Antibiotics in the Chemical Communication of Fungi

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Z. Naturforsch. **59 c**, 816–823 (2004); received September 8, 2004

In dual cultures *Oudemansiella mucida* and *Xerula melanotricha* (basidiomycetes) react to the presence of living *Penicillium notatum* or *P. turbatum* with an increased production of strobilurin A (**1**) or X (**2**). *P. notatum* in turn reacts to the two basidiomycetes or their antibiotic strobilurin A alone with the production of *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**) or chrysogine (**4**). *P. melinii* and *P. urticae* overgrow *O. mucida* due to complete resistance to strobilurin A. *P. brevicompactum*, *P. citrinum*, *P. janczewskii* and the other *Penicillium* strains are all sensitive but apparently do not induce *O. mucida* to produce the amounts of strobilurin A needed to inhibit their growth.

Key words: Strobilurin, Antifungal Antibiotics, Chemical Communication between Fungi

Introduction

Saprophytic and soil inhabiting microorganisms are prolific producers of secondary metabolites with elaborate structures but in many cases with no obvious function for the producers. This is especially true for the numerous compounds with no apparent biological activities. For these a role in the still ongoing evolution of biochemical pathways in an "evolutionary playground" has been proposed by Zähner *et al.* (1983). On the other hand, however, it is conceivable that metabolites with antimicrobial activities might play a role in securing a substrate or habitat against competitors. In nature microbial communities are generally complex and extremely difficult to analyze because many of the participants form microcolonies or can not be grown in culture thus prohibiting a direct access to their metabolites. Among the antibiotic producing microorganisms basidiomycetes offer the advantage that most species produce conspicuous fruiting bodies allowing to trace their mycelia and metabolites in their natural substrates and habitats. In preceding investigations, it was found that among other basidiomycetes *Mycena tintinnabulum* in its natural habitat produced strobilurin D in amounts sufficient to suppress the growth of competing fungi *e.g. Penicillium* ssp. (Engler *et al.*, 1998a, b).

Secondary metabolism requires precursors and energy which are diverted from primary metabolism and thus cannot be used for growth and maintenance. It therefore would be advantageous to produce an antibiotic only when needed, that is in the presence of competitors. In order to investigate this intriguing possibility we analyzed dual cultures of *Oudemansiella mucida*, *Strobilurus tenacellus*, *Strobilurus conigenoides* and *Xerula melanotricha*, all producers of strobilurins and oudemansins (Anke *et al.*, 1977, 1979, 1983; Vondracek *et al.*, 1983), and several *Penicillium* species. The strobilurins and oudemansins belong to an interesting group of antifungal antibiotics, the biological activity of which depends on the presence of an (*E*)-β-methoxyacrylate moiety. The strobilurins and oudemansins selectively inhibit the ubiquinol-oxidation center of the mitochondrial bc_1 complex. Synthetic analogues have become widely used agricultural fungicides (for reviews see Anke and Steglich, 1999; Sauter *et al.*, 1999).

Material and Methods

Fungi

O. mucida 96006, *O. mucida* 76042, *S. conigenoides* 83070, *S. tenacellus* 80012, *X. melanotricha* 78215 and *P. notatum* KL1 are strains from our culture collection. *P. notatum* Flem. TEA 015 is the original strain isolated by Fleming in 1928; it was obtained from Dr. R. W. S. Weber, Kaiserslautern. *P. melinii* ATCC 13351, *P. turbatum* ATCC

0939–5075/2004/1100–0816 $ 06.00

28797 and *P. urticae* ATCC 48165 were bought from LGC Promochem (Wesel, Germany), *P. brevicompactum* DSM 2215 from DSMZ GmbH (Braunschweig, Germany), and *P. janczewskii* CBS221.28 from CBS (Baarn, The Netherlands). *P. citrinum* (048-93), *Penicillium* ssp. SE 14-93, HA062-92 and HA009-93 were gifts of Prof. H. Anke, IBWF.

