSI1*-Promoter Sequence was amplified from pJET-*OSI1p* using primers designed for Infusion Cloning (Takara Bio Europe, Saint-Germain-en-Laye, France) (YKLpGREG fwd GGG AAC AAA AGC TCA TGA GAT TAG TAA TGC ATA GCG G and YKLpGREGLuc rev TTT GGC GTC TTC CAT GAT CGA CTT TGT TTG CTT AGA ATT C). Luciferase was amplified from the pPZP221 plant transformation vector [59] using primers also designed for In-Fusion® Cloning (Takara Bio Europe, Saint-Germain-en-Laye, France) LucipGREG576fwd 5′-GGG

AAC AAA AGC TGG TCA TGG AAG ACG CCA AAA ACA T-3′ and LucipGREG576rev 5′-AAT TAC ATG ACT GCT TCT TGG CCT TTA TGA GGA-3′. The plasmid pGREG576 [60] was linearized by digesting with *Sac*I and *Xho*I before the OSI1-promoter and the luciferase was incorporated into the linearized vector by In-Fusion® Cloning. The resulting plasmid (*pOSI1*promoter::luciferase reporter) contained a fragment of the OSI1 promoter upstream of the ATG start codon and was amplified in *E. coli* DH5α and verified by colony PCR, restriction digestion and sequencing the plasmid, transformed into yeast cell by electroporation and plated out on uracil-lacking CSM medium.

4.4. OSI1 overexpression

The pDH20 plasmid (*OSI1* overexpression construct) was kindly supplied to us by Dr. Dominik Heer, Institute of Molecular Systems Biology, ETH Zürich and detail can be found in [34].

4.5. Luciferase assay

Luciferin (Diagonal, Münster, Germany) was dissolved in 0.1 M sodium citrate, pH = 5 (Carl Roth, Karlsruhe, Germany) to a final concentration of 1 mM luciferin. 100 µL of an overnight-culture was adjusted to an $OD_{600} = 0.5$ and (in CSM-medium) was treated with 25 µL of aqueous solution of either allicin or H_2O_2 and 25 µL of luciferin-solution in black flat-bottomed 96 well microtiter plates. Plates were cultivated and luminescence measured in a Tristar^{2}S plate reader (Berthold, Bad Wildungen, Germany).

4.6. Glutathione measurements

Glutathione was measured using a glutathione-reductase based enzymatic assay, performed as described in [16]. Overnight cultures in CSM medium were diluted to OD = 1.0 and 20 mL were centrifuged (5 min × 3000*g*), washed by resuspension and recentrifugation in phosphate buffer (48 mM Na_2HPO_4, 95 mM NaH_2PO_4, 6.3 mM EDTA. pH 7.5) and the pelleted cells resuspended in 1 mL buffer and vortexed (3x for one minute with cooling on ice in between) and centrifuged in a bench top centrifuge. The supernatants were transferred into new reaction tubes. Cell lysate (12.5 µL) were given into a 1 mL cuvette before adding 737.5 µL of glutathione-reductase master mix {5 µL glutathione-reductase from yeast (Sigma, 20 U mL^{-1}), 50 µL 6 mM dithionitrobenzoic acid (DTNB, Carl Roth, Karlsruhe, Germany), 350 µL 0.3 mM NADPH (Carl Roth, Karlsruhe, Germany) all in phosphate buffer, and 332.5 µL H_2O}, final volume 750 µL. Absorbance was measured in 1 min-intervals for 10 min at 412 nm in a spectrophotometer (Beckman DU800). All measurements were performed in triplicate.

4.7. Cysteine exchange constructs

The Wt *YAP1* and cysteine amino acid exchange constructs expressed from pRS316 were as described in [42]. Constructs were checked by sequencing and transformed into the Δ*yap1* background by electroporation according to the protocol of Mapp (2012, http://www.umich.edu/~mapplab/protocols/yeast_transformation.pdf). Successful Yap1 expression was confirmed on Western blots using a commercially available mouse monoclonal antibody to the MYC-tag and decorated with an anti-mouse IgG HRP-linked antibody (data not shown).

4.8. Drop tests

Aliquots (10 µL) of serial dilutions of an overnight culture in CSM medium were plated onto CSM agar containing the final given concentration of allicin or H_2O_2 mixed into the agar at 50 °C and poured immediately. Because the Δ*gsh1* mutant is non-viable in the absence of GSH, these cultures were grown in CSM containing 50 µM GSH and plated onto CSM agar containing 50 µM GSH.

4.9. Growth kinetics

An overnight culture in CSM medium was diluted to $OD_{600} = 0.05$ and grown on to OD_{600} 0.2. Aliquots (100 µL) were pipetted into a 96 well plate (Sarstedt, Germany, flat-bottomed) containing 100 µL of allicin in medium to give the quoted final concentration. Plates were shaken at 28 °C and 180 rpm in a Tristar^{2}S plate reader (Berthold, Bad Wildungen, Germany).

4.10. Petri plate inhibition zone test

A log phase culture of the yeast test strain was adjusted to $OD_{600} = 1.0$ and 100 µL pipetted into a reagent tube to which 10 mL molten CSM agar, kept at 50 °C in a water bath, was added and immediately poured into a 9 cm diameter Petri dish. This ensured a uniform distribution of cells throughout the agar. Wells were cut with a 6 mm diameter cork borer and 25 µL of allicin test solution pipetted into these. Plates were incubated at 28 °C for 2 days.

4.11. Yap1p-translocation

To follow the Yap1-accumulation in the nucleus upon oxidative stress, Δ*yap1* cells were transformed with the pRS-cp-Yap1-HA GFP plasmid [41] by electroporation. The construct was a gift from S. Kuge, University of Tokyo and was kindly forwarded to us by Ms. Alise Ponsero & M. Toledano, CEA Saclay, France). Transformants were selected on CSM lacking histidine. For microscopy, transformants were grown in liquid CSM minus histidine over night at 28 °C (220 rpm) and diluted to $OD_{600} = 1$ and treated with allicin and H_2O_2 as indicated. Pictures were taken at timepoints t = 0 min and t = 10 min after allicin/H_2O_2 treatment; non-treated cells were used as control. Nuclear localization was scored under the fluorescence microscope (Leica DMR, Leica GmbH, Wetzlar, Germany) and photographed using a microscope digital camera (KY-F75U, JVC Deutschland GmbH, Friedberg, Germany) using Discus Software (Carl Hilgers, Technisches Büro, Königswinter, Germany).

For quantitative data, three independent fields of view with at least 300 cells each were counted. The experiment was performed twice with similar results.

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4.3. Publikation III

A Comparison of the Antibacterial and Antifungal Activities of Thiosulfinate Analogues of Allicin

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A Comparison of the Antibacterial and Antifungal Activities of Thiosulfinate Analogues of Allicin

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Allicin (diallylthiosulfinate) is a defence molecule from garlic (*Allium sativum* L.) with broad antimicrobial activities in the low µM range against Gram-positive and -negative bacteria, including antibiotic resistant strains, and fungi. Allicin reacts with thiol groups and can inactivate essential enzymes. However, allicin is unstable at room temperature and antimicrobial activity is lost within minutes upon heating to >80 °C. Allicin's antimicrobial activity is due to the thiosulfinate group, so we synthesized a series of allicin analogues and tested their antimicrobial properties and thermal stability. Dimethyl-, diethyl-, diallyl-, dipropyl- and dibenzyl-thiosulfinates were synthesized and tested *in vitro* against bacteria and the model fungus *Saccharomyces cerevisiae*, human and plant cells in culture and *Arabidopsis* root growth. The more volatile compounds showed significant antimicrobial properties via the gas phase. A chemogenetic screen with selected yeast mutants showed that the mode of action of the analogues was similar to that of allicin and that the glutathione pool and glutathione metabolism were of central importance for resistance against them. Thiosulfinates differed in their effectivity against specific organisms and some were thermally more stable than allicin. These analogues could be suitable for applications in medicine and agriculture either singly or in combination with other antimicrobials.

Garlic has been used since ancient times for its health beneficial properties and modern research has provided a scientific basis for this practice[1–3]. Garlic compounds have been shown to decrease cholesterol and fatty acid levels in the blood[4–6] and lower blood pressure[7–11]; thus, garlic consumption can contribute to the prevention of cardiovascular diseases[12]. Anti-tumour activities of garlic compounds have been demonstrated, providing for a potential use in cancer-therapy and prevention[13,14]. Another very important garlic property is the antimicrobial activity observed in raw garlic extract. The main anti-bacterial compound of fresh garlic is allicin, a thiosulfinate with two allyl groups as carbon chains (diallylthiosulfinate)[15,16]. Besides bacteria, the effects of allicin have been investigated against fungi, protozoa and viruses[17–19]. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were also shown to be susceptible to allicin[20]. Allicin is produced from the non-protein amino acid alliin (S-allylcysteine sulfoxide) upon tissue damage in a reaction that is catalyzed by the enzyme alliinase (Fig. 1). Structurally analogous thiosulfinates are produced in nature by other *Allium* and *Petiveria* spp.[21], and antimicrobial activity has been reported for this group of compounds[22–24]. Unlike conventional antibiotics, allicin is volatile and can kill bacteria via the gas phase[17]. This is particularly interesting since many lung-pathogenic bacteria are susceptible to allicin[25,26]. Although allicin is also toxic to human cells[27,28], the successful treatment of tuberculosis by breathing in the vapour from crushed garlic preparations was reported in the pre-antibiotic era[29,30].

Thiosulfinates are disulfide-S-monoxides and as products of the condensation of two sulfenic acids, can be viewed as 'sulfenic acid anhydrides'[31]. In the laboratory, allicin can be synthesized most effectively by oxidation of diallyldisulfide (DADS) with H_2O_2 in the presence of an organic acid catalyst that is first oxidized to the corresponding peroxy-acid, e.g. performic acid or peracetic acid[23,32].

The reactivity of thiosulfinates towards thiol-groups is an important component of their antimicrobial activity[15,23,33,34]. The electron-withdrawing effect of the *O*-atom creates an electrophilic sulfur centre which reacts readily with thiols, or more specifically, with thiolate ions (Fig. 2), thereby forming an *S*-allylmercapto adduct. Thus, many enzymes with catalytically important thiol-groups are oxidized and inhibited when exposed to allicin[33],

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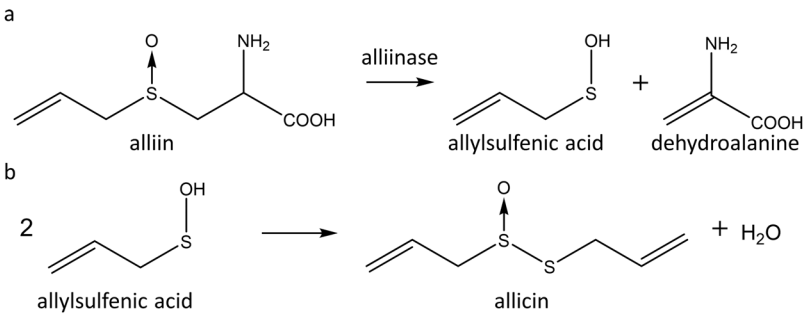


Figure 1. Biosynthesis of allicin from S-allyl cysteine sulfoxide (alliin). The enzyme alliinase (a C-S lyase, E.C. 4.4.1.4.) catalyzes the formation of allylsulfenic acid and dehydroalanine (**a**), whereupon two molecules of allylsulfenic acid condense spontaneously to yield one molecule of allicin (**b**).

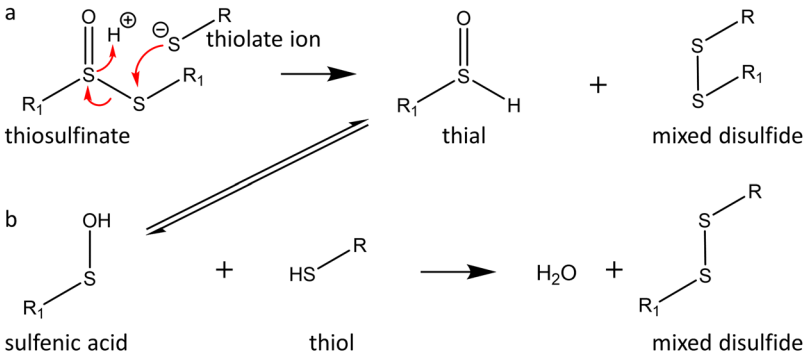


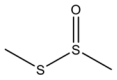
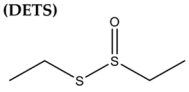
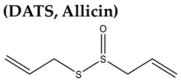
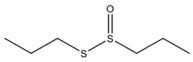
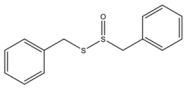
Figure 2. Reaction mechanism of a thiol with a thiosulfinate. The reaction leads directly (**a**), and indirectly (**b**), to the formation of a mixed disulfide, which under some conditions may react further with RSH in a thiol disulfide exchange reaction to form RSSR and R_1SH.

whilst, a few enzymes are activated upon oxidation by allicin, for instance fructose-1,6-bisphosphatase from chicken liver[35].

To understand allicin's cellular mode of action in more detail, a proteome-wide investigation in *Escherichia coli* was performed to identify the proteins oxidized by allicin exposure. After cells were treated with 0.79 mM allicin, 73 *S*-thioallylated proteins were identified, including some essential enzymes of primary metabolism[36]. Furthermore, allicin reacts with low-molecular weight cellular thiols such as glutathione (GSH), shifting the GSH-based cellular redox-potential to a more oxidized state. Indeed, it was recently shown for *Saccharomyces cerevisiae* that treatment with allicin altered the ratio of reduced (GSH) and oxidized glutathione (GSSG) into a range that would predict induction of apoptosis and this was confirmed by cytological and genetic methods[37]. In yeast, GSH synthesis is regulated by the Yap1p transcription factor, which has oxidation-sensitive cysteines and coordinates the oxidative stress response by regulating the expression of response genes. For example, Yap1p regulates the expression of the GSH biosynthetic genes *GSH1*, *GSH2* and glutathione reductase (*GLR1*), which utilizes NADPH to reduce GSSG to GSH. Allicin was shown to oxidize critical cysteines in Yap1p and *Δyap1* and *Δglr1* mutants were shown to be hypersensitive to allicin[38]. This situation is analogous to the essential role of GSH in resistance of yeast to dipyridyl disulfide, which is also a highly specific reagent for thiol groups, showing pronounced antifungal activity[39].

