Pestic. Sci. 1997, **49**, 229–236

Effect of Phenylpyrroles on Glycerol Accumulation and Protein Kinase Activity of *Neurospora crassa*

Christian Pillonel[a]* & Thomas Meyer[b]

[a] Ciba-Geigy Limited, Division Crop Protection, R&D Disease Control, CH-4002 Basle, Switzerland
[b] Ciba-Geigy Limited, Pharmaceutical Division, Research Department, CH-4002 Basle, Switzerland

(Received 31 May 1996; revised version received 20 September 1996; accepted 17 October 1996)

Abstract: The effect of phenylpyrroles on glycerol synthesis in *Neurospora crassa* has been investigated and compared with the mode of action of vinclozolin (a dicarboximide). The results indicated that fenpiclonil, fludioxonil and vinclozolin at concentrations which inhibit growth by 50% induce accumulation of glycerol in the mycelium of *N. crassa*. Furthermore a protein kinase (PK-III) possibly involved in the regulation of the glycerol synthesis is inhibited by phenylpyrroles, whereas vinclozolin is without effect. This implies that the target sites of phenylpyrroles and dicarboximides in the osmosensing signal transmission pathway are different. Comparative experiments with enzymes from human and animal sources revealed that PK-III could be a protein kinase Cδ. It is suggested that inhibition of PK-III activity may result in an increased concentration of a non-phosphorylated regulatory protein which may activate a MAP-kinase cascade of reactions resulting in increased glycerol synthesis.

Key words: phenylpyrroles, vinclozolin, mode of action, *Neurospora crassa*, protein kinase, glycerol synthesis

1 INTRODUCTION

Fenpiclonil and fludioxonil are novel phenylpyrrole fungicides (Fig. 1).[1,2] Biochemical studies revealed that fenpiclonil, at concentrations which reduced growth by about 50%, inhibited the transport of monosaccharides in *Fusarium sulphureum* Schlecht.[3,4] It also stimulates the intracellular accumulation of neutral polyols, such as glycerol and mannitol, whereas nuclear division, respiration, chitin-, ergosterol-, phospholipids-, DNA- and RNA-syntheses are not affected.[4,5] A cascade of events has been postulated which originates in the inhibition of the transport-associated phosphorylation of glucose and leads to the accumulation of polyols and cell death.[4] However, the primary target site of fenpiclonil and the final cause of cell death remain unclear. A direct effect on hexokinase is unlikely, since inhibition of cell-free phosphorylation was not observed.[4] Under laboratory conditions, both phenylpyrroles and dicarboximides select for resistant mutants which are

* To whom correspondence should be addressed.

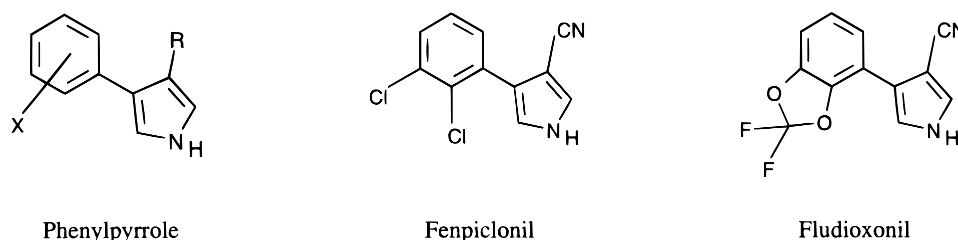

Fig. 1. General structure of phenylpyrrole fungicides and structural formulae of fenpiclonil and fludioxonil.

Pestic. Sci. 0031-613X/97/$09.00 © 1997 SCI. Printed in Great Britain

sensitive to osmotic stress and cross-resistant to both types of fungicides.[4,5] This suggests that osmoregulation is either a common feature in the mechanism of resistance to both fungicides or that phenylpyrroles and dicarboximides interfere with the osmosensing signal transduction pathway. The aim of the present work was to establish the target site of phenylpyrroles in the osmosensing glycerol synthesis regulatory pathway and to compare it with the mode of action of vinclozolin. Sugar transport and osmoregulatory glycerol synthesis in *Neurospora crassa* Shear & Dodge are well documented.[4,6] Therefore, this fungus was used in this study.

2 MATERIALS AND METHODS

2.1 Chemicals

4-(2,3-Dichloro-[U-^{14}C]phenyl)-1*H*-pyrrole-3-carbonitrile (fenpiclonil; sp. act. 0·462 GBq mmol^{-1}), 4-(2,2-difluor-1,3-benzodioxol-4-yl)-1*H*-[4-^{14}C]pyrrole-3-carbonitrile (fludioxonil; sp. act. 0·506 GBq mmol^{-1}) and their non-radioactive forms were synthesized by Ciba (structures are represented in Fig. 1). Radiolabelled 2-deoxy-D-[U-^{14}C]glucose (sp. act. 10·8 GBq mmol^{-1}) and [γ-^{33}P]ATP (37–110 TBq ml^{-1}) were purchased from Amersham. All other chemicals were obtained either from Fluka (CH-9470 Buchs, Switzerland), Merck (CH 8953 Dietikon, Switzerland) or Sigma (St Louis, USA).

