Chemotaxonomy of *Pochonia* and other conidial fungi with *Verticillium*-like anamorphs

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Pochonins are antiviral and antiparasitic resorcylic acid lactones (RAL) structurally related to monorden. They were found in the invertebrate-associated fungus *Pochonia chlamydosporia*. Their production and distribution was studied by means of High Performance Liquid Chromatography with UV-visual and mass spectrometric detection (HPLC-UV/Vis and HPLC-MS) in cultures of *Pochonia* species and further conidial fungi with *Verticillium*-like anamorphs that had until recently been included in *Verticillium* sect. *Prostrata*. The results support the recent generic segregation by Gams, Zare and co-workers because pochonins were found to occur exclusively in species of the genus *Pochonia*. With few exceptions, the production of RAL appeared to be a rather constant feature in cultures of *P. chlamydosporia* from around the world. According to preliminary results, secondary metabolite profiles in strains of allied genera such as *Lecanicillium*, *Haptocillium* and *Rotiferophthora* are different from those encountered in *Pochonia*. The alkaloid pseurotin A was found as main metabolite in several of the *P. chlamydosporia* isolates examined. As inferred from HPLC profiling data, strains of *P. suchlasporia* clustered into at least three chemotypes. The ex-type strain of *P. suchlasporia* var. *catenata* produced monorden, while several other strains produced metabolites whose HPLC-UV and HPLC-MS characteristics were similar to the mycotoxins, aurovertin B and citreoviridin A. Yet different metabolites were detected in a third chemotype of *P. suchlasporia*. Differences in secondary metabolite profiles were also found in two strains of *P. bulbillosa*. While the ex-type strain was found devoid of all aforementioned compounds, CBS 247.68 contained the aurovertin-related metabolites detected in part of the *P. suchlasporia* isolates. The sequence of the ITS nrDNA of CBS 247.68 was different from that of the type strain but identical to the sequences of *P. suchlasporia* var. *catenata*. Several strains of the latter variety showed identical sequences, despite considerable variations in their HPLC metabolite profiles. Minisatellite PCR fingerprinting was found useful to segregate *Pochonia* at species and strain level, pointing toward the existence of further, cryptic species. The possible chemotaxonomical importance and ecological functions of secondary metabolites in these fungi is discussed.

The anamorphic genus *Verticillium* Nees is known to be heterogeneous, comprising fungi with affiliations to Clavicipitaceae and Hypocreaceae inside Hypocreales, and Phyllachorales. They had been divided into four sections and a residual group, mainly based on morphological characters (GAMS & VAN ZAAYEN 1982). Sections *Verticillium* and *Nigrescentia* comprise many saprophytic and plant-associated fungi (among the latter several causal agents of plant diseases). Certain species that attack or colonise a broad range of invertebrates and fungi, implying potential as biological control agents (KERRY & DE LEIJ 1991, KERRY 1995, STENZEL & ANDERSCH 1992), were until recently included in section *Prostrata*. This section comprises species of the Clavicipitaceae. Because of their obvious differences in lifestyle, host range, morphological and cultural characters, parameters that would allow for a more natural classification of *Verticillium* remained to be established.

Molecular studies by MESSNER et al. (1996) had already pointed toward the polyphyletic status of *Verticillium*. More recently, molecular and morphological features of *Verticillium* and allies with emphasis on sect. *Prostrata* were examined (ZARE, GAMS & CULHAM 2000), later including various other Clavicipitaceae (SUNG et al. 2001), and finally resulting in a new generic concept for sect. *Prostrata* (GAMS & ZARE 2001). The majority of these species were thus transferred to *Lecanicillium* W. Gams & Zare. *Cordyceps militaris* (L.: Fr.) Link is so far the only teleomorph known to be associated with *Lecanicillium*. *Simplicillium* W. Gams & Zare was delineated as a sister group, comprising four species with affinities to *Torrubiella* Boudier (ZARE & GAMS 2001a). As type species of these new genera, the tropical *Lecanicillium lecanii* (Zimmerm.) Zare & W. Gams and *Simplicillium lanosoniveum* (Van Beyma) Zare & W. Gams, respectively, were designated. While *Lecanicillium* appeared paraphyletic as inferred from cladistic analyses of ITS, SSU and LSU nrDNA regions, *Simplicillium* and further groups within *Verticillium* sect. *Pro-*

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strata were recognised as monophyletic. Basically in accordance with the aforementioned characters, several further genera were distinguished. One of those is *Haptocillium* W. Gams & Zare (ZARE & GAMS 2001b), which comprises parasites of free-living nematodes that form adhesive conidia and is typified by *Haptocillium balanoides* (Drechsler) Zare & W. Gams. The genus *Pochonia* A.C. Batista & Fonseca was newly circumscribed to accommodate the *Verticillium*-like parasites of nematode eggs and cysts. These fungi, some of which may also colonise mollusc eggs or rotifers, usually form dictyochlamydospores or at least characteristic swollen hyphae besides their verticillate stages. They were previously also referred to as *Dictyoarthrinopsis* A. C. Batista & Ciferri, *Stemphyliopsis* Petch and, most frequently, *Diheterospora* Kamyschko. The teleomorph of the type species, *P. chlamydosporia* (Goddard) Zare & W. Gams, was identified as *Cordyceps chlamydosporia* Evans. The confirmation of morphological data by molecular methods finally led to a user-friendly key to *Pochonia* spp., which is based on cultural and morphological characters. *Verticillium*-like parasites of rotifers that produce flat dictyochlamydospores and appear to be related to *Pochonia* as deduced from morphological similarities and molecular data, remained within the genus *Rotiferophthora* Barron (ZARE, GAMS & EVANS 2001). Several species such as *V. epiphytum* Hansford were retained within the "residual group of *Verticillium*".

Fungi adapted to different lifestyles, in particular those displaying manifold forms of mutualism and parasitism, are likely to have developed chemical agents (i.e., secondary metabolites) to overcome their host's defence or otherwise act as signals in their natural habitats. Numerous examples in the literature revealed that plant parasites produce a great variety of phytotoxic agents [see, e.g., BOTTALICO & LOGRIECO (1998) for *Alternaria* metabolites]. Nematode-destroying fungi were shown to produce nematicidal compounds and antibiotics that have so far not been encountered in other fungi (STADLER et al. 1993b, 1994a and 1994b, ANKE et al. 1995). Likewise, many insecticidal agents have already been discovered from insect-associated fungi (see overview by ANKE & STERNER 2002). Nonetheless, the overproduction of mycotoxins or other secondary metabolites with unfavourable biological effects by fungal biopesticides may actually constitute a prohibitive environmental risk for their development, large-scale field application and marketing. Therefore, the secondary metabolites of candidate biocontrol agents need to be identified and studied for biological activities. Hence, it appeared promising – from a practical standpoint as well as for further clarification of the taxonomy and biology of these fungi – to further elucidate the role of their secondary metabolites as marker compounds. No comprehensive chemotaxonomic studies on secondary metabolites were hitherto carried out with mycophilic or invertebrate-associated clavicipitaceous fungi. Although in some cases a characteristic pigmentation of the cultures (relating to secondary metabolism) was observed in particular species, few attempts have been made so far to actually identify these colouring matters So far only Oosporein was identified from a fungus that currently belongs to *Simplicillium* (treated as *V. lamellicola* by WAINWRIGHT, BETTS & TEALE 1986). A recent study of the antimalarial metabolites of the newly described lepidopteran parasite *Cordyceps pseudomilitaris* Hywel-Jones & Sivichai (JATURAPAT et al. 2001) revealed bioxanthracenes as active principles. Similar compounds had been reported earlier from a *Verticillium* species by TOKI et al. (1992) as modulators of the N-acetyl-D-aspartate receptor. The occurrence of these aromatic compounds was reported to be a constant feature in strains of *C. pseudomilitaris*. These data suggested that it may be possible to further clarify the anamorph-teleomorph relationships in clavicipitaceous fungi by the employment of secondary metabolite profiles. Recently we encountered some well-known and several novel biologically active compounds in cultures of strain P0297 (**1** – **12** in Fig. 1), which gave rise to study their occurrence and chemotaxonomical significance by High Performance Liquid Chromatography (**HPLC**) profiling.

The discovery and characterisation of compounds **1** – **12** was reported separately (HELLWIG et al. 2003), while the results of an intensified evaluation of further strains and species will be presented subsequently. Concurrently, the value of Minisatellite (**MIS**) PCR to discriminate these fungi at species and strain level was evaluated. MIS sequence motifs constitute conventional oligonucleotide probes in hybridisation-based DNA fingerprinting. This technique was, previously employed by LIECKFELD, MEYER & BÖRNER (1993), MEYER (1996) and STADLER et al. (2001) to generate specific PCR fingerprint patterns.

Based on the outcome of these studies, we tried to evaluate whether a chemotaxonomical approach would confirm recent results based on complementary methodology and help to achieve a better understanding as to the ecological role of secondary metabolites in this important group of clavicipitaceous ascomycetes.

Experimental

Unless stated otherwise, culture media ingredients and solvents for chromatography and spectroscopy were obtained from Merck (Darmstadt, Germany), while solid chemicals, including reference compounds such as aurovertin B, citreoviridin A and helvolic acid, were provided by Sigma-Aldrich (Deisenhofen, Germany).

A. Organisms and Fermentation

In the present study we compared representative strains on their metabolite profiles and PCR characteristics. For this purpose, we included several of the cultures that were recently characterised by Gams, Zare and co-workers (ZARE, GAMS & CULHAM 2000, SUNG et al. 2001, ZARE, GAMS & EVANS 2001, ZARE & GAMS 2001a, ZARE & GAMS 2001b). Aside from their

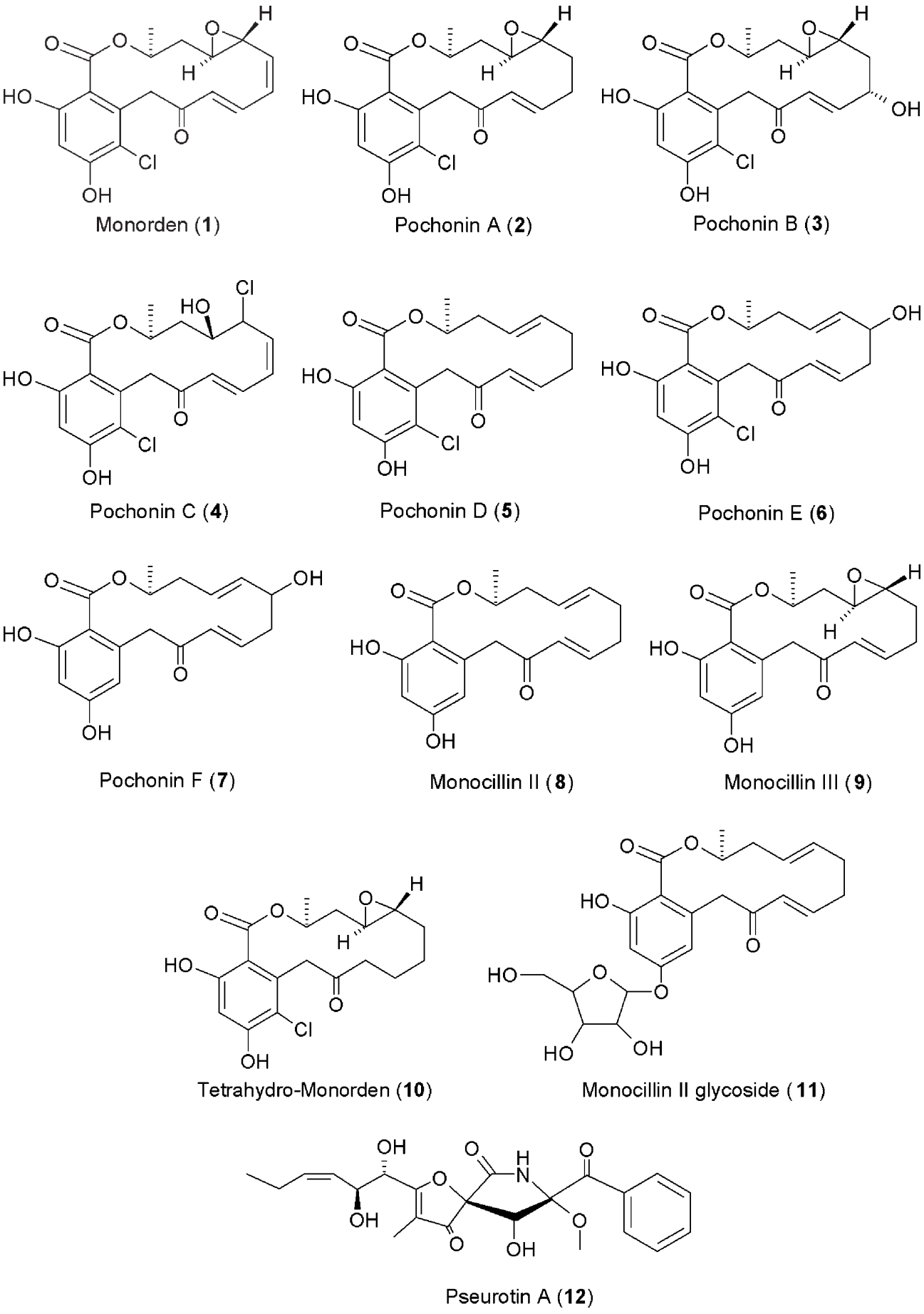


Fig. 1. Chemical structures of metabolites isolated from *P. chlamydosporia* var. *catenulata* strain P0297

materials, which are deposited with CBS, Utrecht, The Netherlands and CABI, Egham, UK, we studied further strains from CBS, MUCL (Louvain-la-Neuve, Belgium), UAMH (Edmonton, Canada) and the Bayer culture collection. The latter strains (P0297, P0452) are maintained under liquid nitrogen in 10% glycerol and on YMG (STADLER et al. 2001) agar slants at the Bayer Research Centre (Wuppertal, Germany). Some data on their taxonomy, geographic origin and collection sites are compiled in Tab. 1. For further information the reader is referred to the respective catalogues of the provider institutions.

