JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Use of a Substrate/Alliinase Combination To Generate Antifungal Activity in Situ

FIONA H. FRY,† NEAL OKARTER,† CAMILLA BAYNTON-SMITH, MICHAEL J. KERSHAW, NICHOLAS J. TALBOT, AND CLAUS JACOB*

School of Biological and Chemical Sciences, University of Exeter, Stocker Road, Exeter, Devon EX4 4QD, United Kingdom

Allicin, an active ingredient of garlic, possesses a range of antimicrobial properties. Unfortunately, certain properties of the compound, such as chemical instability and low miscibility with water, have hampered its practical use in the past. Here, we show that it is possible to use a binary system consisting of the plant enzyme alliinase and its substrate alliin to generate allicin, and hence antifungal activity, in situ. During application, the two inactive components generate compounds that inhibit growth and infection-related development of the rice blast fungus *Magnaporthe grisea*. It is therefore possible to "trigger" biological activity in a controlled, yet effective manner. Apart from circumventing many of the drawbacks of allicin, this binary system has additional important advantages, such as low toxicity of its individual components and selective activation. Importantly, alliinase is also able to use different substrates, therefore paving the way to a range of novel, binary antimicrobial systems with custom-made chemical and biochemical properties.

KEYWORDS: Binary system; allicin; alliinase; disulfide-S-oxide; antifungal activity; *Magnaporthe grisea*; green chemistry

INTRODUCTION

Recent years have seen a growing interest in the use of natural antimicrobial agents. Among the compounds discussed for antibacterial and antifungal use, allicin (allylthiosulfinate, diallyl disulfide-S-monoxide, **Figure 1**), an active ingredient of garlic, has attracted considerable attention (*1*−*16*). Allicin forms part of a natural, primitive antimicrobial defense system in plants, such as garlic (*Allium ursinum*), and studies have revealed that it is active against a wide range of bacteria and fungi, such as *Escherichia coli* and *Candida albicans* (*17*). **Table 1** provides a brief overview of known antimicrobial activities of allicin. Not surprisingly, its antifungal properties, paired with an intrinsically low toxicity toward humans, have already made allicin a potential lead compound for drug design and the development of "benign" agricultural pesticides. In recent years, this interest has increased, because the ability of disulfide-S-monoxides to inhibit a wide range of cysteine-containing enzymes diminishes the risk of microorganisms developing resistance against such an agent. Indeed, the effectiveness of allicin against a range of otherwise drug-resistant bacteria has now been shown (*17*).

Unfortunately, allicin has several properties that make it difficult for the compound to be used in practice. It is chemically unstable at room temperature and readily decomposes to biologically inactive sulfur species (*14*, *18*). Although this might be beneficial in a certain context (for example, if long-term effects of allicin are to be avoided), it makes manufacture,

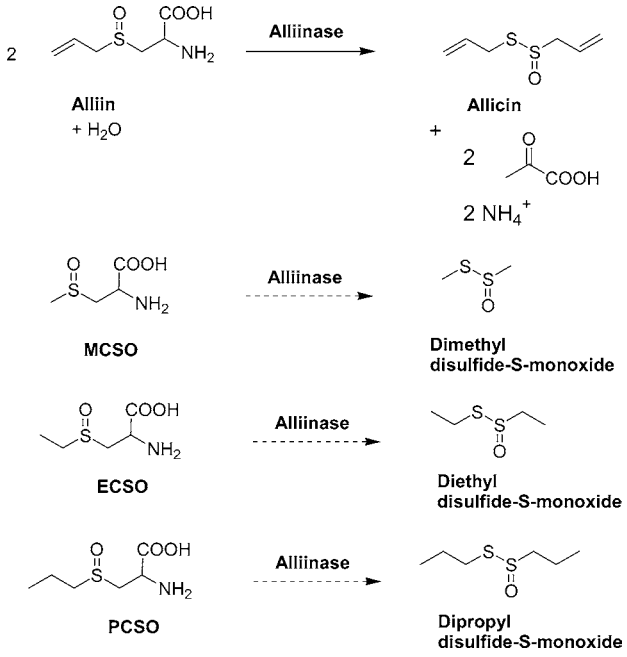

Figure 1. Enzymatic conversion of cysteine-based sulfoxides to disulfide-S-oxides in the presence of alliinase. The chemical structures of substrates used in this study are shown, together with the corresponding disulfide-S-monoxides formed. Abbreviations are explained in the text.

* Author for correspondence. Tel.: +44 1392 263462; fax: +44 1392 263434; e-mail: C.Jacob@ex.ac.uk.
† These authors contributed equally.

10.1021/jf048481j CCC: $30.25 © 2005 American Chemical Society
Published on Web 01/14/2005

Table 1. Selection of Important Antimicrobial Activities of Allicin Known to Date[a]

	IC_{50} value (μg/mL)	ref
Bacteria		
Escherichia coli, MDR and sensitive strain	15	*17*
Staphyloccocus aureus, MDR and senstive strain	12	*17*
Streptococcus pygenes	3	*17*
Pseudomas, senstive to cefprozil	15	*17*
Pseudomas aeruginosa, MDR mucoid strain	>100	*17*
Helicobacter pylori	6	*38*
Yeast		
Aspergillus spp.	8–32	*12*
Candida albicans, clinical isolates	0.8	*17*
Candida neoformans	0.3	*17*
Candida tropicalis	0.3	*17*
Torulopsis glabrata, clinical isolates	1.9	*17*

[a] This list is far from complete. The appropriate references can be consulted for more detailed information regarding individual activities. Examples of antifungal activity of allicin have also been reported (*12–14*).

storage, and maintaining a reliable activity of the sample difficult. Practical use of allicin is further hindered by the compound's pungent smell and its low miscibility with water, which makes application of the compound, especially in larger quantities, rather difficult.