2.2 Organisms

Neurospora crassa (wild-type strain 74-OR8-1) was obtained from the American Type Culture Collection (ATCC 18889). Recombinant baculoviruses for protein kinase C isoenzymes were provided by Silvia Stable, Max-Plank-Gesselschaft, Cologne, Germany. A431 human epithelial carcinoma cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). BALB/c 3T3 cells were kindly provided by C. Stiles (Dana-Farber Cancer Institute, Boston).

2.3 Culture and growth conditions of *Neurospora crassa*

Mycelium was grown as inoculum in Petri dishes at 24°C in the dark for 14 days (medium: Biomalt 10, Difco dry yeast extract 4, glucose 4 and agar 20 g litre^{-1} in water). Macroconidia were collected in sterile water, filtered through glass wool and then transferred into Vogel's liquid minimal medium supplemented with sucrose (20 g litre^{-1}) according to the method described by Favre and Turian.[7] For toxicity tests, the cell density was adjusted to 5×10^4 macroconidia ml^{-1} and 8 ml were pipetted into Petri dishes. Active compounds were added to the medium from 1000-fold concentrated stock solutions in acetone. Control treatments received equivalent quantities of acetone. The cultures were incubated at 24°C and 90% relative humidity in the dark for three days. After incubation the cultures were vacuum-filtered on glass microfibre filters (Whatman GF/C; $d = 4{\cdot}7$ cm), previously dried overnight at 70°C under vacuum and weighed. The filters with mycelium were dried as described above and weighed again. The IC_{50} (concentration of inhibitor required to reduce growth by 50%) was used as a parameter for inhibition. The glycerol, deoxyglucose and fungicide accumulation tests were conducted after incubation of a conidial suspension (6×10^5 ml^{-1}) in Vogel's medium in a rotary shaker at 180 rev min^{-1} and 24°C for 24 h. For the isolation of protein kinases, *N. crassa* was cultivated under the same conditions during 36 h.

2.4 Glycerol accumulation

After pre-incubation of standard mycelial suspensions of *N. crassa* in Vogel's medium (25 ml) depleted of sugar for 1 h, active compounds mixed with glucose (200 μM) were added to the medium from 1000-fold concentrated stock solutions in acetone. Control treatments received equivalent quantities of acetone. These cultures were incubated in a rotary shaker at 180 rev min^{-1} and 240°C. After incubation, mycelium was collected on Millipore SWCW04700 (8 μm) filters, extensively washed with ice-cold water and homogenized in a mortar with ethanol + water (80 + 20 by volume; 5 ml) and extracted at 70°C for 2 min. After centrifugation at 8000*g* the glycerol concentration was measured in the supernatant by using the UV-glycerol assay procedure (Boehringer Mannheim GmbH).

2.5 Deoxyglucose accumulation

Mycelial suspensions of *N. crassa* in Vogel's medium without sucrose (10 ml) were equilibrated for 1 h. Accumulation of 2-deoxy-D-[U-^{14}C]glucose was measured at intervals after addition of the fungicide from 1000-fold concentrated stock solutions according to Jespers *et al.*[3]

2.6 Protein kinases isolation

Mycelium of *N. crassa* (50 g) in the logarithmic phase of growth was harvested by filtration, rinsed with cold water, homogenized with the omnimixer and extracted with 100 ml of buffer A containing Tris-HCl (20 mM; pH 7·6), EGTA (10 mM), EDTA (2 mM), 2-mercaptoethanol (50 mM), sucrose (0·25 M), sodium azide (0·3 mM), benzamidine (10 mM), phenylmethylsul-

fonyl fluoride (1 mM), pepstatin (0·25 μg ml^{-1}), leupeptin (10 μg ml^{-1}) according to Favre and Turian.[7] The homogenate was centrifuged at 20 000g for 20 min. The supernatant was further centrifuged at 100 000g for 1 h. The supernatant was collected through glass wool and solid ammonium sulfate was added to a final level of 50% saturation. The precipitate was recovered by centrifugation at 20 000g for 20 min and resuspended in 2·5 ml buffer B containing Tris-HCl (20 mM; pH 7·6), EGTA (1 mM), EDTA (1 mM), 2-mercaptoethanol (50 mM). The suspension obtained was passed through a Sephadex G-25 PD-10 (50 × 15 mm) equilibrated with buffer B. Fractions (2 ml) were collected and assayed for protein kinase activity. Active fractions were applied to a Bio-Gel TSK DEAE-5-PW column (75 × 7·5 mm) equilibrated with buffer B. The column was extensively washed with the same buffer. Adsorbed proteins were eluted with a linear gradient of sodium chloride (0–0·4 M) added in buffer B. Fractions (2 ml) were collected and assayed for protein kinase activity.