For initial screening fermentation, submerged cultures were prepared by adding 2 ml of a mycelial suspension in 10 % glycerol to 500 ml Erlenmeyer flasks containing 150 ml of Q6/2 medium (D-glucose 0.2 %, glycerol 1 %, cotton seed meal 0.5 %; tap water, pH 7.2) or YMG medium. Shake flasks were sterilised at 1 bar and 121°C for 30 min. For preliminary experiments, YMG solidified by addition of 1.5 % agar-agar, as well as solid and liquid Potato Dextrose media (PDA) and Oatmeal agar (OA) (Difco) were also employed. PDA also was preferably used for morphological studies. YMG and Q6/2 cultures used for secondary metabolite profiling were propagated in the dark on a rotary shaker at 140 rpm at 23 °C and harvested at least one day after the free glucose was depleted. In all cases, the pH of the cultures remained below values of 7 at this stage of fermentation (see Discussion). Fermentation times varied according to the growth characteristics of the cultures (Tab. 2). Immediately after harvest, the cultures were freeze-dried, and the resulting materials were stored at 4 °C until further use.

B. Extraction of fungi and generation of physicochemical data

For extraction, 250 mg of freeze-dried cultures were suspended in 25 ml of H_2O *dest*. in 50 ml Falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 30 min in an ultrasonic bath. Thereafter, 25 ml of ethyl acetate were added. After vigorous shaking, followed by sonification for another 30 min and centrifugation (10 min at 1000 x *g*), 20 ml of the supernatants were withdrawn, dried over Na_2SO_4 and evaporated to dryness *in vacuo* (40 °C). Aliquots of these extracts were dissolved in methanol and analysed immediately by HPLC-UV/visual and HPLC-MS methodology.

The methods for generation of HPLC-UV/visual profiles were previously described in detail (STADLER et al. 2001). HPLC-MS was conducted using a different instrumental equipment: An Agilent (Waldbronn, Germany) HP 1100 coupled to a Micromass-LCT mass spectrometer (Micromass, Manchester, UK) was employed to generate mass spectra in the Electrospray ionisation (ESI) mode. In contrast to previous studies, data were recorded in both the positive and negative ESI mode (range between 150 and 1500 Da). Accordingly, positive [$(M+H)^+$] and negative [$(M-H)^-$] molecular ions and their adducts were observed. For chromatographic separation a Waters (Eschborn, Germany) Symmetry C18 column (particle size: 3.5 μm; column dimensions 2.1 x 50mm) was employed. The mobile phase consisted of H_2O/acetonitrile (ACN); formic acid [0.1 % (v/v)] was added to both eluants as to facilitate ionisation and separation. The following elution profile was employed: 0–1 min: isocratic at 100 % H_2O; 1–7 min: linear gradient from 0% ACN to 90 % ACN; 7–8 min: isocratic at 90 % ACN; 8–8.5 min: gradient from 90 % ACN – 100 % ACN). The following instrumental parameters were employed for generation of HPLC-MS data: Source temperature: 100 °C; desolvation temperature: 200 °C; capillary voltage: 3.2 kV; gas flow (total): 550 l/h; Nebuliser gas: N_2, flow: 3l/h, resolution: 4000; scan time: 1s. Retention times (**Rt**) and characteristic signals of the detected metabolites are summarised in Tab. 3.

C. Minisatellite (MIS) PCR

DNA extraction and MIS PCR were performed in analogy to a previously published method (see STADLER et al. 2001 and MEYER et al. 1996, respectively). For DNA extraction, 100 mg to 200 mg of mycelial material was detached from solid media and transferred into 2 ml screw-cap sample tubes. To achieve lysis of the fungal cells, sea sand and 1 ml of CTAB buffer (2 M NaCl, 20 mM EDTA, 10 mM Tris-HCl, 2 % (w/v) N-acetyl-N,N,N-trimethyl ammonium bromide (CTAB), 2 % polyvinyl pyrrolidone; pH 8.0 were added. The disruption of the cell material was effected by a 30 s treatment in the Fast-Prep Instrument (FP120, BIO101 Savant). Cell debris was sedimented by centrifugation at maximum speed in a microcentrifuge. After centrifugation, 600 μl of the supernatant were extracted with 200 μl phenol/$CHCl_3$ (1:1 (v/v), and the supernatant after centrifugation mixed with an equal volume of 2-propanol to precipitate the nucleic acids. The sediment obtained after centrifugation (15 min at maximum speed in a microcentrifuge) was washed in 70 % ethanol and dissolved in 100 μl of TE buffer. The phenol/$CHCl_3$-extraction step was introduced only recently in order to remove contaminants inhibiting the PCR and/or degrading DNA.

One μl of the DNA preparations were used as template for PCR amplifications, while the remainder was maintained at 4 °C until further use. For PCR amplification and analysis of MIS DNA sequences, an oligonucleotide of the core sequence of the wild type phage M13 (5`GAGGGTGGCGGTTCT 3`) was used as a MIS specific PCR primer. The PCR program consisted of a 180 s initial denaturation at 94 °C, followed by 40 cycles of 40 s at 94 °C, 60 s at 53 °C and 120 s at 72 °C. The reaction was ended with a final extension for 180 s at 72°C. Reaction products were separated on 1 % agarose gels in TRIS-Borate running buffer (TBE) applying a sample buffer containing 1 μl of GelStar (BioWhittaker Molecular Applications, Rockland, U.S.A.) per 500 μl of DMSO. DNA (2 μl – 5 μl) was mixed with 2 μl of sample buffer, incubated for 5 min and loaded onto the gel. Bands were detected by UV illumination and photographed using a video documentation system (Ltf Labortechnik, Wasserburg, Germany).

Tab. 1. List of all strains investigated. Generic classification according to GAMS & ZARE (2001). Some strains have been deposited in several public collections. The collection numbers under which the strains were actually obtained are printed in **bold** typeset. Unless indicated otherwise in the left columns, the strains were also studied by GAMS, ZARE & EVANS (2001) or in other publications of this series, respectively.

Species/Variety/Status/Remarks	Strain No*	Country of Origin	Habitat/Locality	Collector
***Haptocillium balanoides* (Drechsler) Zare & W. Gams**				
ex-type of *V. balanoides* Drechsler; ex-type of *H. balanoides*	**CBS 250.82**	Canada	Winnipeg, ex nematode in soil	J. Reid
***Haptocillium glocklingiae* Zare & W. Gams**				
ex-type of *H. glocklingiae*	**CBS 101434**	Japan	from nematode in rabbit dung	S.L. Glockling, No. S.G.4
***Haptocillium campanulatum* (Glockling) Zare & W. Gams**				
ex-type of *V. campanulatum* Glockling, ex-type of *H. capanulatum*	**CBS 101240**	UK	England, Berkshire, nematodes in muntjae dung, Fagus forest	S.L. Glockling
***Haptocillium sinense* (K.G. Zhang, L. Cao & Z.Q. Liang) Zare & W. Gams**				
ex-type of *V. sinense* K.Q. Zhang et al.; ex-type of *H. sinense*	**CBS 567.95**	China	Guizhou Prov., Danzai county, nematode near root of *Actinidia deliciosa*	K.-Q. Zhang
originally identified as *V. coccosporum* (Drechsler) W. Gams	**CBS 101431**	Japan	ex nematode in rotten tubers	S.L. Glockling
***Haptocillium zeosporum* (Drechsler) Zare & W. Gams**				
ex-type of *H. zeosporum*	**CBS 335.80**	Iraq	from soil nematode	I.S. Damirdagh
***Lecanicillium lecanii* (Zimmerm.) Zare & W. Gams**				
ex-neotype of *L. lecanii*; Teleomorph *Torrubiella confragosa*	**CBS 102067** IMI 331550	Indonesia	Sulawesi, ex *Coccus viridis*	H.C. Evans
***Pochonia bulbillosa* (W. Gams & Malla) Zare & W. Gams**				
ex-type of *V. bulbillosum* W. Gams & Malla; ex-type of *P. bulbillosa*	**CBS 145.70**	Denmark	Hörsholm, conifer forest, root of *Picea abies*	D.S. Malla, No. S9-18
ex-type of *V. cephalosporum* Gams	**CBS 247.68**	Germany	Kiel-Kitzeberg, wheat field soil	W. Gams, No. C1109
***Pochonia chlamydosporia* var. *chlamydosporia* (Goddard) Zare & W. Gams**				
not studied by GAMS, ZARE & EVANS (2001)	**MUCL 8330** CBS 361.64	Netherlands	Wageningen, rhizosphere of *Solanum tuberosum*; sandy soil	J.H. van Emden
ex-type of *Dictyoarthrinopsis kelleyi* Dominik & Majchrowicz	**MUCL10008** CBS 594.66	Guinea	soil	T. Dominik & I. Majchrowicz
ex-type of *Pochonia humicola* Batista & Fonseca	**CBS 429.64** MUCL 9880	Brazil	Ceará Mirim, Estado do Rio Grande do Norte, soil	J. Oliveira da Silva
ex-neotype of *Pochonia chlamydosporia* var. *chlamydosporia*	**CBS 103.65** ATCC 16289	Germany	Kiel-Kitzeberg, soil under *Brassica napus*	W. Gams, No. C 734
	CBS 102066 IMI 321351	UK	ex cyst nematode on *Solanum tuberosum*	D.H. Crump

Tab. 1. Continued

Species/Variety/Status/Remarks	Strain No*	Country of Origin	Habitat/Locality	Collector
authentic strain of teleomorph *Cordyceps chlamydosporia* H.C. Evans	**CBS 101244** IMI 331547	Brazil	Ouro Preto de Oueste, Rondonia state, from mollusc eggs in forest soil	H.C. Evans
not studied by GAMS, ZARE & EVANS (2001)	**UAMH 4324**	not stated	dead leaves of *Euphorbia pulcherrima* (poinsettia)	D.M.Dixon G-7a-1
***Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & W. Gams**				
	MUCL15018 OAC 10474 CBS 397.69	Canada	Guelph, Ontario, soil	G.L. Barron
Single-ascospore isolate from *Cordyceps* species	**CBS 101896** IMI 338017	Ecuador	ex beetle larva (Coleoptera)	H.C. Evans
	CBS 250.83A,	Sweden	Lund, egg of *Heterodera avenae*	C. Dackman, No. 3
Single-ascospore isolate from *Cordyceps* species	**CBS 102064** IMI 331575	Ecuador	ex beetle larva (Coleoptera)	H .C. Evans
ex-type of *P. chlamydosporia* var. *catenulata,* strain obtained from both CBS and UAMH, UAMH 2630 not studied by GAMS, ZARE & EVANS (2001)	**CBS 504.66** UAMH 2630 ATCC 16683 IMI 113164 OAC 10250	Canada	Ontario; Guelph, soil in mixed forest	G.L. Barron
not studied by GAMS, ZARE & EVANS (2001)	**P0297**	Germany	Wuppertal, plant debris	
not studied by GAMS, ZARE & EVANS (2001)	**UAMH 1775**	Canada	soil, British Columbia, Christie Island, Howe Sound	J.W. Carmichael
not studied by GAMS, ZARE & EVANS (2001)	**UAMH 1776**	Canada	soil, British Columbia, Christie Island, Howe Sound	J.W. Carmichael
not studied by GAMS, ZARE & EVANS (2001)	**UAMH 1845**	Canada	not stated	G.F. Orr
***Pochonia gonioides* (Drechsler) Zare & W. Gams**				
ex-neotype of *P. gonioides*	**CBS 891.72**	Germany	Teutoburger Wald, from *Pulcherricium caeruleum*	J.A. Stalpers
	CBS 611.89	Germany	Berlin, litter of *Pinus sylvestris*	G. Kraepelin
obtained as *P. chlamydosporia var. catenulata,* not studied by	**MUCL11413**	France	Paris, no further information given in MUCL catalogue	J. Brun
not studied by GAMS, ZARE & EVANS (2001)	**UAMH 2736**	Mozambique	soil	C. Magalhaes 1062-86
***Pochonia microbactrospora* W. Gams & Zare**				
ex-type of *P. microbactrospora*	**CBS 101433**	Japan	Tsukuba, bdelloid rotifers in litter	S. Glockling, No. S.G. 3
***Pochonia rubescens* Zare, W. Gams & López-Llorca**				
ex-type of *P. rubescens*	**CBS 464.88**	UK	Eastern Scotland, egg of *Heterodera avenae,* characteristic red pigment produced on solid media	L. López-Llorca, No. 10
Pochonia suchlasporia* (W. Gams & Dackmann) Zare & W. Gams var. *suchlasporia				
ex-type of *P. suchlasporia* var. *suchlasporia*	**CBS 251.83** LCP 89.2563	Sweden	Lund, egg of *Heterodera avenae*	C. Dackman
	CBS 816.83	Denmark	København, egg of *Heterodera avenae*	M. Juhl, No. 3,55-3

Tab. 1. Continued

Species/Variety/Status/Remarks	Strain No*	Country of Origin	Habitat/Locality	Collector
Pochonia suchlasporia* (W. Gams & Dackmann) Zare & W. Gams var. *catenata				
ex-type of *V. suchlasporium* var. *catenatum*	**CBS 817.83**	Sweden	Lund, egg of *Heterodera avenae*	C. Dackman, No. 18
ex-type of *P. suchlasporia* var. *catenata*	**CBS 248.83**	Sweden	Lund, egg of *Heterodera avenae*	C. Dackman
obtained from UAMH as *V. coccosporum*, treated as *P. suchlasporia* var. *catenata* by GAMS, ZARE & EVANS (2001)	**UAMH 6596** **CBS 495.90**	Canada	Algonquin Park, Nipissing district, Ontario, decaying mushroom (as stated in UAMH catalogue)	D. Malleau
obtained from MUCL as V. *chlamydosporium* var. *chlamydosporium,* see GAMS (1988)	**MUCL 15046** CBS 551.69	Canada	Puslinch, Ontario, soil; cedar forest	G.C. Bhatt
obtained from CBS as *P. chlamydosporia* var. *catenulata*, not studied by GAMS, ZARE & EVANS (2001)	**CBS 749.83**	Czech Republic	Slovenský Raj, soil in mixed forest	B. Grunda
	CBS 416.95 INIFAT 94/173	Cuba	worm on dead leaf of *Roystonia regia*	R.F. Castañeda
not studied by GAMS, ZARE & EVANS (2001)	**P 0452**	Austria	soil sample	
***Rotiferophthora angustispora* (G.L. Barron) G.L. Barron**				
ex-type of *R. angustispora*	**CBS 101437**	UK	England, from a rotifer in decaying straw	S.L. Glockling
***Rotiferophthora lacrima* S.L. Glockling**				
	IMI 375098	Japan	No further information provided in IMI catalogue	
***Rotiferophthora minutispora* S.L. Glockling**				
ex-type of *R. minutispora*	**CBS 101436** IMI 372229	Japan	Chiba Prefecture, bdelloid rotifers in soil	S.L. Glockling
***Simplicillium lanosoniveum* (van Beyma) Zare & W. Gams**				
ex-type of *S. lanosoniveum*	**CBS 123.42**	Netherlands	ex hair of *Cibotium schiedei* in greenhouse	F.H. van Beyma
	CBS 101241 IMI 317451	Jamaica	ex *Hemileia vastatrix*, single spore isolate of teleomorph *Torrubiella* sp.	C. Prior
***Verticillium epiphytum* Hansford**				
Type of *Cephalosporium pimprinum* Thirumalachar *ined*., producer of antiamoebin, obtained as *V. psalliotae* Treschow	**CBS 650.85** ATCC 16541	India	no further information provided in CBS catalogue	M.J. Thirumalachar
Type of *Cephalosporium curtipes* var. *uredinicola* Sukapure & Thirumalachar, obtained as *V. psalliotae* Treschow	**CBS 154.61** ATCC 14495 IMI 090246	India	Khandala, uredinium of rust	M.J. Thirumalachar

Tab. 2. Growth characteristics of the strains investigated on secondary metabolite production in shake cultures using Q6/2 medium and characteristic secondary metabolites detected by HPLC analysis.
* Estimated yields of compounds **1** and **12**: ++ = over 10 mg/l; + = between 1 and 10 mg/l;
** RAL = Resorcylic acid lactones; for identity of compounds listed under "other metabolites" see Tab. 3.