Using the highly damaging rice blast fungus *Magnaporthe grisea* as a model target (*19*), this paper evaluates "green" alternatives to the use of allicin that avoid these problems and tests the efficacy of utilizing a novel means of allicin generation as a method for controlling plant diseases caused by fungi. First, we will consider the use of alternative disulfide-S-monoxides, such as cystamine-S-monoxide. Second, we will show how a combination of alliinase and different sulfoxide substrates can be used to generate antifungal activity in situ.

MATERIALS AND METHODS

Materials. Alliin and 4-(2-pyridylazo)resorcinol (PAR) were purchased from Fluka (Gillingham, U.K.). Diallyl disulfide, S-methyl cysteine, S-ethyl cysteine, hydrogen peroxide, peracetic acid, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (Poole, U.K.). The ConA-Sepharose column and the PD-10 gel filtration column were purchased from Pharmacia (Buckinghamshire, U.K.). Cd,Zn-metallothionein was purchased from Sigma, and Zn_7-MT was reconstituted and purified according to a standard method (*20*).

MilliQ water was used throughout the experiments. All experiments were performed at 25 °C unless stated otherwise. UV/vis spectra were recorded on a Cary50*Bio* spectrophotometer (Varian). Kinetic traces were obtained using recording intervals of 10 s or less (continuous monitoring). ^{1}H NMR spectra were recorded on a 300 MHz NMR machine (Bruker). IR spectra were obtained on a FT-IR instrument (Nicolet Avatar). All experiments were performed in triplicate.

Synthesis of S-Oxides and Allicin. Cystamine S-monoxide was synthesized from cystamine according to the procedure by Steinman and Richards (21). L-Cystine-S-monoxide was synthesized from L-cystine according to the procedure by Wälti and Hope (*22*). S-Methyl-cysteine sulfoxide (MCSO) and S-ethyl-cysteine sulfoxide (ECSO) were synthesized according to the procedure by Stoll and Seebeck (*23*). Allicin was synthesized according to a procedure described by Block et al. (*24*). Analytical results for all of these compounds (melting points, elemental analysis, ^{1}H NMR, IR spectra) were in accordance with literature values. All sulfoxides were subsequently used as a racemic mixture.

Metallothionein Oxidation Assay. The MT assay can be used to monitor the general effects of oxidizing agents on cysteine proteins in vitro (*25*). In accordance with literature, reconstituted zinc-MT (0.5 μM) was incubated with the chromophoric dye PAR (100 μM) in 20 mM HEPES−Na^+ buffer (pH 7.4) (*25*, *26*). Oxidative zinc release from MT was monitored spectrophotometrically at 500 nm ($\epsilon_{500}(ZnPAR_2)$ = 65 000 $M^{-1}cm^{-1}$) for 30 min. Total zinc release after 30 min was compared to an ebselen control (*25*).

Alliinase Extraction, Purification, and Characterization. Alliinase was extracted from commercially available garlic cloves according to a modified literature procedure (*27*). In short, fresh garlic cloves were peeled and ground with buffer A in a blender (Warring model 35BL59) until the mixture was homogeneous (*27*). Purification of the mixture involved filtration, centrifugation, and precipitation, followed by resuspension, further filtration, affinity column chromatography (ConA-Sepharose), and finally a gel-filtration column (PD-10). For full details of the purification, see Kuettner et al. (*27*) with the following modifications: Since the enzyme extract was used for the generation of antifungal activity, the enzyme preparation had to be modified to avoid the presence of sodium azide, Perfabloc (a commonly used protease inhibitor), and sodium phosphate. Extraction and purification of the alliinase enzyme were therefore done with the following modifications: Buffer A contained no sodium azide or Perfabloc; buffer B contained no Perfabloc; buffer C contained no Perfabloc; buffer D contained no sodium phosphate or sodium azide. Alliinase purity was established using SDS PAGE and the enzyme was pure. The concentrations in the individual fractions were determined using a standard Bradford assay. Alliinase activity was measured using the recently proposed method of Rabinkov et al., where the oxidation of 4-mercaptopyridine (4-MP) by the enzymatically formed disulfide-S-monoxides is monitored spectrophotometrically (ϵ_{343} = 19 800 $M^{-1}cm^{-1}$) (*28*). Initial, zero-order rates for the enzymatic formation of disulfide-S-monoxides were calculated for 0−2 min.

Fungal Isolates. Strains of *M. grisea* used in this study are stored at the University of Exeter. The fungus was grown at 24 °C on complete medium following a standard procedure described previously (*29*, *30*). For long-term storage of *M. grisea*, the fungus was grown through sterile filter paper disks; the latter were then desiccated for 48 h and stored at −20 °C until further use.