Fermentation and isolation

Dual cultures of basidiomycetes and *Penicillium* strains

Basidiomycetes were maintained on agar slants with YMG medium (g/l): yeast extract (4), malt extract (10), glucose (4), pH 5.6. For submerged cultivation 250 ml of YMG medium were inoculated with 25 pieces of mycelium taken from one agar plate and incubated for 5 d on a rotary shaker at 22 °C and 120 rpm. 10 and 15 ml of that culture were used to inoculate 1 or 2 l of cornmeal medium (g/l): cornmeal (10), glucose (10), KH_2PO_4 (1.5), KCl (0.5), $NaNO_3$ (0.5), $MgSO_4 \times 7\ H_2O$ (0.5), pH 5.8, in 2 or 5 l Erlenmeyer flasks. After 4 d of growth at 22 °C and 120 rpm on a rotary shaker 2×10^8 spores/l or 10 mg of vegetative mycelia/l of the *Penicillium* strains were added. The dual cultures were grown until the free glucose in the medium was used up. At this time strobilurin-synthesis stopped and the *Penicillium* strains started to grow conspicuously.

Strobilurin A (**1**), strobilurin X (**2**), *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**) and chrysogine (**4**) from dual cultures

During fermentation 100 ml samples were taken every other day. The culture fluid was separated from the mycelia by filtration and then extracted two times with an equal volume of ethyl acetate. The mycelia were extracted with 200 ml of methanol/acetone (1:1). The extracts were evaporated to dryness and then taken up in 1 ml of methanol. 25 μl of these solutions were assayed by analytical HPLC for their content of strobilurin A (**1**), strobilurin X (**2**), *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**) and chrysogine (**4**), respectively. **1** and **2** were identified by HPLC-MS and comparison with authentic samples (see Fig. 1 for structures).

1, **3** and **4** containing extracts were applied onto a silica gel column (Merck 60, 18 × 2.5 cm). Elution with cyclohexane/ethyl acetate (9:1) yielded **1**, while **3** and **4** were eluted with ethyl acetate. Pure **1** was obtained by preparative HPLC (Shimadzu) on Nucleosil Diol (7 μm; column 250 × 25 mm; Merck; flow 5 ml/min; elution with cyclohexane/*tert*-butyl methyl ether, 84:16 v/v; yield: 5,9 mg/l). **3** and **4** were obtained by preparative HPLC (Jasco) on Nucleosil RP18 (5 μm; column 250 × 25 mm; Merck, Darmstadt, Germany; flow 7.5 ml/min; elution with water/methanol, 45:55 v/v and 38:62 v/v, yielding **3** and **4**; yield: 2 mg/l and 4,2 mg/l).

Strobilurin A acid (**5**) from *Penicillium urticae* ATCC 48165

P. urticae was maintained on agar slants with YMG medium. For submerged cultivation 1 l of cornmeal medium was inoculated from an agar plate and incubated on a rotary shaker at 22 °C and 120 rpm. After 48 h 10 mg of pure strobilurin A (**1**) were added and the culture incubated for another 24 h. Then, the culture fluid was separated from the mycelia by filtration and extracted two times with an equal volume of ethyl acetate. The extract was evaporated to dryness, taken up in 1 ml of methanol and assayed for the content of strobilurin A (**1**), strobilurin A acid (**5**) (for structure see Fig. 4) and other metabolites.

Purification of **5** was achieved by preparative HPLC (Jasco) on Nucleodur RP18 (7 μm; column 250 × 21 mm; Macherey-Nagel; flow 28 ml/min; elution with water/acetonitril, 35:65 v/v; yield: 3 mg/l).

Analytical methods

For analytical HPLC a Hewlett-Packard 1090 series II instrument or a HP Series 1100 LCD-MSD HPLC-MS set-up (Hewlett-Packard, Waldbronn, Germany), for preparative HPLC a Shimadzu Model SCL 10 AVP with a multiwavelength-detector SPD-M10 AVP (Shimadzu, Duisburg, Germany) or a Jasco Model PU-980 with a multiwavelength-detector MD-910 (Jasco, Groß-Umstadt, Germany) were used. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using

sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra were recorded with a LC-MS (HP 1100; APCI, negative mode) and a Jeol SX102 spectrometer, while the UV and the IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer. The optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Biological assays

Antifungal, antibacterial (Anke *et al.*, 1989), cytotoxic (Zapf *et al.*, 1995), phytotoxic or nematicidal (Stadler *et al.*, 1993; Anke *et al.*, 1995) activities were assayed as described previously.