Besides being redox active, allicin is also quite lipophilic. The calculated log*P* value of allicin is 1.35, indicating that allicin is membrane permeable and its antimicrobial activity is certainly facilitated by its ready entry into cells[40]. At the same time, it has been shown that allicin is able to form transient pores in artificial and in bio-membranes, which perhaps accounts for its reported synergy with membrane-active antibiotics such as amphotericin B and polymixin B[41].

Overall, this magnitude of data shows that allicin is on the way to become a well characterized natural product with potential to be used both in medicine and agriculture. Allicin is unstable in storage and degrades rapidly at temperatures above 80 °C[17,42]. However, allicin and its derivatives have been discussed as lead compounds for new antibiotics[24,43], but very little is known about the biological activities of other thiosulfinates. Nevertheless, there is some promising albeit limited data on some allicin-derivatives which have been tested as inhibitors for cysteine proteases in the parasitic protozoa *Plasmodium falciparum* and *Trypanosoma brucei*[44]. Furthermore, along with allylisothiocyanate (AITC), DMTS (frequently referred to as methyl methanethiosulfinate, MMTSO) was described as one of the most important antimicrobial compound in cabbage plants for defence against microbial pathogens[45,46]. In garlic therefore, thiosulfinates other than allicin, although quantitatively more minor, may still be of considerable biological activity and interest.

Compound	M_r	log*P**	Relative Hydrophobicity **	Relative Hydrophilicity **	Miscibility with water	Thermal stability ***
Dimethylthiosulfinate (DMTS) 	110.19	-0.21	+	+++++	Yes	100%
Diethylthiosulfinate (DETS) 	138.24	0.64	++	++++	Yes	94%
Diallylthiosulfinate (DATS, Allicin) 	162.26	1.35	+++	+++	Yes	57%
Dipropylthiosulfinate (DPTS) 	166.29	1.61	++++	++	Yes	75%
Dibenzylthiosulfinate (DBTS) 	234.33	3.43	+++++	+	Immiscible	11%

*log*P* values were calculated using Chemdraw Professional 15.1 software (Cambridge Software).

**On a relative scale from + to +++++

***Proportion not degraded after 10 min at 99°C, determined by HPLC

Figure 3. Physical properties of different thiosulfinates.

In the work reported here we have synthesized a series of simple thiosulfinates, based on allicin as the lead compound, and evaluated their chemical stability and antimicrobial effectivity. Here, dimethyl- (DMTS), diethyl- (DETS), diallyl- (DATS, allicin), dipropyl- (DPTS) and dibenzyl- (DBTS) thiosulfinates form a series of increasing molecular mass and hydrophobicity which would be expected to affect physical characteristics such as rate of diffusion, volatility and membrane accessibility/permeability, all of which may be expected to have an influence on the biological properties of the molecules. We showed that some of the thiosulfinates are active as a vapour and that as little as one hour exposure to allicin vapour was inhibitory to microbial growth. We investigated a series of yeast mutants affected in GSH metabolism and protein disulfide reduction and showed similar responses to those for allicin, suggesting a similar mode of action. We confirm that the thiosulfinate moiety is important for antimicrobial activity but that this activity is modified by the surrounding substituent groups. The compounds tested are all quite 'allicin-like' in structure and mostly naturally occurring, and the results are discussed in terms of the merits and the potential of such thiosulfinates for applications in medicine and agriculture.

Results and Discussion

Comparison of the physical properties of DMTS, DETS, DATS, DPTS and DBTS. The structures and the physical properties of the test substances dimethyl- (DMTS), diethyl- (DETS), diallyl- (DATS, allicin), dipropyl- (DPTS) and dibenzyl- (DBTS) thiosulfinate are summarized in Fig. 3. As an indication of the relative membrane permeabilities the calculated log*P* values, also known as log K_{ow} for the log of the partition coefficient between octanol and water, were calculated using the software Chemdraw Professional 15.1. DMTS was the most hydrophilic compound tested (log $P = -0.21$) while DBTS was the most hydrophobic (log $P = 3.43$). DBTS was immiscible with water, methanol and ethanol and decomposed in DMSO, but was found to be soluble and stable in dimethyl formamide (DMF), in which it was dissolved for use in experiments (see Materials and Methods). DMTS, DETS, DATS and DPTS were sufficiently water soluble to be used in aqueous solutions.

Comparison of MICs, MBCs and MFCs for DMTS, DETS, DATS and DPTS. We set out to survey a range of microorganisms with respect to the effects of the thiosulfinates and based on our previous experience with allicin we chose *E. coli* as an example of an enteric bacterium, *P. fluorescens* and *P. syringae 4612* as examples of relatively allicin-resistant and allicin-susceptible strains, respectively, *M. luteus* as a Gram-positive example and the Euroscarf yeast reference strain BY 4742 as a model fungus. International standard EUCAST

		Gram-positive			Gram-positive	Yeast
		Escherichia coli K12 Ec	*Pseudomonas fluorescens Pf—01*	*Pseudomonas syringae pv. phaseolicola 4612 Ps4612*	*Micrococcus luteus Ml*	*Saccharo-myces cerevisiae* BY4742 *Sc*
Thio-sulfinate	Conc. (μg mL^{-1})					
DMTS	MIC MBC	64 64	16 32	16 16	64 64	16 MFC 16
DETS	MIC MBC	64 64	128 256	8 16	32 32	8 MFC 8
DATS	MIC MBC	32 32	128 256	16 16	16 32	2 MFC 4
DPTS	MIC MBC	32 32	256 256	32 64	32 32	2 MFC 4

Table 1. MIC and MBC values (μg mL^{-1}) for the different thiosulfinates in the bacteria tested and MIC and MFC for yeast. The values are given as the highest value out of three replicates.

test procedures were used to determine the minimal inhibitory concentration (MIC) and minimal bactericidal or fungicidal concentration (MBC, MFC), respectively, for the different thiosulfinates with selected bacteria and *Saccharomyces cerevisiae*. It should be noted that the use of μg mL^{-1}, rather than equimolar concentrations, is an unfortunate anachronism of the internationally used standard test protocol[47]. MICs for the bacteria tested ranged from 8–256 μg mL^{-1} and MBCs from 16–256 μg mL^{-1} for the various thiosulfinates (Table 1).

As is apparent from Table 1, the model fungus *S. cerevisiae* (*Sc*, baker's yeast) was more susceptible to thiosulfinates when compared to the bacteria tested. At the same time, DATS (allicin) and DPTS were the most effective compounds, with a MIC of 2 μg mL^{-1} and a MFC = 4 μg mL^{-1} for both thiosulfinates.

Indeed, with only few exceptions, DATS was the thiosulfinate most effective against all test organisms; however, in some cases other thiosulfinates showed equal or marginally better activity in the EUCAST test procedure. Nonetheless, our data does not reveal any universal trends of antibiotic effectivity, such as a distinctive structure-activity relationship or a strong correlation between log*P* and activity, as may have been anticipated. Thus, for *E. coli* there seemed to be a slight increase in effectivity from 64 to 32 μg mL^{-1} (MIC = MBC) as one progresses along the M_r series DMTS, DETS, DATS and DPTS whereas for *Pseudomonas fluorescens* the reverse trend was apparent with a decreasing effectivity from DMTS, DETS, DATS to DPTS (Table 1). Interestingly, DMTS was more active against *P. fluorescens* than DATS, with a MIC of 16 μg mL^{-1} and a MBC of 32 μg mL^{-1}-compared to DATS with a MIC of 128 μg mL^{-1} and a MBC = 256 μg mL^{-1}. Notably, most of the thiosulfinates were active in the low micromolar to low millimolar range, e.g. the MIC for allicin and yeast was 2 μg mL^{-1} (=12 μM) and for *P. fluorescens* was 128 μg mL^{-1} (=0.8 mM).

Comparison of antibacterial activities of thiosulfinates using a Petri-plate-diffusion test. In this part of the investigation, bacteria-seeded agar was used. A bacterial suspension was mixed in the agar medium just above gelling temperature and then poured rapidly into the Petri-plate to give an even distribution of cells throughout. Holes were cut with a cork borer and 20 μL of test solution were pipetted into each well. The usefulness of this test for assessing antimicrobial activity of novel test substances is described in[48]. A crucial prerequisite for this assay is adequate water-solubility of the compound, so it is able to diffuse through the water-based agar-solidified medium. Since DBTS is not water-soluble and needs to be dissolved in DMF, we could not perform the plate-inhibition zone assay with it. DMTS, DETS, DATS and DPTS showed antibiotic activity against all bacteria in this study, resulting in clear inhibition zones, the size of which was dose-dependent (Fig. 4A,B).

Each test substance will diffuse into the agar from the central well and establish a concentration gradient over the time of the experiment. Thus, each substance can be compared for relative efficacy against different test organisms, but the efficacy of the substances cannot be compared with each other because of their assumedly different diffusion rates. A further cautionary note is, that since *E. coli* cells are cultivated at 37 °C and all other organisms at 28 °C, the diffusion behaviour of the test substances for *E. coli* will also not be comparable to that in tests with the other organisms. Still, within the constraints outlined above, it can be seen that, for each given test substance, relative antibiotic effectivity differed between the different organisms exposed to them. Therefore, considering antibacterial activity first, DMTS was most effective in this test system against *P. syringae 4612* and least effective against *P. fluorescens* whereas DATS was most effective against *Micrococcus luteus* and least effective against *P. fluorescens* (Fig. 4A,B). All the thiosulfinates proved very effective against yeast BY4742 cells and resulted in relatively large inhibition zones in comparison to those for bacteria. This is in agreement with the results of the EUCAST procedure, which also showed yeast to be more sensitive to thiosulfinates when compared to the bacteria tested (Table 1). In control plates without thiosulfinate, bacteria grew up to the edge of the well as a continuous lawn.

Antimicrobial effects of thiosulfinates via the gas phase. To investigate the antibiotic activity of DMTS, DETS, DATS, DPTS and DBTS via the gas phase, a 20 μL drop of 80 mM test solution was placed in the centre of a Petri-dish lid and the Petri-dish base, containing medium seeded with bacteria, was inverted above the lid as previously described[17]. Thus, there was no contact between the test solution and the agar itself except by diffusion through the air. With the exception of DBTS, which was presumably not sufficiently volatile to achieve inhibitory concentrations, all thiosulfinates produced an inhibition zone above the droplet in the Petri plate lid

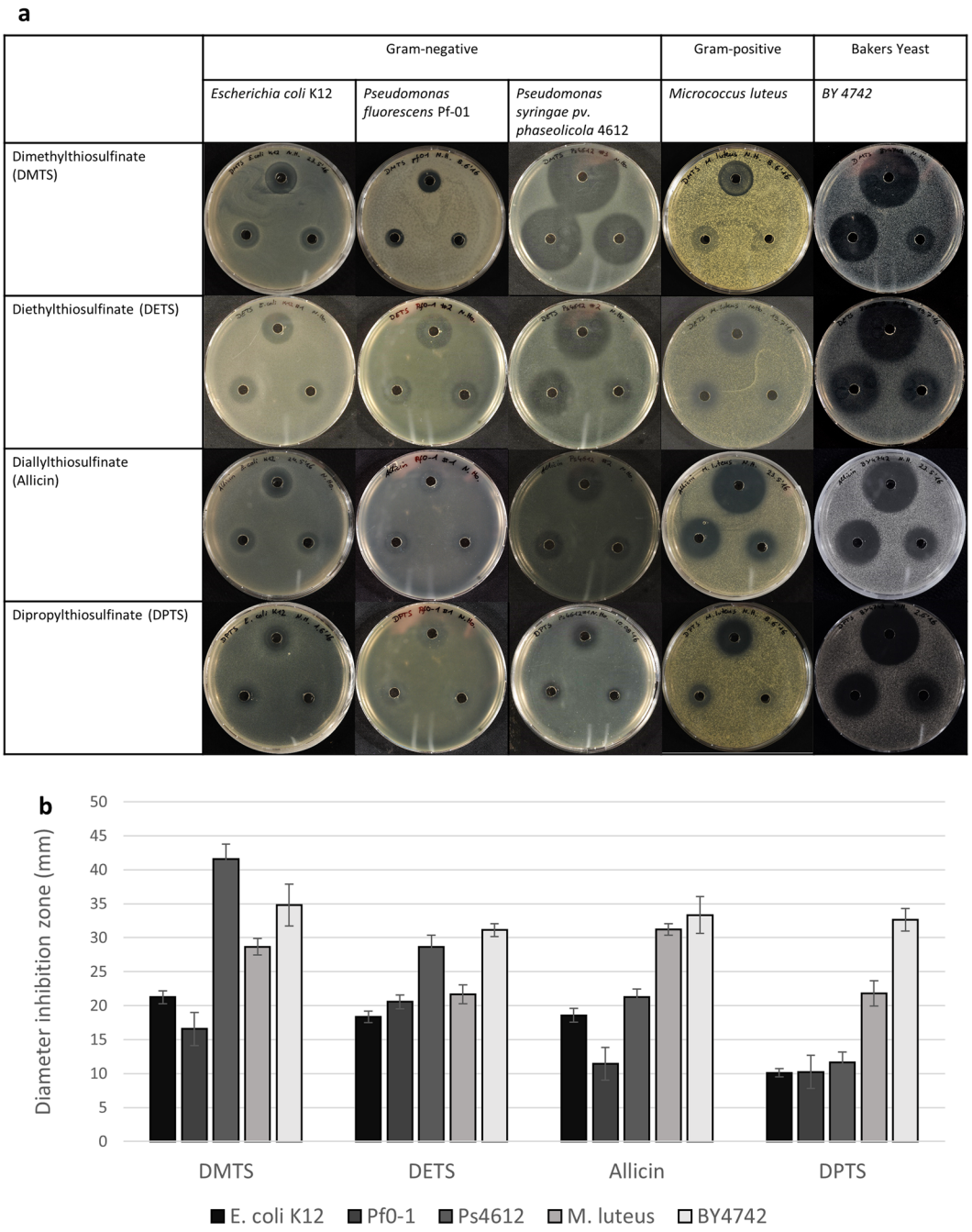


Figure 4. Plate inhibition zone assay showing the antimicrobial activity of different thiosulfinates against various bacteria and yeast. (**a**) Representative pictures for Gram-negative bacteria (*E. coli* K12, *Pseudomonas* spp.), Gram-positive bacteria (*M. luteus*) and yeast BY4742 cells. Cells were incorporated in 50 °C warm agar and poured into a Petri-dish. The upper well contained 20 µL of 8 mM solution, the hole at the left side of each Petri-dish contained 20 µL of 4 mM solution and the hole at the right side of the Petri-dish contained 20 µL of 2 mM solution of each compound tested. (**b**) The diameter of inhibition zone was measured for the hole containing 20 µL of 8 mM solution of the test compound. Error bars show standard deviation about the mean, n = 9.

for *E. coli*, *P. syringae 4612* and *M. luteus*. Interestingly, only DMTS, which is presumably the most volatile of the thiosulfinates under investigation, was able to inhibit *P. fluorescens* via the gas phase (Fig. 5).

