high as the initial one (fig. 3). While, in general, any increase in optical density of a bacterial culture reflects an increase in the number of cells, such an increase occurs with gram-positive bacteria growing in the presence of CAP on different grounds. It is well known that wall synthesis in gram-positive microorganisms is not suppressed when protein synthesis is blocked [10], and, more recently, we could demonstrate by combined electron microscopic and spectrophotometric studies that the increase in optical density measured with CAP-treated staphylococcal cultures was not due to an increased number of cells but to the drug-induced growth of thickened cell walls. Obviously this phenomenon may also influence the MIC determination, which depends on optical properties of the bacterial suspension, as well. However, since at present the only MIC data examined are for our SG 511-strain (and two other staphylococcal strains with basically the same CAP sensibility), further investigations are required to prove the correctness of this assumption for staphylococcal strains with lower MICs for CAP also.

Since cell wall thickening can be induced by other bacteriostatic antibiotics, too (i.e., tetracyclines [11] or macrolides [12]), the possibility of false data must be taken into account when conventional MIC tests are performed with these drugs. Altered MIC definitions, considering a certain increase in cell number or in colony-forming units (CFU test), may deliver more realistic results but are laborious and slow as compared to microcalorimetry; moreover, it is only possible to obtain a single result at the end of an experiment, whereas with flow microcalorimetry inhibition effects are recorded continuously. Therefore, particularly when developing new chemotherapeutic agents or for clinical studies of bacterial susceptibilities, the microcalorimetric assay of the reduction, delay and loss of efficiency of microbial metabolism may yield better evaluation criteria for the antibiotic activity of a drug than do MIC techniques or photometric methods alone.

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Fungitoxicity of *m*-fluorophenylalanine-containing peptides towards *Pythium ultimum*

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Summary. The tripeptide L-*m*-fluorophenylalanyl-L-alanyl-L-alanine was much more fungitoxic towards *Pythium ultimum* than the dipeptide L-*m*-fluorophenylalanyl-L-alanine or *m*-fluorophenylalanine. The fungitoxicity of the tripeptide was reduced by L-alanyl peptides and phenylalanine, but not by other amino acids. In contrast, the fungitoxicity of *m*-fluorophenylalanine was unaffected by peptides, and was antagonized by several amino acids. These results suggest the effective delivery of *m*-fluorophenylalanine into the cell by a tripeptide carrier.
Key words. Amino acid transport; fluorophenylalanine; peptide transport; *Pythium ultimum.*

Peptide permeases generally exhibit less specific substrate range than amino acid permeases [1]. This characteristic can be exploited to facilitate entry of impermeant amino acid analogs, or other molecules, into the cell by delivery in the form of a peptide which may then undergo intracellular hydrolysis. In various natural [2–5] and synthetic [1, 6–9] antimicrobial peptides this concept of 'illicit transport' is utilized to enable a toxophore to reach its intracellular site of action. In *Candida albicans* [10], for example, fungitoxicity of the amino acid toxophore *m*-fluorophenylalanine (*m*-FPhe) is greatly enhanced by delivery as the di- or tripeptides, L-*m*-fluorophenylalanyl-L-alanine (L-*m*-FPhe-L-Ala) and L-*m*-fluorophenylalanyl-L-alanyl-L-alanine (L-*m*-FPhe-L-Ala-L-Ala).

Information about the existence and properties of peptide permeases in plant pathogenic fungi would be helpful in the design of agricultural fungicides which could be taken up by peptide transport. Unfortunately, the limited evidence for such permeases derives from studies of polyoxins, which are transported by peptide permeases in

Alternaria kikuchiana[4], and *Pellicularia sasakii*[11], and act by inhibiting chitin synthesis. Polyoxins are inactive against oomycete fungi, which include many important plant pathogens, since this taxonomic group does not have a chitinous cell wall. To our knowledge, peptide permeases have not been demonstrated in oomycetes.

To explore the feasibility of the peptide transport concept in oomycete fungi we have studied the fungitoxicity of *m*-FPhe and the di- and tripeptides L-*m*-FPhe-L-Ala and L-*m*-FPhe-L-Ala-L-Ala against *Pythium ultimum*, the causal agent of damping off and seedling blight diseases in various crops.

Materials and methods. Pythium ultimum (ATCC 26083) was obtained from the American Type Culture Collection, Rockville, Maryland, and was maintained on potato dextrose agar at 25 °C via serial transfer every 3–4 days.

