The Himanimides, New Bioactive Compounds from *Serpula himantoides* (Fr.)Karst.

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Himanimides, Antibiotics, Basidiomycete

In a screening of basidiomycete cultures from Chile for the production of antibiotics we identified a *Serpula himantoides* strain as a producer of metabolites inhibiting the growth of bacteria and fungi. Bioactivity guided purification resulted in the isolation of four new antibiotics. Their structures were elucidated by spectroscopic methods. All four compounds are succinimide and maleimide derivatives, of which two are N-hydroxylated.

Introduction

Serpula himantoides (*Coniophoraceae*), a resupinate member of the aphyllophorales is of widespread occurrence in the temperate zones of Europe and the Americas. It causes an intensive brown rot on gymnosperms and more rarely on angiosperms. *S. lacrymans*, a closely related species causes the destructive dry rot of wooden structures in buildings. Both species contain the pulvinic acid derivatives xerocomic acid, variegatic acid, and variegatorubin, pigments typically encountered within the boletes (Gill and Steglich, 1987). The production of polyacetylenes by *S. lacrymans* has been reported (Jones and Thaller, 1984).

Materials and Methods

General

For analytical HLPC, a Hewlett Packard 1090 series II instrument (column: Merck LiChrocart 125–4 filled with LiChrospher 100 RP18) was used. Preparative HPLC was performed with a Jasco model PU-980 system with diode array detector. TLC analyses were performed on Macherey-Nagel Alugram Sil G/UV254 precoated plates and visualised with anisaldehyde/sulfuric acid 1:1 (5% in ethanol) and heating up to 120 °C. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in CDCl3, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra were recorded with a Jeol SX102 spectrometer, while the UV spectra were obtained with a Perkin Elmer λ16 and the IR spectra with a Bruker IFS 48 instrument. The optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Producing organism and fermentation

Fruiting bodies of *Serpula himantoides* were collected in Concepcion, Chile, growing on *Eucalyptus globulus* in autumn 1995. Mycelial cultures of strain 95099 were derived from the spore print of a fruting body. A voucher specimen of the fungus is deposited in the herbarium of the Department

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Biotechnology, University of Kaiserslautern. The strain 95099 is kept on YMG agar containing (g/liter): Glucose 4, malt extract 10, yeast extract 4, agar 20, pH 5.5. Fermentation was carried out in YMG medium at 24 °C in a 100 liter fermentor (Braun Biostat U) with aeration (15 liter air/min) and agitation (120 rpm); 10 liters of a well-growth culture (10 days) in the same medium were used as inoculum. During fermentation, aliquots of the culture fluid (200 ml) were extracted with ethyl acetate. The combined extracts were dried with Na_2SO_4 and concentrated *in vacuo.* (40 °C). The extracts were disolved in methanol and this solution (100 μg/ml) was used to determine the antimicrobial activity against bacteria and fungi. The fermentation was stopped after 240 h when the antibacterial (*Bacillus brevis, B. subtilis*) activity of the extract had reached its peak.

Isolation

The compounds were extracted from the culture filtrate by absortion onto Mitsubishi DIAION HP-21 resin. The resin was washed with methanol and the himanimides eluted with acetone yielding 4.13 g of crude product. This was applied onto a column (25 × 250 mm) containing silica gel (0.063–0.2 mesh, Merck 60). Elution with cyclohexane-ethylacetate 1:1 v/v yielded 615.4 mg of an enriched product which was applied onto another silica gel column. (15 × 145 mm, 0.063–0.2 mesh, Merck 60). From this column 64,7 mg of 95099–11 were obtained by elution with cyclohexane-ethylacetate 8:2 v/v. Elution with cyclohexane-ethylacetate 7:3 v/v yielded 198 mg of a himanimide A (**1**) containing fraction. From this 5.6 mg of pure **1** was obtained by preparative HPLC (column: Macherey and Nagel, 250 × 21.2 mm containing Nucleosil C18 (7 μm) flow rate: 5 ml/min; Gradient 0–70% MeOH in 40 min; R_t **1**: 24 min.

Elution of the silica gel column with cyclohexane-ethylacetate 3:7 v/v yielded 1633 mg of a fraction containing himanimide B (**2**) and himanimide D (**4**). A repetition of silica gel chromatography as described above resulted in 167.6 mg of pure 2 and 6 mg of pure **4**.

Himanimide A (**1**) (3-[4-(3-methylbut-2-enoxy)-phenyl-4-phenylmethyl-2,5-dioxo-3-pyrroline) was obtained as a yellow oil. UV (MeOH), λmax (log ε): 230 nm (4.1) and 356 nm (3.6). IR (KBr): 3450, 2925, 1735, 1600, 1510, 1465, 1380, 805, 745 and 700 cm^{-1}. See Tables I and II for NMR data. FABHRMS: (348.1598, $M+H^+$, $C_{22}H_{22}O_3N$ requires 348.1600).

Himanimide B (**2**) (3-[4-(2,3-dimethyl-3-methylbutoxy)phenyl-4-phenylmethyl-2,5-dioxo-3-pyrroline) was obtained as a yellow oil. UV (MeOH), λmax (log ε): 229 nm (4.3) and 356 nm (3.8). IR (KBr): 3445, 2930, 1715, 1605, 1510, 1455, 1350, 1255, 1180, 1085, 1030, 840, 745 and 700 cm^{-1}. See Tables I and II for NMR data. FABHRMS: (382.1659, $M+H^+$, $C_{22}H_{24}O_5N$ requires 382.1654).