It is perhaps surprising in this experiment to see such clear zones of inhibition with fairly sharp borders. We interpret this as reflecting the concentration gradient of the thiosulfinates diffusing away from the central drop into the still air above it in the closed Petri dish, and a tight threshold inhibition concentration.

To test how much time was required for allicin to diffuse through the gas phase and achieve an inhibitory concentration at the seeded agar, a time-resolved experiment with allicin and *E. coli* was carried out. After a given exposure time the seeded agar plate was placed over a new lid without allicin solution and incubated overnight. The results show that as little as one hour of exposure to allicin already leads to an effective growth inhibition

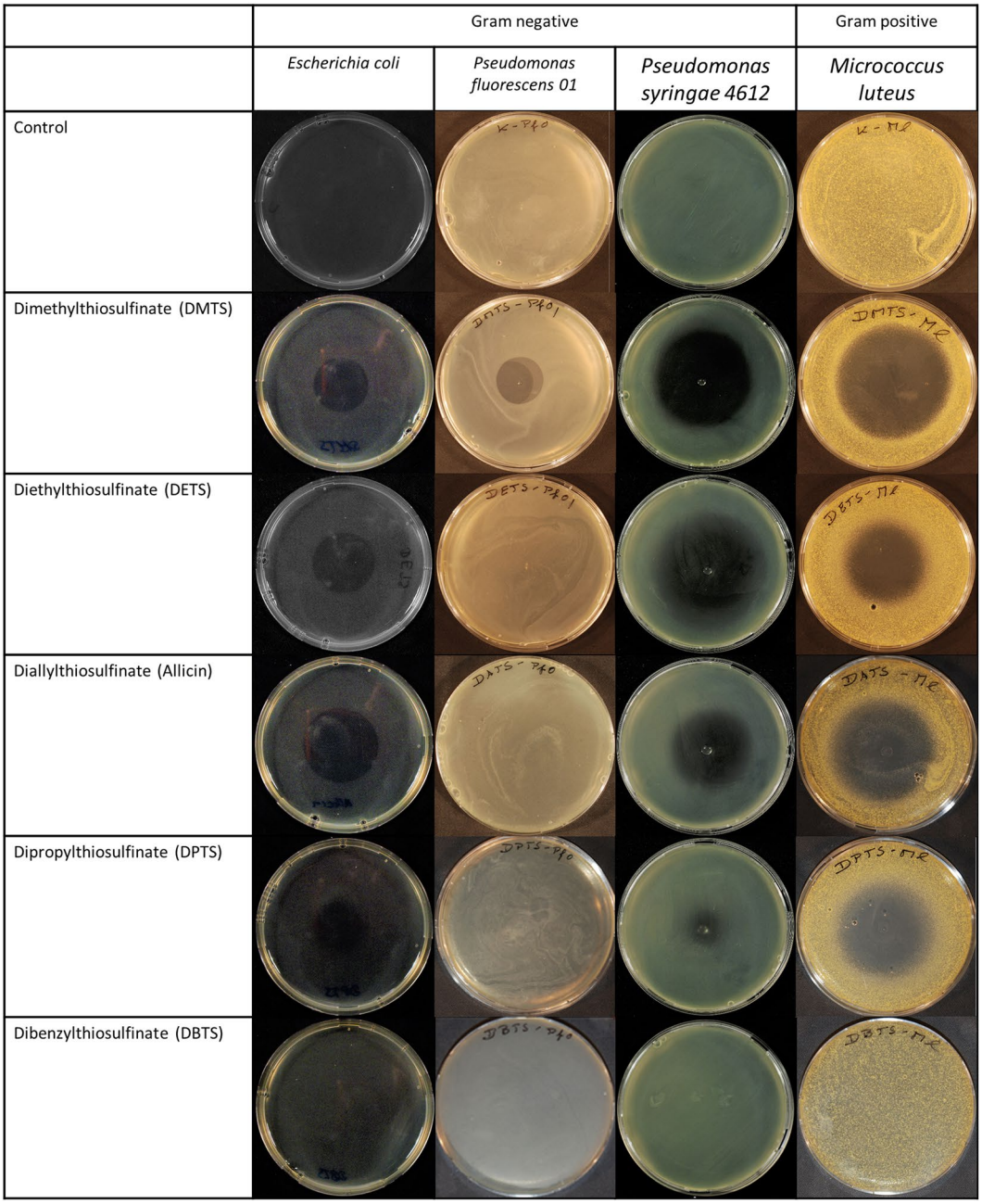


Figure 5. DMTS, DETS, DATS, DPTS, but not DBTS, show antibiotic activity via the gas phase. A 20 µL drop of an 80 mM solution of the test thiosulfinate was placed in the lid of a Petri-dish and the base with bacteria-seeded agar inverted over it. Thus, the agar did not come into contact with the droplet. Inhibition of growth was visible as a halo with reduced bacterial growth.

of bacteria above the drop. An exposure of only four hours was sufficient to achieve maximum inhibition, since exposure for 20 hours did not result in a bigger inhibition zone (Fig. 6).The complete lack of an inhibition zone for the 15, 30 and 45 minute time points presumably reflects the 'deadtime' required for the allicin wave to diffuse across the airspace between the drop and the agar surface and reach the minimum inhibitory concentration. Just 15 minutes later at the 1 h time point an inhibition zone approximately one third of the maximum diameter was achieved.

Comparison of antibacterial activities of thiosulfinates in drop tests. In this procedure, the test substance was incorporated at a given concentration into medium kept just above gelling temperature and the plates were poured immediately. Aliquots (10 µL) of a 10^n dilution series of a log-phase culture of the test bacteria were pipetted as discrete spots onto the medium. Inhibition can be seen in comparison to growth on control plates without test substance. *E. coli* cells (Gram-negative) were hardly inhibited by 100 µM DMTS, DPTS or DBTS whereas allicin and DETS caused a high degree of inhibition after 36 h. In the agar diffusion test, *E. coli* cells

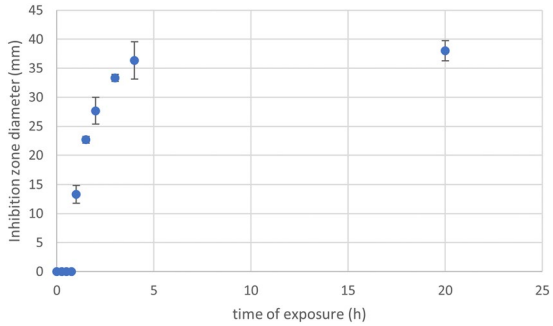


Figure 6. Allicin (20 µL droplet of 80 mM solution) shows inhibitory effects against *E. coli* in this test dependent on exposure time, with maximum inhibition reached after only four hours exposure.

were inhibited by all test substances and this result demonstrates the importance of not relying on the conditions of a single test when assessing the antimicrobial effectivity of test compounds. Thus, the standard EUCAST procedure uses a low titre of cells in stationary culture, the agar diffusion test works with a concentration gradient, the drop test incorporates the substance at fixed concentrations and different cell densities are tested, whereas shake culture exposes the test cells under conditions of continuous agitation and high aeration. Furthermore, divergent results for the different test scenarios, illustrate that only the relevant test situation in the real world (clinical or agricultural situation) will be definitive.

In the drop tests, Gram-negative *P. syringae* 4612 cells were inhibited strongly by all thiosulfinates up to 48 h after plating out (Fig. 7). Here, DETS, DMTS, allicin and DBTS appeared more effective than DPTS. Gram-positive *Micrococcus luteus* was inhibited strongly by all thiosulfinates up to 48 hours after plating but after longer incubation growth resumed and after 7 days, for instance, DMTS was hardly different to the control. This result suggests that the effect of DMTS at the test concentration is primarily bacteriostatic rather than bactericidal. For the other thiosulfinates, both bactericidal and bacteriostatic effects were apparent and allicin and DBTS showed the highest antibacterial effects overall (Fig. 7).

Antifungal activity of thiosulfinates in drop tests. As discussed already, the thiosulfinates seemed to be particularly active against the model fungus *S. cerevisiae*. This is rather fortunate, as a plethora of viable mutants of this eukaryote are available which can be used for chemogenomic profiling studies. Essentially, such studies investigate the divergent sensitivities of the wildtype and different mutants against given compounds and subsequently provide a glimpse into the possible mode(s) of action of those agents[48]. Therefore, this part of the study was designed to test whether the other thiosulfinates might have a similar mode of action to allicin, which is known to target the GSH pool and GSH metabolism[36,38].

Saccharomyces cerevisiae was used as a model fungus in drop tests on agar medium containing the test substance and in shake culture in 96 well plates (see next section) to assess the antimycotic activity of thiosulfinates. In the drop test, 10 µL of 10-fold serial dilutions were plated out onto control medium, or medium containing 5 µM of the test thiosulfinate. The ability of wildtype (wt) BY4742 cells to grow in the presence of thiosulfinates was compared with the ability of *Δyap1*, *Δglr1*, *Δzwf1*, *Δgnd1*, and *Δtrx2* yeast mutants. Yap1p is a transcription factor that coordinates the oxidative stress response in yeast[49] and which is activated by direct *S*-thioallylation of specific cysteines by allicin in the C-term of the protein[38]. Yap1p controls the expression of several oxidative stress response genes including *GLR1* and *TRX2*. Glutathione reductase (Glr1p) is an NADPH-dependent enzyme which reduces GSSG back to GSH and Trx2p, which is the major yeast thioredoxin, reduces protein disulfides (PSSP) and glutathiolated proteins (PSSG) back to thiols utilizing thioredoxin reductases that are NADPH-dependent[31]. The major source of NADPH for metabolic reactions in cells is the first two reactions of the oxidative pentose phosphate pathway (PPP), catalysed by glucose-6-phosphate dehydrogenase (Zwf1p) and 6-phosphogluconate dehydrogenase (Gnd1p), respectively. Thus, the mutants chosen are all appropriately relevant for testing and comparing the mechanism of action of allicin in relation to GSH metabolism, with respect to the other thiosulfinates (Fig. 8).

In the absence of thiosulfinate in the medium the wt and mutant yeast strains grew equally well down to the 10^{-6}-fold dilution. DMTS at 5 µM did not affect the growth of the wt or any of the mutant cells (Fig. 9). DETS at 5 µM was not inhibitory for BY4742 wildtype, but clearly inhibited *Δyap1*, *Δglr1*, *Δzwf1*, *Δgnd1* cells at this concentration. Allicin was the first compound in the series that inhibited the wildtype at 5 µM in addition to showing a greater inhibition in *Δyap1 Δglr1*, *Δzwf1*, *Δgnd1* and marginally in *Δtrx2* cells. Interestingly, the drop test did not resolve any differential toxicity for DPTS at 5 µM between the wt and most mutants. However, see Fig. 10 in the next section with respect to growth kinetics. DBTS showed the highest toxicity to yeast of the thiosulfinates tested and again the drop test did not resolve any differential toxicity for DPTS at 5 µM between the wt and the mutants. However, see Fig. 11 in the next section with respect to growth kinetics.

