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Antimicrobial intermediates of the general phenylpropanoid and lignin specific pathways

Mark S. Barber*, Victoria S. McConnell, Brian S. DeCaux

School of Biological Sciences, University of Southampton, Southampton, SO16 7PX, UK

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Abstract

The minimum inhibitory concentration (MIC) of the major intermediates of the general phenylpropanoid and lignin specific pathways of plants were determined employing a range of yeasts and bacteria. Of the three main classes of compounds tested, the hydroxycinnamaldehydes were the most effective, possessing notable antifungal and antibacterial activity. Determination of the minimum killing concentration (MKC) of the hydroxycinnamaldehydes revealed MIC/MKC ratios suggesting these compounds to be fungicidal, but not bactericidal, in their mode of action. In contrast, the hydroxycinnamic acids and hydroxycinnamyl alcohols possessed little antimicrobial activity, with the exception of the hydroxycinnamic acids, which were antibacterial.

Keywords: Antimicrobial activity; Hydroxycinnamaldehydes; Hydroxycinnamyl alcohols; Hydroxycinnamic acids; Lignin

1. Introduction

The intermediates of the general phenylpropanoid pathway (GPP) and lignin specific pathway (LSP) are naturally occurring hydroxycinnamates commonly found in higher plants (Fig. 1). Material entering the GPP via the deamination of L-phenylalanine or tyrosine leads to the formation of a series of hydroxycinnamic acids and hydroxycinnamoyl-CoA esters, varying in their degrees of hydroxylation and *O*-methylation. The shift into the LSP occurs via the reduction of the hydroxycinnamoyl-CoA esters to their corresponding hydroxycinnamaldehydes and hydroxycinnamyl alcohols. The hydroxycinnamyl alcohols are usually incorporated into lignin, which is often associated with defence, but the role of the various GPP and LSP intermediates in defence is less clear. Several of the intermediates of the GPP have been reported to possess antimicrobial activity (Bell, 1970; Baranowski et al., 1980; Shuen and Buswell, 1992; Snook et al., 1992; Tuncel and Nergiz, 1993) and some LSP intermediates are potential phytoalexins (Keen and Littlefield, 1979). Unfortunately, the multiplicity of assays employed by different investigators to assess antimicrobial activity does not easily allow direct comparison between studies and there has yet to be a comprehensive study. To clarify this situation, the antimicrobial properties of the major intermediates of the GPP and LSP have been investigated employing three yeasts: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Sporobolomyces roseus*, and three bacteria: *Bacillus subtilis* subsp. *Niger*, *Escherichia coli* and *Pseudomonas syringe*.

2. Results and discussion

The MIC of the major intermediates of the GPP and LSP were determined by serial dilution (Table 1). Of the three main classes of intermediate, the hydroxycinnamaldehydes were the most effective,

* Corresponding author. Tel.: +44-1703-592024; fax: +44-1703-594-319.

E-mail address: m.s.barber@soton.ac.uk (M.S. Barber).

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Table 1
Minimum inhibitory concentration (MIC) of the major intermediates of the general phenylpropanoid and lignin specific pathways. Percentage inhibition at 8.0 mM is given in parentheses, where MIC > 8.0 mM