Genus	Species	Strain	Time [h]	pH	Monorden* (1)	Pseurotin A* (12)	Other metabolites
Haptocillium	*balanoides*	CBS 250.82	168	5,9	–	–	HZ1, HZ2
	glocklingiae	CBS 101434	144	5,6	–	–	HZ1, HZ2
	campanulatum	CBS 101240	192	6,7	–	–	HZ1, HZ2
	sinense	CBS 567.95	192	5,9	–	–	HZ1, HZ2
	sinense	CBS 101431	336	5,2	–	–	HZ1, HZ2
	zeosporum	CBS 335.80	288	6,8	–	–	HZ1, HZ2
Lecanicillium	*lecanii*	CBS 102067	192	6,5	–	–	LL1, LL2, LL3
Pochonia	*bulbillosa*	CBS 145.70	168	5,7	–	–	-
	bulbillosa	CBS 247.68	168	5,9	–	–	PS3, PS4-
	chlamydosporia var. *cat.*	MUCL 15018	168	6,2	–	–	PS2, PM3
	chlamydosporia var. *cat.*	CBS 250.83A	168	6	+	–	RAL** (**2** - **6**)
	chlamydosporia var. *cat.*	CBS 101896	168	6,3	++	+	RAL (**2** - **6**)
	chlamydosporia var. *cat.*	CBS 504.66	168	5,9	++	–	RAL (**2** - **6**)
	chlamydosporia var. *cat.*	CBS 102064	168	6,4	++	+	RAL (**2** - **6**)
	chlamydosporia var. *cat.*	P0297	168	5.7	++	+	RAL (**2** - **6**)
	chlamydosporia var. *cat.*	UAMH 1775	192	6.2	++	+	RAL (**2** - **6**)
	chlamydosporia var. *cat.*	UAMH 1776	192	5.6	++	+	RAL (**2** - **6**)
	chlamydosporia var. *cat.*	UAMH 1845	192	5.6	++	+	RAL (**2** - **6**), PR2
	chlamydosporia var. *chlam.*	CBS 429.64	168	6,1	++	–	RAL (**2** - **6**)
	chlamydosporia var. *chlam.*	CBS 102066	168	6,5	++	+	RAL (**2** - **6**)
	chlamydosporia var. *chlam*	CBS 101244	144	5,5	++	+	RAL (**2** - **6**)
	chlamydosporia var. *chlam*	MUCL 10008	168	6,4	+	(+)	PC1
	chlamydosporia var. *chlam.*	MUCL 8330	168	5,3	+	(+)	PC1
	chlamydosporia var. *chlam.*	CBS 103.65	192	6,8	–	–	PC1
	chlamydosporia var. *chlam.*	CBS 429.64	168	6,1	++	–	RAL (**2** - **6**)
	chlamydosporia var. *chlam.*	UAMH 2736	192	5.8	++	++	RAL (**2** - **6**)
	chlamydosporia var. *chlam.*	UAMH 2439	192	6,0	++	++	RAL (**2** - **6**)
	gonioides	CBS 611.89	168	5,9	–	–	-
	gonioides	CBS 891.72	168	6,2			-
	gonioides (cf.)	MUCL 11413	168	5,9	–	–	-
	microbactrospora	CBS 101433	336	3,5	–	–	PM1, PM2
	rubescens	CBS 464.88	96	5,1	+	–	RAL? (PR1, PR2)
	suchlasporia var. *cat.*	MUCL 15046	168	5,9	–	–	PS1
	suchlasporia var. *cat.*	CBS 495.90	192	6,8	–	–	PS1, PS2
	suchlasporia var. *cat.*	CBS 248.83	168	5,6	++	(+)	RAL (**2** – **6**)
	suchlasporia var. *cat.*	CBS 817.83	168	6,6	–	–	PS1
	suchlasporia var. *cat.*	CBS 749.83	168	6,4	–	–	PS3, PS4
	suchlasporia var. *cat.*	CBS 416.95	192	6,6	–	–	PS3, PS4
	suchlasporia var. *cat.*	P 0452	192	7,3	–	–	PS3, PS4
	suchlasporia var. *suchl.*	CBS 251.83	168	5,9	–	–	PS2
	suchlasporia var. *suchl.*	CBS 816.83	168	6,4	–	–	PS1, PS2
Rotiferophthora	*angustispora*	CBS 101437	300	5,4	–	–	RM2
	lacrima	IMI 375098	168	4,9	–	–	RM1, RM2
	minutispora	CBS 101436	336	4,3	–	–	RM1, RM2
Simplicillium	*lanosoniveum*	CBS 123.42	192	6	–	–	SL1, SL2
		CBS 101241	192	5.9	–		SL1, SL2
Verticillium	*epiphytum*	CBS 650.85	168	6,5	–	–	VE1
	epiphytum	CBS 154.61	192	5,7	–	–	VE1

D. ITS nrDNA sequencing

In analogy to the method used by ZARE, GAMS & CULHAM (2000), PCR-based amplification and sequencing of the nearly complete ITS nrDNA region was done with some strains of taxa where HPLC-profiles and MIS PCR data had disagreed with the characteristics of the respective type strains. The ITS region was amplified in a PCR using primers ITS1 and ITS2 (WHITE et al. 1990), applying the same thermal profile as used for the Minisatellite PCR. Amplification products were purified using DNA binding paramagnetic beads (Mag Prep PCR Clean Up Kit, Tecan Schweiz AG, Hornbrechtikon, Switzerland) using the protocol supplied by the manufacturer. Nucleotide sequences were obtained by cycle sequencing using the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Freiburg, Germany) and the LI-COR 4200 Genetic Analyzer (LI-COR Inc. Lincoln, Nebraska, USA).

Since sequencing was done merely to verify species identity, only one strand was sequenced using primer ITS1. A search for sequences similar to the ones determined was conducted and accompanying alignments with the best matches were obtained by FASTA, provided as an on-line service by the Europaean Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/fasta33/). Sequences were compared with those deposited in the EMBL/Genbank databases.

ITS nrDNA sequences were obtained from the following strains: CBS 145.70, CBS 247.68, CBS 749.83, CBS 495.90, P0452, CBS 416.95 and UAMH 1845.

E. Natural Products Databases

The following databases on secondary metabolites were available:

Antibase-a database of natural compounds from microorganisms and higher fungi. Update 1/2001. University of Göttingen and Chemical Concepts, Weinheim, Germany. This database focuses on microbial metabolites.

BNPD: Bioactive Natural Products Database. Founded by L. Bèrdy in Budapest, Hungary. The contents of this database have meanwhile been taken over into DNP; the BNPD database is no longer available as independent feature.

DNP: Chapman & Hall's Dictionary of natural products on CD-ROM. Distributed by CRC Press. Update 1/2002. This database contains data on all biological sources, including fungi and other micro-organisms.

These databases allowed us to search for entries on previously isolated compounds from the fungal genera and species treated herein, including some invalid and formerly misapplied synonyms. The information provided in these databases helped substantially to screen for original literature.

Results

A. Taxonomy, fermentation and secondary metabolite profiles

All strains of *Pochonia* and other genera listed in Tab. 1 were propagated in submerged cultures. Their extracts were prepared and analysed by HPLC-based methodology as described in the Experimental section. So far, the raw data recorded were mainly checked for occurrence of compounds **1** – **12** (Fig. 1). For comparison, the physicochemical data of other prominent secondary metabolites are included in Fig. 2 and Tab. 3 as well. The growth characteristics of all strains investigated and information on the presence of compounds **1** – **6** and **12** in Q6/2 medium are summarised in Tab. 2. For HPLC characteristics of compounds **2** – **11** see (HELLWIG et al. 2003). In Tab. 3 and Fig. 2, only the characteristic Rt and spectra of monorden (**1**) and pseurotin A (**12**) are pointed out, along with those of yet unidentified compounds. Some strains produced a great variety of secondary metabolites with very similar polarity. In gradient systems optimised for characterisation of compounds **1** – **12**, the correspondence of HPLC-UV/visual and HPLC-MS data was not always clear. Frequently more than one potential molecular peak was observed upon analysis of the crude extracts at a given Rt. The HPLC-MS data for unknown compounds are thus given in Tab. 3 only if their detection was unambiguous, e.g., by matching signals corresponding to a particular mass in both the positive $(M+H)^+$ and negative $(M-H)^-$ electrospray mode and relative intensities of at least 80 %.

Compounds **1** – **11** belong to the same chemical type of secondary metabolites and are referred to as resorcylic acid lactones (RAL) further below. In all RAL producing strains, monorden (**1**) was detected as a major metabolite among other compounds of this type. Minor components such as **2** – **7** and **10** – **11** were frequently detected only upon HPLC-UV analyses at higher concentrations or by the more sensitive HPLC-MS methodology. In accordance with the findings on *P. chlamydosporia* var. *catenulata* P0297 (HELLWIG et al. 2003) during optimisation of production of antiparasitic and antiviral agents, HPLC analyses of extracts from fermentations in YMG medium did not reveal any additional main secondary metabolites as compared to those derived from Q6/2 medium. Production of RAL was always noted in YMG medium if the concurrent extract from Q6/2 contained these metabolites as well. None of the strains produced detectable amounts of Pseurotin A (**12**) in YMG. Therefore, the data obtained with extracts from YMG are not shown in Tab. 2. Especially monorden (**1**) decomposed after longer fermentation times (see Discussion). The non-halogenated monocillins (**8** and **9**, see AYER et al. 1980) and the new metabolites, pochonin F (**7**), tetrahydromonorden (**10**) and monocillin II glycoside (**11**), were only produced as main metabolites by P0297 upon modification of culture media by addition of bromide salts (HELLWIG et al. 2003). They were not found in any of the examined strains under regular fermentation conditions.

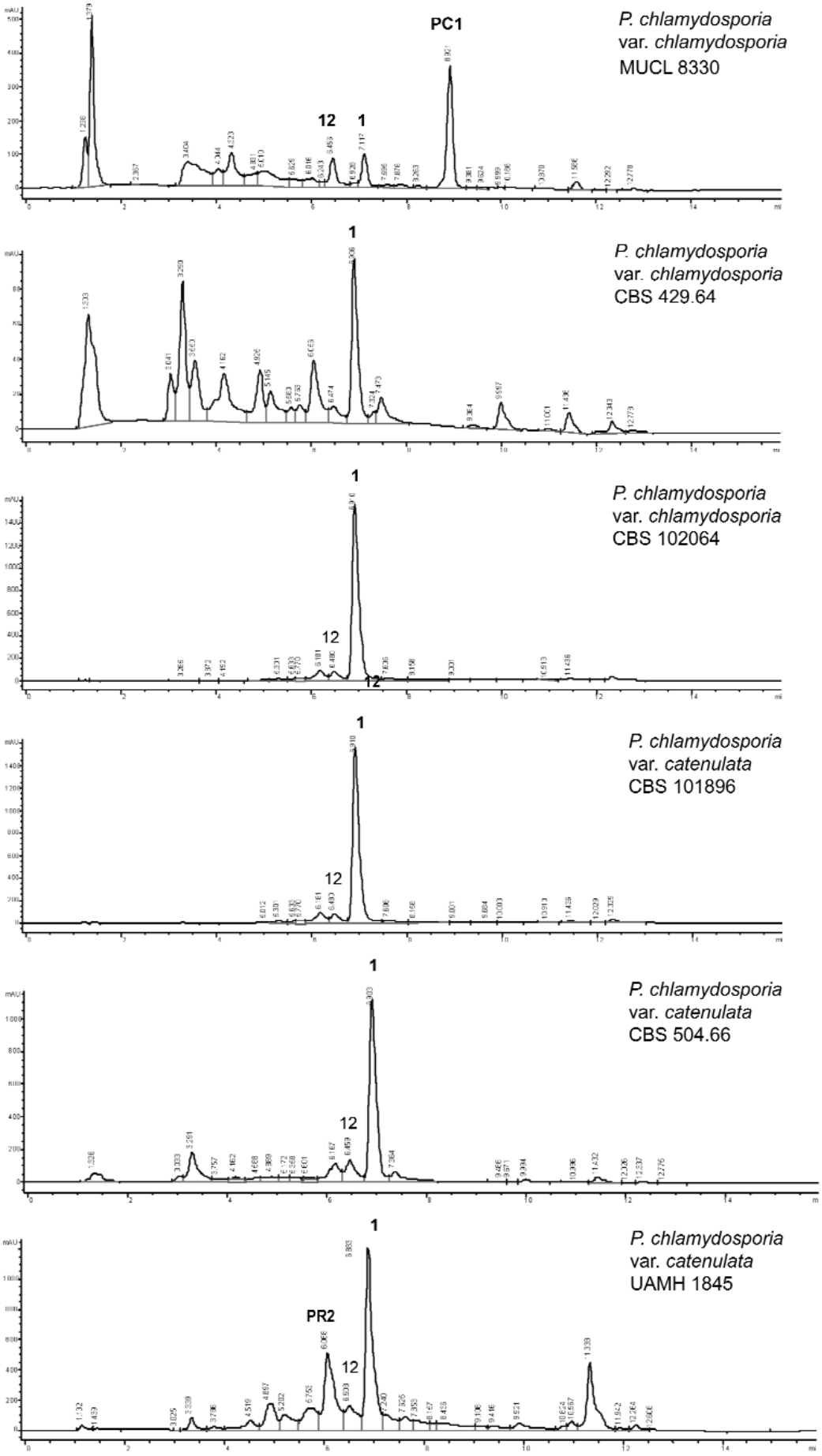


Fig. 2a. HPLC-UV chromatograms (210 nm) of extracts derived from several strains of *Pochonia chlamydosporia*