These findings, albeit of a preliminary nature, are rather intriguing as they (a) confirm the considerable toxicity of the various thiosulfinates towards yeast and perhaps fungi in general, (b) support the overall impression that allicin, DPTS and DBTS are more active compared to the shorter chain analogues and (c) also point towards similar underlying mode(s) of action against which GSH metabolism plays an important role. As mentioned in the introduction, induction of oxidative stress is probably just one aspect of antimicrobial activity, and it would

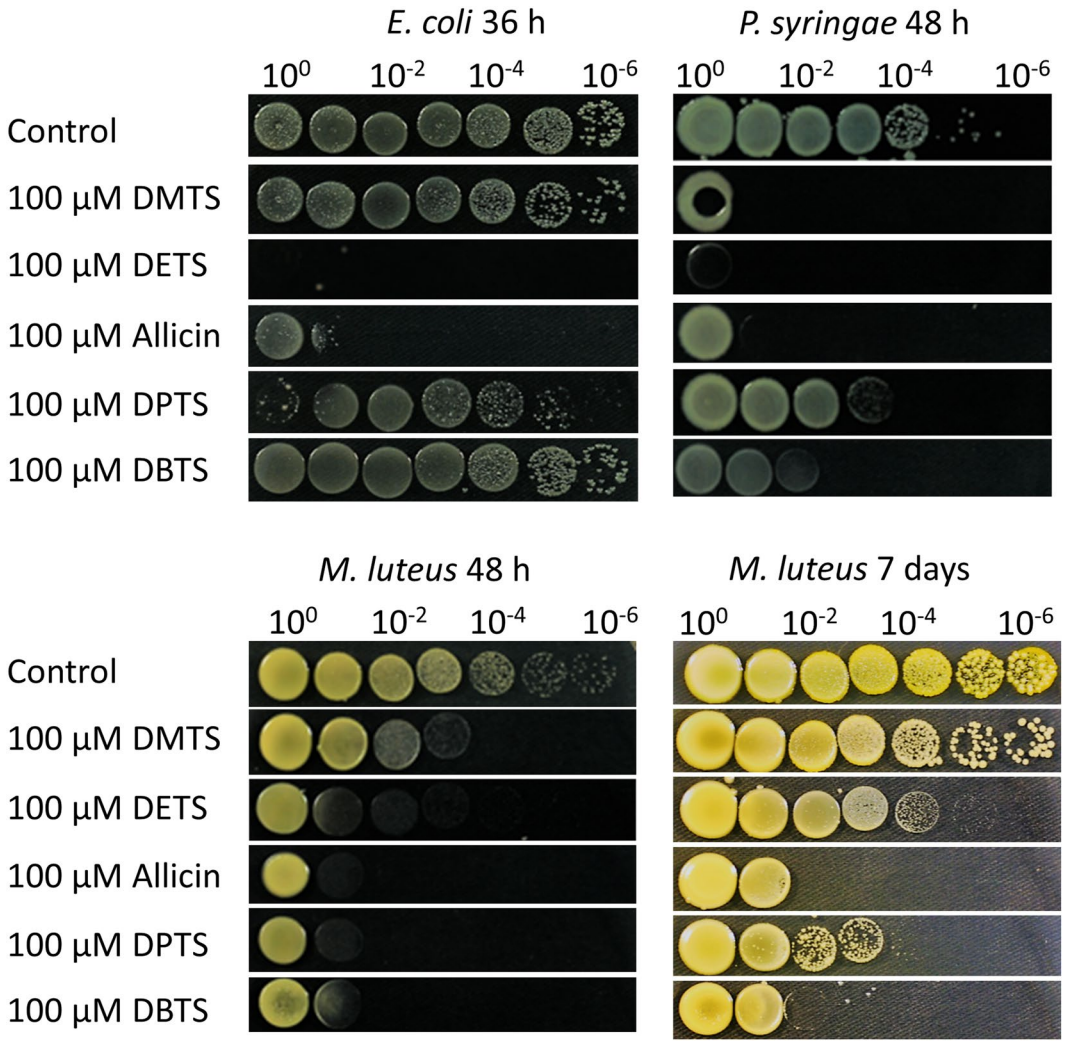


Figure 7. Drop test illustrating the relative inhibitory activities of the thiosulfinates incorporated into growth medium at 100 µM on *E. coli, P. syringae* 4612 and *M. luteus*, respectively. *M. luteus* is shown after 48 h and 7 days of incubation.

not be surprising if some of the test compounds had additional mechanisms. For instance, highly toxic benzylthiol may be formed as part of DBTS intracellular redox transformations.

Antifungal activity of thiosulfinates in shake culture. As is apparent from the results of the antimicrobial assays described so far, the relative sensitivity of an organism to an antibiotic is test-dependent and threshold inhibitory concentrations can vary depending on the conditions. Furthermore, the ability of a particular test to resolve different sensitivities between isolates also varies. Therefore, the effects of thiosulfinates on wt and *Δyap1*, *Δglr1*, *Δzwf1*, *Δgnd1*, and *Δtrx2* yeast mutants were also investigated in shake culture because this has the additional advantage of providing relative growth kinetics and not just an end-point result[48]. In contrast to the stationary culture conditions in the MIC and MBC tests, or in drop tests where cells are plated onto medium containing the test substance, in shake culture cells tend to grow more robustly and generally tolerate higher concentrations of antibiotics. Previous experiments had shown that 50 µM allicin reduced the growth rate of wt BY4742 cells by approximately 50% at the end time point of 15 h in shake culture in CSM medium. Therefore, in these experiments we exposed the cells to 50 µM of thiosulfinates for comparison.

The growth of BY4742 wildtype and the mutants in CSM medium controls was similar, showing no variation in the timing of the start of exponential growth, the rate of growth or end point cell titre reached after 15 h. Figure 10a shows a representative plot chosen from 4 replicates.

DMTS at 50 µM led to a delay of a few hours in the start of exponential growth of the wt but in contrast to the drop test (Fig. 8) the effect on the mutants was very pronounced, with longer lag phases until the resumption of growth and a much-reduced cell titre after 15 h. Furthermore, at 50 µM DMTS completely inhibited growth of the *Δglr1* mutant (Fig. 10b).

DETS at 50 µM led to a slight delay in the start of exponential growth of the wt but completely inhibited growth of the *Δyap1*, *Δglr1*, *Δzwf1* mutants up to the end of the experiment (Fig. 10c).

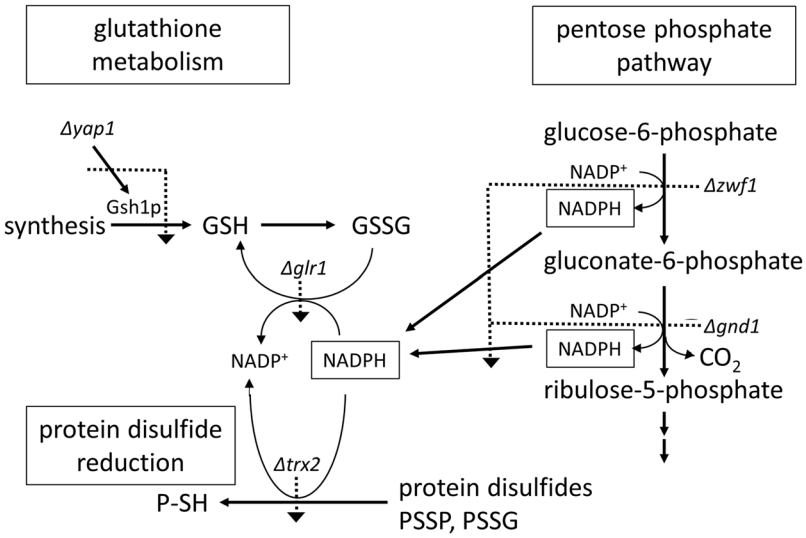


Figure 8. Scheme showing how the various deletion mutants affect GSH synthesis (*Δyap1*) and GSSG reduction, either directly (*Δglr1*), or by blocking the production of NADPH reducing equivalents (*Δzwf1*, *Δgnd1*), or by supressing the NADPH-dependent reduction of protein disulfides (*Δtrx2*, indirectly *Δzwf1*, *Δgnd1*). Dotted lines show the metabolic lesions caused by the deletion mutants.

In contrast to DMTS and DETS, allicin at 50 µM caused a marked inhibition of the wt, delaying the start of exponential growth and leading to a much-reduced endpoint cell titre. Growth of all of the mutants was completely inhibited up to the end of the experiment (Fig. 10d).

DPTS at 50 µM was more inhibitory than allicin to the wt and again completely inhibitory to all of the mutants (Fig. 10e).

Taken together the results of the shake culture experiments show a gradient of increasing inhibitory activity from DMTS < DETS < allicin < DPTS, corresponding to the increasing log*P* along the thiosulfinate series. This might be coupled with the relative ease with which the compounds can traverse the membrane to gain access to the cells. Allicin, for instance (log*P* = 1.35), is readily membrane permeable and indeed causes transient pore formation in biological and artificial membranes[40,41]. This activity series can also be seen in the drop test, but the increased sensitivity of the mutants to DPTS compared to the wt was not resolved (Fig. 9).

Because DBTS is insoluble in water, it was dissolved in dimethyl formamide (DMF) which was present at 0.5% v/v in the final test solutions. DMF at this concentration showed no significant effect on growth of either the wt or the mutants in comparison to the CSM controls (Fig. 11a,b). However, a dose-dependent inhibition of wt and mutants can be seen at 5, 10 and 25 µM, with the latter concentration being completely inhibitory to the growth of all yeast strains (Fig. 11c–e). It can clearly be seen that the *Δglr1* mutant is the most sensitive and this was completely inhibited for the duration of the experiment at 5 µM DBTS in 0.5% DMF. This very high degree of inhibition compared to the other thiosulfinates, suggests that DBTS had the greatest activity of the thiosulfinate series against *Saccharomyces cerevisiae*, a result which corresponds to the drop test results shown in Fig. 9. Yet this result must be viewed with caution. Notably, a synergistic effect was observed between DMF and allicin, which at 25 µM showed a lesser inhibitory effect without DMF than with 0.5% DMF where it also completely inhibited the growth of wt and all mutant cells (Fig. 11f,g). Whilst DMF alone had no effect on cell growth, there was most likely also a synergistic effect between DBTS and DMF. These findings caution against the often naïve use of common solvents such as DMF or DMSO to enhance the solubility of refractory test substances.

To sum up the results of the chemogenetic profiling, the observation that the chosen mutants were generally more sensitive to thiosulfinates than the wt suggests that the other thiosulfinates are probably acting similarly to allicin and targeting the cellular GSH pool and GSH metabolism as well as resulting in protein thiol oxidation[3,20,36,38]. The strongly susceptible phenotype of the *Δglr1* mutant in comparison to the weaker susceptibility of the *Δtrx2* mutant is particularly informative in this regard. Thus, it seems that GSH is the first line of cellular defence and the ability of the cells to reduce GSSG to GSH is crucial for the cells's resistance to allicin and the other thiosulfinates. Oxidizing accessible protein thiols to PSSP and PSSG is also part of allicin's mode of action[36] and this is reflected in the sensitivity of the *Δtrx2* mutant, a trait also shown for the analogue thiosulfinates and thus supporting a similar mode of action as for allicin (Figs. 9–11). The importance of Zwf1p and Gnd1p activities is clear because these enzymes are the major source of NADPH to provide reducing potential needed for Glr1p to reduce GSSG, and Trx2p activity via NADPH-dependent thioredoxin reductases.

Effect of thiosulfinates on the viability of human lung epithelial carcinoma cells (A549). If an antibiotic is to be used in the treatment of patients, then the differential susceptibility of mammalian cells and target pathogen cells should be as high as possible. Allicin is a biocide and kills mammalian cells as well as bacteria and fungi in a dose-dependent manner. The effects of allicin on a range of mammalian cell lines has been investigated[29], but the mammalian cell toxicity of the other thiosulfinates is unknown. In the work reported here,

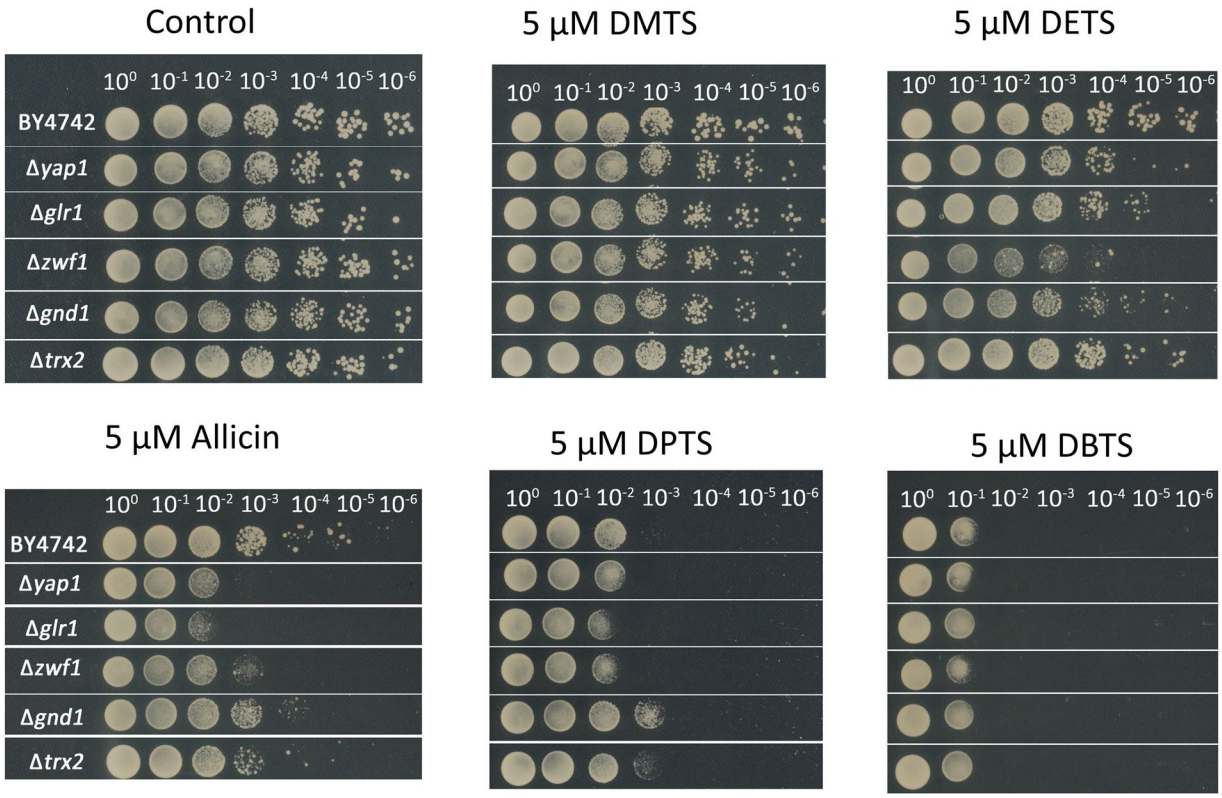


Figure 9. Drop test of *S. cerevisiae* cells on CSM medium containing test thiosulfinates at 5 µM. The wt BY4742 was compared with the *Δyap1* and other mutants. Drops (10 µL) of serial dilutions up to 10^{-6} were plated out. In the control both wt and mutant cells behaved similarly and grew down to the 10^{-6} dilution. The effects of the various thiosulfinates are shown in the remaining panels.

an MTT test for cell viability with human alveolar basal epithelial adenocarcinoma (A549) cells was performed. However, because allicin reduces the adherence of cultured cells, and this leads to variable losses during the usual washing procedure, any unreacted thiosulfinate was titrated out by adding excess cysteine. Cysteine itself, added to medium without cells, did not lead to a reduction of MTT.