Results and Discussion

*Structure elucidation of N-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**), chrysogine (**4**) and strobilurin A acid (**5**)*

N-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**): $[\alpha]_D^{22}$ + 114 to + 116° (in benzene). – UV (MeOH): λ_{max} = 215, 251 and 291 nm (log ε 3.83, 3.59 and 3.16). – IR (KBr): ν = 3355, 1664, 1590, 1524, 1450, 1393, 1304, 1122, 758 and 630 cm^{-1}. – As **3** is insoluble in chloroform the NMR experiments were recorded in DMSO-d_6, and the 1D data are given in Table I. The corresponding 2D NMR correlations were observed for **3** as for **4**, and the comparison with literature data identified the structure of the product.

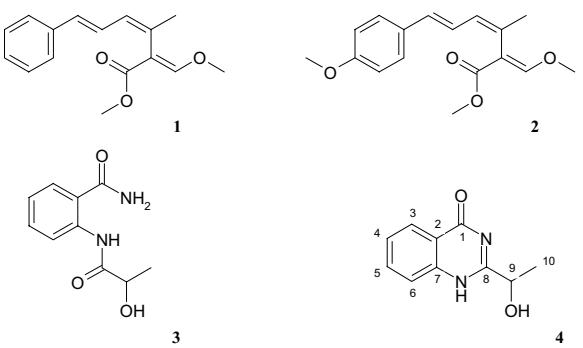

Fig. 1. Structures of strobilurin A (**1**), strobilurin X (**2**), *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**) and chrysogine (**4**).

Chrysogine (**4**): $[\alpha]_D^{22}$ + 183 to + 186° (in ethyl acetate). – UV (MeOH): λ_{max} = 224, 263 and 302 nm (log ε 4.05, 3.56 and 3.28). – IR (KBr): ν = 3424, 2930, 1677, 1610, 1470, 1376, 1332, 1252, 1139, 984 and 775 cm^{-1}. – The structure of chrysogine (**4**) was determined by spectroscopic techniques. The

Table I. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (for **3**) and chrysogine (for **4**). The spectra were recorded in DMSO-d_6 (**3**) and $CDCl_3$ (**4**), and the chemical shifts (δ) are given in ppm relative to the solvent signals (7.26 ppm for $CHCl_3$ and 2.50 for DMSO-d_6 in ^{1}H NMR and 77.0 for $CDCl_3$ and 39.51 for DMSO-d_6 in ^{13}C NMR). The coupling constants *J* are given in Hz. The multiplicity of the ^{13}C NMR signals were determined from the HMQC data.

C	**3** ^{1}H (δ; mult.; *J*)	^{13}C (δ; mult.)	**4** ^{1}H (δ; mult.; *J*)	^{13}C (δ; mult.)
1	–	162.3; s	–	170.2; s
2		121.0; s	–	120.9; s
3	8.21; d; 8.0	126.4; d	7.74; dd; 1.4, 7.8	128.4; d
4	7.44; t; 8.0	126.7; d	7.11; dt; 1.1, 7.8	122.3; d
5	7.73; t; 8.0	134.8; d	7.45; ddd; 1.4, 7.8, 8.4	131.8; d
6	7.63; d; 8.0	126.7; d	8.57; dd; 1.1, 8.4	119.8; d
7	–	148.5; s	–	138.7; s
8	–	159.0; s	–	174.1; s
9	4.72; q; 6.7	67.0; d	4.10; dq; 3.8, 6.8	67.9; d
10	1.55; d; 6.8	22.2; q	1.30; d; 6.8	20.8; q
1-NH[a]	–		8.14; brs	
1-NH[b]	–		7.57; brs	
7-NH	2.85; brs		11.98; s	
9-OH	2.77; brs		5.95; d; 3.8	

[a,b] Two discernible signals.

LC-MS data clearly showed that the molecular weight of the compound is 190, and the observation of the signals for 10 carbon atoms and 10 protons in the NMR spectra recorded in $CDCl_3$ indicated that the elemental composition is $C_{10}H_{10}N_2O_2$. The 1D NMR data of **4** are given in Table I, and are in agreement with those already published. For *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**), the LC-MS data suggested that it weighs 208, and the spectroscopic resemblance between the two compounds suggested that it is a hydrated version of **4**. This was supported by the presence of signals for 10 carbon atoms in the ^{13}C NMR spectrum and 12 protons in the ^1H NMR spectrum. The 1D NMR data of **3** and **4** are given in Table I, and are in agreement with those already published (Dai *et al.*, 1993; Niederer *et al.*, 1992).