Minimum inhibitory concentration (MIC) values were determined using a microtiter plate assay with twofold serial dilutions of the test sample (100 µl) in asparagine-sucrose medium[12]. In antagonism tests, the medium was amended with the potential antagonist. Inoculum was prepared by transferring mycelial plugs, 7 mm in diameter, to 9-cm petri plates containing 20 ml of liquid asparagine-sucrose medium. The plates were incubated for 48 h at 25 °C on a rotary shaker at 50 rpm, then homogenized by means of four 5-s bursts at maximum speed in a Waring blender. The homogenate was centrifuged at 2000 × g for 5 min and the mycelial pellet resuspended in 10 times the original culture volume of medium. Microtiter plates were inoculated with 100 µl of mycelial suspension per well, then incubated at 25 °C for 48 h after which MIC values were determined by visual inspection. Poison agar assays were performed by adding test compounds and antagonists dissolved in sterile water to 20-ml aliquots of asparagine-sucrose medium containing 0.8% agar at 50 °C, then pouring the molten agar into 9-cm petri dishes. After the agar had hardened the plates were inoculated in the center with 7-mm diameter plugs taken from the growing edges of 2-day-old cultures grown on asparagine-sucrose agar. Colony diameters were measured after growth for 24 h at 25 °C, and the diameter of the inoculum plug was substracted. Two measurements were taken from each plate, and two replicate plates were used for each treatment.

m-FPhe-containing peptides were generously provided by Dr W. D. Kingsbury of Smith Kline and French Laboratories, Philadelphia. D,L-*m*-FPhe, all other amino acids and peptides were from the Sigma Chemical Co., St. Louis, Mo.

Results and discussion. The tripeptide L-*m*-FPhe-L-Ala-L-Ala was 16-fold more fungitoxic then *m*-FPhe towards *P. ultimum*, however the dipeptide L-*m*-FPhe-L-Ala-L-Ala was inactive (table 1). These results differ to some extent from those reported for *Candida albicans*, in which both the tri- and dipeptides were found to be significantly more active than *m*-FPhe[10]. In *C. albicans*, the higher activity of the peptides has been attributed to efficient delivery of the toxophore into the cell by a peptide permease, and subsequent intracellular hydrolysis to release the toxophore. As found for *C. albicans*, the tripeptide containing the D-isomer of *m*-FPhe was inactive towards *P. ultimum*.

In order to evaluate whether L-*m*-FPhe-L-Ala-L-Ala and *m*-FPhe are taken up by different transport systems in *P. ultimum*, we examined the ability of peptides and amino acids to influence their fungitoxicity. Tables 2 and 3 demonstrate that the fungitoxicity of the tripeptide was significantly reduced by L-alanyl peptides but not by D-alanyl peptides or by L-alanine itself. In contrast, the fungitoxicity of *m*-FPhe was unaffected by peptides. The effect of L-alanyl peptides on fungitoxicity of L-*m*-FPhe-L-Ala-L-Ala was examined both in the microtiter assay and in a poison agar assay, which is more sensitive, and thus more reliable for detecting small differences in fun-

Table 1. Fungitoxicity of *m*-fluorophenylalanyl peptides

Compound	MIC (µM)
D,L-*m*-FPhe	125
L-*m*-FPhe-L-Ala-L-Ala	7.8
D-*m*-FPhe-L-Ala-L-Ala	> 500
L-*m*-FPhe-L-Ala	> 500
D-*m*-FPhe-L-Ala	> 500

Table 2. Effect of peptides on the fungitoxicity of L-*m*-FPhe-L-Ala-L-Ala and D,L-*m*-FPhe

Peptide	Concentration (mM)	MIC (µM) L-*m*-FPhe-L-Ala-L-Ala	D,L-*m*-FPhe
None		7.8	125
L-Ala	10	7.8	125
$L\text{-}Ala_2$	1	15.6	125
	10	125	125
$L\text{-}Ala_3$	1	62.5	125
	10	250	125
$L\text{-}Ala_4$	1	31.2	125
	10	125	125
$L\text{-}Ala_5$	1	31.2	125
$D\text{-}Ala_2$	1	7.8	125
	10	7.8	125
$D\text{-}Ala_3$	1	7.8	125
	10	7.8	125

Table 3. Antagonism of L-*m*-FPhe-L-Ala-L-Ala activity by peptides in a poison agar assay