Expression and partial purification of PKC subtypes were carried out using the baculovirus vector system as described by Marte *et al.*[8] The c-AMP-dependent protein kinase isolated from rabbit was a gift of Dr Brian Hemmings, FMI Basle. The CDK-1 (p34^{cdc2}/cyclin B) isolated from starfish oocytes was obtained by Dr Laurent Meijer, CNRS, Station Biologique, Roscoff, France and purified as described.[9]

2.7 Protein kinase activity measurement

Protein kinase C activity was assayed as described by Meyer *et al.*[10] and carried out in a reaction mixture (100 μl) containing Tris-HCl (20 mM; pH 7·4), protamine sulfate (20 μg), magnesium nitrate (10 mM), ATP (10 μM) (0·2 μCi [γ-^{33}P]ATP), sodium azide (1 mM), okadaic acid (0·04 μM), 20 μl of enzyme-containing fractions and various concentrations of inhibitors from 20-fold concentrated stock solutions in dimethyl sulfoxide. Control treatments received equivalent quantities of dimethyl sulfoxide. Incorporation of [γ-^{33}P] onto protamine sulfate was determined by spotting aliquots (50 μl) onto P81 chromatography paper (Whatman). The IC_{50} value was used as parameter for inhibition. For the measurement of the substrate and calcium dependence, the medium was modified by replacing protamine sulfate by histone (type III-S) or α-casein dephosphorylated and by adding calcium chloride (0·4 mM), phosphatidylserine (1 μg) and diolein (0·1 μg). The phosphorylation of endogenous substrate was tested under the same conditions without substrate added. Assays for cAMP-dependent kinase were carried out using Kemptide as substrate according to Meyer *et al.*[10] CDK-1 was tested as described by Rialet and Meijer.[11] Epidermal growth factor (EGF)- and platelet-derived growth factor (PDGF)-stimulated total cellular tyrosine phosphorylation were measured according to Trinks *et al.*[12]

2.8 Fenpiclonil and fludioxonil accumulation

The uptake of [^{14}C]fenpiclonil and [^{14}C]fludioxonil in *N. crassa* was assessed by using the procedure described by Jespers *et al.*,[13] except that the mycelium was washed with water (50 ml) after incubation. In order to determine the intracellular concentration of the fungicide, the protoplasmic water content was determined from the differences between fresh weights and dry weights of mycelial pads according to the methods described by Ellis and Grindle.[14]

3 RESULTS

3.1 Effect on in-vitro growth

N. crassa was exposed to increasing concentrations of inhibitors to establish the dosage response of growth. The results indicated that the in-vitro growth of this fungus is strongly inhibited by fenpiclonil ($IC_{50} = 0{\cdot}17$ μM), fludioxonil ($IC_{50} = 0{\cdot}06$ μM), vinclozolin ($IC_{50} = 9$ μM), cyprodinil ($IC_{50} = 0{\cdot}3$ μM), fenpropidin ($IC_{50} = 13$ μM) and propiconazole ($IC_{50} = 1{\cdot}8$ μM). The effect of various phenylpyrroles is listed in Table 1.

3.2 Glycerol accumulation studies

Standard mycelial suspensions of *N. crassa* were incubated with glucose in the presence or absence of fenpiclonil (0·3 μM) up to 240 min and the glycerol in mycelial extracts was measured. The results indicated that *N. crassa* accumulated substantial quantities of glycerol when incubated with fenpiclonil, whereas a small transient accumulation, due to the addition of glucose, was observed in the control assay (Fig. 2). Comparative experiments with other fungicides (fludioxonil, cyprodinil, fenpropidin and propiconazole) indicated that the stimulation of the glycerol synthesis is specific to phenylpyrroles and dicarboximides (Fig. 3).

3.3 Deoxyglucose accumulation

Fenpiclonil, fludioxonil and vinclozolin tested up to 100 μM did not affect the uptake of radioactive 2-deoxyglucose in *N. crassa* (data not shown). This result indicated that transport-associated phosphorylation of sugars is not the primary target of the phenylpyrroles and dicarboximides in *N. crassa*.