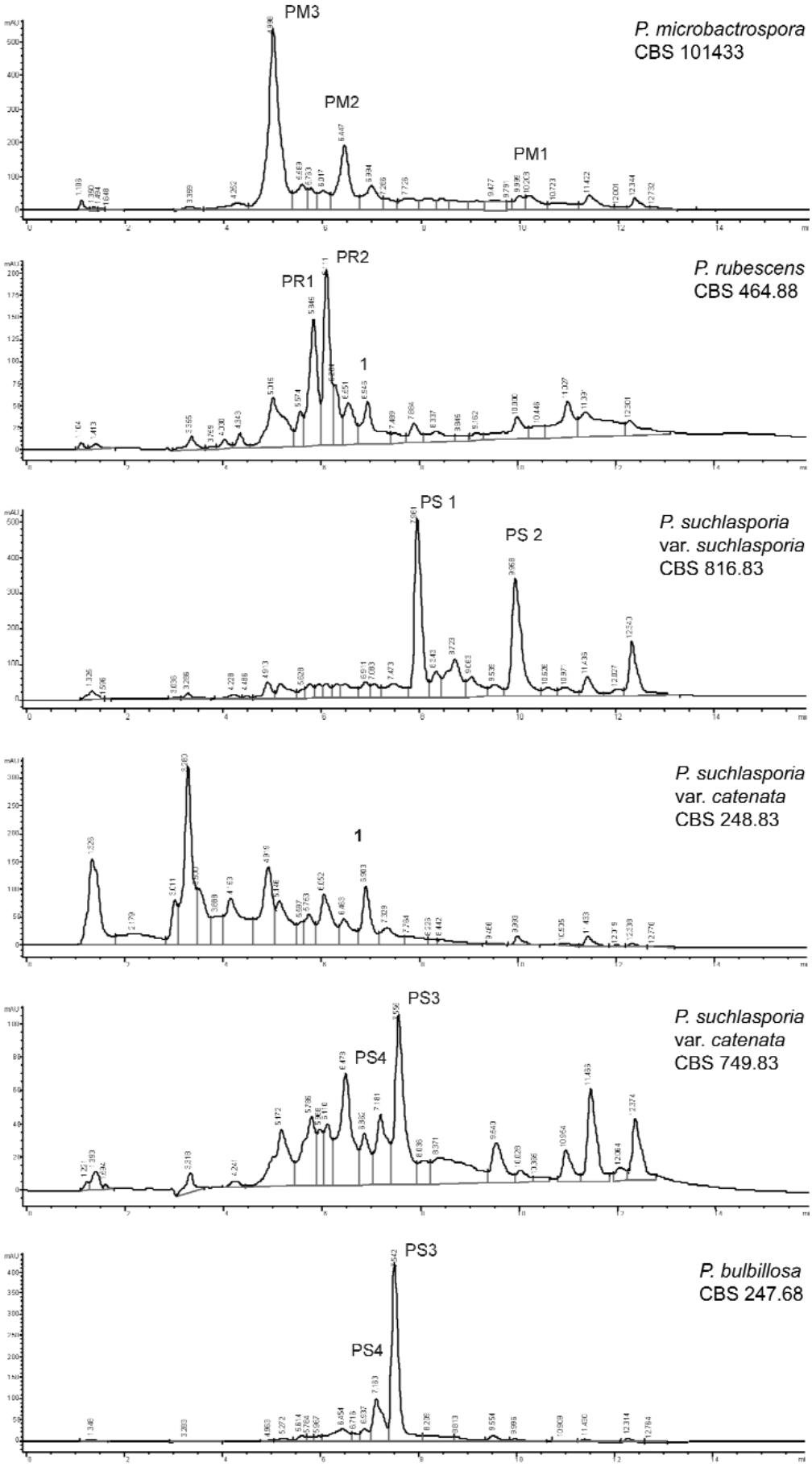


Fig. 2b. HPLC-UV chromatograms (210 nm) of extracts derived from several strains of *Pochonia* species

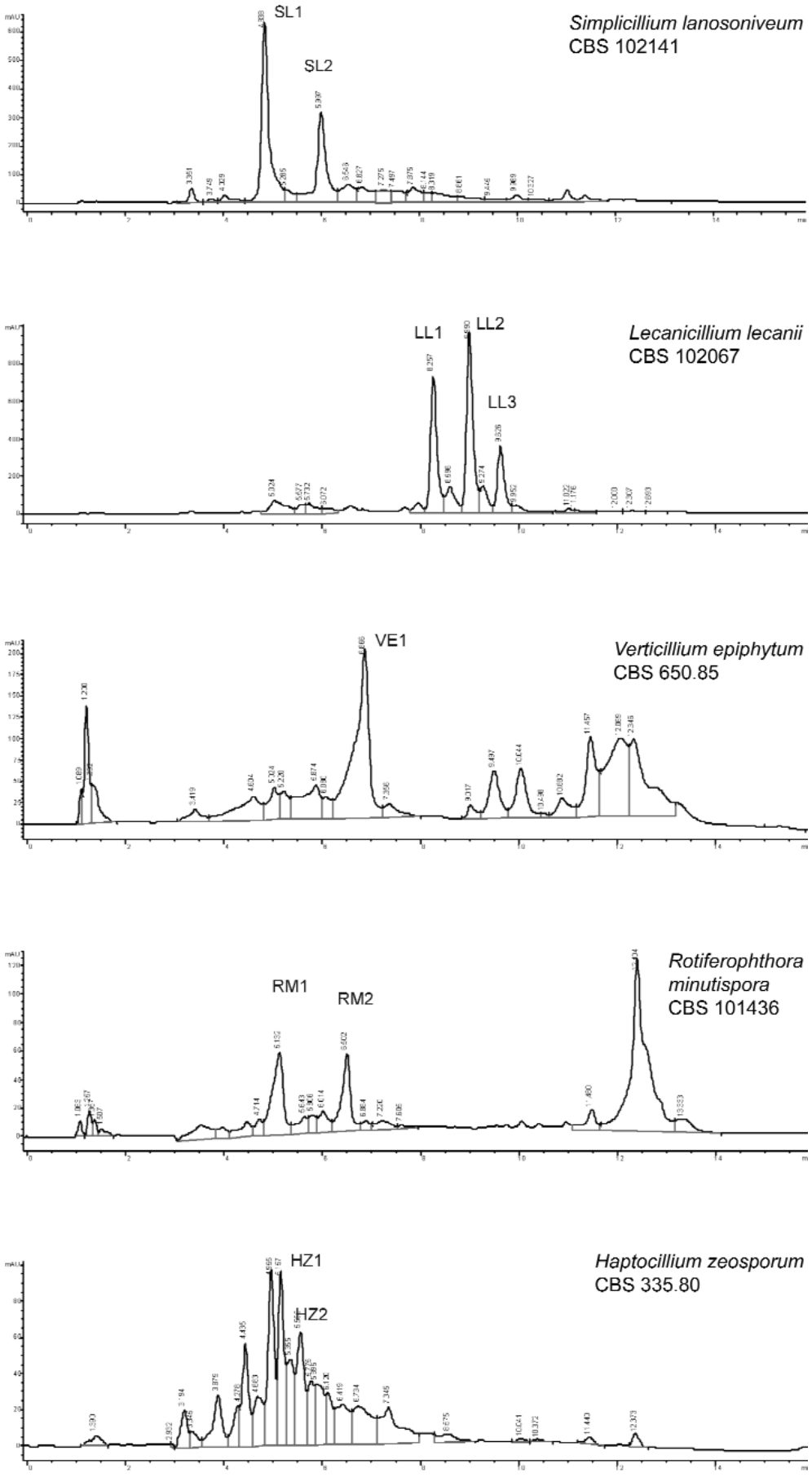
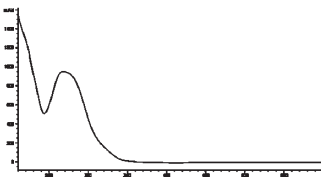
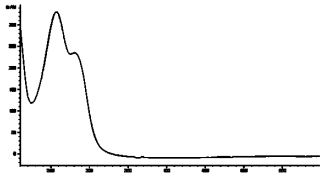
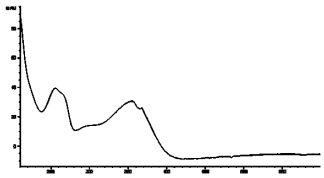
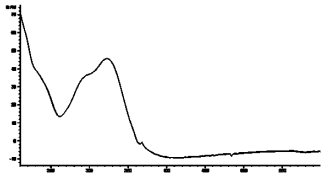
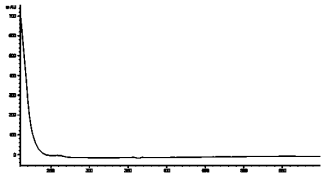
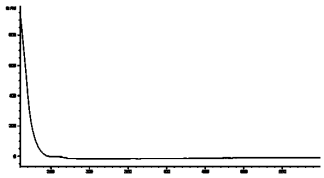
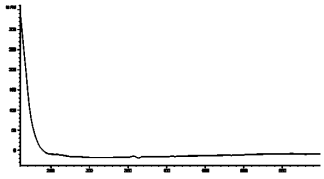
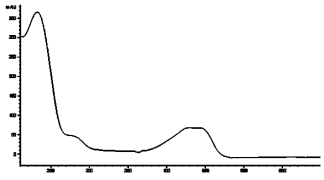
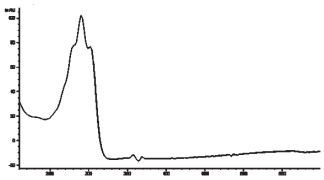
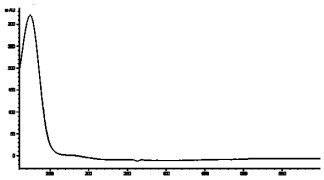
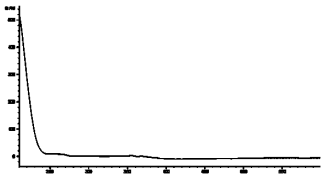
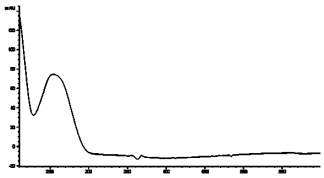
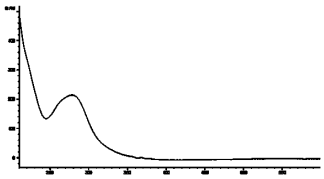
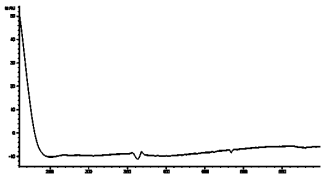
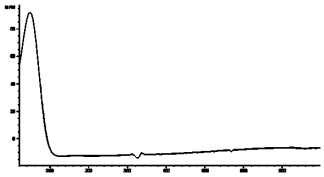
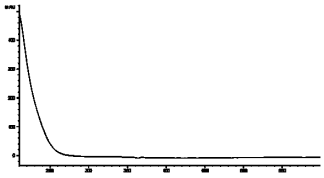
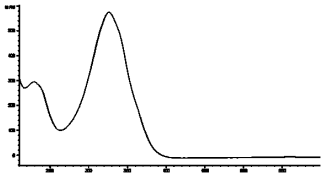


Fig. 2c. HPLC-UV chromatograms (210 nm) of extracts derived from several strains of genera allied to *Pochonia*

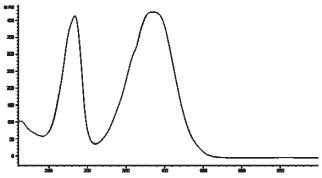
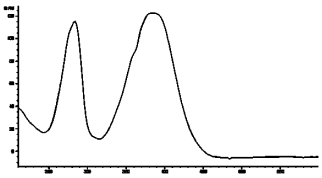
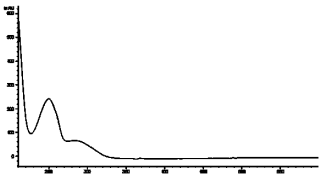
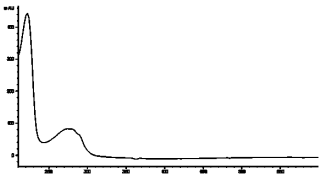
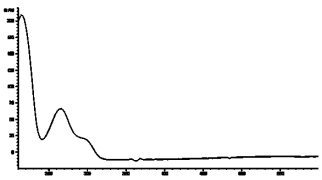
Tab. 3. Retention times (Rt) of characteristic secondary metabolites detected in the crude extracts, including corresponding HPLC-UV spectra and corresponding mass peaks observed by HPLC-MS.
n.d. = not determined or not safely assigned because of possible overlays in HPLC-MS. For HPLC characteristics of pochonins, tetrahydromonorden and monocillins (**2**–**11**) see HELLWIG et al. (2003).