Strobilurin A acid (**5**): UV (MeOH): λ_{max} = 229 and 294 nm (log ε 4.16 and 4.23). – IR (KBr): ν = 3430, 2934, 1678, 1624, 1450, 1384, 1241, 1129, 962, 750 and 693 cm^{-1}. – The structure was determined by NMR in $CDCl_3$ (solvent signals at 7.27 and 77.0, respectively). The presence of a monosubstituted benzene ring was evident from the proton signals at 7.35 ppm [d, J = 7.6 Hz, integrating for 2 protons, 1-H (numbering according to Steglich *et al.*, 1997)], 7.28 ppm (J = 7 Hz, integrating for 2 protons, 2-H) and 7.19 ppm (t, J = 7 Hz, integrating for 1 proton, 1-H), and the corresponding carbon signals were obtained from a HMQC spectrum as 126.3 ppm (C-1), 128.5 ppm (C-2) and 127.2 ppm (C-3). C-6 appeared at 137.8 ppm, indicated by the HMBC correlation between 2-H and this signal. 7-H appeared as a doublet at 6.51 ppm with a coupling constant (15.5 Hz) characteritic for a *trans* double bond, 8-H was a double doublet at 6.64 ppm while 9-H was a doublet at 6.30 ppm (J = 10.6 Hz). 8-H gave strong COSY correlations to both 7-H and 9-H. The corresponding carbon signals we are found at 131.5 ppm (C-7), 126.4 ppm (C-8) and 130.2 ppm (C-9), while C-10 appeared at 130.9 ppm. H-8 gave HMBC correlations to both C-6 and C-10, while the C-10 methyl substituent (2.00/23.5 ppm) gave HMBC correlations to C-9, C-10 and C-11. C-11, with the characteristic low chemical shift for an enol beta-carbon atom (110.0 ppm), showed a HMBC correlation with 13-H, appearing as a singlet at 7.52 ppm (the corresponding carbon signal for C-13 is found at 160.6 ppm). The methoxy substituent (3.88/62.1 ppm) at C-13 was demonstrated by the HMBC correlation from the methoxy protons to C-13. 13-H, and to a small extent also the C-10 methyl protons, gave a HMBC correlation to C-12 (171.0), which was a free carboxylic acid. The NOESY correlations observed were all in agreement with the structure presented.

Effect of different Penicillium species and strains on strobilurin production and growth of Oudemansiella mucida 96006

Dual cultures of *O. mucida* 96006 and *Penicillium* strains were grown in cornmeal medium as described in Materials and Methods. The optimal time for the addition of the *Penicillia* was 96 h after inoculation of the *O. mucida* 96006 cultures. As shown in Fig. 2, 2×10^8/l of *P. notatum* KL1 spores or 10 mg/l of vegetative mycelia were able to induce strobilurin A (**1**) production over 20 fold.

Next, the influence of other *Penicillium* species on the strobilurin production of *O. mucida* 96006 was tested. Under the same fermentation conditions the addition of *P. notatum* Flem. TEA 015 spores lead to an increased strobilurin A (**1**) production comparable to the effect caused by *P. notatum* KL1 spores. In the presence of *P. turbatum* ATCC 28797 a remarkable increase in strobilurin X (**2**) production was observed (see Fig. 3). Apparently, the induction of strobilurin production in *O. mucida* 96006 is, with regard to these *Penicillia*, neither strain nor species specific. On the other hand different *Penicillium* strains induce different strobilurin derivatives.

P. brevicompactum DSM 2215, *P. citrinum* 048-93, *P. janczewskii* CBS221.28, *P. melinii* ATCC 13351, *P. urticae* ATCC 48165 and the *Penicillium* ssp. SE 14-93, HA062-92 and HA009-93 grew very fast and *O. mucida* 96006 was not able to out compete them.

The antifungal activity of strobilurin A (**1**) on all *Penicillium* strains used in this study was tested. While *P. melinii* and *P. urticae* were highly resistant to strobilurin A (MIC's 100 μg/ml) *P. brevicompactum*, *P. citrinum*, *P. janczewskii* and the other *Penicillium* strains were all sensitive but apparently did not induce *O. mucida* to produce the amounts of strobilurin A needed to inhibit their growth. In the case of *P. urticae* ATCC 48165 the reason for its resistance was a rapid degradation of strobilurin A (**1**) to the inactive acid **5** (100% within 24 h; see Fig. 4). This is interesting since in many cases strobilurin resistance has been found to be due to the replacement of amino acids within