All the thiosulfinates caused a dose-dependent decrease in the viability of cultured human A549 cells, pivotal over the 0.625–1.25 mM range (Fig. 12). There was some variation in the relative activities of different thiosulfinates but these were not consistently statistically significant between experiments. Nevertheless, the tendency that DMTS was least toxic and allicin most toxic to A549 cells was a clearly visible trend in all the experiments and the data confirm that the analogues are of similar toxicity to allicin. Therefore, like allicin, because of a relatively low differential toxicity between bacteria and mammalian cells, thiosulfinates might be better used at low concentration in combination with other clinically proven antibiotics, for example against MDR strains where allicin has generally been shown to be effective[25]. Because thiosulfinates are titrated out by GSH, oral use is in any case likely to be precluded because it will not be possible to achieve therapeutic concentrations via the oral route[50]. This is clear because a single clove of garlic can produce up to 5 mg of allicin, a substance toxic to cells in µg amounts, and yet, garlic is consumed worldwide without detrimental effects to the consumer. In this regard, specialist applications must be considered. Thus, the shortage of volatile antibiotics, coupled with reports from the pre-antibiotic era of successful treatment of tuberculosis patients by garlic vapour inhalation[29,30], indicate such a potential and emphasize the fact that cells in suspension culture do not have the same environment as cells in the body. In the intact organism, with a circulating blood supply and continual replenishment of GSH, particularly in the lungs which are continually exposed to oxidative stress, the volatile thiosulfinates may be able to play a role against lung-pathogenic organisms via the direct pulmonary route, either alone or in combination with conventional antibiotics taken orally[25]. This important point needs addressing in the future in long-term animal studies, which are beyond the scope of the present study.

Effect of thiosulfinates on the viability of tobacco bright-yellow-2 (BY-2) cells and Arabidopsis seedling root growth. We have previously shown that although allicin traverses biological and artificial membranes easily[41], it does not penetrate the wax plates of the plant cuticle efficiently and when sprayed onto leaves up to 2.4 mM showed no plant toxicity, making it a good contact fungicide, comparable in effectivity to the commercial fungicide Cuprozin™ against cucumber downy mildew[51]. Furthermore, allicin was shown to be as effective as the commercial fungicide Aatiram® in sanitizing carrot seed infested with *Alternaria* spp. and may be suitable for controlling other seed-borne diseases[52]. However, young seedling roots do not have a cuticle and we

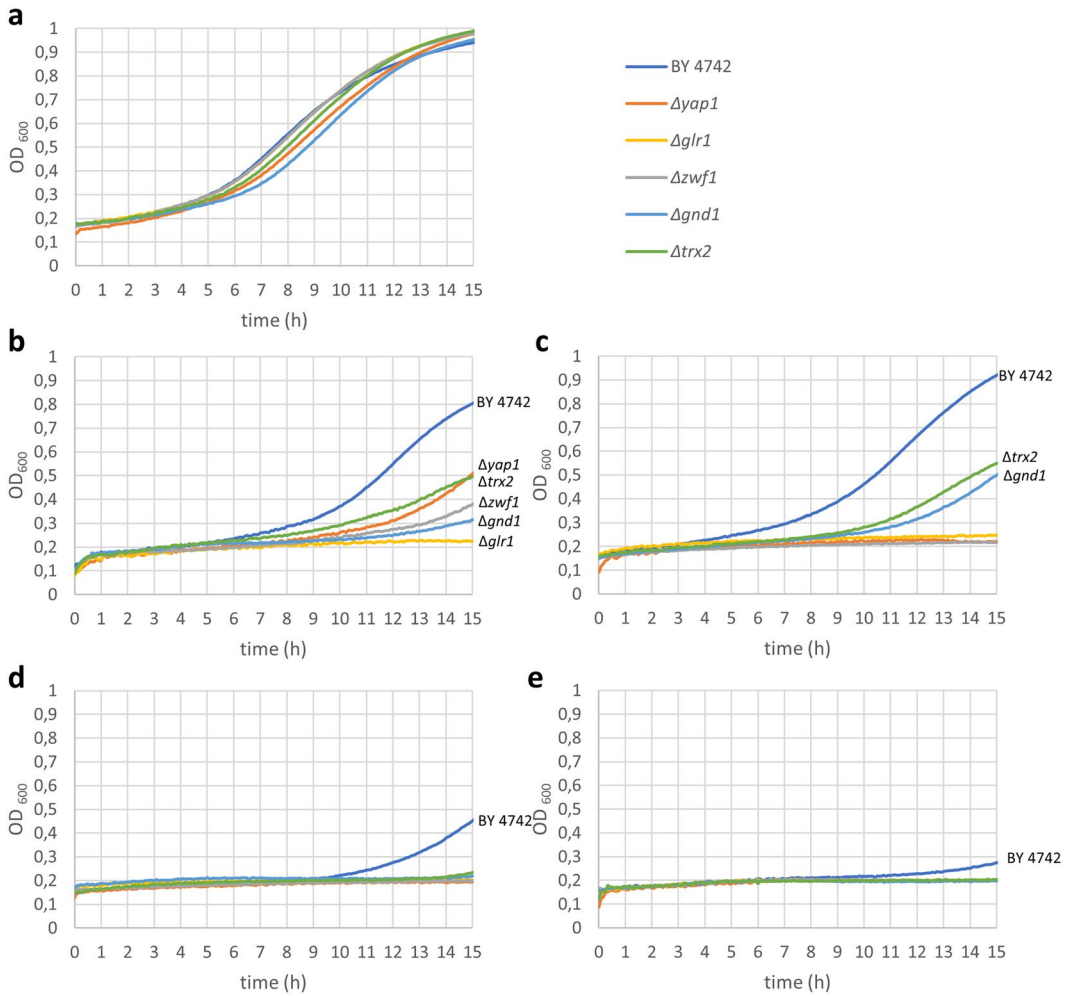


Figure 10. Effects of 50 µM thiosulfinates on the growth in shake culture of wt BY4742 and *Δyap1*, *Δglr1*, *Δzwf1*, *Δgnd1*, and *Δtrx2* mutant yeast cells in CSM. (**a**) CSM alone (control); (**b**) DMTS; (**c**) DETS; (**d**) DATS (allicin); (**e**) DPTS. The experiments were repeated twice with similar results.

have previously shown that allicin over the 25–100 µM range progressively inhibited root growth in *Arabidopsis* seedlings and at > 500 µM caused extensive bleaching of the cotyledons[3]. Plant toxicity data for the other thiosulfinates is lacking, therefore we tested the effect of a one hour exposure to thiosulfinates on the viability of tobacco bright yellow 2 (BY-2) cell cultures[53], and the effect on *Arabidopsis* root growth.

Evans Blue was used to stain dead BY-2 cells[54] and the trend observed with A549 adenocarcinoma cells, that DMTS was least toxic and DATS most toxic, was clearly reiterated (Fig. 13).

We also compared the effect of thiosulfinates on the growth of *Arabidopsis* seedling roots. Seeds were allowed to germinate for three days before placing on medium containing thiosulfinate. Root length was measured after three days of continual exposure. *Arabidopsis* Col-0 wt and *pad2* and *gr1* mutants in the Col-0 background were tested. The *pad2* line is mutated in the glutamate cysteine ligase gene and has only approximately 20% of the GSH level found in the wt[55]. The *gr1* mutant line is a knockout mutant of glutathione reductase and has a higher proportion of GSSG in the glutathione pool because it cannot reduce GSSG back to GSH[56].

As can be seen in Fig. 14a, the mutants *pad2* and *gr1*, compromised respectively in GSH synthesis and GSSG reduction, are more sensitive to thiosulfinate treatment than the Col-0 wt. Root growth was impaired and, interestingly, approximately 30% of the mutant seedlings exposed to 50 µM DPTS showed a branched root phenotype. This was not observed in the wt and this phenomenon may be worthy of further investigation. The concentration-dependent inhibition of root growth by thiosulfinates is shown in Fig. 14b. The enhanced sensitivity of GSH metabolism mutants compared to the Col-0 wt confirms the results of the chemogenetic screen with yeast mutants (Figs. 9–11) and again points to the central role of cellular GSH in the resistance of cells to thiosulfinates.

Materials and Methods

Materials. MSDS was purchased from Acros Chemicals (Geel, Belgium). DADS (80%), DEDS, DPDS and DBDS were purchased from Sigma Aldrich (Munich, Germany). Formic acid (98%, p.a.) was purchased from Carl Roth (Karlsruhe, Germany). H_2O_2 (30%) was purchased from Merck (Darmstadt, Germany). Acetic acid

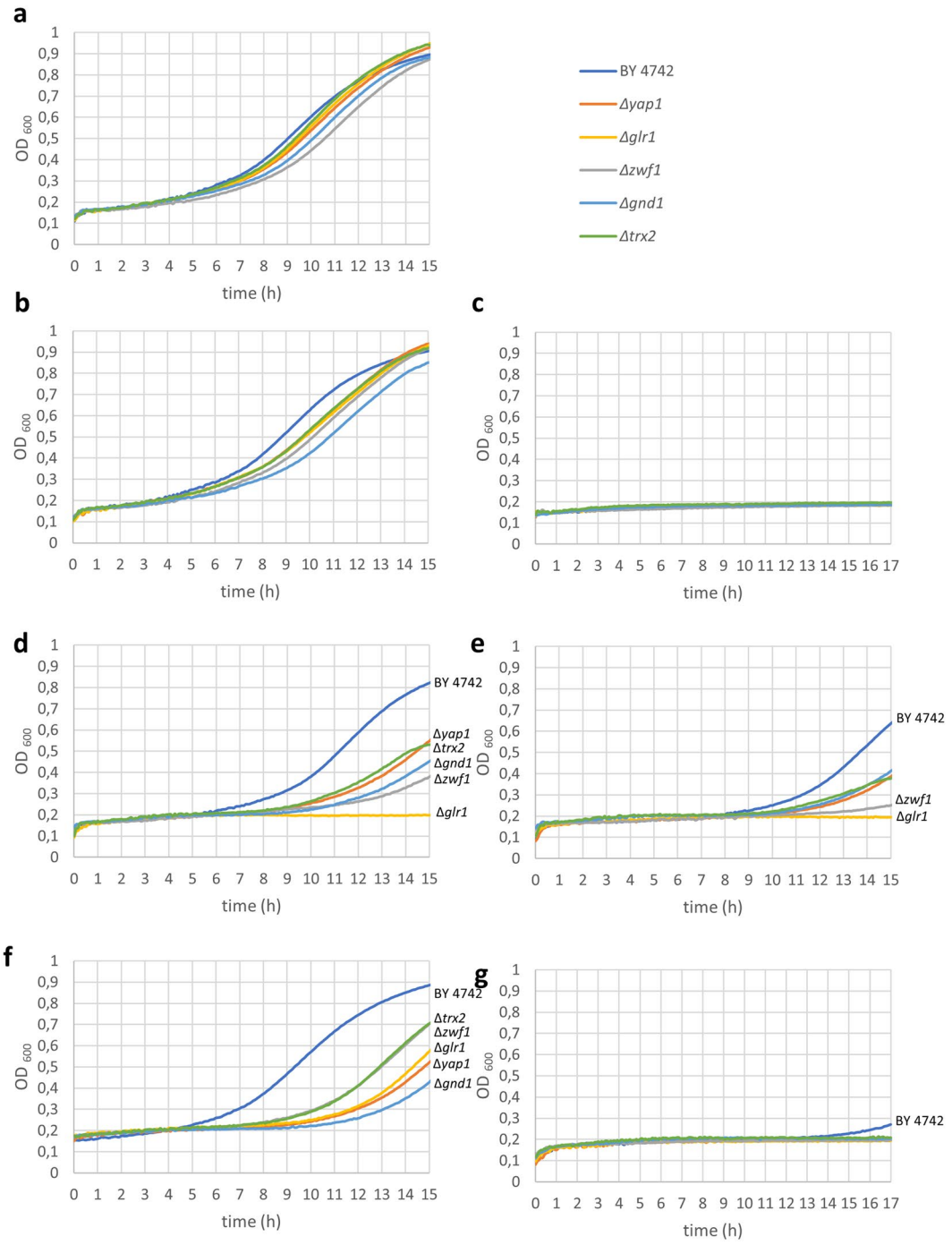


Figure 11. Effect of DBTS on the growth in CSM of wt BY4742 and *Δyap1*, *Δglr1*, *Δzwf1*, *Δgnd1*, and *Δtrx2* mutant yeast cells in CSM and the synergistic effect of DMF with allicin. (**a**,**b**) are controls in the absence and presence of 0.5% DMF. (**c**) 5 µM; (**d**) 10 µM and (**e**) 25 µM DBTS in the presence of 0.5% DMF, respectively. (**f**) 25 µM allicin and (**g**) 25 µM allicin in 0.5% DMF. The experiments were repeated twice with similar results.