Compound/occurrence	HPLC-UV/Vis			LC-MS	
	UV/Vis-spectrum	Rt [min.]	Rt [min]	ESI pos. [*m/z* (%)]	ESI neg. [*m/z* (%)]
Monorden (1) *Pochonia chlamydosporia* (most strains), *P. rubescens, P. suchlasporia* var. *catenulata* (ex-type strain CBS 248.83 only)		6.85-7.05	4.86-4.90	347, 349 [M^+-H_2O+H] 365, 367 [M^++H] 711, 713 [$2M^+$-H_2O+H] 729, 731 [$2M^+$+H] (Chlorine pattern)	363, 365 [M^--H] (Chlorine pattern)
Pseurotin A (12) *Pochonia chlamydosporia* (several strains)		6.05–6.14	4.68	433 [M^++H]	431 [M^--H]
HZ1 *Haptocillium* species		4.92–4.95	n.d.	n.d.	n.d.
HZ2 *Haptocillium* species		5.35–5.39	n.d.	n.d.	n.d.
LL1 *Lecanicillium lecanii*		8.22–8.27	5.51–5.55	609 [M^++H]	607 [M^--H]
LL2 *Lecanicillium lecanii*		8.97–9.04	5.87–5.91	637 [M^++H]	635 [M^--H]
LL3 *Lecanicillium lecanii*		9.62–9.65	6.22–6.24	665 [M^++H]	663 [M^--H]
PC1 *Pochonia chlamydosporia* (some strains only)		8.87–8.94	6.05–6.09	425 [M^+-H_2O+H] 443 [M^++H]	441 [M^--H]

Tab. 3. Continued

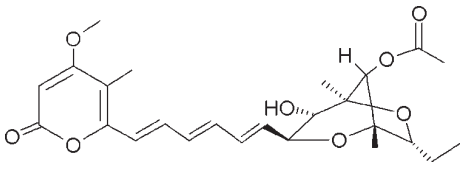
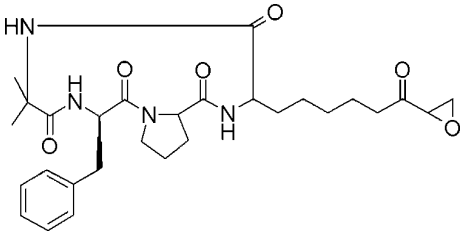
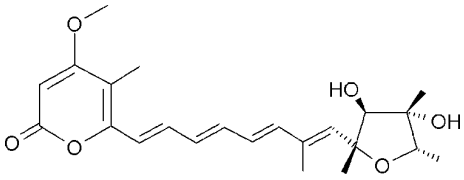
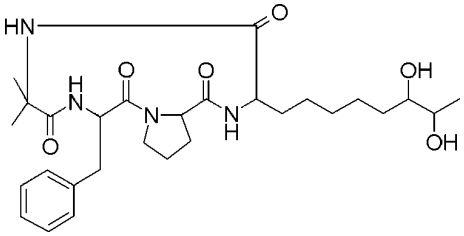
Compound/occurrence	HPLC-UV/Vis		LC-MS		
	UV/Vis-spectrum	Rt [min.]	Rt [min]	ESI pos. [*m/z* (%)]	ESI neg. [*m/z* (%)]
PM1 *Pochonia microbactrospora*		10.2–10.32	n.d.	n.d.	n.d.
PM2 *Pochonia microbactrospora*		6.42–6.5	n.d.	n.d.	n.d.
PM3 *Pochonia microbactrospora* and *P. chlamydosporia var. catenulata* (MUCL 15018 only)		4.92–5.0	n.d.	n.d.	n.d.
PR1 *Pochonia rubescens*		5.79–5.83	n.d.	n.d.	n.d.
PR2 *Pochonia rubescens; P. chlamydosporia var. catenulata* (UAMH 1845 only)		6.15–6.17	4.81–4.83	381, 383 [M^++H] 363, 365 [M^+-H_2O+H] (Chlorine pattern)	379, 381 [M^--H] (Chlorine pattern)
RM1 *Rotiferophthora* species		5.28–5.32	3.46–4.48	n.d.	n.d.
RM2 *Rotiferophthora* species		6.49–6.55	4.67–4.70	n.d.	n.d.
PS1 *Pochonia suchlasporia*		7.95–8.00	5.37–5.4	345 [M^++H]	343 [M^--H]
PS2 *Pochonia suchlasporia*		9.93–9.97	6.21–6.24	466 [M^++H]	464 [M^--H]

Tab. 3. Continued

Compound/occurrence	HPLC-UV/Vis		LC-MS		
	UV/Vis-spectrum	Rt [min.]	Rt [min]	ESI pos. [*m/z* (%)]	ESI neg. [*m/z* (%)]
PS3 *Pochonia suchlasporia var. catenata Chemotype 2*		7.48–7.54	5.17–5.21	419 [M^++H]	417 [M^--H]
PS4 *Pochonia suchlasporia var. catenata Chemotype 2*		7.08–7.14	5.00–5.03	419 [M^++H]	417 [M^--H]
SL1 *Simplicillium lanosoniveum*		4.82–4.85	3.92–3.96	n.d.	n.d.
SL2 *Simplicillium lanosoniveum*		5.96–5.99	4.39–4.44	n.d.	n.d.
VE1 *Verticillium epiphytum*		6.8–6.92	4.75–4.82.	223 [M^++H]	221 [M^--H]

RAL were observed in most strains of both varieties of *P. chlamydosporia,* as well as in *P. rubescens* and in *P. suchlasporia* var. *catenata* CBS 248.83. Neither the remaining strains of *P. suchlasporia* nor the representatives of *P. microbactrospora, P. gonioides, P. bulbillosa* and all other genera contained such compounds. Pseurotin A (**12**) was detected only in *P. chlamydosporia* but not in other *Pochonia* species or allied genera. Within *P. chlamydosporia*, some differences were noted. Cultures of CBS 102064, CBS 102066, CBS 429.64 and all UAMH strains of this variety (Tab. 1), as well as all strains of the variety *catenulata* but MUCL 15018 yielded rather large amounts of RAL and pseurotin A (**12**). Monorden (**1**) was always identified as main metabolite (Fig. 2a). As compared to the aforementioned strains, the HPLC profiles of MUCL 8330 and MUCL 10008 (ex-type of *Dictyoathrinopsis kelleyi*) contained only rather small amounts of these characteristic compounds. A main component with a rather characteristic UV spectrum (**PC1**) was observed (Fig. 2b, Tab. 3) in the two latter strains. The ex-neotype strain of *P. chlamydosporia* var. *chlamydosporia* (CBS 103.65) also did not yield RAL and Pseurotin A (**12**), despite showing the typical morphological characters as described previously (Gams 1988, Zare, Gams & Evans 2001). The culture yielded rather small amounts of extractable material as compared to the other strains of this species. Its only main metabolite detected in other strains as well was **PC1** (see above). Further studies on its secondary metabolism are ongoing. Interestingly, the production of monorden (**1**) appeared to be closely correlated with the ability of the strains of both varieties of the type species to produce dictyochlamydospores, according to preliminary results. These morphological features were also seen during microscopic control of samples taken from cultures of P0297 during fermentation in Q6/2 medium, while in the other strains of *P. chlamydosporia* examined, such correlations are only deduced from the amounts of dictyochlamydospores produced by a certain strain on OA or PDA plates.

Tab. 4. Chemical structures and some biological data on aurovertin B, citreoviridin A and some characteristic secondary metabolites from fungi formerly classified in *Verticillium* Sect. *Prostrata*

Trivial name (chemical type)	Chemical structure	Source and important biological activities	Reference(s)
Aurovertin B (polyketide)		*Calcarisporium arbuscula* *"Verticillium sulphureum"* Anthelmintic, antiparasitic, Mycotoxin (ATPase inhibitor)	CUTLER (1988)
Chlamydocin (lipopeptide)		*Diheterospora* (*Pochonia?*) species Cytostatic, immunomodulatory	CLOSSE & HUGUENIN (1974)
Citreoviridin A (polyketide)		*Penicillium* species (*P. toxicarium, P. citreo-viride, P. ochrosalmoneum, P. pulvillorum, P. fellutanum*) Mycotoxin (ATPase inhibitor)	CUTLER (1988)
Diheteropeptin (lipopeptide)		*Diheterospora* (*Pochonia??*) species Cytostatic neuronal injury inhibitor	MASUOKA et al. (1997)

Strain UAMH 1845 differed from all other *P. chlamydosporia* isolates by the production of **PR2** (see *P. rubescens* below), along with large amounts of monorden (**1**). At least in **PR2**, the MS signals revealed the typical fragmentation pattern of a chlorine-containing metabolite, which was also observed in monorden (**1**). This data, along with a rather similar UV spectrum, pointed toward the identity of **PR2** with a monorden analogue.

The HPLC data of MUCL 15018 deviated from other strains of *P. chlamydosporia* var. *catenata*. According to preliminary results, its main detectable metabolites were **PM3** (also found in *P. microbactrospora*) and **PS2** (also observed in some strains of *P. suchlasporia*). This strain is also extant as CBS 367.69 and was included in the studies of ZARE, GAMS & EVANS (2001), where its affinities to *P. chlamydosporia* var. *catenulata* were confirmed by RFLP and other methods. For the time being, MUCL 15018 is therefore treated here under this taxon, but studies on its correspondence are pending (see also PCR characteristics below).

Few similarities in secondary metabolite profiles were noted in the isolates of *P. suchlasporia* examined. Only CBS 248.83 produced monorden and other RAL (Fig. 2b), while the other strains studied were found devoid of these metabolites. Two prominent peaks (**PS1** and **PS2**) were detected in extracts of CBS 816.83 and CBS 251.83. MUCL 15046 was provided as "*V. chlamydosporium* var. *chlamydosporium*" but also did not contain any of the compounds typically observed in *P. chlamydosporia*. Strain CBS 551.69 is listed as identical to MUCL 15046 in the CBS catalogue and was treated as *V. suchlasporium* var. *catenatum* by GAMS (1988). We found that MUCL 15046 resembled UAMH 6596/CBS 495.90 with respect to all discriminative morphological characters available and therefore included it as *P. suchlasporia* var. *catenata*, in agreement with GAMS (1988). Its secondary metabolite profiles resembled those of CBS 816.83. Strains CBS 416.95 and CBS 495.90 had formerly been classified as *V. coccosporum* (Drechsler) W. Gams (GAMS 1988), and both were only recently included in *P. suchlasporia* var. *catenata* (ZARE, GAMS & EVANS 2001). CBS 749.83 (originating from the Czech Republic and obtained as *P. chlamydosporia*) had not been re-examined by ZARE, GAMS & EVANS (2001). Its metabolite profile resembled the ones of some strains of *P. suchlasporia* var. *catenata* (CBS 416.95, CBS 495.90/UAMH 6596 and P0452). Similar compounds were also found in CBS

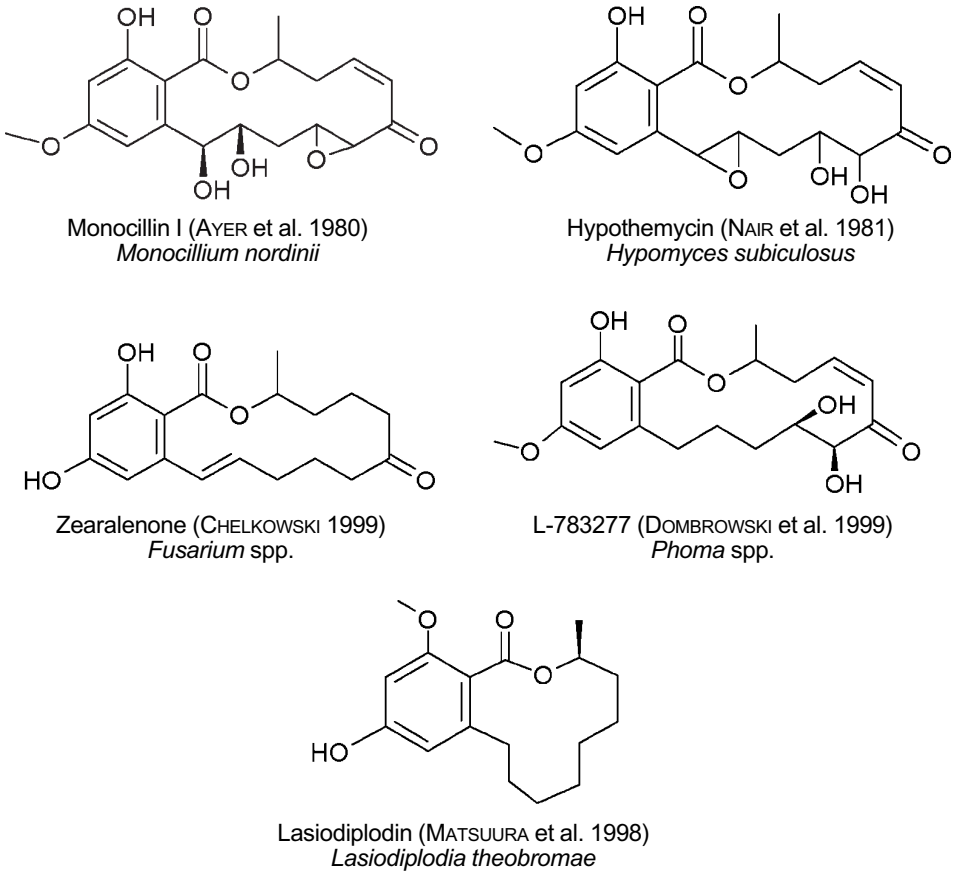
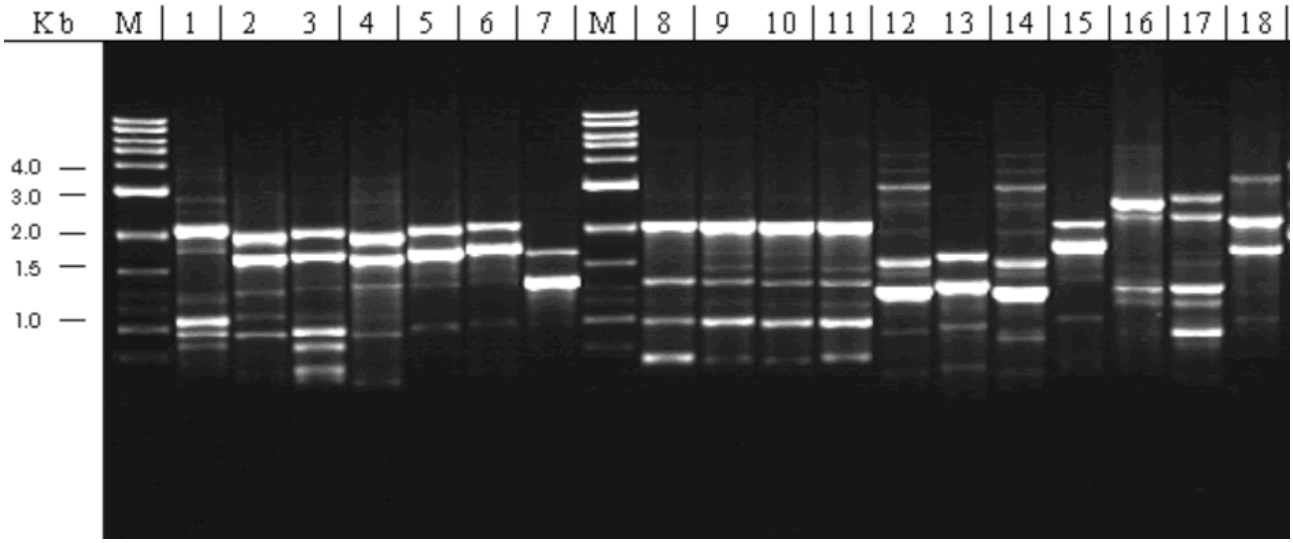