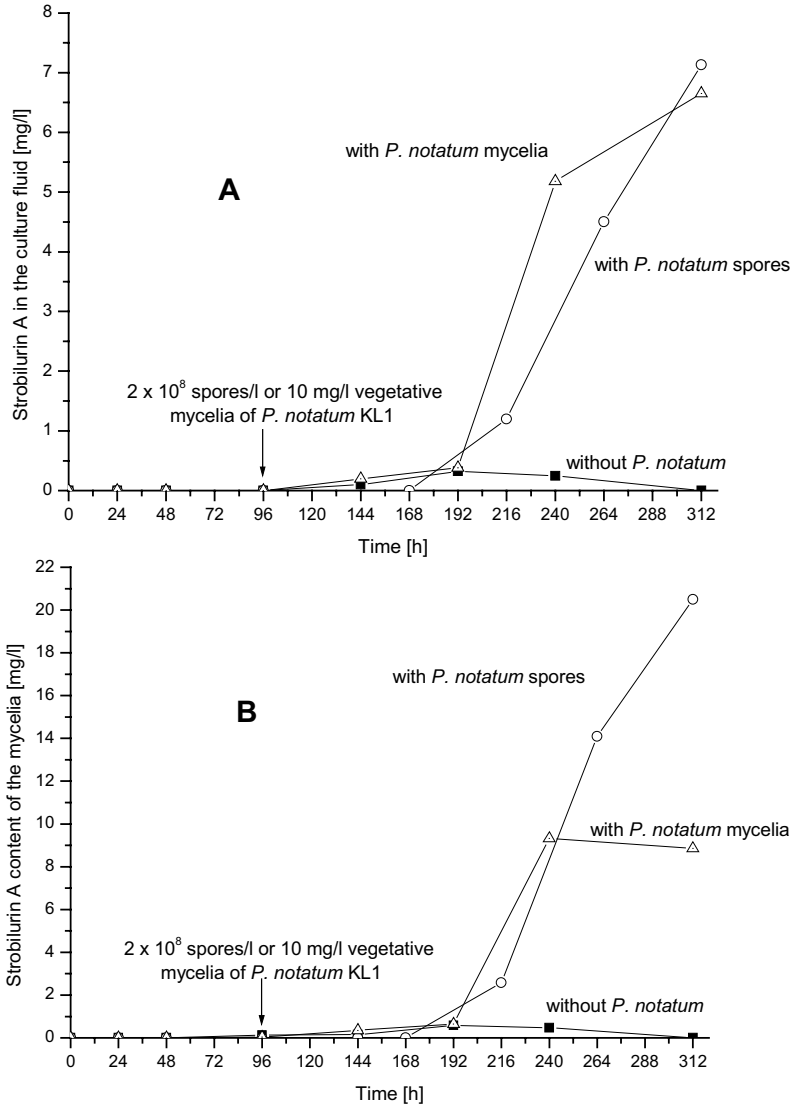

Fig. 2. Induction of strobilurin A production in *O. mucida* 96006 by *P. notatum* KL1 spores or mycelia. The strobilurin A content is given for the culture fluid (A) and mycelia (B).

the binding side in the mitochondrial bc_1 comlex (Kraiczy *et al.*, 1996).

Effect of P. notatum KL1 on other strobilurin producers

Under the same fermentation conditions as described above the addition of *P. notatum* KL1 spores led to an increased strobilurin A (**1**) production in *X. melanotricha* strain 78215 (see Fig. 5), whereas no effect was detected in *S. conigenoides* 83070, *S. tenacellus* 80012 and *O. mucida* 76042. In all these cases no growth of *P. notatum* KL1 could be observed in the dual cultures. Apparently, the amounts of strobilurin A (**1**) produced constitutively (culture fluid: 1–5 mg/l; mycelia: 3–20 mg/l) were sufficient to inhibit *P. notatum* KL1 effectively.