TABLE 1
Effect of Various Phenylpyrroles on In-vitro Growth and Protein Kinase Activity (PK-III) of *Neurospora crassa*

Fungicide			IC_{50} (μM)[a]	
			In vitro growth	*PK-III*
Phenylpyrroles	X[b]	R[b]		
I (Fenpiclonil)	2,3-Cl_2	CN	0·17	2
II	3-Cl	CN	0·4	9
III (Fludioxonil)	2,3(-O-CF_2-O-)	CN	0·06	20
IV	2,4-Cl_2	CN	1·5	20
V	4-F	CN	7	30
VI	2-Cl	CN	0·9	70
VII	3-CF_3	CN	3	85
VIII	3-CH_3	CN	2·5	100
IX	4-Cl	CN	7	100
X	2-OCH_3	CN	25	>100
XI	2,3-Cl_2	$COCH_3$	>100	>100
XII	2,3-Cl_2	$CONH_2$	>100	>100
Other fungicides				
Vinclozolin			9	>100
Cyprodinil			0·3	>100
Fenpropidin			13	>100
Propiconazole			1·8	>100

[a] IC_{50} values based on two replicate experiments. Variation in repetitions less than 5%.
[b] See Fig. 1.

3.4 Effect on protein kinases

Upon chromatography on Bio-Gel TSK DEAE-5PW of the 100 000*g* supernatant of *N. crassa*, three protein kinase peaks (designated PK-I, PK-II and PK-III) have been resolved by using protamine sulfate as substrate (Fig. 4). These peaks correspond to three protein kinases of *N. crassa* (active with histone as substrate) already described in the literature, namely a c-AMP-dependent protein kinase (peak I) and two c-AMP- and calcium-independent protein kinases (peaks II and III).[15] Addition of fenpiclonil in the enzyme assay mixture revealed the phenylpyrrole sensitivity of the protein kinase PK-III and to a lower extent of PK-II. Among the tested fungicides, fenpiclonil was the most

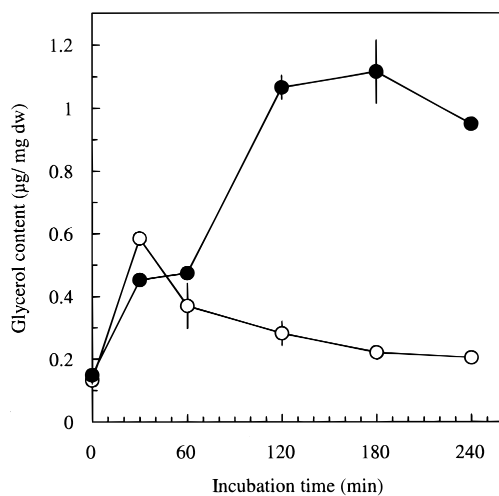

Fig. 2. Intracellular glycerol content of *Neurospora crassa* mycelium incubated (dark symbols) in the presence and (white symbols) in the absence of fenpiclonil (0·3 μM). Error bars indicate the deviation of the mean of two determinations.

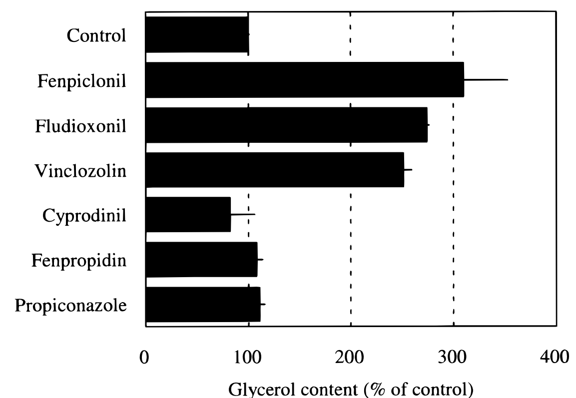

Fig. 3. Effect of fenpiclonil (0·17 μM), fludioxonil (0·06 μM), vinclozolin (9 μM), cyprodinil (0·3 μM), fenpropidin (13 μM) and propiconazole (1·8 μM) on the glycerol content of *Neurospora crassa* after incubation with glucose for 2 h. Error bars indicate the standard deviation of the mean of three determinations.

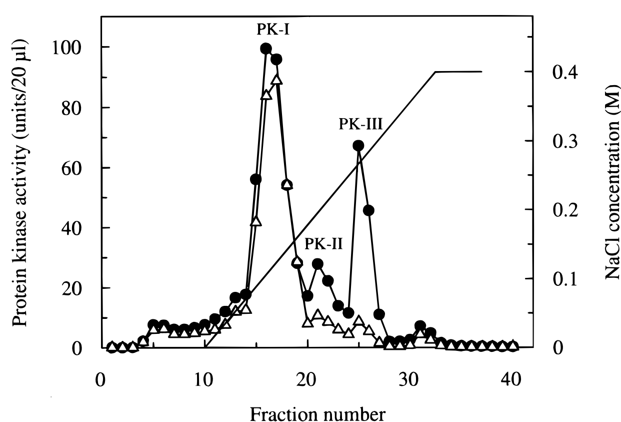

Fig. 4. Separation of protein kinases from *Neurospora crassa* by ion exchange chromatography. Protein kinase activity was measured with protamine sulfate as substrate (dark symbols) in the absence and (white symbols) in the presence of 100 μM fenpiclonil. (—) NaCl gradient (0–0·4 M).

active inhibitor of PK-III ($IC_{50} = 2$ μM) and a significant correlation has been observed between the intrinsic activity of the phenylpyrroles and their effect on the growth of *N. crassa* ($n = 12$; $r^2 = 0{\cdot}739$; $r^2_{cv} = 0{\cdot}628$) (Table 1). Comparative experiments with non-phenylpyrrole fungicides indicated that vinclozolin, cyprodinil, fenpropidin and propiconazole are completely inactive up to 100 μM, whereas the potent and rather unselective protein kinase inhibitor staurosporine inhibited the activity of PK-III ($IC_{50} = 1$ nM) strongly (data not shown).