Fig. 3. Chemical structures of further fungal metabolites of the resorcylic acid lactone type

247.68 from Germany. This fungus was originally designated as type strain of *Verticillium cephalosporum* Gams (GAMS 1971) and only later included in *P. bulbillosa* (GAMS 1988). In all these strains, characteristic peaks corresponding to yet unknown compounds named **PS3** and **PS4** (Tab. 3 and Fig. 2b) and further metabolites with similar UV spectra and molecular masses were detected, revealing the presence of a different chemotype. While CBS 749.83 produced about ten different congeneric compounds of this type, the other producers contained **PS3** and **PS4** as major components. According to a search in a HPLC-UV library (based on the HP/Agilent ChemStation software and established at Bayer) containing UV spectra and standardised Rt of numerous fungal metabolites, the closest resemblance to **PS3** and **PS4** was found in citreoviridin A and aurovertin B (structures see Tab. 4). However, a comparison of HPLC-MS data with standards of both compounds obtained from Sigma-Aldrich revealed different molecular weights (418 Da observed in **PS3** and **PS4** as compared to 460 Da for aurovertin B and 402 Da for citreoviridin A). Furthermore, **PS3** and **PS4** eluted at earlier Rt than the standards employed. This phenomenon was further studied by PCR-based methods (see below). As the morphological data of these *P. suchlasporia* isolates were quite in agreement with those reported by GAMS (1988) and ZARE, GAMS & EVANS (2001), no parameters are available as now that would justify segregation of these chemotypes into different species.

The ex-type strain of *P. bulbillosa* (CBS 145.70) and both examined strains of *P. gonioides* (chromatograms not shown) yielded small amounts of detectable metabolites. Their HPLC profiles were rather similar but showed only a rather broad range of peaks in the hydrophilic polarity range. None of the known or prominent metabolites from other strains were identified. Hence, strong deviations between the two examined isolates of *P. bulbillosa* were noted. Similar metabolite profiles and morphological characters as in *P. gonioides* were observed with MUCL 11413, which is therefore listed as *P.* cf. *gonioides* (rather than as *P. chlamydosporia* var. *catenulata*) in Tab. 1.

P. rubescens was expected to differ from other species within the genus in its secondary metabolite profiles because of a characteristic pigmentation in solid culture reported previously (LÓPEZ-LLORCA & OLIVARES-BERNABEU 1998, ZARE, GAMS & EVANS 2001). Upon fermentation in Q6/2 medium, the type strain produced monorden (**1**) and further compounds with monorden-like UV spectra (**PR1** and **PR2**; see Fig. 2b and Tab. 3), which were not seen in *P. chlamydosporia,* apart from the occurrence of **PR2** in UAMH 1845. Neither pseurotin A (**12**) nor other RAL were detected. These findings

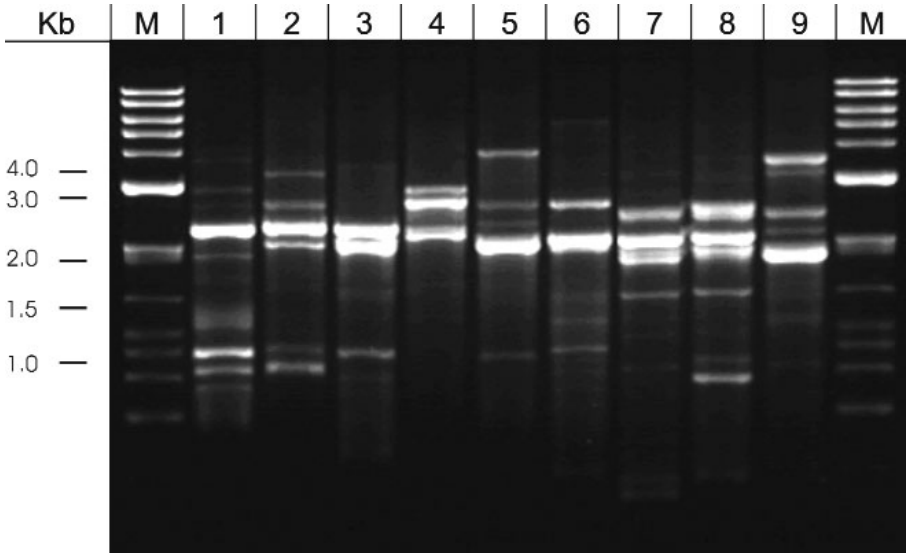


Tracer	Species/Variety	Strain
1	*Pochonia bulbillosa*	CBS145.70
2	*P. chlamydosporia* var. *chlamydosporia*	CBS103.65
3	*P. chlamydosporia* var. *chlamydosporia*	CBS101244
4	*P. chlamydosporia* var. *chlamydosporia*	CBS429.64
5	*P. chlamydosporia* var. *chlamydosporia*	UAMH2736
6	*P. chlamydosporia* var. *chlamydosporia*	UAMH2439
7	*P. chlamydosporia* var. *catenulata*	P0297
8	*P. chlamydosporia* var. *catenulata*	UAMH1845
9	*P. chlamydosporia* var. *catenulata*	UAMH1776
10	*P. chlamydosporia* var. *catenulata*	UAMH1775
11	*P. chlamydosporia* var. *catenulata*	CBS250.83A
12	*P. chlamydosporia* var. *catenulata*	CBS101896
13	*P. chlamydosporia* var. *catenulata*	CBS504.66
14	*P. chlamydosporia* var. *catenulata*	CBS102064
15	*P. chlamydosporia* var. *catenulata*	MUCL15018
16	*P. gonioides*	CBS891.72
17	*P. microbactrospora*	CBS101433
18	*P. rubescens*	CBS464.88
19	*P. suchlasporia* var. *catenata*	CBS248.83
20	*P. suchlasporia* var. *suchlasporia*	CBS251.83
M	1 kb ladder	

Fig. 4a: MIS-PCR electrophorograms (1 % agarose gels) of various strains of *Pochonia* species

point toward a different pattern of side metabolites but a similar secondary metabolism in *P. rubescens* in submerged culture as compared to the type species of the genus and *P. suchlasporia* var. *catenata* CBS 248.83. During fermentation of *Pochonia* species in Q6/2, no red pigments were released into the culture media. The production of characteristic pigments of this species was studied in solid culture (LÓPEZ-LLORCA, MOYA & LLINARES 1994), but their chemical structures remain to be identified.

Neither strain belonging to the allied genera *Haptocillium, Lecanicillium, Rotiferophthora* and *Simplicillium* nor *V. epiphytum* contained RAL or pseurotin A. One of the *V. epiphytum* strains (CBS 650.85) was previously reported to produce antiamoebin, but due to the limited detection range no signal revealing the expected molecular weight (1675 Da) could be seen by HPLC-MS. However, both isolates of *V. epiphytum* studied contained a specific, yet unidentified main metabolite (**VE1** in Fig. 2c). The mycotoxin, helvolic acid [found in *V. epiphytum* according to an unconfirmed and unpublished reference given in TURNER & ALDRIDGE (1983)] was excluded in any strain examined by comparison with an authentic standard provided by Sigma-Aldrich. *Simplicillium lanosoniveum* and *Rotiferophthora* species produced two prominent metabolites each, which were also not encountered in fungi of other genera (**SL1**/ **SL2** and **RM1**/**RM2**, respectively). In the ex-neotype strain of *L. lecanii*, rather characteristic but yet unknown metabolites showing only end absorption by UV-Vis detection were detected as main metabolites. Out of at least seven congeners, the data for the three main components **LL1**, **LL2** and **LL3** are provided in Tab. 3. Their molecular weights were determined to be in the range of 600 – 650 Da. So far, no metabolites with such characteristics have been re-



Trace	Species	Chemotype	Strain
1	*Pochonia bulbillosa*	1	CBS 145.70
2	*P. bulbillosa*	2	CBS 247.68
3	*P. suchlasporia var. catenata*	2	CBS 749.83
4	*P. suchlasporia var. catenata*	2	UAMH 6596/CBS 495.90
5	*P. suchlasporia var. catenata*	3	CBS 817.83
6	*P. suchlasporia var. suchlasporia*	3	CBS 251.83
7	*P. suchlasporia var. catenata*	2	CBS 416.95
8	*P. suchlasporia var. catenata*	2	P 0452
9	*P. suchlasporia var. catenata*	4	CBS 248.83
M	1 kb ladder		

Fig. 4b: MIS-PCR electrophorograms (1 % agarose gels) of various strains of *Pochonia bulbillosa* und *P. suchlasporia* var. *catenata*

ported from *Verticillium* species, according to a search in Antibase, BNPD and DNP.

Metabolite profiles of *Haptocillium* species differed from other genera studied in the general presence of numerous peaks of rather high polarity, which eluted in the range of Rt = 3–6 min (see for example *H. zeosporum* in Fig. 2c) . The gradient systems employed were not suited to achieve a good resolution, and it was difficult to interpret the HPLC data. However, two compounds, one of which constituted a minor metabolite, were detected in all *Haptocillium* species by their characteristic HPLC-UV/Vis spectra in conjunction with matching Rt. The identity of these compounds (main component **HZ1** and minor component **HZ2**) remains to be clarified.

B. MIS PCR and sequence analyses of ITS nrDNA

The results of electrophoretic separations of the MIS PCR products are depicted in Figs. 4a (several *Pochonia* species in concert) and 4b (various strains of *P. suchlasporia* and *P. bulbillosa*).

The evaluation of some species of genera other than *Pochonia* was attempted as well. However, problems such as the presence of PCR inhibitors in the starting materials (especially in *Haptocillium* species) and a decay of DNA and PCR products (possibly due to the presence of DNAses) prevented the straightforward application of this method. Hence, the results on MIS PCR data presented here are restricted to *Pochonia*, while an optimisation of methods is ongoing to overcome the problems encountered in related genera. Only recently, the DNA extraction method was optimised (introducing a phenol extraction step as described in the methods section). The major fragments detected allowed for visual comparison and manual alignment of data. One strain each was so far examined of the species *P. microbactrospora* and *P. rubescens*, and only one trace each is shown of *P. gonioides*. Thus, the patterns of *P. gonioides* (including strain MUCL 11413) and *P. microbactrospora* were found rather specific, while *P. rubescens* showed specific MIS data with similarities to some isolates of *P. suchlasporia* (Figs. 4a and Fig. 4b), in accordance with morphological characters and other PCR-based methods (Zare, Gams & Evans 2001).

In most strains of *P. chlamydosporia*, MIS PCR profiles were fairly in agreement with secondary metabolite profiles and previous results reported by Zare, Gams & Evans (2001). Within *P. chlamydosporia* var. *catenulata*, two different main profile types were observed. P0297 clustered with CBS 101896, CBS 102064 and CBS 504.66 (traces 7 and 13–15 in Fig. 4a), all of which also produced pseurotin A and large

amounts of RAL and showed a characteristic group of two fragments of similar size. A second group within this variety, consisting of strains CBS 250.83A, UAMH 1775 and UAMH 1776 (traces 9,10 and 11 in Fig. 4a), showed clearly distinct MIS profiles. UAMH 1845 (trace 8 in Fig. 4a) showed a MIS pattern similar to the latter group but the minor bands were lacking. As this strain also had shown a deviating metabolite profile, its ITS nrDNA was sequenced and found to be 100 % in agreement with that of the ex-type strain of *P. chlamydosporia* var. *catenulata* (see ZARE, GAMS & EVANS 2001). In agreement with morphological data, its correspondence to this variety was thus confirmed.

In addition to showing deviating metabolite profiles (see above), MUCL 15018 also differed in its MIS PCR profile from the other strains of this variety examined. Its electrophoretic band pattern was even reminiscent of *P. chlamydosporia* var. *chlamydosporia* (compare traces 2–6 and 15 in Fig. 4a). Their patterns also matched, only revealing different intensities of the two main and some minor bands. The data obtained with *P. chlamydosporia* var. *chlamydosporia* MUCL 8330 and MUCL 10008 (not shown) were found quite similar to the one of CBS 103.65, CBS 101244 and the examined UAMH strains of this variety. This was in agreement with morphological characters but not always with the HPLC profiling data (*vide supra*).

The MIS profiles of both varieties of *P. suchlasporia* differed from those of the other species, including *P. chlamydosporia*. However, we found that this species and especially variety *catenata* also showed the highest intraspecific variation (Fig. 4b), which was again in conformity with their deviating metabolite profiles in culture. Those strains that produced the aurovertin-like compounds (Fig. 4b, traces 3–4, 7 and 8) differed clearly from the monorden-producing ex-type strain of *P. suchlasporia* var. *catenulata* (trace 9), and again other patterns were observed in strains CBS 817.83 and CBS 251.83 that produced yet different secondary metabolites. The profile of the ex-type strain of *P. bulbillosa* was lacking two minor bands that were observed in CBS 247.68, i.e., the strain that produced the same aurovertin-like metabolites encountered in part of *P. suchlasporia* var. *catenata*.