*Effect of O. mucida, X. melanotricha and strobilurin A on the production of N-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**) and chrysogine (**4**) by P. notatum KL1*

While *O. mucida* 96006 reacts in the presence of *Penicillium* spp. with an increased strobilurin A (**1**)

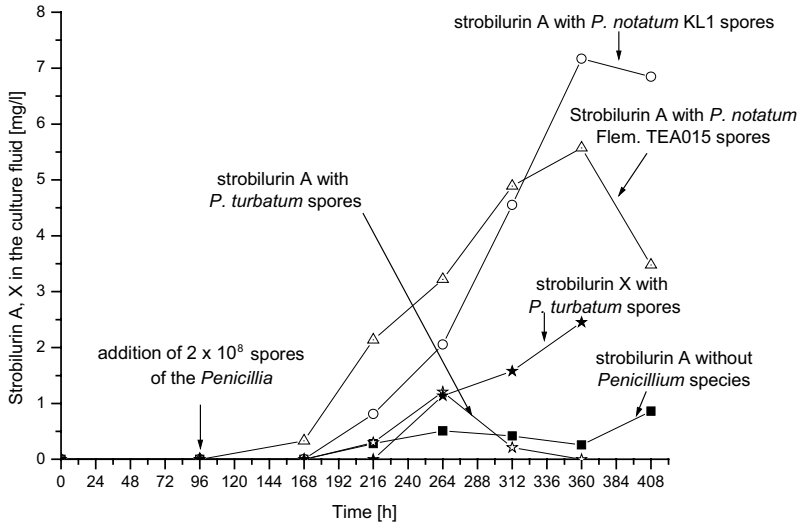

Fig. 3. Induction of strobilurin production in *O. mucida* 96006 by *P. notatum* KL1, *P. notatum* Flem TEA015 and *P. turbatum* ATCC 28797.

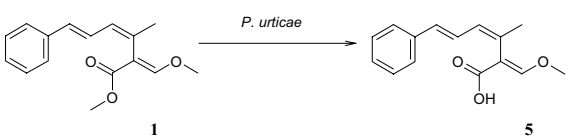

Fig. 4. Degradation of strobilurin A (**1**) to its inactive acid **5** by *P. urticae* ATCC 48165 within 24 h. Both compounds were analyzed by HPLC-DAD/MS.

production, *P. notatum* KL1 in turn reacts with an increased synthesis of *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**), a non antifungal metabolite. This mutual induction of secondary metabolites in dual culture is shown in Fig. 6. The same effect could be observed in dual cultures of *X. melanotricha* 78215 with *P. notatum* KL1 (see Fig. 5). As shown in Fig. 7 *P. notatum* KL1 mono cultures react to the addition of pure strobilurin A with an increased synthesis of chrysogine (**4**), presumably derived from the biosynthetic precur-

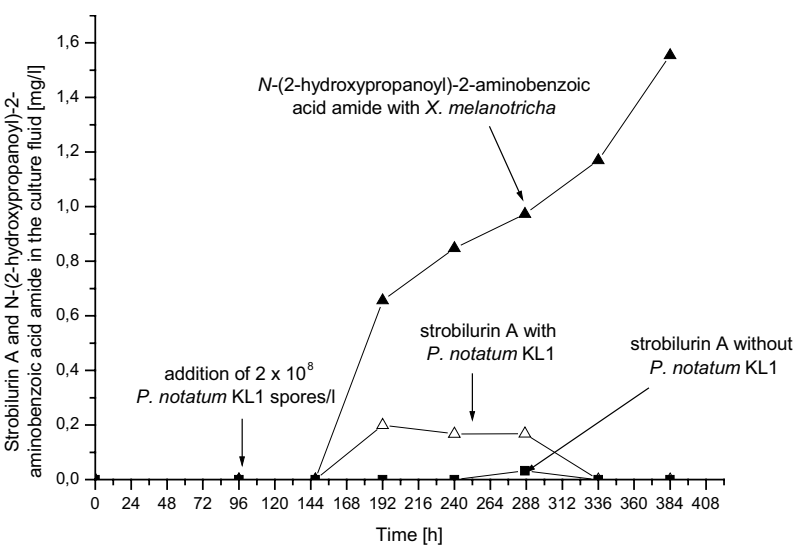

Fig. 5. Induction of strobilurin A (**1**) production by *P. notatum* KL1 and induction of *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**) production by *X. melanotricha* 78215