In order to study the protein kinase PK-III in more detail, the substrate specificity and the effect of calcium and cyclic AMP on its activity have been investigated. The results indicated that protein kinase III of *N. crassa* phosphorylated protamine sulfate (and histone to a lower extent) in a calcium- and phospholipid-independent manner, whereas casein and kemptide (substrate of c-AMP-dependent kinase) were not accepted as substrate and PK-III was not autophosphorylated (data not shown).

Furthermore, comparative experiments were carried out with different protein kinases isolated from animal sources, namely the protein kinases C-α (bovine), the protein kinase C-δ (rat), the cyclin-dependent kinase CDK-1 (starfish) and the cAMP-dependent kinase (rabbit). In addition, epidermal growth factor (EGF)- and platelet-derived growth factor (PDGF)-stimulated total cellular tyrosine phosphorylation were measured in A431 human epithelial carcinoma cells and mouse BALB/c 3T3 fibroblasts, respectively. The results indicated a selective effect of the phenylpyrroles tested on the protein kinase C-δ and the cyclin-dependent kinase, whereas vinclozolin was without effect on the enzymes tested (Table 2).

3.5 Fungicide accumulation studies

According to the literature, fenpiclonil accumulates to high levels in the mycelium of *F. sulphureum* and artificial liposomes.[13] In order to determine the accumulation level of fenpiclonil and fludioxonil in *N. crassa*, mycelial suspensions were incubated with radioactive fenpiclonil and fludioxonil at different concentrations for 30 min. The radioactivity accumulated was measured after extensive washing of the mycelium with water. The results indicated that the accumulation of fenpiclonil and fludioxonil in the intracellular space of *N. crassa* was linearly related with the initial concentration of the fungicides in the medium up to a concentration of 20 μM. Based on the determination of the protoplasmic water content, the internal cell volume of *N. crassa* was assessed to be 1·88 μl mg^{-1} mycelial dry weight. The internal fungicide concentration calculated

TABLE 2
Effect of Fenpiclonil, Fludioxonil and Vinclozolin on Different Protein Kinases from Animal Cells

		IC_{50} (μM)[b]		
Enzyme[a]	*Source of enzyme*	*Fenpiclonil*	*Fludioxonil*	*Vinclozolin*
PKC-α	Bovine	>100	>100	>100
PKC-δ	Rat	3·7	36	>100
CDK-1	Starfish	10·4	27·5	>100
PKA	Rabbit	>100	>100	>100
EGF-R	Human	>100	>100	>100
PDGF-R	Mouse	>10	>10	>10

[a] PKC: Protein kinase C; CDK-1: Cyclin-dependent kinase 1; PKA: c-AMP-dependent kinase; EGF-R: Tyrosin-kinase-specific activity of epidermal growth factor receptor; PDGF-R: kinase activity of the platelet-derived growth factor receptor.
[b] IC_{50} values based on two replicate experiments. Variation in repetitions less than 5%.

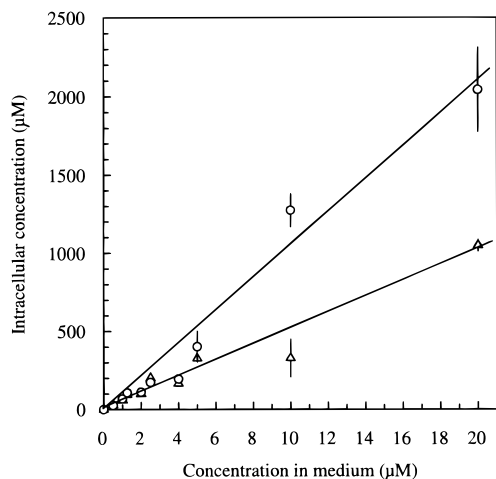

Fig. 5. Relation between the initial concentration in medium and the intracellular concentration of (△) fenpiclonil and (○) fludioxonil in mycelium of *Neurospora crassa* after incubation of the fungus with the fungicide for 30 min. Error bars indicate the standard deviation of the mean of three replicates.