Appressorium Development Assay. Appressorium development by *M. grisea* was studied on plastic coverslips as described by Hamer et al. (*31*). Briefly, a 100-μL drop of a conidial suspension at a concentration of 10^5/mL with the compound or enzyme was placed on the surface of a plastic coverslip (Gelbond, FMC) and left in a humid environment at 24 °C overnight. The frequency of appressorium formation was determined by the number of appressoria which had been elaborated from counting 300 conidia (*29*). In the "trigger assays", either the enzyme or the substrate was added to the conidial suspension after 30 min of incubation.

Toxicity Assays. The toxicity of the alliin, alliiinase, and the binary system was determined by incubating 550 μL of a conidia suspension of *M. grisea* containing 10^6 conidia/mL with 200 μL of 75 mM alliin or 100 μL of alliinase (264 μM) or water (control), injecting the enzyme to the solution at various time points. After incubating for an hour, the conidia were recovered by centrifugation (13 000*g*, 5 min) and washed with water (1 mL). The conidia were resuspended in water (1 mL) and 200 μL of the solution was spread onto the surface of a complete medium agar plate (*29*). The plate cultures were incubated at 24 °C for 4 days and the number of conidial colonies was compared to the control containing only water and conidia ("plate experiments").

RESULTS

Allicin, Cystamine-S-Monoxide, and L-Cystine-S-Monoxide. The MT oxidation assays were concerned with the question of whether allicin could be substituted for other, more stable disulfide-S-oxides. The results indicate that the disulfide-S-monoxides allicin, L-cystine-S-monoxide, and cystamine-S-monoxide all readily release zinc ions from MT (43, 41, and 21%, respectively), while neither alliin nor diallyl disulfide (the enzymatic and chemical precursors of allicin, respectively) interacts with MT with a zinc release of less than 5%. Although there is some variation in the activity of the individual disulfide-

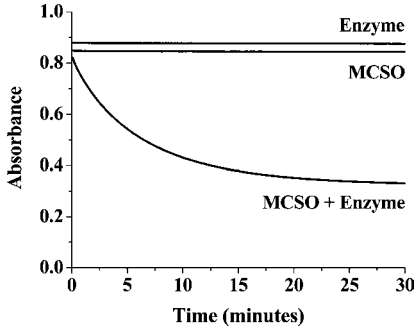

Figure 2. Enzymatic formation of dimethyl disulfide-S-monoxide (dimethylthiosulfinate) from MCSO (510 μM) in the presence of alliinase (13 μM). Similar activity was observed for alliin and ECSO in the presence of alliinase. S-Methyl cysteine and S-ethyl cysteine had no significant activity at these concentrations. The conversion was continuously monitored in a spectrophotometer cuvette, with readings taken every 10 s. Reproducibility of the kinetic trace was within a 5% experimental error. Initial rates for the overall reaction are given in the text.

S-monoxides, the MT assay confirms the ability of all of the disulfide-S-monoxides to attack cysteine-containing proteins.

In contrast, the results obtained in the fungal assays clearly indicate that allicin, but not cystamine-S-monoxide, possesses effective antifugal activity. (No reliable results were obtained for L-cystine-S-monoxide in this assay, because acetic acid had to be used to solubilize the compound, and the resulting drop in pH observed under these conditions also prevented conidial germination by *M. grisea*.) Allicin was able to almost completely (99%) inhibit spore germination by the rice blast fungus *M. grisea* at a concentration of 50 μg/mL (309 μM), while neither cystamine-S-monoxide nor alliin had any significant effect at concentrations up to 500 μg/mL (3.0 mM and 2.8 mM, respectively). Properties such as polarity and hydrophobicity of the molecules, rather than simple chemical reactivity, might therefore play an important role in biological activity of different disulfide-S-monoxides. This might give allicin, and chemically related compounds, an advantage over other, more polar disulfide-S-monoxides.

Alliin/Allinase System. As a consequence, allicin was used as a focus for the second set of experiments. It is well known that allicin is formed in vivo from alliin by the enzymatic action of alliinase (**Figure 1**). This natural binary system can therefore be modeled to induce or "trigger" antifungal activity in situ. Initial in vitro assays with isolated alliinase indicated that catalytic amounts of the enzyme were able to convert alliin, MCSO, and ECSO within minutes to reactive disulfide-S-oxides, which in turn were able to oxidize 4-MP. **Figure 2** shows a representative kinetic trace illustrating that the mixture of MCSO and alliinase, but not the individual components, leads to 4-MP oxidation (via the formation of dimethyl disulfide-S-monoxide, **Figure 1**). Very similar rates of about 3−6 μM s^{-1} were observed for all three substrates (510 μM sulfoxide substrate, 13 μM enzyme, respectively), while neither the substrates nor the enzyme itself was active (rates between 0 and 0.03 μM s^{-1}). Racemic mixtures of the sulfoxides were used, and the enantionmerically pure, (+)-forms of the sulfoxides, such as (+)-alliin, that is, the preferred substrates for alliinase, might give even higher turnover rates.

As would be expected from the enzyme mechanism, alliinase does not significantly convert the sulfide precursors of the sulfoxides, that is, S-methyl cysteine and S-ethyl cysteine, in these assays, and rates for the sulfide/enzyme combination were about 0.1−0.4 μM s^{-1}.