	Minimim inhibitory concentration (mM)					
	Sa. cerevisiae	*Sc. pombe*	*Sp. Roseus*	*B. subtilis*	*E. coli*	*P. syringae*
p-Coumaric acid	> 8.0 (93)	8.0	8.0	2.0	2.0	2.0
Caffeic acid	> 8.0 (8)	> 8.0 (25)	> 8.0 (5)	4.0	8.0	4.0
Ferulic acid	4.0	8.0 (8.0)	2.0	2.0	2.0	2.0
Sinapic acid	> 8.0 (51)	> 8.0 (0)	> 8.0 (15)	2.0	2.0	4.0
p-Coumaraldehyde	2.0	2.0	2.0	2.0	2.0	2.0
Coniferaldehyde	0.5	1.0	2.0	4.0	2.0	2.0
Sinapaldehyde	4.0	4.0	4.0	8.0	4.0	4.0
p-Coumaryl alcohol	> 8.0 (45)	> 8.0 (0) >	8.0 (0)	8.0	> 8.0 (80)	> 8.0 (72)
Coniferyl alcohol	> 8.0 (33)	> 8.0 (90)	> 8.0 (50)	> 8.0 (84)	> 8.0 (0)	> 8.0 (55)
Sinapyl alcohol	> 8.0 (32)	> 8.0 (51)	> 8.0 (80)	> 8.0 (80)	> 8.0 (81)	> 8.0 (37)
Tolnaftate[a]	0.5	1.0	0.5			
Eugenol[a]	2.0	2.0	1.0	4.0	4.0	4.0

[a] Positive antimicrobial agent controls.

possessing both antifungal and antibacterial activity. Coniferaldehyde was the most antifungal compound (mean MIC 1.2 mM) and *p*-coumaraldehyde, the most antibacterial (mean MIC 2.0 mM). Determination of the MKC of the hydroxycinnamaldehydes revealed MIC/MKC ratios of less than five for the yeasts, and in excess of eight for the bacteria (Table 2). This suggests that the hydroxycinnamaldehydes are fungicidal, but not bactericidal, in their mode of action.

The hydroxycinnamic acids were generally antibacterial (mean MIC 3.0 mM), but were only weakly antifungal (MIC $\geq$ 8 mM) with the possible exception of ferulic acid, which had a MIC of 4.0 mM against *Sa. cerevisiae*. Baranowski et al. (1980) previously reported ferulic acid to be antimicrobial towards *Sa. cerevisiae*, causing complete inhibition at a much lower concentration of 0.25 mM. Differences in growth conditions, particularly pH, are the most likely explanation to account for this discrepancy. At the comparative low pH of Baranowski et al. (1980) experiments (pH 3.5), ferulic acid may well be more effective due to its increased membrane permeability in the undissociated state.

The least antimicrobial class of hydroxycinnamates were the hydroxycinnamyl alcohols, which possessed relatively weak antibacterial (mean MIC $\geq$ 8 mM) and antifungal (mean MIC > 8.0 mM) properties.

A largely unanswered question is whether any of the intermediates reach concentrations in plants sufficient to account for the cessation of potential microbial pathogens? Unfortunately, the lack of reliable and complete information regarding the levels of hydroxycinnamates in plants negates the possibility of answering this question with any authority. Free phenols are rarely found in plants, more often occurring as glycosides or esters. The release of free hydroxycinnamates from these stores following microbial infection is clearly possible, as is their renewed synthesis. The best example of hydroxycinnamate accumulation to date occurs in flax, where coniferaldehyde and coniferyl alcohol levels increase at sites of infection in incompatible interactions with the flax pathogen *Melampsora lini* (Keen and Littlefield, 1979). In terms of the con-

Table 2
Minimum killing concentration (MKC) of selected intermediates of the general phenylpropanoid and lignin specific pathways. The MKC/MIC ratio is given in parentheses

	Minimum killing concentration (mM)					
	Sa. cerevisiae	*Sc. pombe*	*Sp. roseus*	*B. subtilis*	*E. coli*	*P. syringae*
p-Coumaric acid				> 8.0	> 8.0	> 8.0
Caffeic acid				> 8.0	> 8.0	> 8.0
Ferulic acid				> 8.0	> 8.0	> 8.0
Sinapic acid				> 8.0	> 8.0	> 8.0
p-Coumaraldehyde	4.0 (2.0)	4.0 (2.0)	4.0 (2.0)	> 8.0	> 8.0	> 8.0
Coniferaldehyde	2.0 (4.0)	2.0 (2.0)	2.0 (1.0)	> 8.0	> 8.0	> 8.0
Sinapaldehyde	8.0 (2.0)	8.0 (2.0)	4.0 (1.0)	> 8.0	> 8.0	> 8.0

centration of these compounds accumulating in the plant, typical values were in the order of 6 μg/g fresh weight for coniferaldehyde and 12 μg/g fresh weight for coniferyl alcohol. However, how these values relate to the actual level of coniferaldehyde and coniferyl alcohol experienced by the invading pathogens is not known. Nevertheless, there is good correlative evidence to support the role of coniferaldehyde and coniferyl alcohol as a phytoalexin in this system.