(100%, p.a.) was purchased from Carl Roth. TLC was performed using Merck TLC Silica gel 60 F254 with concentration zone. Solvent A (n-hexane 99% p.s.) was purchased from Carl Roth. Solvent B (ethyl acetate 99.5% p.s.) was purchased from Carl Roth. Liquid chromatography was performed using silica gel 60 (0.04–0.063 mm (230–400 mesh)) purchased from Carl Roth.

The synthetic procedure for thiosulfinates was after Albrecht *et al.*[32].

Synthesis of Dimethylthiosulfinate (DMTS). Dimethyldisulfide (1.3 g; 13.8 mmol) was dissolved in 5 mL formic acid and stirred for 5 minutes on ice. H_2O_2 (30%; 2.4 mL; 23.7 mmol) was added slowly to the mixture. HPLC and TLC confirmed the reaction had reached completion after 90 minutes. The reaction was stopped

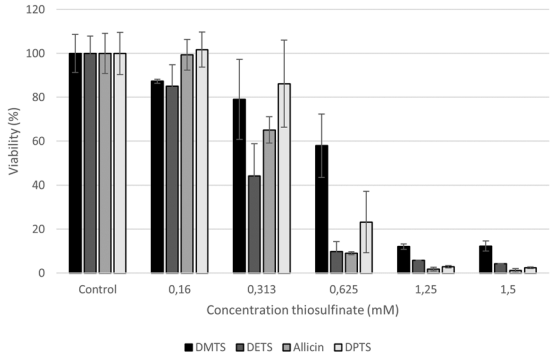


Figure 12. Dose-dependent effect of thiosulfinates on the viability of cultured human A549 cells. The experiment was repeated 4 times (n = 4–8) with similar results and a set of representative data is shown.

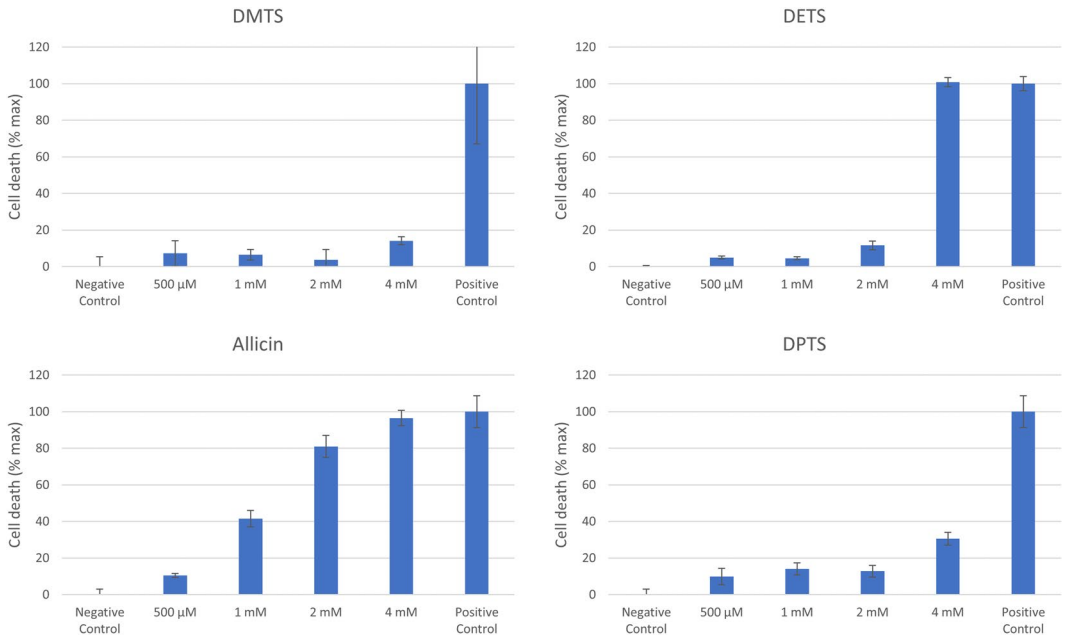


Figure 13. Effect of thiosulfinates on the viability of tobacco BY-2 cells in shake culture. Cells were exposed to the stated thiosulfinate concentration for one hour, stained with Evans blue, and bound dye measured at A_{600}.

by addition of 25 mL H_2O. The mixture was extracted 3 times with 30 mL dichloromethane (DCM) and the organic layer was washed with saturated Na_2CO_3 solution until the acid was completely neutralized. The organic layer was separated, dried with Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure at room temperature to yield a clear oily substance of characteristic smell. Yield: 1.44 g; 13 mmol. DMTS synthesis was stoichiometric, yielding a pure product with a single HPLC peak and no further purification was carried out.

$$^{1}\text{H NMR (500 MHz, } CDCl_3)\ \delta\ 3.29\ (s,\ 3H),\ \delta\ 2.69(s,\ 3H)$$

$$^{13}\text{CNMR (125 MHz, } CDCl_3)\ \delta\ 18.3,\ 48.9$$

Synthesis of Diethylthiosulfinate (DETS). Diethyldisulfide (2.07 g, 16.96 mmol) was dissolved in 5 mL formic acid and stirred on ice for 5 minutes. H_2O_2 (3 mL) was slowly added to the mixture. HPLC analysis indicated the reaction had gone to completion after 90 minutes. The reaction was stopped by addition of 25 mL H_2O and the organic compounds were extracted by washing the mixture 2 times with 10 mL DCM. The solvent was evaporated under reduced pressure at room temperature and the residue was dissolved in an ethyl acetate: n-hexane mixture (1:2). The product was purified on a silica gel 60 column with the same mixture used as mobile phase. The product-containing fractions were combined, and the solvents were removed by rotary evaporation at room temperature. The product was a clear oil with a pungent smell. Yield: 1.7 g; 12.3 mmol

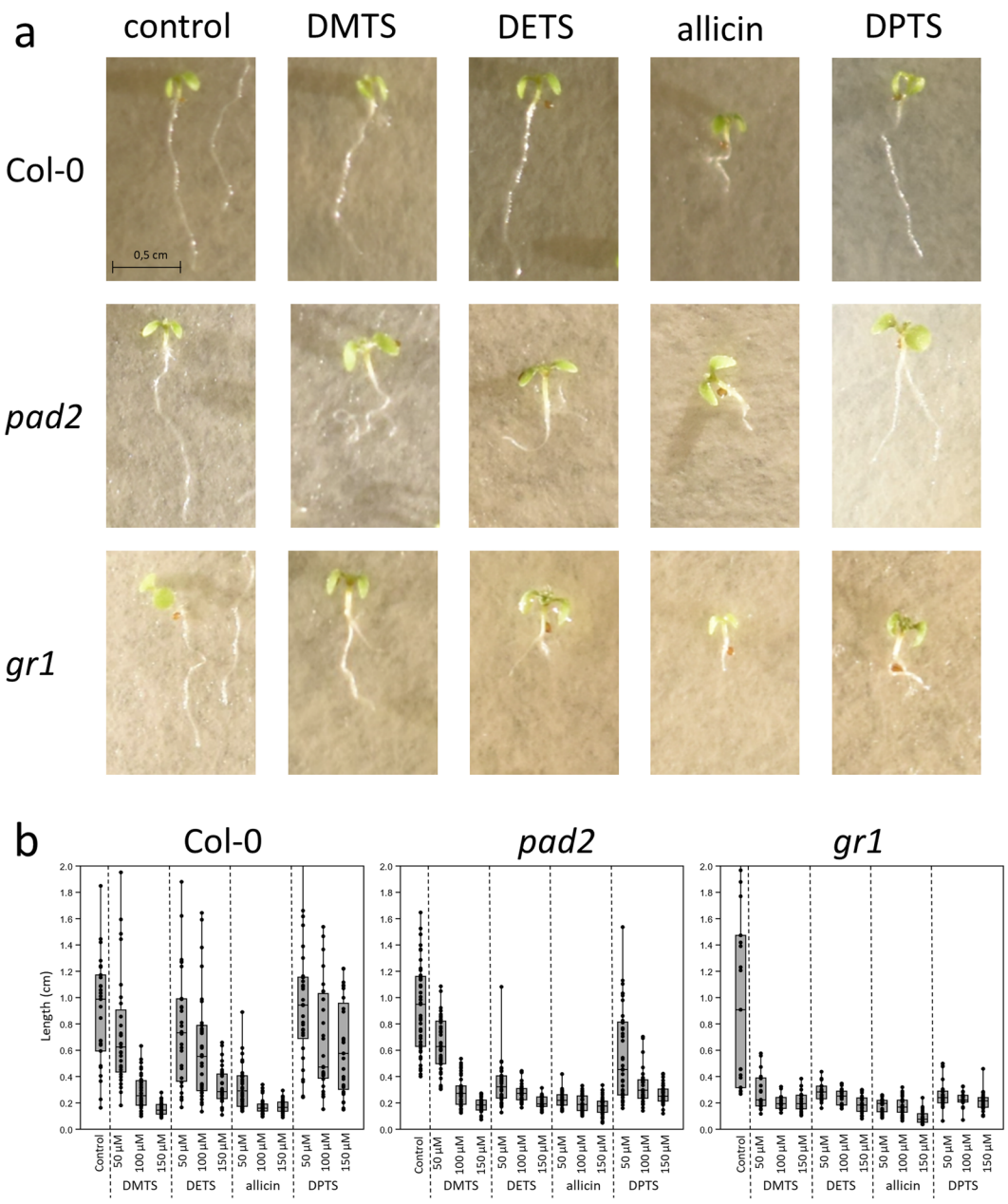


Figure 14. Effect of thiosulfinates on *Arabidopsis* root growth. (**a**) shows the phenotypes of Col-0 (wt), *pad2* and *gr1* seedlings observed after three days of continual exposure to 50 µM thiosulfinate in the growth medium. Scale bar = 5 mm. (**b**) shows the effect of continual exposure to 50, 100 and 150 µM thiosulfinate on root growth of Col-0 (wt), *pad2* and *gr1* seedlings. The box plots show the range of individual measurements, the mean +/− standard deviation, and the median value indicated as a horizontal line.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.34 − 1.37 (t, 3H), δ 1.39 − 1.43 (t, 3H), δ 3.03 − 3.17 (m, 4H)

^{13}CNMR (125 MHz, $CDCl_3$) δ 7.7, 16.1, 26.9, 49.8

Synthesis of Dipropylthiosulfinate (DPTS). Dipropyldisulfide (2.07 g, 13.8 mmol) was dissolved in 5 mL formic acid and stirred on ice for 5 minutes. H_2O_2 (30%, 3 mL) was slowly added to the mixture. The reaction was complete after 4 hours. To stop the reaction 25 mL of H_2O were added and the organic components were extracted by washing 3 times with DCM. The combined organic phases were washed with saturated Na_2CO_3 solution, dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure at room temperature to yield a clear oil. Yield: 2.26 g; 13.6 mmol. DPTS synthesis was stoichiometric, yielding a pure product with a single HPLC peak and no further purification was carried out.

^{1}H NMR (500MHz, $CDCl_3$) δ 0.99 – 1.10 (m, 6H), δ 1.75 – 1.88 (*m*, 4H), δ 3.02 – 3.16 (m, 4H)

^{13}C NMR (125 MHz, $CDCl_3$) δ 13.2, 17.2, 24.3, 34.9, 58.0

Synthesis of di-2-propenethiosulfinate (Allicin). Diallyldisulfide (DADS; 2 g, 13.7 mmol) was mixed in 5 mL formic acid and stirred for 5 minutes at 0 °C. H_2O_2 (30%; 3 mL, 29.6 mmol) was added slowly to the mixture. The reaction was stopped by addition of 25 mL H_2O after approx. 4 hours and the mixture was extracted 3 times with DCM. The solvent was removed under reduced pressure and the product was dissolved in the eluent, a mixture of n-hexane and ethyl acetate (2:1). Separation was performed *via* liquid chromatography using 150 mm silica gel 60 in a column with a diameter of 30 mm. Fractions were collected into tubes cooled in an ice bath and TLC was used to identify fractions containing only allicin. Those fractions were combined, dried with anhydrous sulfates (e.g. Na_2SO_4, $MgSO_4$ or $CuSO_4$) and filtered. The solvents were removed under reduced pressure at RT to yield a clear, oily substance that smells like garlic. Yield: 1.64 g, 10.1 mmol, 73.7%.

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.70 – 3.75 (m, 4H) δ 5.14 – 5.42 (m, 4H) δ 5.68 – 5.88 (m, 2H)

^{13}C NMR (125 MHz, $CDCl_3$) δ 35.1, 59.8, 119.1, 124.1, 125.8, 132.9

Synthesis of Dibenzylthiosulfinate (DBTS). Dibenzyldisulfide (0.51 g, 2.03 mmol) was suspended in 5 mL acetic acid. Cold H_2O_2 (0.23 g, 30%) was added dropwise to obtain an equimolar concentration of the reactants. After 5 h the mixture was extracted two times with DCM, the organic phases combined, and the solvent removed under reduced pressure to yield a white solid substance. The substance was dissolved in a mixture of ethyl acetate and n-hexane (1:2) and purified on a silica gel column. The product-containing fractions were combined, and the solvents removed under reduced pressure at RT. The residual white powder was characterized by NMR as dibenzylthiosulfinate. Yield: 0.15 g, 0.58 mmol.