The sulfoxide/alliinase combinations were therefore tested in a biologically more relevant scenario using *M. grisea*, an agriculturally important, highly damaging fungus, as a target organism. We were particularly interested in determining whether infection-related development of the fungus could be inhibited. *M. grisea* produces specialized cells called appressoria which are able to generate turgor pressure and allow the fungus to breach the plant cuticle and gain entry to leaf tissue (*19*, *29*−*31*). Appressoria can be induced to form on hydrophobic plastic surfaces, and **Figure 3** illustrates the results obtained in the coverslip experiments for different sulfoxide substrates in the absence and presence of alliinase. The figure shows that neither alliin, MCSO, nor ECSO inhibited germination or appressorium formation (**Figure 3a**). Likewise, alliinase on its own had only a minor effect on fungal growth, with over 96% of conidia germinating and just 3.8% nongerminated conidia (although there was a slight reduction of appressorium formation to around 10%). In stark contrast, a combination of alliinase and either alliin, MCSO, or ECSO effectively abolished germination and appressorium formation (**Figure 3b**). Complete loss of spore germination was observed, for example, at sulfoxide concentrations of around 500 μM (472 μM for alliin, 511 μM for MCSO, and 477 μM for ECSO) and catalytic alliinase concentrations of 12 μM.

These substrate/alliinase concentrations reflect the ones used in the 4-MP oxidation assay, and the sulfoxide concentrations used are in the range of the (chemically prepared) allicin concentration required to fully abolish germination (309 μM, see previous), indicating extensive in situ enzymatic activation of the substrates by alliinase in the coverslip experiments.

To confirm that the absence of germination and appressorium formation presented in **Figure 3** was due to irreversible damage of the conidia and not just a temporary effect, toxicity assays were performed. The results for the alliin/alliinase combination are summarized in **Table 2**. While neither alliin nor alliinase affected *M. grisea* vegetative development on their own, a combination of the two effectively prevented fungal growth. Interestingly, alliin and alliinase on their own even slightly increased colony formation, possibly by providing a feedstock for the fungus.

The plate assay was then used in a time-course study to investigate if the enzyme could be added to the conidia/alliin incubation at different time intervals, or if up-front mixing of sulfoxide and alliinase was required for antifungal activity. While the alliin (and alliinase) controls did not prevent colony formation, injection of alliiinase to the alliin/conidia incubation mixture at 0, 0.5, 1.5, and 4 h almost completely inhibited fungal growth. The enzyme could therefore be used as a trigger and could be added to the substrate at various times during incubation, without loss of antifungal activity (**Table 2**).

DISCUSSION

The results obtained in the MT assay and experiments on infection-related development of *M. grisea* underline the exceptional activity of allicin, when compared to other sulfoxides and disulfide-S-monoxides. Compared to the sulfoxides and diallyl disulfide, allicin is considerably more active in the MT assay and in the *M. grisea* spore germination and appressorium development assay. This is hardly surprising, because disulfide-S-monoxides, but not sulfoxides, are able to undergo a rapid cascade of thiol-specific oxidation reactions (*26*). Although disulfides such as diallyl disulfide can undergo thiol/disulfide exchange reactions, their reactivity as oxidants is usually considerably lower than the one of the corresponding disulfide-