In summary, we observed deviations in MIS profiles that were in some way correlated with differences of HPLC profiles in *P. suchlasporia* and *P. bulbillosa*. Upon comparison with previously published data (ZARE, GAMS & EVANS 2001), we noted that many of these strains had only been characterised morphologically and by RFLP. At least the RFLP data of the mtDNA reported for this variety by the latter workers had already shown variations. Sequences of their ITS nRDNA had only been published for CBS 248.83 and CBS 789.85 (the latter of which was not included in our study). We therefore obtained the ITS nRDNA sequences of the strains in question and compared them with those published by ZARE, GAMS & EVANS (2001). In addition, previous results of these authors on the sequences of the ex-type strains CNS 145.70 and CBS 248.83 (which were sequenced along with the other strains as "standards") were confirmed.

Strains CBS 416.95, CBS 495.90 and CBS 749.83 and P0452, but even *P. bulbillosa* CBS 247.68 had ITS nrDNA sequences 100% identical to the one of the monorden-producing ex-type strain of *P. suchlasporia* var. *catenata,* CBS 248.83, while the sequence of CBS 145.70 was in full agreement with the one deposited in Genbank by ZARE, GAMS & EVANS (2001). This means that ITS nrDNA sequences revealed significant differences of the two examined strains *of P. bulbillosa* and were not suited to further segregate strains of *P. suchlasporia* var. *catenata*. These findings were in accordance with the high morphological similarities of these fungi but disagreed with the variability observed in both HPLC and MIS-PCR profiles.

Discussion

The work presented here was aimed at the extension of a chemotaxonomical methodology that had been developed during previous studies on *Daldinia* (STADLER et al. 2001) to another group of fungi with different taxonomic position, lifestyle - and metabolite profiles. In this polyphasic approach, morphological studies and PCR-fingerprinting were carried out to verify the results obtained by HPLC profiling. Some characteristic metabolites were available as standards to facilitate their detection in the crude extracts of the strains investigated. In contrast to the Xylariaceae study, *i)* only cultures and no stromata were studied, *ii)* the standard compounds were not found in the cultures after prolonged fermentation and *iii)* in addition to reliable information on the morphology and ecology of these fungi, substantial data on their molecular characteristics was available, due to the recent efforts of Gams, Zare and other authors. Both Xylariaceae cultures and the fungi presently treated have in common that they are generally not easy to identify based on morphological grounds and that both are well-known to be highly creative secondary metabolite producers.

In the following we wish to discuss our findings with regard to *i)* the adaptation of the methodology to *Pochonia* species and allies, *ii)* the possible chemotaxonomical significance of known and yet unidentified metabolites, *iii)* correlations of HPLC profiles and PCR data, and *iv)* the possible impact of this work to further improve our understanding of the biology and taxonomy of conidial fungi with *Verticillium*-like anamorphs and their relatives.

A. Chemotaxonomical methodology and time course of production of RAL

As discussed by FRISVAD, THRANE & FILTENBORG (1998), protocols for fermentation, extraction and detection of secondary metabolites for chemotaxonomic purposes should be standardised, whenever possible. In the current study, this was ac-

complished by propagation of the strains under investigation in standardised submerged cultures. Some solid cultures and agar plates were also extracted during preliminary experiments. Monorden (**1**), for instance, was found on liquid PD and OA media as well as in solid state OA, YMG and PD agar plates but it was preferably produced in liquid submerged culture (HELLWIG et al. 2003). Our studies on P0297 and other monorden-producing fungi had revealed that the compound did not accumulate in the cultures and was never found in cultures grown for more than ten days, when the pH generally turned alkaline. Solid fermentations were finally abandoned because the onset as well as the endpoint of secondary metabolite production were difficult to determine. Physico-chemical studies on monorden had pointed toward its instability. For instance, monorden immediately decayed in methanolic 1N NaOH and was also found to be unstable upon evaporation of acidic preparative HPLC fractions (HELLWIG et al 2003), or if exposed to light at room temperature in either methanol or H_2O acidified to pH 2.5 with HCl for three days. Hence, fermentation conditions adapted to optimal production of monorden appeared be suited best for a chemotaxonomic study on its occurrence in allied taxa. Accordingly, the glucose content of the media and the pH value were measured as surrogate parameters to follow the time course of secondary metabolite production. In several monorden-producing strains, comparisons of HPLC profiles during fermentation revealed that the production of RAL and most other main detectable metabolites started after 72-96h and stagnated about one day after the free glucose in the culture medium was depleted. Thereafter, the pH value of the culture broth increased, along with disappearance of the RAL and, to some extent, also pseurotin A (**12**). These observations pointed toward the destruction of the RAL, e.g., by esterases produced by the fungus after the stationary growth phase, or merely due to the instability of these lactones when exposed to alkaline conditions (see also *P. rubescens* below). Consequently, all fermentations were terminated at least one day after depletion of free glucose and at pH values in the neutral or the mildly acidic range.

Some strains grew rather slowly and were thus propagated for more than 300 h, but most cultures showed similar growth parameters under the chosen conditions, and all had produced sufficient biomass and consumed their main carbon source at the stage of harvest (Tab. 2). The methodology was thus optimised for production and detection of RAL and pseurotin A. Characteristic pigments produced by particular species such as *P. rubescens* are only observed in solid culture. Remarkably, MIRRINGTON et al. (1964) had noted in the course of their chemical studies on monorden (**1**) that this compound turned to a red enolate pigment under alkaline conditions. On the other hand, monorden was also found unstable under acidic conditions and it might therefore also decay in solid culture. Hence, further studies on *P. rubescens* and other species should be carried out, using a wider range of culture conditions.

In some instances, deviations in HPLC profiles were confirmed by morphological re-examination and PCR data, resulting in the re-classification of the respective strains according to the current taxonomy. In other cases (e.g. CBS 103.65, MUCL 15018 and chemotypes of *P. bulbillosa* and *P. suchlasporia,* and *P. rubescens*, where only one strain was so far included), further studies are needed. For this purpose, we have obtained further extant strains of these taxa, whose characteristics will be the subject of a subsequent paper.

Because of the remarkable diversity of secondary metabolites observed in *Pochonia* and allies, new species may be soon classified on a basis of these "chemotypes" and supported by other characters (see below). During HPLC profiling studies on fungal cultures, it is generally difficult to discuss the lack of production of a given compound in a chemotaxonomical context (as it is always hard to prove a negative). For example, it cannot be excluded that in some strains the regulation of secondary metabolite production may be triggered by processes different from those encountered in P0297. The lack of secondary metabolite production may be due to the fact that the respective producer strains lost their biosynthetic capabilities after repeated subculture and resulting degeneration, which is commonly noted in fungi. In *Pochonia*, loss of dictyochlamydospore production is a commonly observed sign of degeneration (GAMS, ZARE & EVANS 2001), and the same may be true for loss of the ability to produce otherwise typical secondary metabolites.

Moreover, some further characteristic compounds may not have been found because of, e.g., overproduction of a prevailing metabolite in the crude extract, which generally will complicate the detection of minor components. For example, the novel pochonins **4** - **6** were not detected in the crude extracts of *P. chlamydosporia*. Even in strain P0297, their characteristic signals only became clearly detectable after enrichment in preparative HPLC fractions. Upon HPLC analyses of crude extracts, their corresponding HPLC-UV and HPLC-MS signals had still been overlaid by those of the main component, monorden (**1**), which gave a rather broad peak. To verify the results of the present study and to reach further conclusions, selected strains therefore should be subjected to large scale fermentation and preparative HPLC of their crude extracts, followed by analyses of the resulting fractions.

Alternative extraction procedures and analytical protocols might result in the improved detection of, e.g., hydrophilic metabolites of *Haptocillium* species. However, the procedure employed should be regarded as adequate to detect almost any secondary metabolites of medium to low polarity range and low molecular weight.

B. PCR-based data, HPLC profiles and the taxonomy of *Pochonia* and allies

With few exceptions, production of RAL was found to be a rather constant feature in isolates of *Pochonia chlamydosporia* obtained from soil, plant material, nematodes and molluscs, independent from their geographic origin. Monorden (**1**), the prevailing metabolite, also occurred in other species

and varieties of *Pochonia* (*P. suchlasporia* var. *catenata* CBS 248.83, *P rubescens*) but was so far not encountered in *P. bulbillosa*, *P. gonioides* and *P. microbactrospora* or in strains of the allied genera *Haptocillium*, *Rotiferophthora*, *Lecanicillium* and *Simplicillium*.

While the intensified evaluation of the latter genera is pending, *Verticillium epiphytum* was already included in the current study. This mycoparasite of rust belongs to the so-called residual group of *Verticillium* sect. *Prostrata*, i.e., it was not yet assigned to any of the new genera established by GAMS & ZARE (2001) because molecular data had not clarified its affinities. The status of this species as a member of the Clavicipitaceae was supported in the molecular study by SUNG et al. (2001), although these authors stated that its relationships to other teleomorphs and anamorphs remained poorly resolved. From a chemotaxonomical point of view, the fact that the peptaibol, antiamoebin (THIRUMALACHAR 1972) is the only compound previously identified from this species actually may raise the question whether or not *V. epiphytum* constitute a true member of the Clavicipitaceae. Peptaibols are oligopeptides containing large amounts of the rare amino acid aminoisobutyric acid. Fide Antibase and DNP, these metabolites are widespread in *Trichoderma* Pers., *Hypomyces* (Fr.) Tul., *Sepedonium* Link and other genera belonging to the Hypocreaceae but have rarely been encountered from other fungi, including Clavicipitaceae. Out of a total of 158 metabolites of this type that are currently listed in Antibase, more than 140 were reported from *Trichoderma* (101 entries) or other Hypocreaceae (42 entries). Antiamoebin itself was also reported from *Emericellopsis* J.F.H. Beyma (Bionectriaceae) and *Stilbella* Lindau (mitosporic Hypocreales; THIRUMALACHAR 1972, ANKE & STERNER 1997), both of which also belong to the Hypocreales but are not members of the Clavicipitaceae (KIRK et al. 2002). In the current study, two strains of *V. epiphytum* failed to produce any of the typical metabolites of either *Pochonia* or *Rotiferophthora* and produced a characteristic compound (**VE1**). The identity of this metabolite with antiamoebin remained unclear because its molecular weight (1675 Da) was slightly higher than the range allowed for by the instrumental parameters employed by us using HPLC-MS (up to 1500 Da). This phenomenon was beyond the scope of the present study. As will be discussed below, there are several metabolites which are produced by a wide range of species within the Hypocreales, but there is little information as to whether the production of these compounds is restricted to individual taxa.

The UV-visual spectra of the characteristic compounds found in most strains of *P. suchlasporia* var. *catenata* and *P. bulbillosa* CBS 248.83 resembled those of aurovertin B and citreoviridin A (Tab. 4). The molecular masses of the metabolites **PS3** and **PS4**, however, were different from those of the standard compounds, and they eluted at earlier Rt than standards of the known compounds. The citreoviridins were previously described from *Aspergillus* and *Penicillium* species, while aurovertins are known from *Calcarisporium arbuscula* (see overview by TURNER & ALDRIDGE 1983). Citreoviridins and aurovertins exhibit very strong adverse effects in biological systems, including phytotoxic (CUTLER 1988), antiparasitic (BASCO & LE BRAS 1994, CATALDI DE FLONBAUM & STOPPANI 1981) broad-spectrum cytotoxic (RICHARD 1990) and antibiotic activities. Such compounds also were likely to affect, e.g., invertebrate or fungal hosts of *P. bulbillosa* and *P. suchlasporia*. Their most important biological target sites are probably the mitochondrial ATPases of various eukaryotic organisms (LOWE & BEECHEY 1986). However, neurotoxic effects, possibly relating to their modulation of g-aminobutyric acid metabolism (DATTA & GHOSH 1983) were also ascribed to this class of mycotoxins.

The results of the MIS PCR investigations appear promising for strain identification purposes of *Pochonia* species, even though more isolates of several species need to be studied. Eventually, the method could possibly serve as a prerequisite for chemotaxonomical and phylogenetic studies based on large strain collectives. DNA sequencing, microscopic work or preparation of samples by fermentations and extraction are generally more time-consuming and work-intensive, and MIS PCR appears to be a method of choice for pre-selection.

For example, the concurrent variability seen in the MIS and HPLC profiles of both varieties of *P. chlamydosporia* points toward the presence of further subgroups (chemotypes). This should be confirmed by evaluation of a larger number of species and isolates whether the correlations between MIS data and HPLC profiles in *Pochonia* species coincided. If this were the case, MIS data would possibly also be valuable as a parameter to monitor population genetic studies on these fungi and even help to discover potentially further, yet undescribed species. As compared with the findings of the RFLP techniques employed by ZARE, GAMS & EVANS (2001), the MIS PCR data appear to be either in agreement or may even provide a better resolution. The results also stand in contradiction to those reported by ARORA, HIRSCH & KERRY (1996), who could not discriminate their strains of *Verticillium chlamydosporium* by PCR-based methods. This suggests that their fungi should be re-examined by either the methods used by GAMS, ZARE & EVANS (2001), and/or by the ones presented in the current paper. However, the limitations of MIS PCR also became apparent by the fact that allied genera were not quite as easy to study, and the mode of sample preparation for PCR yet remains to be optimised. Furthermore, the handling, alignment and analysis of such data should be facilitated by further standardisation of this technique, possibly by a transfer of the bands observed by electrophoresis into a format allowing for computerised image analyses (see ARDRA in STADLER et al. 2001).

The variability in *P. suchlasporia* regarding MIS-PCR and HPLC profiles despite having identical ITS nrDNA sequences and similar morphological characters also raises the question as to the existence of "cryptic" species within this taxon.

It was shown that *P. suchlasporia* var. *catenata* can be divided into (at least) three chemotypes. These are represented by *i)* the monorden-producing ex-type strain (CBS 248.83; chemotype 1), *ii)* by CBS 817.83 and MUCL 15046, which contain compounds similar to *P. suchlasporia* var. *suchlasporia* (chemotype 2) and *iii)* by the remaining strains of this taxon listed in Tab. 1, which produce the aureovertin-like compounds **PS3** and **PS4** (chemotype 3).

Here, the phylogenetic species concept (TAYLOR et al. 2000) can apparently not yet find application. The molecular data available do obviously not reflect the differences that can easily be observed by chemotaxonomical methodology in *Pochonia* species.