The possibility of artificially raising the levels of hydroxycinnamaldehydes in plants has arisen indirectly from attempts to alter plant lignification. Much work has focused on cinnamyl alcohol dehydrogenase (CAD), the enzyme responsible for the conversion of hydroxycinnamaldehydes to hydroxycinnamyl alcohols, prior to their incorporation into lignin. Abnormally high levels of hydroxycinnamaldehyde incorporation into lignin have been reported in naturally occurring CAD mutants and in plants where CAD has been deliberately down regulated (Bucholtz et al., 1980; Halpin et al., 1994; MacKay et al., 1997; Stewart et al., 1997; Ralph et al., 1998; Yahiaoui et al., 1998). Pool levels were not measured in these plants, but this approach may well allow hydroxycinnamaldehydes to be artificially raised to antimicrobial levels, which may have a significant impact on plant resistance.

3. Experimental

3.1. Test microorganisms

The yeasts: *Sa. cerevisiae* (019 391), *Sc. pombe* (039 917) and *Sp. roseus* (043 529) were from the International Mycological Institute and maintained on YEP-glucose agar at 20°C. The bacteria: *B. subtilis* subsp. *niger* (8649), *E. coli* (12210) and *P. syringae* (649) were from the National Collections of Industrial and Marine Bacteria, and maintained on nutrient agar at 30°C.

3.2. Growth media

Unless otherwise stated, all growth media components were obtained from Oxoid. All media was adjusted to pH 7.1 with 0.1 M NaOH or HCl prior to autoclaving at 121°C for 15 min. YEP-glucose agar: 0.5% (w/v) yeast extract, 0.5% (w/v) mycological peptone, 1.0% (w/v) glucose (Merck) and 1.5% (w/v) technical agar No. 3. YEP-glucose broth: 0.5% (w/v) yeast extract, 0.5% (w/v) mycological peptone and 1.0% (w/v) glucose. Nutrient agar: 1.3% (w/v) nutrient broth and 1.5% (w/v) bacteriological agar No. 1. Nutrient broth: 1.3% (w/v) nutrient broth.

3.3. Test compounds

Unless otherwise stated, all chemicals were obtained from the following divisions of the Sigma-Aldrich. Sigma: *p*-coumaric acid, caffeic acid, ferulic acid, coniferyl alcohol, tolnaftate and eugenol. Aldrich: coniferaldehyde, sinapaldehyde and sinapyl alcohol. Fluka: sinapic acid. *p*-Coumaryl alcohol and *p*-coumaraldehyde were synthesised by the method of Quideau and Ralph (1992). All compounds were recrystallised from appropriate solvent and checked for purity by TLC. Stock solutions of the test compounds were initially prepared at 400 mmol l^{-1} in sterile distilled water and dissolved by boiling. Serial two-fold dilutions of the stock solutions were prepared in sterile distilled water, followed by a 40-fold dilution with the appropriate media prior to autoclaving.

3.4. MIC determination

MIC values were determined in sterile multiwell plates using YEP-glucose broth for the yeasts and nutrient broth for the bacteria. Each well contained 180 μl of growth media containing appropriate amounts of each test compound and 20 μl of log phase cells (1.0×10^6 cells ml^{-1}). Each compound was tested at six concentrations (8.0, 4.0, 2.0, 1.0, 0.5 and 0.25 mM), including a sterile distilled water control, using three replicates at each concentration. Plates were incubated at 20°C for the yeasts and 30°C for the bacteria, and growth was assessed by the change in absorbance at 630 nm after 36 h for the yeasts (48 h for *Sp. roseus*) and 24 h for the bacteria. The lowest concentration of each compound that reduced the change in absorbance relative to the sterile distilled water control by greater than 95% was recorded as the MIC for that compound.