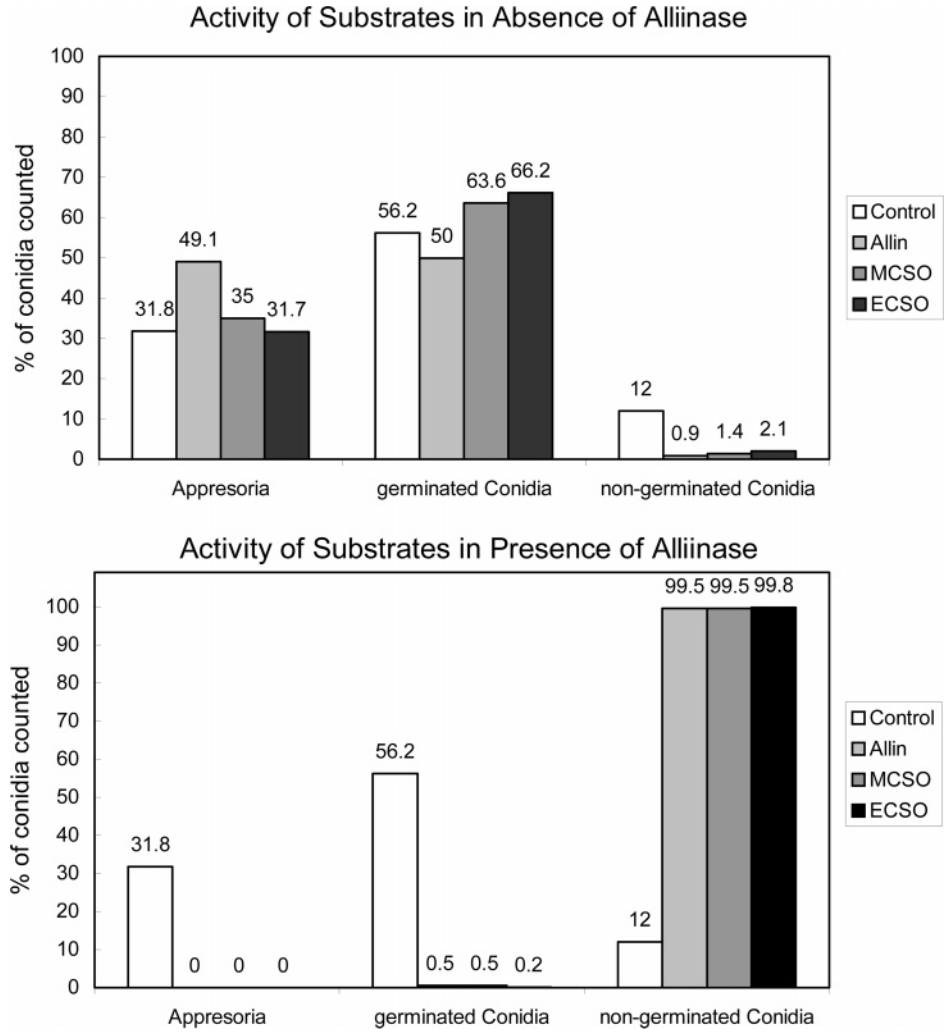

Figure 3. Use of a binary substrate/enzyme system to prevent germination and appresoria formation of *M. grisea* spores in the coverslip experiments. While the substrates on their own did not prevent germination and appressoria formation, a combination of substrate and enzyme effectively abolished germination and appressoria formation. This effect was observed for all three substrates tested, that is, alliin, MCSO, and ECSO, and complete inhibition of germination in these assays was achieved in assays typically containing around 500 μM substrate and 12 μM enzyme. At this concentration, alliinase on its own had no effect on germination, with just 3.8% of conidia not germinated.

Table 2. *M. grisea* Colony Formation after Incubation of the Conidia with Alliin (950 μM), Alliinase (48 μM), and an Alliin/Alliinase Combination of the Same Concentrations[a]

	rel no. of colonies (control = 1)	
treatment	0 min	30 min
time lag (alliinase injection)		
control (water)	1.0	1.0
alliin	1.3	1.9
alliinase	2.0	3.0
alliin/alliinase	0.1	0

[a] Changes in alliinase injection time (0 and 30 min shown here) had little effect on antifungal activity. The plate experiments generally required higher concentrations of both allicin and allin/allininase mixtures to show full antimicrobial effect.

S-monoxides (*32*). As far as reactivity toward cysteine proteins is concerned, all three disulfide-S-monoxides tested, that is, allicin, cystamine-S-monoxide, and L-cystine-S-monoxide, showed comparable reactivity in the MT assay. In contrast, there was a dramatic difference in activity between allicin and cystamine-S-monoxide in the *M. grisea* spore germination assays. While allicin abolished conidial germination at around 50 μg/mL, cystamine-S-monoxide was unable to inhibit spore germination at concentrations as high as 500 μg/mL. At first this might be rather surprising, because their chemical reactivity toward cysteine residues seems to be similar to that of allicin. Both cystamine-S-monoxide and L-cystine-S-monoxide are, however, considerably more polar than allicin, and this might reduce their biological activity. It has already been shown by Miron et al. that the activity of allicin as an antimicrobial agent might be linked to its high lipophilicity, that is, its ability to penetrate bacterial or fungal cell walls and membrane (*33*). Although other underlying biological factors cannot be ruled out, this difference might well explain the exceptionally high activity of allicin in the spore germination assays. As a consequence, allicin might be considered as a lead compound for novel antibacterial and antifungal agents.

Unfortunately, the chemical properties of allicin make it difficult to apply this compound on a larger scale. An agent with chemical properties similar to cystamine-S-monoxide, but a biological activity comparable to allicin, would therefore be superior to both the polar S-monoxides and the unstable allicin. Interestingly, plants such as garlic using the allicin defense system are faced with similar problems and encounter the additional difficulty of using allicin without oxidizing their own,

cysteine-containing plant enzymes. As a consequence, allicin is not stored in the plant but is enzymatically synthesized "on demand" by the alliin/alliinase substrate/enzyme couple (*34*).

The results shown in **Figures 2**−**4** indicate that it is possible to mimic this natural substrate/enzyme defense system using chemically synthesized substrates and catalytic amounts of isolated alliinase. Like its natural analogue, this semisynthetic bicomponent system provides a means to effectively generate antifungal disulfide-S-oxides in situ. Enzymatic activation of an unreactive sulfoxide, such as MCSO (**Figure 2**), results in a disulfide-S-monoxide that is then able to oxidize thiol compounds such as 4-MP and also cysteine residues in proteins (as already shown for allicin). This reactivity would be specific for thiol groups in peptides and proteins but not specific for individual cysteine proteins. As a consequence, the bicomponent system may provide broad-spectrum antimicrobial activity, similar to that of naturally occurring allicin (**Table 1**).

The in vitro findings reported here are supported by our experiments with *M. grisea*. While both individual components of the substrate/enzyme system show little antimicrobial activity, a mixture of both almost completely abolished germination and appressorium formation by *M. grisea* (**Figures 3** and **4**). As **Table 2** shows, this process is irreversible and the conidia have been effectively disabled. It is likely that this process involves the oxidation of cysteine residues in fungal proteins to disulfides. Earlier studies have shown that disulfide-S-monoxides readily react with a range of different thiol groups, such as MT, where each disulfide-S-monoxide can oxidize a total of four cysteine residues (via sulfenic acids and disulfides), making this class of sulfur compounds highly effective oxidizing agents (*26*). Interestingly, a recent report has demonstrated that *M. grisea* secretes an MT-like protein to the cell wall during appressorium formation and this is required for plant infection to occur (*35*). This redox-sensitive protein may provide one of the many potential targets that an agent such as allicin can react with.