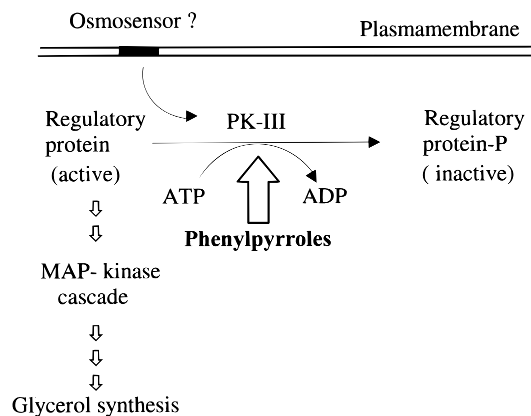

Fig. 6. Model of a possible glycerol synthesis signal transduction system in *Neurospora crassa* indicating the target site of phenylpyrroles.

indicated 109- and 49-fold accumulation of fludioxonil and fenpiclonil, respectively, as compared with the concentration in the external medium (Fig. 5).

4 DISCUSSION

As described for *Fusarium sulphureum* treated with fenpiclonil, *N. crassa* accumulated substantial quantities of glycerol when incubated with fenpiclonil, fludioxonil or vinclozolin at the concentration producing 50% growing inhibition (Figs 2 & 3).[3] Comparative experiments with other fungicides (cyprodinil, fenpropidin and propiconazole) indicated that the stimulation of glycerol synthesis by phenylpyrroles and dicarboximides is a specific process which can be compared to the response of cells under osmotic shock.[14,16–19]

In *Saccharomyces cerevisiae* Mayer ex Hansen, hypertonic stress results in the activation of a two-component system that regulates an osmosensing mitogen-activated protein (MAP) kinase cascade. An integral plasmamembrane sensor protein (first component), containing a cytosolic transmitter domain, acts as a histidine-phosphorylating autokinase when activated by an environmental signal (low osmolarity). The phosphoryl group of the activated transmitter is transferred to a regulator protein (second component). Under conditions of elevated osmotic pressure, the regulator protein remains unphosphorylated and transduces a signal that stimulates the MAP-kinase cascade, leading to the synthesis of glycerol.[18,19] In *N. crassa* and other fungi the existence of the osmosensing glycerol synthesis regulating system has never been demonstrated. However, the sensitivity of the protein kinase PK-III to phenylpyrroles (Fig. 4, Table 1) suggests the involvement of this enzyme in the regulation of the glycerol synthesis in *N. crassa* as described in Fig. 6 and a relationship between the fungitoxicity of the phenylpyrrole fungicides and their effect on PK-III.

According to our results, the isolated PK-III is calcium- and cyclic-AMP-independent and is more active with protamine sulfate than histone, casein or kemptide. Furthermore, comparative experiments with enzymes from human and animal sources revealed that PKC-δ and CDK-1 are most sensitive to phenylpyrroles (Table 2). In cells of higher organisms, PKC-δ and CDK-1 play key roles in signal transduction and cell cycle control mechanisms, respectively.[20–24] Both enzymes belong to the large family of serine/threonine-specific protein kinases, but only PKC-δ accepted protamine sulfate as substrate. In addition, among the PKC subtypes tested, PKC-δ was rather selective for the inhibition by phenylpyrroles. These compounds did not inhibit either the conventional, calcium-dependent PKC subtypes (PKC-α, -βI, -βII and -γ) or the non-conventional, calcium-independent PKC-isoforms (PKC-ε, -η and -θ) or the atypical, calcium-independent and unresponsive PKC-ζ (data not shown). Together, these data suggest a relation between PK-III and PKC-δ. In this respect it is interesting to note that Itoh *et al.*[17] have already suggested that MAP-kinase is activated in a protein kinase C-dependent manner. A possible homology between PK-III and the histidine kinase described in yeast as well as the identity of the substrate of PK-III remain unknown.[18]

Concerning the interaction of dicarboximides with the osmosensing signal transduction pathway, the lack of effect of vinclozolin on PK-III suggests either the presence of an alternative osmosensor (a different osmosensing signal transmission pathway) or an interaction between vinclozolin and another step of the same pathway. In this respect, our result is a strong indication that the target sites of dicarboximides and phenylpyrroles in the osmosensing signal transmission pathway are different. Under laboratory conditions both phenylpyrrole and dicarboximide fungicides select

for resistant mutants of *Botrytis cinerea* Pers., which are osmotically sensitive, cross-resistant to both classes of fungicides and not pathogenic; whereas under field conditions *B. cinerea* acquires resistance only against dicarboximides and the resistant isolates are not osmotically sensitive.[4,5] Furthermore Orth *et al.*[25] reported a c-AMP-dependent serine (threonine) protein kinase which confers vinclozolin resistance in laboratory mutants of the phytopathogenic fungus *Ustilago maydis* (DC) Corda. Together with our results these findings suggest that this c-AMP-dependent kinase is not the direct target site of vinclozolin, but that it is part of a resistance mechanism occuring only under laboratory conditions.