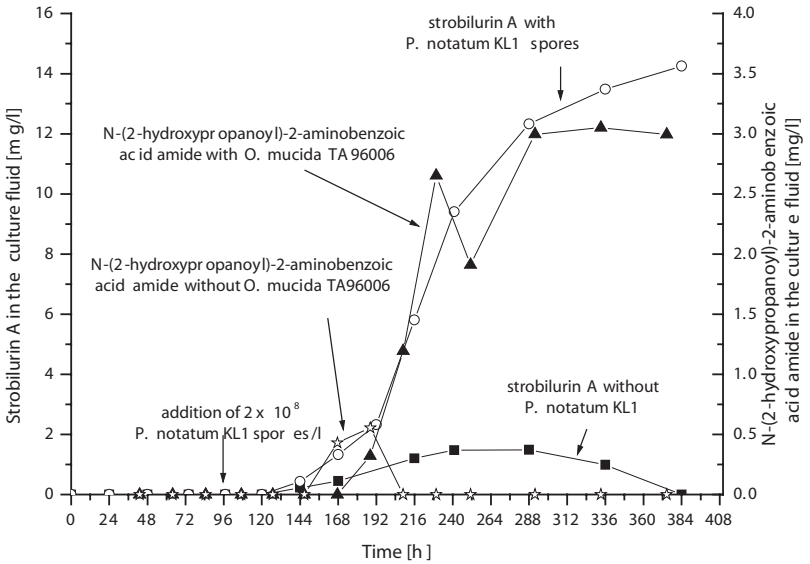

Fig. 6. Induction of strobilurin A (**1**) production by *P. notatum* KL1 and induction of *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**) production by *O. mucida* 96006.

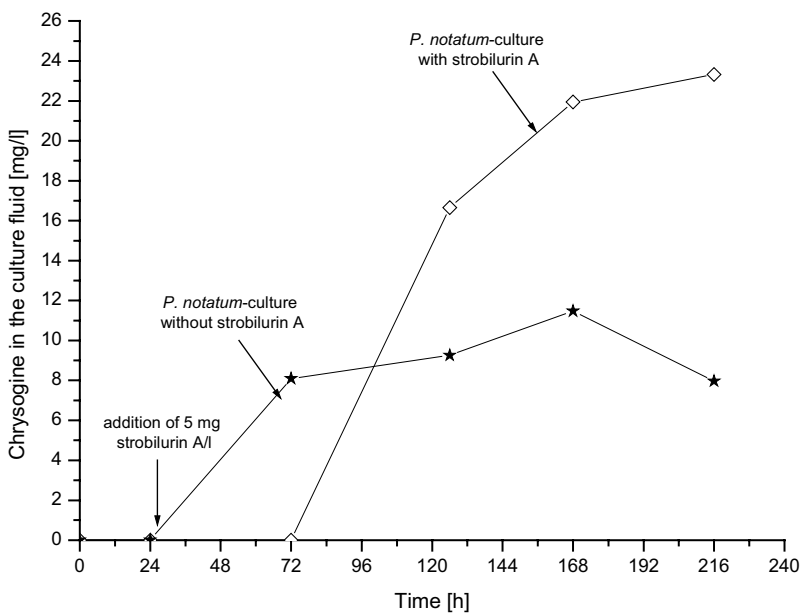

Fig. 7. Induction of chrysogine (**4**) production in *P. notatum* KL1 due to strobilurin A. 2.1×10^8 spores/l of *P. notatum* KL1 were used to inoculate 1 l of cornmeal medium. After 24 on a rotary shaker at 22 °C and 120 rpm 5 mg of strobilurin A (**1**) was added. 100 ml samples were taken every other day and analyzed by HPLC.

sor **3**. In dual cultures with *O. mucida* 96006 chrysogine was detected only in trace amounts.

In *O. mucida* 96006 mono cultures the addition of purified *N*-(2-hydroxypropanoyl)-2-aminobenzoic acid amide (**3**) has no effect on strobilurin A (**1**) synthesis. Since in our biological assays no antifungal, antibacterial, cytotoxic, phytotoxic or nematicidal activity could be detected for **3** its possible role for the producing fungus remains elusive. The same seems to be true for **4** which exhibited no antimicrobial and only very weak cytotoxic activities [IC_{80} = 50 μg/ml for Jurkat cells (DSMZ ACC 282)].

Apparently non-parasitic saprophytic fungi sharing the same substrate have developed mechanisms to react to the presence of competitors. In dual cultures *O. mucida* and *P. notatum* react by an increased synthesis of secondary metabolites.

In *P. notatum* the inducing signal is an antifungal agent produced by the other fungus growing in the same culture. How *O. mucida* becomes aware of the presence of *P. notatum* is not clear. So far no *Penicillium* metabolite could be found to be involved in the induction of strobilurin A synthesis. Filter sterilized culture fluid of *P. notatum*, dead spores or mycelia or methanol extracts thereof all had no effect on *O. mucida.*

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft for financial support.

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