In Basidiomycete and Xylariaceae taxonomy, pigments and other secondary metabolites are traditionally used to segregate species, and even in conidial fungi, deviations in secondary metabolite production frequently proved to be indicators for the existence of undescribed species (FRISVAD, THRANE & FILTENBORG 1998). Only recently, PCR-based methods have been engaged to study such occurrences. For instance, recent studies on *Stachybotrys chartarum* (Ehrenberg ex Link) Hughes strains from indoor environments (ANDERSEN, NIELSEN & JARVIS 2002) revealed the presence of cryptic species within this taxon, which were found to differ in their secondary metabolite profiles. Particular isolates were found to preferably produce alkaloids of the atranone type, while others contained instead macrocyclic trichothecene mycotoxins. PELTOLA et al. (2002) later showed that these isolates also differed in their RAPD patterns and on the presence of the trichodiene synthase (*Tri*5 gene). From independent studies by CRUSE et al. (2002), employing sequencing and phylogenetic analyses of three polymorphic protein loci (including trichodiene synthase), the existence of cryptic species in *Stachybotrys chartarum* was postulated as well. The genes encoding for secondary metabolite production have so far been rarely included in molecular phylogenetic studies. It may be especially useful to include them in a molecular phylogeny of those fungi that are likely to have gained a selective advantage because of their lifestyle and evolution. So long as these genes have not been studied, HPLC profiling to detect and analyse their distribution will in our opinion remain to be the method of choice.

Another matter is the occurrence of two chemotypes *in P. bulbillosa*: In agreement with the morphological studies by GAMS (1988) and RFLP data by GAMS, ZARE & EVANS (2001)], the morphological characters of CBS 247.68 did not deviate much from those of the ex-type strain, CBS 145.70. We found the ITS sequence of CBS.247.68 identical to the ones of *P. suchlasporia* var. *catenata*. CBS 247.68 had already shown deviations to both *P. suchlasporia* and other strains of *P. bulbillosa* when examined by RFLP of the mtDNA (ZARE, GAMS & EVANS 2001). It had originally been reported by GAMS (1971) as a separate species, *Verticillium cephalosporum,* which he then characterised by the complete absence of dictyochlamydospores and only later included in *V. bulbillosum* by GAMS (1988), even though he stated that its conidia were somewhat atypical of *V. bulbillosum*. Interestingly, some isolates that had been regarded as *V. cephalosporium* by Gams (1971) were later transferred into *P. suchlasporia*, despite they did not form dicytochlamydospores in culture (GAMS 1988). During our own studies we only observed occasional production of swollen hyphae but no dictyochlamydospores in the culture of CBS 247.68. We also failed to observe conidial chains that would be typical for *P. suchlasporia* var. *catenata* in this strain as well as in some of the *P. suchlasporia* isolates. In these species it is apparently not easy to find parameters that allow for clear segregation of taxa. Expression of typical morphological characters may degenerate during preservation, and even molecular methods may provide insufficient information, as already seen in the phylogenetic studies by ZARE, GAMS & EVANS (2001). In their analysis of the 5.8S/ITS nrDNA regions, *P. chlamydosporia* appeared clearly distant from the other species and appeared related to *Rotiferophthora* species. The sequences of the remaining species (including *P. bulbillosa* and *P. suchlasporia*) were found rather similar, and those of *P. bulbillosa* and *P. gonioides* were almost identical, despite the fact that the latter two species can be easily distinguished by morphological characters.

It has been shown before that different species of conidial fungi may have identical ITS nrDNA regions, while their morphological characters are in agreement with secondary metabolite profiles. For instance, the 5.8S/ITS sequences of *Penicillium solitum* Westling, *P. echinulatum* Raper & Thom and *P. discolor* Frisvad & Samson, were also found 100 % identical. While *P. solitum* differs from the other two species in having smooth conidia and all are distinguished by other, less discriminative morphological and cultural characters, their secondary metabolite profiles are highly species-specific (SKOUBOE al. 1999 and references therein). Aside from several types of metabolites that are common in all three species, *P. solitum* produces the HMG reductase inhibitors of the compactin type, whereas tremorgenic mycotoxins are found in *P. echinulatum* and the highly cytotoxic chaetoglobosins in *P. discolor*. Further examples for *Penicillium* spp. with highly similar 5.8S/ITS nrDNA sequences that differ in their secondary metabolite profiles are (according to J.C. Frisvad, pers. comm.) the ochratoxin-producing species related to *P. verrucosum* Dierckx studied by LARSEN, SVENDSEN & SMEDSGAARD (2001). On the other hand, both features appear to be in accordance in the *P. simplicissimum* (Oudemans) Thom complex (TUTHILL, FRISVAD & CHRISTENSEN 2001), where a fair resolution into species was achieved by concordant sequence data and secondary metabolite profiles.

The aim of the present study was not the description of new species. However, the aforementioned data, as well as the current characterisation of *Pochonia* emphasise the advantage of a polyphasic species concept for segregation of morphologically similar groups of conidial fungi.

C. Occurrence of RAL and pseurotin A in phialidic hyphomycetes

Pseurotin A (**12**) has previously been reported from a *Diheterospora* species (KOMAGATA & HAYAOKA 1996), but was earlier also discovered in *Pseudeurotium ovale* Stolk (BLOCH & TAMM 1976) and *Aspergillus fumigatus* Fr. (WINK et al. 1992; WENKE, ANKE & STERNER 1993). Pseurotin A is also frequently found in particular species of *Penicillium* and is apparently widespread in Eurotiales (J.C. Frisvad, pers. comm.). The status of *Pseudeurotium* and the family Pseudeurotiaceae has recently been reconsidered from the outcome of molecular studies, which resulted in their recognition as a polyphyletic group with little or no affinities to the Eurotiales (SUH & BLACKWELL 1999). Interestingly, the type strain of *Pseudeurotium ovale* was originally isolated from a cyst of the nematode *Globodera rostochiensis*. The distribution of Pseurotin A in aforementioned taxa should therefore be examined systematically. The report by WENKE, ANKE & STERNER (1993) on *in vitro* inhibition of chitin synthase from *Coprinus cinereus* (Schff. ex Fr.) S.F. Gray may give rise to speculate whether this compound will mediate the development of nematode cysts, where chitin biosynthesis is essential. However, pseurotin A was found devoid of nematicidal effects in a motility assay using the free-living nematode species, *Caenorhabditis elegans* by Wenke and co-workers. It has never been reported to exhibit any significant insecticidal or fungicidal activities.

Monorden (**1**) is already known to occur in egg parasites of nematodes. The compound was repeatedly reported from fungi identified as "*Diheterospora"* or *"Verticillium chlamydosporium"* in accordance with the results presented here (ESPENSHADE & CALTON 1978, MYERS-KEITH 1986, LEINHOS & BUCHENAUER 1992, KHAMBAY et al. 2000). In addition, it has been found several times in the extracts of other fungi. *Monocillium* S.F. Saksena is an anamorph of *Niesslia* Auerswald, a genus of the Niessliaceae (Hypocreales). The mycophilic fungus *Monocillium nordinii* (Bouchier) W. Gams was the first species from which the monocillins had been isolated as co-metabolites of monorden (AYER et al. 1980). Monorden had also been reported under the synonym radicicol in cultures of *Nectria* (anam. *Cylindrocarpon) radicicola* Gerlach & L. Nilsson (DELMOTT & DELLMOTT-PLAQUEE 1953, MIRRINGTON et al. 1964), and in *Neocosmospora tenuicristata* S. Ueda & Udagawa (SHIMADA et al. 1995). The former species is now classified as *Neonectria radicicola* (MANTIRI et al. 2001). Further RAL with chemical structures very similar to monorden appear to be common in the Hypocreales. The zearalenones, which are oestrogenic mycotoxins, also belong to the chemical type of RAL and are widespread in *Fusarium* Link (CHELKOWSKI 1998). The related *Hypomyces trichothecoides* Tubaki [syn. *H. subiculosus* (Berk. & M. A. Curtis) Höhnel according to W. Gams, pers. comm.] was reported to produce the analogous hypothemycin (NAIR, CAREY & JAMES 1981). Several additional derivatives of zearalenones have been reported from *Cochliobolus* (Pleosporales) and other fungi according to TURNER & ALDRIDGE (1983) and ANKE (1997). Hypothemycin and related compounds were also found in *Aigialus* J. Kohlmeyer et S. Schatz (Massarinaceae, Pyrenulales) by ISAKA et al. (2002) and in the basidiocarps of a *Coriolus* Quélet species (AGATSUMA et al. 1993). The latter report gives rise to assume that it may be involved in parasitism by a mycophilic species because it would be most unusual to encounter such a compound in healthy basidiomycete fruitbodies. Finally, species of *Humicola* Traaen (TANAKA et al. 1998, WICKLOW et al. 1998), *Penicillium* Link (NOSAWA & NAKAYIMA 1979) and *Phoma* Sacc. (DREYFUSS et al. 1994, ZAO et al. 1999) were also reported to produce monorden and/or other RAL with similar structural properties. Notably, monorden has become famous because its production was increased by solid fermentation of a *Humicola* species as compared to conventional procedures during space flight (LAM et al. 1998). Lasiodiplodins are structurally similar metabolites with a smaller lactone ring than the typical RAL that were reported from plant-pathogenic species of *Lasiodiplodia* Ellis & Everh. (Dothideales) by MATSUURA et al. (1998).

From these data it is deduced that RAL, including monorden, may occur in many ascomycete orders, while particular species within Hypocreales and Clavicipitaceae appear to be especially rich in such metabolites. Because of the obvious benefit which may be involved in their possession, analogies in biogenetic pathways leading to bioactive agents should not be presumed rare in mitosporic fungi.

D. Other compounds from species formerly included in *Verticillium* sect. *Prostrata*

Numerous peaks corresponding to yet unknown compounds were observed as major and minor components in the extracts examined. This prompted us to conduct a query in natural products databases (Antibase, DNP, BNPD) on previously published metabolites from taxa that are now included in *Pochonia*. These databases allow for a search on generic names such as "*Verticillium"* and some synonyms, as well as some species names in conjunction with meanwhile outdated generic names (e.g., "*Diheterospora chlamydosporia", Verticillium coccosporium*"). The structures of these metabolites, some references and comments on their biological activities are listed in Tab. 4.

"Diheterospora" (= *Pochonia*) species were reported to produce diheterosporin (MASUOKA et al. 1997) and chlamydocin (CLOSSE & HUGUENIN 1974), two peptides with cytostatic and further interesting biological activities. A structurally similar oligopeptide with a molecular weight of 528 Da was reported from a fungus named *V. coccosporum* by GUPTA et al. (1994). This organism was probably misidentified. As stated by GAMS, ZARE & EVANS (2001), at present no strain is available that exactly matches this species originally described by DRECHSLER (1941). We did not yet study *Pochonia* species on the occurrence of such peptidic compounds, even though this may be feasible, once standards for HPLC studies will be made available. These examples only give a brief over-

view on the metabolic diversity of fungi formerly classified in *Verticillium* sect. *Prostrata*, and it would probably lead too far to discuss all further previous publications. On the other hand, there are whole genera such as *Rotiferophthora*, whose secondary metabolism has apparently not been studied at all. These fungi may harbour a great diversity of previously untapped metabolites, and further studies like the present one will hopefully be rewarding.

One question that could not be answered as yet is whether these metabolites play a role in the actual process of parasitism. KHAMBAY et al. (2000) evaluated biological activities of an extract from *P. chlamydosporia* against *Meloidogyne incognita*, an important plant pathogenic nematode, by bioassay-guided fractionation of the crude extracts. This root-knot nematode was not affected under the chosen conditions by a fraction consisting of monorden. Instead the main nematicidal principle was identified as phomalactone (a simple reactive lactone not detected in the present study). However, monorden (**1**) has indeed been reported to possess nematicidal, antiparasitic and various other biological activities against other organisms (for overviews see ANKE & STERNER 2002, HELLWIG et al. 2003). During our studies RAL were located in the mycelia as well as released into the culture broth of *P. chlamydosporia*. Regarding the apparent instability of monorden in solution, the compound may only exhibit its activity upon direct contact of the fungal mycelium with the nematode hosts. It might have decayed under the assay conditions employed by KHAMBAY et al. (2000). As deduced from data on other biological activities described in the literature (e.g., cytostatic effects), cyclopeptides previously isolated from fungal taxa synonymous of *Pochonia* – such as chlamydocin and diheteropeptin (see above) – would also be likely to play a role in the host-parasite interactions. To the best of our knowledge these metabolites were never studied on insecticidal or nematicidal effects. Likewise, if the chemical similarities of the yet unknown compounds produced by most examined strains of *P. bulbillosa* with the mycotoxins of the aurovertin/citreoviridin group were verified, it would not be difficult to explain their biological function from a chemoecological view because their target sites in biological systems are well-known. According to Antibase, Aurovertin B was also reported from an ominous fungus named "*Verticillium sulphureum*". Neither the literature referring to the isolation of this compound (given in Antibase as TURNER & ALDRIDGE 1983) from this biological source nor any reference as to the correspondence to this organism was located as now.

Despite the various biological effects reported for these secondary metabolites, their actual involvement in the process of parasitism remains to be proven in either case. Considering large scale field application of such organisms in biological control, strains should be selected that affect the target host species effectively but do not overproduce mycotoxins. The available HPLC methodology would probably facilitate the detection of such compounds even in samples taken from mixed cultures of nematodes and fungi or even from the environment, again provided that standards are available.

In conclusion, metabolite profiling and PCR fingerprinting proved to be useful tools for further characterisation of the fungi studied. Secondary metabolites did also basically reflect the taxonomy established by Gams, Zare and co-workers as far as the genus *Pochonia* and its affinities to allied genera are concerned, while those genera themselves (i.e. *Lecanicillium*, *Haptocillium* and *Simplicillium*) are in need of further evaluation. It remains to be seen whether the results and methodology presented here will eventually lead to further deductions as to their classification or help to further elucidate the chemoecological role of the metabolites produced by these organisms. For the time being, the data presented here should be regarded as preliminary. Nonetheless, a further refinement of the techniques and the evaluation of a wider range of species and strains appears feasible. Future work will also show whether this approach proves valuable for applied purposes such as dereplication and diversification of microbial libraries established for industrial screening, as well as for the characterisation of fungal biopesticides.

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