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.15 – 4.28 (m, 4H) δ 7.12 – 7.36 (m, 10H)

^{13}C NMR (125 MHz, $CDCl_3$) δ 36.1, 62.2, 127.7, 128.7, 128.8, 129.1, 129.9, 130.3, 136.6

Analysis of thiosulfinates by HPLC. A Jasco HPLC system with a UV-2077 multichannel detector and a PU-980 pump was used. The separation was performed on a Prontosil Kromaplus column (150 × 4.6 mm, 5 µm) at 25 °C and the UV detector was operated at 254 nm. The flow rate was 1 mL min^{-1}. The mobile phase consisted of H_2O (A) and methanol. The following gradient was used: 56% A (0 min) 53% A (10 min) 7% A (15 min) 7% A (30 min) 56% A (31 min) 56% (35 min).

Thermal stability of thiosulfinates. Aqueous solutions (500 µL, 10 mM) of DMTS, DETS, DPTS, and allicin were heated in sealed tubes in a thermostated shaker for 10 minutes at 99 °C and cooled on ice afterwards. A 10 mM DBTS solution in aqueous 10% DMF was treated similarly. The chemical decomposition of the compounds was analysed with HPLC using UV detection.

Test microorganisms used in this study. *E. coli* K12, *Pseudomonas fluorescens* and *Pseudomonas syringae* pv. *phaseolicola* 4612 were used as representative Gram-negative bacteria. *E. coli* was grown on LB-medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 15 g L^{-1} agar for solid medium, all chemicals from Carl Roth, Karlsruhe) without selection and incubated at 37 °C. *Pseudomonas* isolates were grown at 28 °C on King's B medium (20 g L^{-1} peptone, 12.6 g L^{-1} glycerol, 1.5 g K_2HPO_4 (anhydrous); upon autoclaving, 1.5 g L^{-1} $MgSO_4$ (heptahydrate) was added. For solid medium, 15 g L^{-1} agar was added; all chemicals were purchased from Carl Roth, Karlsruhe, Germany).

***Micrococcus luteus* (*Ml*), grown on LB-medium at 28 °C, was used as a representative Gram-positive bacterium.** The haploid *Saccharomyces cerevisiae* yeast strain BY4742 (Matα; his3Δ1;leu2Δ0, lys2Δ0, ura3Δ0) was used as a model fungus in tests with different thiosulfinates. The BY4742 mutant Δ*yap1* (YML007w) used in this study lacks a redox-sensitive transcription factor that is important for oxidative stress response. All mutants were obtained from the EUROSCARF Collection, University of Frankfurt (Main), Germany (http://www.euroscarf.de/).

Yeast was grown in CSM medium (0.79 g L^{-1} CSM Drop-Out: Complete [ForMedium, Norwich, United Kingdom]; 6.9 g/l Yeast Nitrogen Base [ForMedium, Norwich, United Kingdom]; 40 g L^{-1} D-Glucose [Carl Roth, Karlsruhe, Germany], 15 g L^{-1} agar for solid medium.

Determination of minimal inhibitory concentration (MIC) and minimal bacteriocidal (MBC) concentration. Susceptibility testing was performed following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines using the broth dilution method in 96-well microtiter plate format[47]. Bacteria (*Escherichia coli*, *Pseudomonas syringae* pv. *phaseolicola* 4612, *Pseudomonas fluorescens Pf*-01, *Micrococcus luteus*) were grown over-night in media and temperatures as described above. The flasks were shaken at 220 rpm. At the next day, the bacteria were diluted to an OD_{600} of 0.3 and used in a dilution of 1:234. The

thiosulfinates were dissolved in deionized water to a final concentration of 2,048 µg mL^{-1}. In a 96-well plate, a 2-power dilution series was performed in 50 µL volume and 50 µL of the diluted bacteria culture were added. The concentrations varied therefore from 1024 µg mL^{-1} to 1 µg mL^{-1} and one well was reserved for the control. The microtiter plates were incubated for 20 h at the optimal temperatures for the bacteria, as mentioned above. The lowest concentrations that showed no visible growth of bacteria is the minimal inhibitory concentration (MIC).

To determine the minimal bactericidal concentration, 10 µL out of each well was inoculated onto a plate with the optimal medium for the particular bacterium and incubated for 24 h. The lowest concentrations that showed no growth after 24 h gave the MBC value.

Plate inhibition zone assay to test for antimicrobial activity against bacteria and yeast. An overnight culture of the bacteria grown in LB-medium under optimal conditions (*E. coli*: 220 rpm, 37 °C, other bacteria: 220 rpm, 28 °C) was diluted to about $OD_{600} = 0.1$ and further grown to $OD_{600} = 0.5$. Temperature of LB-agar was equilibrated in the water bath to 50 °C. 50 µL of bacteria suspension is added to 10 mL of medium, mixed in a falcon tube and poured into a petri-dish with 9 cm diameter. Upon solidification holes were punched out using a cork borer (6 mm diameter). Each hole was filled with 20 µL of thiosulfinate solution (8 mM, 4 mM and 2 mM). Plates were grown for 20 h. The diameter of the inhibition zone was measured and plates were photographed.

An overnight culture of yeast in CSM medium was diluted to about $OD_{600} = 0.1$ and grown again at 28 °C and 220 rpm to $OD_{600} = 0.5$. 50 µL were added to 10 mL of 50 °C warm CSM medium and mixed in a falcon tube. Further procedure was as with bacteria (see above).

Antimicrobial effects of thiosulfinates via the gas phase. Bacteria were seeded into LB-medium as described above. The different thiosulfinates were diluted with water to a final concentration of 80 mM. DBTS was first solved in DMF and subsequently also diluted to 80 mM with a final concentration of 5% DMF. Drops of 20 µL were placed in the center of the Petri-dishes lid. The Petri dish was incubated upside down over night at the particular temperature (*E. coli* 37 °C, other bacteria 28 °C). For a time resolved test the droplet was removed after following hours passed: 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 20. The plates were incubated for 20 hours at 37 °C.

Drop-test using yeast wildtype and mutant strains. A yeast-overnight culture in CSM (see above) was diluted to $OD_{600} = 0.8$. Solid CSM medium was equilibrated to a temperature of 50 °C and thiosulfinates were added to a final concentration of 5 µM. Medium was poured in a quadrangular Petri-dish (10 × 10 × 2 cm). Dilutions (10 µL) of the yeast suspension (10^0–10–7 in medium) were spotted onto the solid medium and plates were incubated at 28 °C for 44 h. It was optically evaluated to which dilution yeast was able to grow.

Yeast growth kinetics. The compounds tested were diluted in CSM medium to final concentrations as indicated in the figure. A yeast overnight-culture was adjusted to $OD_{600} = 0.9$. 158 µL medium containing were mixed with 7 µL diluted culture per well. Growth was monitored in a plate reader (Berthold TriStar^{2}S LB 942) at 600 nm over a time period of 15 h at 28 °C and shaking.

MTT test for viability of human cells. Human lung epithelial carcinoma cells (A549), a cell line developed in 1972 and widely used as an *in vitro* model (ATCC-CLL 185), were incubated for 2 weeks at 37 °C and 5% atmospheric CO_2 in 96-well plates. Cells were cultivated in 100 µL DMEM medium with penicillin/streptomycin 1% (v/v) (each 10,000 U/mL, Lonza, Verviers, Belgium) and fetal bovine serum (FBS) 10% (v/v) (Sigma-Aldrich, St. Louis, USA). After removal of the medium by aspiration, cells were exposed to thiosulfinates (dissolved in medium) for 1 h (controls with medium only). Thiosulfinate concentrations between 0.16 and 2.5 mM (two-power dilution series) were tested. After incubation, unreacted thiosulfinate was titrated out by the addition of 100 µL 6 mM cysteine (AppliChem GmbH, Darmstadt, Germany) dissolved in phosphate-buffered saline (PBS). The cells were incubated for a further 24 hours for recovery. Medium was removed, and cell viability was tested with MTT (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide, Carl Roth GmbH, Karlsruhe, Germany). MTT (100 µL 0.5% (w/v)) dissolved in phosphate-buffered saline (PBS) was added to each well and the plate incubated for 3 h at 37 °C and 5% CO_2. Cells were lysed by adding 100 µL isopropanol and the A_{630} subtracted from A_{570} automatically in the plate reader (TriStar2 LB942, Berthold Technologies, Bad Wildbad, Germany). Medium containing 6 mM cysteine did not cause any reduction of MTT.

BY-2 Tobacco cells. BY-2 cells (kindly provided by Dr. C. Langenbach, Institut f. Biologie III, RWTH Aachen University) were grown in modified MS Medium (4.3 g L^{-1} MS basal salt mixture (Duchefa Biochemie, Haarlem, Netherlands), 30 g L^{-1} sucrose, 0.2 g L^{-1} KH_2PO_4, 0.2 mg L^{-1} 2,4-Dichlorophenoxyacetic acid, 1 mg L^{-1} Thiamin Hydrochloride, 100 mg L^{-1} Myo-Inositol, pH = 5.8 with KOH (all chemicals were purchased from Carl Roth, Karlsruhe, Germany)) shaken in the dark at 90 revolutions min^{-1} for 7 days. Cells were treated with thiosulfinates for 1 hour and then incubated for 15 min with 0. 5% Evans Blue (Sigma), unbound dye was removed by washing. Dye bound to dead cells was solubilized in 50% methanol with 1% SDS for 30 min at 50 °C and quantified by absorbance at 600 nm in a plate reader. Negative controls were not treated with thiosulfinates and positive controls were heated to 99 °C for 30 minutes[54]. Negative and positive controls were set as 0% and 100%, respectively. Data are presented as means with standard deviations of four replicates.

***Arabidopsis* seedling root assay.** *Arabidopsis* seedling root assay was performed after Borlinghaus *et al.*[3] Surface-sterilized *Arabidopsis thaliana* seeds (Col-0, *pad2* and *gr1*) were sown on sterile filter papers and placed on Murashige & Skoog (MS) solid medium. The Petri plates were tilted to an angle of approx. 70° to ensure root growth according to root gravitropism. After three days of cultivation, filter papers were transferred to MS

medium that contained different amounts of thiosulfinates. After 3 days treatment seedlings were photographed and the root length was measured.

Conclusions and Perspective

Based on allicin (diallylthiosulfinate, DATS) as the lead compound, a comprehensive investigation of the antibiotic properties of molecules with a thiosulfinate functional group and symmetrical side chains modifying their physical properties (log *P*, diffusivity) showed that:

- All thiosulfinates tested exhibit a degree of antimicrobal activity comparable to allicin, but absolute activities depended on the conditions used in the individual tests.
- Except for DBTS all the thiosulfinates tested were more heat-stable than allicin.
- For allicin, activity by diffusion through the gas phase could be detected after as little as one hour of exposure. This may suggest a possible use for the treatment of lung diseases because conventional antibiotics are not volatile. Furthermore, uses in agriculture, e.g. fumigation of soils and treatment of seeds against seed-borne diseases can be considered.
- Antimycotic activity was higher than antibacterial activity for the thiosulfinates tested.
- The hypersusceptibility of the *Saccharomyces cerevisiae* Δ*yap1*, Δ*glr1*, Δ*zwf1*, Δ*gnd1*, and Δ*trx2* and the *Arabidopsis pad2* and *gr1* mutants suggests that, like allicin, the other thiosulfinates may attack the glutathione pool and glutathione metabolism in target cells.
- The synthesis method employed[32] can be used for very many natural and non-natural disulfides to create the more reactive electrophilic sulfur centre of the thiosulfinate functional group and opens up the possibility to investigate very many novel compounds, with potentially improved properties compared to the lead substance allicin.

Although allicin and the analogue thiosulfinates are natural products, nearly all to be found to greater or lesser extent in the common foodstuff garlic, any strategies to develop thiosulfinate-containing formulations for use in medicine and agriculture must of course be accompanied by detailed economic feasibility analysis and toxicology testing, not least of all from the view of potential exposure of agricultural workers when plants are being treated on a field scale. Certainly, consuming garlic itself is generally considered to be health-promoting and the low levels of oxidative stress which result through consumption cause activation of phase 2 protection enzymes, such that garlic is considered physiologically to be an antioxidant foodstuff[2]. The results presented here contribute to knowledge of the effects of thiosulfinates on a wide range of organisms including procaryotes and eucaryotes and suggest that thiosulfinates, alone or possibly in combination with other substances, could be developed for specific applications in medicine or agriculture.

Data availability statement. Data will be made available upon request.

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Author Contributions

R.L. performed the experiments, prepared the Figures and wrote parts of the manuscript, N.H. performed some experiments, C.J. provided N.M.R. facilities and supervised R.L., M.C.H.G. wrote parts of the manuscript, A.J.S. wrote the manuscript and supervised R.L.

Additional Information

Competing Interests: The authors declare no competing interests.

5. Diskussion

Nachfolgend werden die Ergebnisse der drei Publikationen noch einmal zusammenfassend erörtert und diskutiert.

Zu Beginn dieser Forschungsarbeit galt es, die Synthese und Aufreinigung des relativ reaktiven Naturstoffes Allicin zu verbessern, um spätere Versuche mit einer hochreinen Substanz durchführen zu können. Seit Stoll und Seebeck 1947 [98] erstmals eine Synthese von Allicin ausgehend vom Diallyldisulfid beschrieben haben, gab es immer wieder Berichte von Synthesen mit unterschiedlichen Oxidationsmitteln und unterschiedlichen Aufreinigungsmethoden. Diese beschriebenen Methoden hatten allerdings den Nachteil, dass nur geringe Ausbeuten erreicht werden konnten und die Aufreinigung nur mit erheblichem Aufwand möglich war.