3.5. MKC determination

Samples (20 μL) from the MIC experiments were plated out on YEP-glucose agar for the fungi and nutrient agar for the bacteria. Plates were incubated at 20°C for 72 h for the yeasts and 30°C for 48 h for the bacteria. Growth was assessed by visual inspection and the lowest concentration of each compound that totally inhibited growth was recorded as the MKC for that compound.

References

Baranowski, J.D., Davidson, P.M., Nagel, C.W., Branen, A.L., 1980. Inhibition of *Saccharomyces cerevisiae* by naturally occurring hydroxycinnamates. Journal of Food Science 45, 592–594.

Bell, A.A., 1970. 4-Hydroxybenzaldehyde and vanillin as toxins

formed in leaf wound sap of *Phaseolus lunatus*. Phytopathology 60, 161–165.

Bucholtz, D.L., Cantrell, R.P., Axtell, J.D., Lechtenberg, V.L., 1980. Lignin biochemistry of normal and brown midrib mutant sorghum. Journal of Agricultural Food Chemistry 28, 1239–1241.

Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert, B., Tollier, M.T., Schuch, W., 1994. Manipulation of lignin quality by down-regulation of cinnamyl alcohol-dehydrogenase. Plant Journal 6 (3), 339–350.

Keen, N.T., Littlefield, L.J., 1979. The possible association of phytoalexins with resistance gene expression in flax to *Melampsora lini*. Physiological Plant Pathology 14, 265–280.

MacKay, J.J., Omalley, D.M., Presnell, T., Booker, F.L., Campbell, M.M., Whetten, R.W., Sederoff, R.R., 1997. Inheritance, gene expression, and lignin characterisation in a mutant pine deficient in cinnamyl alcohol dehydrogenase. Proceedings of the National Academy of Sciences of the United States of America 94 (15), 8255–8260.

Quideau, S., Ralph, J., 1992. Facile large scale synthesis of coniferyl, sinapyl and para coumaryl alcohol. Journal of Agricultural and Food Chemistry 40 (7), 1108–1110.

Ralph, J., Hatfield, R.D., Piquemal, J., Yahiaoui, N., Pean, M., Lapierre, C., Boudet, A.M., 1998. NMR characterisation of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. Proceedings of the National Academy of Sciences of the United States of America 95 (22), 12803–12808.

Shuen, S.K., Buswell, J.A., 1992. Effect of lignin derived phenols and their methylated derivatives on the growth of *Lentinus spp*. Letters in Applied Microbiology 15 (1), 12–14.

Snook, M.E., Csions, S., Chortyk, O.T., 1992. Inhibition of growth of *Phytophthora parasitica* var *nicotianae* by aromatic acids and coumarins in a laboratory bioassay. Journal of Chemical Ecology 18 (8), 1287–1297.

Stewart, D., Yahiaoui, N., McDougall, G.J., Myton, K., Marque, C., Boudet, A.M., Haigh, J., 1997. Fourier-transform infrared and Raman spectroscopic evidence for the incorporation of cinnamaldehydes into the lignin of transgenic tobacco (*Nicotiana tabacum* L) plants with reduced expression of cinnamyl alcohol dehydrogenase. Planta 201 (3), 311–318.

Tuncel, G., Nergiz, C., 1993. Antimicrobial effect of some olive phenols in a laboratory medium. Letters in Applied Microbiology 17 (6), 300–302.

Yahiaoui, N., Marque, C., Myton, K.E., Negrel, J., Boudet, A.M., 1988. Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. Planta 204 (1), 8–15.