Having established the interaction between phenylpyrroles and PK-III, the key question is what the primary mode of action of these fungicides can be. Direct comparison between the IC_{50} values of phenylpyrroles on PK-III activity and growth inhibition does not allow a conclusion about the lethal effect of protein kinase inhibition. However, the correlation between the intrinsic activity and the growth inhibitory values suggests that the protein kinase PK-III is the target site of phenylpyrroles (Table 1). On the other hand, fungicide accumulation studies indicated 109- and 49-fold accumulation of fludioxonil and fenpiclonil respectively, as compared with the concentration in the external medium (Fig. 5). Considering a possible underestimation of the accumulation, due to the extensive washing of the mycelium after incubation with radiolabelled fungicide, the important intracellular concentration of fenpiclonil and fludioxonil accounts for their relatively low intrinsic activity.[13] In a recent publication Jespers & de Waard[4] suggested that the primary effect of fenpiclonil in *F. sulphureum* is the inhibition of the transport-associated phosphorylation of glucose. According to our results, the accumulation of deoxyglucose in intact cells of *N. crassa* is not inhibited by phenylpyrroles (data not shown). This result can be accounted for by a different sugar transport mechanism. As described in the literature, the sugar transport system in *N. crassa* comprises a low-affinity facilitated diffusion system and a repressible high-affinity active transport, whereas in *Fusarium*, *Aspergillus* and yeast the main accumulation system seems to be a transport-associated phosphorylation.[26–28] Finally the protein kinases have multiple functions and a protein kinase-mediated sugar transport (in *Fusarium*) is possible. The existence of a substrate site for a protein kinase in the sugar transporter of yeast supports this hypothesis.[29] The exact nature of PK-III and its implication in the regulation of the glucose transport are now under investigation in our laboratories. To our knowledge, phenylpyrroles are the first commercial fungicides that interfere with pathogens through the inhibition of a protein kinase. This finding underlines the value of fenpiclonil and fludioxonil as new elements of anti-resistance strategies. Advantage has been taken of this situation by developing a mixture of fludioxonil and cyprodinil, a compound with another new mode of action, as a sustainable solution for efficient *Botrytis* management.[30,31]

REFERENCES

1. Nevill, D., Nyfeler, R. & Sozzi, D., CGA 142705: a novel fungicide for seed treatment. *Proc. Brighton Crop Prot. Conf. Pests Dis. 1988*, **1** (1988) 65–72.
2. Gehmann, K., Nyfeler, R., Leadbeater, A. J., Nevill, D. & Sozzi, D., CGA 173506: a new phenylpyrrole fungicide for broad-spectrum disease control. *Proc. Brighton Crop Prot. Conf. Pests Dis. 1990*, **2** (1990) 399–406.
3. Jespers, Ad B. K., Davidse, L. C. & de Waard, M. A., Biochemical effects of the phenylpyrrole fungicide fenpiclonil in *Fusarium sulphureum* (Schlecht). *Pestic. Biochem. Physiol.*, **45** (1993) 116–29.
4. Jespers, Ad B. K. & de Waard, M. A., Effect of fenpiclonil on phosphorylation of glucose in *Fusarium sulphureum. Pestic. Sci.*, **44** (1995) 167–75.
5. Leroux, P., Lanen, C. & Fritz, R., Similarities in the antifungal activities of fenpiclonil, iprodione and tolclofos-methyl against *Botrytis cinerea* and *Fusarium nivale. Pestic. Sci.*, **36** (1992) 255–61.
6. Grindle, M., Isolation and characterization of vinclozolin resistant mutants of *Neurospora crassa. Trans. Brit. Mycol. Soc.*, **82** (1984) 635–43.
7. Favre, B. & Turian, G., Identification of a calcium- and phospholipid-dependent protein kinase (protein kinase C) in *Neurospora crassa. Plant Sci.*, **49** (1987) 15–21.
8. Marte, B. M., Meyer, T., Stabel, S., Gesche, J. R., Jaken, S., Fabbro, D. & Hynes, N. E., Protein kinase C and mammary cell differentiation: involvement of protein kinase Cα in the induction of β-casein expression. *Cell Growth Diff.*, **5** (1994) 239–47.
9. Azzi, L., Meijer, L., Reed, S. I., Pidikiti, R. & Tung, N. Y. L., Interaction between the cell cycle control proteins $p34^{cdc2}$ and $p9^{CKShs2}$: evidence of two cooperative binding domains in $p9^{CKShs2}$. *Eur. J. Biochem.*, **203** (1992) 353–60.
10. Meyer, T., Regenass, U., Fabro, D., Alteri, E., Rösel, J., Müller, M., Caravatti, G. & Matter, A., A derivative of staurosporine (CGP 41251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vivo* anti-tumor activity. *Int. J. Cancer.*, **43** (1989) 851–6.
11. Rialet, V. & Meijer, L., A new screening test for antimitotic compounds using the universal M phase-specific protein kinase $p34^{cdc2}$/cyclin B^{cdc13}, affinity-immobilized on $p13^{suc1}$-coated microtiter plates. *Anticancer Res.*, **11** (1991) 1581–90.
12. Trinks, U., Buchdunger, E., Furet, P., Kump, W., Mett, H., Meyer, T., Müller, M., Regenass, U., Rihs, G., Lydon, N. & Traxler, P., Dianilinophthalimides: potent and selective, ATP-competitive inhibitors of the EGF receptor protein tyrosine kinase. *J. Med. Chem.*, **37** (1994) 1015–27.
13. Jespers, Ad B. K., Davidse, L. C. & de Waard, M. A., Interaction of the phenylpyrrole fungicide fenpiclonil with membrane-related processes in *Fusarium sulphureum. Pestic. Sci.*, **40** (1994) 133–40.
14. Ellis, S. W. & Grindle, M., Effect of osmotic stress on yield and polyol content of dicarboximide-sensitive and -resistant strains of *Neurospora crassa. Mycol. Res.*, **95** (1991) 457–64.