In der entwickelten Methode wurde anstelle von Essigsäure als Katalysator Ameisensäure genutzt. Dies führte zu einer Reihe von Verbesserungen. So findet die durch Ameisensäure katalysierte Reaktion schon bei niedrigeren Temperaturen statt und kann somit mit höherer Reproduzierbarkeit im Eisbad bei 0°C durchgeführt werden. Auch bei 0°C ist die Reaktionsgeschwindigkeit bei der Nutzung der Ameisensäure im Vergleich zur Essigsäure höher. Daraus resultiert der große Vorteil dieser Methode: Durch die niedrigere Reaktionstemperatur und die geringere Reaktionsdauer kommt es zu einem geringeren Abbau des Produktes, damit zur Bildung von weniger Nebenprodukten und somit zu höherer Reinheit bei insgesamt höherem Umsatz. Ein weiterer Vorteil dieser Methode wird bei der Aufreinigung des Produktes offensichtlich: Die Ameisensäure hat einen geringeren Dampfdruck als die Essigsäure und lässt sich unter vermindertem Druck somit schneller entfernen. Auch dieser Zeitgewinn führt zur Bildung von weniger Nebenprodukten.

Sollte Allicin entweder als pharmazeutischer Wirkstoff oder aber als landwirtschaftliches Fungizid eingesetzt werden, sind die Produktionskosten von großem Interesse. Die Nutzung von organischen Säuren wie Essigsäure und Ameisensäure ist dann natürlich eine relativ preisgünstige Alternative. Dazu kommt, dass diese Verbindungen gewissermaßen Naturstoffe sind und eine bessere Umweltverträglichkeit besitzen als z.B. chlorierte Persäuren.

Im Rahmen dieser wissenschaftlichen Publikation konnte außerdem der Beweis für den von Nikolic postulierten Reaktionsmechanismus der Oxidation des Diallylsulfids

zum Allicin erbracht werden [98]. Nikolic nahm an, dass die Oxidation in zwei Schritten stattfinden würde: Im ersten Schritt Bruch der Disulfidbindung unter Entstehung einer Allylsulfensäure und im zweiten Schritt Kondensation zweier Moleküle Allylsulfensäure zum Allicin unter Abspaltung von Wasser. Durch die Oxidation von Diallyldisulfid und Dipropyldisulfid in einem Reaktionsgefäß und der Entstehung von Allicin, Dipropylthiosulfinat sowie beider möglicher asymmetrischer Thiosulfinate konnte gezeigt werden, dass der Reaktionsmechanismus in der Tat über die Sulfensäure verläuft und nicht über eine alternative direkte Oxidation des Disulfides. Sie ist also gewissermaßen analog zur Biosynthese, bei der im ersten Schritt ebenfalls die Allylsulfensäure entsteht, welche im zweiten Schritt zum Allicin kondensiert. Mit diesem Experiment konnte auch gleichzeitig gezeigt werden, dass die Methode auch zur Synthese verschiedener kurzkettiger Thiosulfinate genutzt werden kann.

Bei der Aufklärung von biochemischen Wirkmechanismen solcher Substanzen ist der Modellorganismus Hefe ein wichtiges Instrument. Die Abwesenheit bestimmter Gene führt zu erhöhter Anfälligkeit eines mutierten Hefestammes gegenüber spezifischen Reaktiven Sauerstoff Spezies (ROS). Ein solches Gen ist das YAP1 (Yeast AP-1), das einen redoxregulierten Transkriptionsfaktor codiert, der eine zentrale Rolle bei der antioxidativen Antwort der Hefe spielt. Das Yap1p reguliert die Expression von verschiedenen antioxidativen Genen, z.B. den Glutathion Synthetasen, den Gluta- und Thioredoxinen und den Glutathion- und Thioredoxin-Reduktasen. Es selbst wird durch Oxidation im Zellkern aktiviert.

In unseren Studien wurde ein Reporterkonstrukt entwickelt, das Yap1 abhängig ist, und die Anwesenheit von aktiviertem/ oxidierten Yap1p durch Lichtabgabe anzeigt. Damit konnte gezeigt werden, dass sowohl H_2O_2 als auch Allicin das Yap1p aktivierten, Allicin schon bei geringeren Konzentrationen. Die Aktivierung des Yap1p und die damit einhergehende Aufkonzentration im Zellkern konnte auch durch ein Yap1-GFP Konstrukt optisch nachgewiesen werden.

Da H_2O_2 ebenfalls das Yap1p aktivieren kann, die Oxidation dabei über die Proteine Gpx3p und Ybp1p verläuft, wurde diese Route in Gpx3p- und Ybp1p- defizitären Mutanten verfolgt. Es zeigte sich, dass H_2O_2 ohne diese beiden Proteine das Yap1p nicht aktivieren kann, Allicin hingegen schon. Dies liegt daran, dass Allicin mit Thiol-gruppen reagieren kann und es zu einer *S*-thioallylierung des Schwefelatoms kommt.

Die Aktivierung des Yap1p durch H_2O_2 verläuft so, dass Disulfidbrücken zwischen den Cysteinen C303 / C598 und den Cysteinen C310 / C629 des Proteins gebildet werden. Dadurch ist es sterisch am Export aus dem Zellkern gehindert. Die relevanten Cysteine bei einer Oxidation durch Allicin wurden dadurch bestimmt, dass die Cysteine des Yap1p durch Alanin ausgetauscht wurden. Es hat sich gezeigt, dass zumindest eines der beiden Cysteine C598 und C620 vorhanden sein muss, damit Yap1p durch Allicin aktiviert werden kann, bei einem Doppelaustausch der Cysteine kommt es zu keiner Aktivierung mehr.

Somit konnte eindrucksvoll demonstriert werden, dass Allicin, obwohl es chemisch genauso wie H_2O_2 ein Oxidationsmittel ist, biochemisch einen unterschiedlichen Wirkmechanismus hat. Die zelluläre Antwort von Hefe auf OS, sowohl durch H_2O_2 als auch durch den Naturstoff Allicin, verläuft aber über den Transkriptionsfaktor Yap1p.

Diese Ergebnisse sind besonders interessant mit Blick auf das Konzept des zellulären Thiolstats, da gezeigt wird, dass einige Thiole bei Oxidation durch die RSS Allicin wie ein Schalter wirken können. Diese Selektivität bezüglich spezifischer Thiolgruppen ist Grundlage des Konzeptes des zellulären Thiolstats.

Da die Reaktivität des Allicins mit Thiol-gruppen durch die funktionale Gruppe des Thiosulfinates begründet ist, wurden Thiosulfinat-Derivate mit verschiedenen Resten synthetisiert und gegen verschiedenste Modellorganismen getestet. So konnten weitere Untersuchungen über den biochemischen Wirkmechanismus der Thiosulfinate durchgeführt werden und der Einfluss der Seitenketten studiert werden. Nachdem erfolgreich Dimethyl-, Diethyl-, Diallyl-, Dipropyl- und Dibenzyl-thiosulfinat synthetisiert worden sind, wurden diese Substanzen auf ihre thermische Stabilität und ihre antimikrobiellen Eigenschaften hin untersucht.

Es konnte gezeigt werden, dass alle getesteten Thiosulfinate antimikrobielle Aktivität aufwiesen, die relativen Aktivitäten sich allerdings in Abhängigkeit von Testsystem und Modellorganismus hin unterscheiden. Die gesättigten Thiosulfinate wiesen eine höhere Wärmestabilität als Allicin und Dibenzylthiosulfinat auf. Bei den Stoffen Allicin, Dimethyl-, Diethyl- und Dipropyl- thiosulfinat konnte Aktivität selbst über die Gasphase nachgewiesen werden. Im Fall von Allicin war eine Stunde Einwirkzeit ausreichend, um eine antimikrobielle Wirkung nachzuweisen. Darin unterscheiden sich die Thiosulfinate von herkömmlichen Antibiotika, deren Dampfdruck zu gering ist, um über die Gasphase wirksam sein zu können. Dadurch können Thiosulfinate von Interesse

für die Bekämpfung von Infektionen der Lunge sein oder aber potenziell zur Begasung von Böden in der Landwirtschaft genutzt werden. Dies ist speziell von Interesse, da bei den Studien gezeigt wurde, dass die antifungale Aktivität der Thiosulfinate höher ist als die antibakterielle.

Mithilfe eines chemogenetischen Screenings mit ausgewählten Allicin-sensitiven Hefe-Mutanten konnte demonstriert werden, dass der Wirkmechanismus der verschiedenen Thiosulfinate dem des Allicins ähnlich ist und sowohl der Glutathion-Pool als auch der Glutathion-Metabolismus von zentraler Bedeutung für die Wirkung der Thiosulfinate und ebenso für die Resistenz gegenüber diesen ist.

6. Zusammenfassung und Ausblick

In der vorliegenden Dissertation werden verschiedene neue Erkenntnisse im Hinblick auf die Wirkungsweise des schwefelhaltigen Naturstoffes Allicin beschrieben.

Es wird zuerst eine neu entwickelte Methode zur Synthese des Allicins beschrieben und ihre Vorteile werden diskutiert. Diese Methode kann ebenfalls zur Synthese anderer, symmetrischer wie asymmetrischer Thiosulfinate genutzt werden.

Der Wirkmechanismus von Allicin wird am Modellorganismus Bierhefe (*Saccharomyces cerevisiae*) untersucht. Dabei zeigt sich, dass die Konzentration von Glutathion in der Zelle mit dem Wachstum unter Allicin-Stress korreliert. Dies ist damit zu erklären, dass die antioxidative Antwort von Hefe gegenüber Allicin vom Transkriptionsfaktor Yap1p abhängig ist. Dessen Oxidation führt zur Expression von Genen, die wiederum die Bildung von antioxidativen Enzymen und Molekülen bewirkt.

Des Weiteren wird die Wirkung von strukturell dem Allicin ähnlichen Thiosulfinaten auf verschiedene Modellorganismen untersucht. Es wird gezeigt, dass die verschiedenen Organismen gegenüber unterschiedlichen Thiosulfinaten anfällig sind, und dass manche Thiosulfinate aufgrund ihrer hohen Volatilität sogar über die Gasphase wirken können. Die Daten legen nahe, dass der Wirkungsmechanismus der untersuchten Thiosulfinate dem des Allicins ähnlich ist.

Insgesamt ist Allicin ein sehr vielversprechendes Molekül mit starken antimikrobiellen Eigenschaften. Es zeigt ein sehr breites Wirkungsspektrum und eine hohe Reaktionsbereitschaft. Es besitzt keine spezifischen Target-Proteine, sondern reagiert entsprechend dem Konzept des zellulären Thiolstats selektiv mit den oxidationsbereitesten Thiolen.

Durch diese hohe Reaktivität ist ein Einsatz als orales Antibiotikum wenig wahrscheinlich. Von großem Interesse ist aber der Einsatz als antibakterieller Wirkstoff in der Lunge über die Gasphase [100] oder als antimikrobieller Wirkstoff bei der Begasung von Böden in der Landwirtschaft.

Auch wenn Allicin als Inhaltsstoff von Knoblauch von Menschen konsumiert wird, sind weitere Studien zur differenziellen Toxizität notwendig, bis der Wirkstoff in Form eines Präparats in der Medizin eingesetzt werden kann. Auch die Formulierung von Allicin wird in zukünftigen Studien eine wichtige Rolle spielen, damit das fertige Präparat

möglichst lange haltbar ist und der Wirkstoff erst am Ort und Zeitpunkt der Bestimmung freigesetzt wird. Sollte Allicin in der Landwirtschaft eingesetzt werden, müssen Studien die Unbedenklichkeit des Kontakts mit Allicin feststellen.

Bis zum Einsatz in Medizin und Landwirtschaft wird Allicin hauptsächlich in Form von Knoblauch oral eingenommen und wirkt dabei als Oxidant. Wie in Hefe auch, löst die direkte chemische Oxidation eine indirekte, aber auch umfassende antioxidative Antwort aus und führt zu einer verstärkten Bildung von Antioxidantien. Somit ist Knoblauch nicht nur köstlich als Zutat zu Speisen, sondern auch ein sehr interessanter Naturstoff mit oxidativen und antioxidativen Eigenschaften.

7. Summary and Outlook

The thesis at hand gives an overview over the various gains in scientific knowledge about the sulfur containing natural product allicin.

First, a novel method for the synthesis of allicin is described and the advantages thereof discussed. This new method can also be applied to the synthesis of other, symmetric and asymmetric thiosulfinates.

The mode of action of allicin is investigated in the model organism brewer's yeast (*Saccharomyces cerevisiae*). We could show that the concentration of glutathione inside the cell correlates with the cell growth under conditions of allicin stress. This phenomenon is caused by the fact, that the oxidative stress response of yeast against allicin is dependent on the transcription factor YAP1p. The oxidation of this protein leads to an expression of specific genes, which again leads to an enhanced production of antioxidative enzymes and molecules.

Furthermore, we studied the activity of thiosulfinates with an allicin resembling structure against various model organisms. The results show, that different organisms are hypersusceptible towards different thiosulfinates. Due to their high volatility some tested compounds even show antimicrobial activity through the gas phase. The data suggest that the mode of action of the tested thiosulfinates is similar to the one of allicin.

All things considered is allicin a promising molecule with strong antimicrobial properties. It has a broad range of efficacy and highly reactive. Instead of having specific target proteins its reactivity follows the thiolstat concept and it reacts with thiols which are easiest oxidized.

Due to its high reactivity a usage as an oral antibiotic does not seem likely. An interesting application of allicin could be as a volatile antibiotic for the lung or as an antimicrobial ingredient for the fumigation of soils in the agricultural sector.

Even though allicin is consumed by humans as an ingredient of garlic further studies of its differential toxicity are essential before it can be used in medicine. The formulation also needs to be a target of future studies in order to increase the shelf life of an allicin containing drug and to ensure the release at the desired site of action. Should allicin be used in agriculture, effects of exposure to allicin on a larger scale must be studied.

Until allicin is applied in medicine or agriculture, it is mostly consumed orally in form of garlic and acts as an oxidant. As in yeast, the direct chemical oxidation triggers an indirect biochemical antioxidative response and leads to an increased production of antioxidants. Therefore, garlic must be considered not only delicious but also a fascinating natural product with oxidative and antioxidative properties.

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