This rapid and effective oxidation of cysteine residues is matched by the efficiency of the substrate/enzyme system itself. As an enzyme-driven, catalytic process, the latter is able to convert significant quantities of sulfoxides to disulfide-S-monoxides within minutes. As the germination process of *M. grisea* conidia takes between 1 and 4 h, activation is sufficiently fast to allow the enzyme to effectively trigger antimicrobial activity at a desired point in time. This is also consistent with the time-dependence study, where alliinase was able to trigger disulfide-S-oxide formation and subsequently prevent fungal growth even 4 h after the conidia were incubated with sulfoxide solution.

Unlike the natural defense system, the isolated, semisynthetic bicomponent system described here has an additional, major advantage: Alliinase is not substrate-specific and is able to activate a range of "synthetic" substrates (*23*, *36*). This opens the door to different antimicrobial agents that can all be enzymatically synthesized from harmless sulfoxides in situ. The chemical properties and biological activity of the resulting, active disulfide-S-monoxides can be controlled by changing the structure of the sulfoxide substrates, making the alliinase-based bicomponent approach widely applicable.

Taken together, these results have several important implications for the practical use of disulfide-S-oxides as antimicrobial agents.

First, the use of the bicomponent system avoids many of the problems associated with allicin. Cysteine-based sulfoxides mostly are chemically stable solids without noticeable odor or toxicity toward humans and can easily be synthesized from the cysteine. Since alliinase is also rather stable, and is only required in catalytic amounts, the bicomponent system is relatively cheap, effective, and easy to handle when compared to allicin. Because chemically pure substrates can be used, the bicomponent approach might also be superior to extracted or chemically synthesized allicin that would be likely to contain impurities and decomposition products. Interestingly, a related system based on alliinase/antibody hybrids has recently been considered in a medical context (*37*).

Second, the substrate/enzyme system is not limited to the alliin/alliinase couple, and the methyl and ethyl disulfide-S-monoxides generated as part of this study are chemically distinctively different from allicin. While their enzymatic generation and practical use requires further investigation, additional substrates, such as S-propyl cysteine sulfoxide (PCSO), have already been reported in the literature (*36*), supporting the notion that there is considerable flexibility in the bicomponent system.

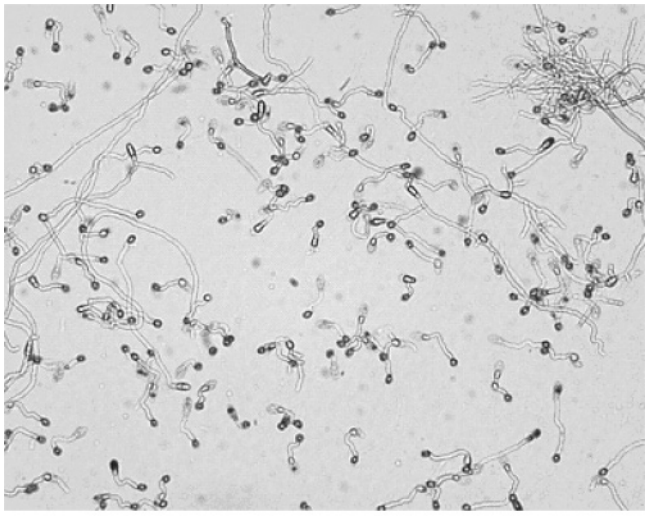

Figure 4. Representative images of conidia incubated with alliin (468 μM) in the absence (**a**) and presence (**b**) of alliinase (2 μM). The images were taken with a Zeiss Axioskop 2 mot plus microscope/camera with Zeiss AxioVisiona 3.0 software at 10 times magnification. Experimental details are in the text. Higher concentrations of alliinase completely abolish germination.

Third, the trigger assays indicate that it is possible to apply the sulfoxides on their own and then use small amounts of an enzyme formulation to trigger activity at the exact place and time where and when antimicrobial activity is required. This aspect might become important if plants were used that actually express enzymes with alliinase-like activity. In this case, antimicrobial activity might simply be triggered by the (injured) plant itself, as a response to injury or infection. Amazingly, in this case the "fungicide" to be applied, that is, the sulfoxide, would be completely nontoxic on its own. This would allow an approach toward antimicrobial treatment far superior to most available agents by merely mimicking a naturally occurring defense system that has been successfully operating in many plants for millions of years. This has important consequences for limiting the emergence of resistance in the field and for public acceptance of such a disease-control strategy. If alliinase activity were provided by the infected plant itself, this would also reduce the overall costs of the bicomponent approach and make it economically more competitive than a system based on purified enzyme.

In summary, the studies presented here have shown that it is possible to mimic the natural antimicrobial defense of *Allium* species to inhibit germination and appressorium formation of fungal pathogens such as *M. grisea*. A triggered antimicrobial system based on different sulfoxide substrates and alliiinase might be superior to the application of conventional fungicides or allicin itself. Although higher concentrations of this natural antimicrobial system might have to be used, its intrinsically low toxicity toward humans, biodegradability, and low costs make it an interesting alternative to current agents, especially in developing countries, where *M. grisea* poses a major problem. Although extensive future studies will be necessary to evaluate and refine such a system in practice, the idea of mimicking a successful, bicomponent system with broad-spectrum antimicrobial activity deserves further attention.