Treatment	Colony diameter (mm ± SEM)
Control	51.4 ± 0.5
L-*m*-FPhe-L-Ala-L-Ala	16.0 ± 0.2
L-*m*-FPhe-L-Ala-L-Ala + L-Ala	15.6 ± 0.4
L-*m*-FPhe-L-Ala-L-Ala + $L\text{-}Ala_2$	28.6 ± 0.5
L-*m*-FPhe-L-Ala-L-Ala + $L\text{-}Ala_3$	45.8 ± 0.5
L-*m*-FPhe-L-Ala-L-Ala + $L\text{-}Ala_4$	40.3 ± 0.3
L-*m*-FPhe-L-Ala-L-Ala + $L\text{-}Ala_5$	42.0 ± 0.4
L-*m*-FPhe-L-Ala-L-Ala + $D\text{-}Ala_2$	14.2 ± 0.3
L-*m*-FPhe-L-Ala-L-Ala + $D\text{-}Ala_3$	14.4 ± 0.3

L-*m*-FPhe-L-Ala-L-Ala was tested at 5 µM, and the potential antagonists at 1 mM. Results are the mean ± SEM.

Table 4. Effect of amino acids on the fungitoxicity of D,L-*m*-FPhe and L-*m*-FPhe-L-Ala-L-Ala

Amino acid	Concentration (mM)	MIC (μM) D,L-*m*-FPhe	L-*m*-FPhe-L-Ala-L-Ala
None		125	7.8
L-Phe	0.1	250	31.2
	1.0	2000	500
	10.0	>2000	>500
L-Met	0.1	250	7.8
	1.0	500	7.8
	10.0	1000	7.8
L-Ile	0.1	125	7.8
	1.0	250	7.8
	10.0	500	7.8
L-Leu	0.1	125	7.8
	1.0	250	7.8
	10.0	1000	7.8
L-Trp	0.1	250	7.8
	1.0	500	7.8
	10.0	1000	7.8
L-Val	0.1	125	7.8
	1.0	250	7.8
	10.0	500	7.8
L-Tyr	0.1	125	7.8
	1.0	250	7.8

Fungitoxicity was unaffected by L-Gly, L-Ala, L-Ser, L-Thr, L-Asp, L-Asn, L-Glu, L-Gln, L-Lys, L-His, L-Arg, L-Cys, L-Pro, L-Hyp, at 10 mM.

gitoxicity. Very similar results were obtained using the two methods, and the tripeptide L-Ala_3 was found to be the most effective antagonist. In amino acid antagonism experiments, L-Phe was the only amino acid which antagonized L-*m*-FPhe-L-Ala-L-Ala, whereas the fungitoxicity of *m*-FPhe was reduced by all 3 aromatic amino acids (L-Phe, L-Tyr and L-Trp) and the neutral amino acids L-Met, L-Ile, L-Leu and L-Val (table 4). Similar antagonism of L-*m*-FPhe-L-Ala-L-Ala by L-Phe was reported in *Candida albicans*[10], and was interpreted to indicate competition with L-*m*-FPhe at its intracellular site of action following hydrolysis of the tripeptide.

These results support the hypothesis that L-*m*-FPhe-L-Ala-L-Ala is transported into *P. ultimum* by a peptide permease, whereas *m*-FPhe is delivered by an amino acid transport system. Results of the amino acid antagonism experiments (table 4) suggest that *P. ultimum* has a very similar transport system for aromatic and neutral amino acids to that described for *Achlya*[13], another oomycete. The ineffectiveness of D-Ala_2 and D-Ala_3 as antagonists of L-*m*-FPhe-L-Ala-L-Ala and the lack of fungitoxicity of D-*m*-FPhe-L-Ala-L-Ala are consistent with the specificity of peptide permeases in general for L-amino acid containing peptides.

In conclusion, our results strongly suggest that *P. ultimum* has an operational tripeptide transport system, and that the concept of illicit transport can be applied in the design of fungicides active against oomycete fungi.

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Carboxylesterases of high molecular weight in the hemolymph of *Locusta migratoria*

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Summary. The main carboxylesterase in the hemolymph of the migratory locust, *Locusta migratoria*, is a protein of high molecular weight; about 700–750 kDa. This esterase hydrolyzes juvenile hormone III, α-naphthylacetate and β-naphthylacetate. The carboxylesterase dissociates to give an esterase of molecular weight 148 kDa after treatment of the hemolymph with mercaptoethanol.
Key words. Carboxylesterase; juvenile hormone esterase; juvenile hormone binding protein; hemolymph proteins; migratory locust.

Hemolymph proteins of insects have many physiological functions. Analysis of the protein composition by either gel permeation chromatography or non-denaturing polyacrylamide gel electrophoresis (PAGE) revealed that many proteins in the hemolymph have high molecular weights. In the migratory locust, *Locusta migratoria*, for instance, almost 80% of the proteins from the hemolymph of adult males have molecular weights above