15. Judewicz, N. D., Glikin, G. C. & Torres, H. N., Protein kinase activities in *Neurospora crassa*. *Arch. Biochem. Biophys.*, **206** (1) (1981) 87–92.
16. Kelly, J. A. & Budd, K., Polyol metabolism and osmotic adjustment in the mycelial ascomycetes *Neocosmopora vasinfecta* (E. F. Smith). *Exper. Mycol.*, **15** (1991) 55–64.
17. Itoh, T., Yamauchi, A., Miyai, A., Yokoyama, K., Kamada, T., Ueda, N. & Fujiwara, Y., Mitogen-activated protein kinase and its activator are regulated by hypertonic stress in Madin-Darby canine kidney cells. *J. Clin. Invest.*, **93** (6) (1994) 2387–92.
18. Maeda, T., Wurgler-Murphy, S. M. & Salto, H., A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* (*London*), **369** (1994) 242–5.
19. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. & Gustin, M. C., An osmosensing signal transduction pathway in yeast. *Science* (*Washington*), **259** (1993) 1760–3.
20. Parker, P. J., Protein kinase C: a structurally related family of enzymes. In *Protein kinase C: Current Concepts and Future Perspectives*, ed. D. Lester and R. Epand. Ellis Horwood Series in Biochemistry and Biotechnology, Chichester, UK, 1992, pp. 3–25.
21. Nishizuka, Y., Studies and perspectives of protein kinase C. *Science* (*Washington*), **233** (1986) 305–12.
22. Jaken, S., PKC interactions with intracellular components. In *Protein kinase C: Current Concepts and Future Perspectives*, ed. D. Lester and R. Epand. Ellis Horwood Series in Biochemistry and Biotechnology, Chichester, UK, 1992, pp. 237–55.
23. Borner, C. & Fabbro, D., PKC and cellular transformation. In *Protein kinase C: Current Concepts and Future Perspectives*, ed. D. Lester and R. Epand. Ellis Horwood Series in Biochemistry and Biotechnology, Chichester, UK, 1992, pp. 297–354.
24. Nigg, E. A., Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bioassays*, **17** (1995) 471–80.
25. Orth, A. B., Rzhetskaya, M., Pell, E. J. & Tien, M., A serine (threonine) protein kinase confers fungicide resistance in the phytopathogenic fungus *Ustilago maydis*. *Appl. Environ. Microbiol.*, **61** (6) (1995) 2341–5.
26. Romano, A. H., Sugar transport systems of baker's yeast and filamentous fungi. In *Carbohydrate Metabolism in Cultured Cells*, ed. M. J. Morgan. Plenum Press, New York, 1986, pp. 225–44.
27. Wright, J. K., Seckler, R. & Overath, P., Molecular aspects of sugars: ion co-transport. *Ann. Rev. Biochem.*, **55** (1986) 225–48.
28. Vojtek, A. B. & Fraenkel, D. G., Phosphorylation of yeast hexokinases. *Eur. J. Biochem.*, **190** (1990) 371–5.
29. Szkutnicka, K., Tschopp, J. F. Andrews, L. & Cirillo, V. P., Sequence and structure of the yeast galactose transporter. *J. Bacteriol.*, **171** (8) (1989) 4486–93.
30. Forster, B., Heye, U., Pillonel, Ch. & Staub, T., Methods to determine the sensitivity to cyprodinil, a new broad spectrum fungicide. In *Botrytis cinerea. 11th Symposium Rheinardsbrunn* (*Germany*) **00** (1995) 365–76.
31. Masner, P., Muster, P. & Schmid, J., Possible methionine biosynthesis inhibition by pyrimidinamine fungicides. *Pestic. Sci.*, **42** (1994) 163–6.