SAFETY

This work has no wider safety implications. None of the sulfoxides, alliinase, or disulfide-S-oxides used are harmful to humans in the concentrations used here. The *M. grisea* spores used in some of the experiments are not dangerous to animals or humans.

ACKNOWLEDGMENT

We thank Dr. Andrea Holme for the purification of alliinase and technical assistance. We also thank the *ECOspray* Ltd. for helpful discussions.

LITERATURE CITED

(1) Adetumbi, M. A.; Lau, B. H. S. Allium-Sativum (Garlic) - a Natural Antibiotic. *Med. Hypotheses* **1983**, *12*, 227−237.
(2) Agarwal, K. C. Therapeutic actions of garlic constituents. *Med. Res. Rev.* **1996**, *16*, 111−124.
(3) Ankri, S.; Miron, T.; Rabinkov, A.; Wilchek, M.; Mirelman, D. Allicin from garlic strongly inhibits cysteine proteinases and cytopathic effects of *Entamoeba histolytica*. *Antimicrob. Agents Chemother.* **1997**, *41*, 2286−2288.
(4) Feldberg, R. S.; Chang, S. C.; Kotik, A. N.; Nadler, M.; Neuwirth, Z.; Sundstrom, D. C.; Thompson, N. H. In Vitro Mechanism of Inhibition of Bacterial-Cell Growth by Allicin. *Antimicrob. Agents Chemother.* **1988**, *32*, 1763−1768.
(5) Davis, L. E.; Shen, J. K.; Royer, R. E. In Vitro Synergism of Concentrated Allium-Sativum Extract and Amphotericin-B against *Cryptococcus-Neoformans*. *Planta Med.* **1994**, *60*, 546−549.
(6) Krest, I.; Keusgen, M. Quality of herbal remedies from Allium sativum: Differences between alliinase from garlic powder and fresh garlic. *Planta Med.* **1999**, *65*, 139−143.
(7) Ross, Z. M.; O'Gara, E. A.; Hill, D. J.; Sleightholme, H. V.; Maslin, D. J. Antimicrobial properties of garlic oil against human enteric bacteria: Evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. *Appl. Environ. Microbiol.* **2001**, *67*, 475−480.
(8) Lun, Z. R.; Burri, C.; Menzinger, M.; Kaminsky, R. Antiparasitic Activity of Diallyl Trisulfide (Dasuansu) on Human and Animal Pathogenic Protozoa (*Trypanosoma Sp*, *Entamoeba-Histolytica* and *Giardia-Lamblia*) *in-Vitro*. *Ann. Soc. Belg. Med. Trop.* **1994**, *74*, 51−59.
(9) Rabinkov, A.; Miron, T.; Mirelman, D.; Wilchek, M.; Glozman, S.; Yavin, E.; Weiner, L. S-Allylmercaptoglutathione: the reaction product of allicin with glutathione possesses SH-modifying and antioxidant properties. *Biochim. Biophys. Acta-Mol. Cell Res.* **2000**, *1499*, 144−153.
(10) Yoshida, H.; Katsuzaki, H.; Ohta, R.; Ishikawa, K.; Fukuda, H.; Fujino, T.; Suzuki, A. Antimicrobial activity of the thiosulfinates isolated from oil- macerated garlic extract. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 591−594.
(11) Canizares, P.; Gracia, I.; Gomez, L. A.; de Argila, C. M.; Boixeda, D.; Garcia, A.; de Rafael, L. Allyl-thiosulfinates, the bacteriostatic compounds of garlic against *Helicobacter pylori*. *Biotechnol. Prog.* **2004**, *20*, 397−401.
(12) Shadkchan, Y.; Shemesh, E.; Mirelman, D.; Miron, T.; Rabinkov, A.; Wilchek, M.; Osherov, N. Efficacy of allicin, the reactive molecule of garlic, in inhibiting *Aspergillus* spp. *in vitro*, and in a murine model of disseminated aspergillosis. *J. Antimicrob. Chemother.* **2004**, *53*, 832−836.
(13) Yamada, Y.; Azuma, K. Evaluation of the in vitro antifungal activity of allicin. *Antimicrob. Agents Chemother.* **1977**, *11*, 743−9.
(14) Block, E. The Organosulfure Chemistry of the Genus *Allium* - Implications for the Organic Chemistry of Sulfur. *Angew Chem., Int. Ed. Engl.* **1992**, *31*, 1135−1178.
(15) Davis, L. E.; Shen, J. K.; Cai, Y. Antifungal activity in human cerebrospinal fluid and plasma after intravenous administration of *Allium sativum*. *Antimicrob. Agents Chemother.* **1990**, *34*, 651−3.
(16) Ghannoum, M. A. Studies on the anticandidal mode of action of *Allium sativum* (garlic). *J. Gen. Microbiol.* **1988**, *134* (Pt 11), 2917−2924.
(17) Ankri, S.; Mirelman, D. Antimicrobial properties of allicin from garlic. *Microbes Infect.* **1999**, *1*, 125−129.
(18) Krest, I.; Keusgen, M. Stabilization and pharmaceutical use of alliinase. *Pharmazie* **1999**, *54*, 289−293.
(19) Talbot, N. J. On the trail of a cereal killer: Exploring the biology of *Magnaporthe grisea*. *Annu. Rev. Microbiol.* **2003**, *57*, 177−202.
(20) Vaŝák, M. Standard Isolation Procedure for Metallothionein. *Methods Enzymol.* **1991**, *205*, 41−44.
(21) Steinman, H. M.; Richards, F. M. Participation of cysteinyl residues in the structure and function of muscle aldolase. Characterization of mixed disulfide derivatives. *Biochemistry* **1970**, *9*, 4360−4372.
(22) Wälti, M.; Hope, D. B. Synthesis of the isomers of the mono- and di-hydroxy-analogues of cystine and comparison with metabolites excreted in the urine. *J. Chem. Soc., Perkin 1* **1971**, *12*, 2326−2328.
(23) Stoll, A.; Seebeck, E. Die Synthese des natürlichen Allins und seiner drei optisch aktiven Isomeren. *Helv. Chim. Acta* **1951**, *34*, 481−486.
(24) Block, E.; Ahmad, S.; Catalfamo, J. L.; Jain, M. K.; Apitzcastro, R. Antithrombotic Organosulfur Compounds from Garlic - Structural, Mechanistic, and Synthetic Studies. *J. Am. Chem. Soc.* **1986**, *108*, 7045−7055.
(25) Jacob, C.; Maret, W.; Vallee, B. L. Ebselen, a selenium-containing redox drug, releases zinc from metallothionein. *Biochem. Biophys. Res. Commun.* **1998**, *248*, 569−573.

(26) Giles, G. I.; Tasker, K. M.; Collins, C.; Giles, N. M.; O'Rourke, E.; Jacob, C. Reactive sulphur species: an *in vitro* investigation of the oxidation properties of disulphide S-oxides. *Biochem. J.* **2002**, *364*, 579−585.

(27) Kuettner, E. B.; Hilgenfeld, R.; Weiss, M. S. Purification, characterization, and crystallization of alliinase from garlic. *Arch. Biochem. Biophys.* **2002**, *404*, 339−339.

(28) Miron, T.; Shin, I.; Feigenblat, G.; Weiner, L.; Mirelman, D.; Wilchek, M.; Rabinkov, A. A spectrophotometric assay for allicin, alliin, and aliinase (alliin lyase) with a chromogenic thiol: reaction of 4- mercaptopyridine with thiosulfinates. *Anal. Biochem.* **2002**, *307*, 76−83.

(29) Talbot, N. J.; Ebbole, D. J.; Hamer, J. E. Identification and characterization of MPG1, a gene involved in pathogenicity from the rice blast fungus Magnaporthe grisea. *Plant Cell* **1993**, *5*, 1575−90.

(30) Crawford, M. S.; Chumley, F. G.; Weaver, C. G.; Valent, B. Characterization of the heterokaryotic and vegetative diploid phases of *magnaporthe-grisea*. *Genetics* **1986**, *114*, 1111−1129.

(31) Hamer, J. E.; Howard, R. J.; Chumley, F. G.; Valent, B. A mechanism for surface attachment in spores of a plant pathogenic fungus. *Science* **1988**, *239*, 288−290.

(32) Giles, G. I.; Tasker, K. M.; Jacob, C. Hypothesis: The role of reactive sulfur species in oxidative stress. *Free Radical. Biol. Med.* **2001**, *31*, 1279−1283.

(33) Miron, T.; Rabinkov, A.; Mirelman, D.; Wilchek, M.; Weiner, L. The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity. *Biochim. Biophys. Acta-Biomembr.* **2000**, *1463*, 20−30.

(34) Lancaster, J. E.; Collin, H. A. Presence of Alliinase in Isolated Vacuoles and of Alkyl Cysteine Sulfoxides in the Cytoplasm of Bulbs of Onion (Allium- Cepa). *Plant Sci. Lett.* **1981**, *22*, 169−176.

(35) Tucker, S. L.; Thornton, C. R.; Tasker, K.; Jacob, C.; Giles, G.; Egan, M.; Talbot, N. J. A fungal metallothionein is required for pathogenicity of *Magnaporthe grisea*. *Plant Cell* **2004**, *16*, 1575−1588.

(36) Keusgen, M.; Junger, M.; Krest, I.; Schoning, M. J. Development of a biosensor specific for cysteine sulfoxides. *Biosens. Bioelectron.* **2003**, *18*, 805−12.

(37) Miron, T.; Mironchik, M.; Mirelman, D.; Wilchek, M.; Rabinkov, A. Inhibition of tumor growth by a novel approach: in situ allicin generation using targeted alliinase delivery. *Mol. Cancer Ther.* **2003**, *2*, 1295−301.

(38) O'Gara, E. A.; Hill, D. J.; Maslin, D. J. Activities of garlic oil, garlic powder, and their diallyl constituents against *Helicobacter pylori*. *Appl. Environ. Microbiol.* **2000**, *66*, 2269−2273.

Received for review September 10, 2004. Revised manuscript received November 12, 2004. Accepted November 13, 2004. This work was in part funded by the University of Exeter and Exeter Antioxidant Therapeutics Ltd. Work on rice blast at the University of Exeter is authorized by the Plant Health division of the Department for Environment, Food, and Rural Affairs PHL 204/4560.

JF048481J