Himanimide C (**3**) (*N*-hydroxy-3-[4-(3-methylbut-2-enoxy)phenyl-4-phenylmethyl-2,5-dioxo-3-pyrroline) was obtained as a yellow oil. UV (MeOH), λmax (log ε): 232 nm (4.3), 290 nm (3.7) and 374 nm (3.6). IR (KBr): 3470, 2925, 1720, 1605, 1510, 1250, 1180, 1080, 840 and 700 cm^{-1}. See Tables I and II for NMR data. FABHRMS: (364.1546, $M+H^+$, $C_{22}H_{22}O_4N$ requires 364.1549).

Himanimide D (**4**) (*N*-hydroxy-3-[4-(3-methylbut-2-enoxy)phenyl-4-phenylmethyl-2,5-dioxo-3-pyrrolidine) was obtained as a colourless oil, $[\alpha]_D$ 0° (*c* 0.5 in $CHCl_3$). UV (MeOH), λmax (log ε): 225 sh (4.1), 277 nm (3.4). IR (KBr): 3450, 2925, 1710, 1610, 1510, 1455, 1385, 1230, 1060, 1005, 835, 750 and 700 cm^{-1}. See Tables I and II for NMR data. FABHRMS: (366.1729, $M+H^+$, $C_{22}H_{24}O_4N$ requires 366.1705).

Biological assays

Antimicrobial activitiy was determined in the serial dilution assay or the plate diffusion assay as described by Anke *et al.* (1989). Test for cytotoxicity towards HL-60 cells (human promyelocytic leukemia, ATCC CCL 240), Colo 320 (human colon adenocarcinoma, DSMZ ACC 144) and L1210 (lymphocytic leukemia, mouse, ATCC CCL219) was determined as reported by Zapf *et al.* (1995). Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested as described by Anke *et al.* (1989). Inhibition of appressorium formation in germinating conidia of *Magnaporthe grisea* strain P1 was measured as described previously by Thines *et al.* (1997).

Results and Discussion

Fermentations were harvested 190–200 h after inoculation when the antibiotic content of the cul-

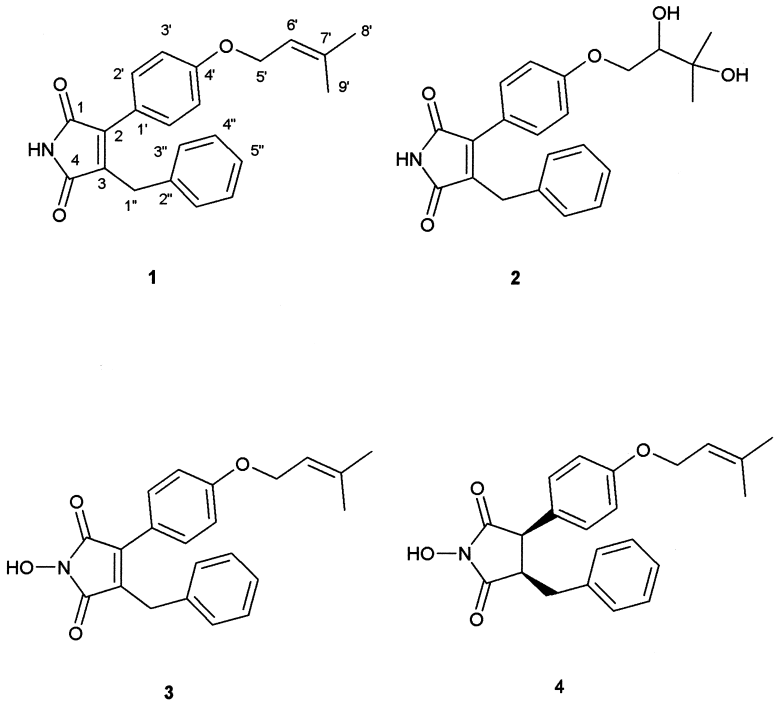

Fig. 1. The structures of himanimides A (**1**), -B (**2**), -C (**3**) and -D (**4**).

ture broth had reached its maximum. The compounds were isolated from the culture fluid as described in the experimental section. The mycelia did not contain antibiotic activity and were discarded. The structures (see Fig. 1) of the four metabolites were determined by spectroscopic techniques. High resolution MS experiments revealed the elemental composition of the four metabolites, and these were in agreement with the ^{1}H and ^{13}C NMR data (presented in Tables I and II). 2D NMR experiments, COSY NOESY, HMQC and HMBC, were used to establish the structures and to assign all signals. The composition of himanimide A (**1**) is $C_{22}H_{22}O_3N$, and the unsaturation index is consequently 13. The presence of two benzene rings, one as a benzyl group and the other as a 1,4-disubstituted benzene, together accounting for eight unsaturations, was indicated by the NMR data, and the ninth is situated in the 3-methylbut-2-enoxy substituent on C-4′ (positioned by the HMBC correlation between 5′-H_2 and C-4′). The remaining part, consisting of C_4HO_2N and accounting for four unsaturations, is substituted at C-2 by the *para*-substituted benzene (as indicated by the HMBC correlation from 2′-H to C-2) and at C-3 by the benzyl group (indicated by the HMBC correlations from 1″-H_2 to C-2, C-3 and C-4). The remaining proton, which is not attached to a carbon according to the HMQC spectrum, appears as a broad singlet at 7.39 ppm in the ^{1}H NMR spectrum and correlates to C-1, C-2, C-3

Table I. ^{1}H (500 MHz) NMR data (δ; multiplicity; *J*) for himanimide A (**1**), B (**2**), C (**3**) and D (**4**). The spectra were recorded in $CDCl_3$, and the solvent signal for $CHCl_3$ (7.26 ppm) was used as reference. The coupling constants *J* are given in Hz.

H	**1**	**2**	**3**	**4**
2	–	–	–	3.58, d, 3.6
3	–	–	–	3.11, m
2′	7.53, d, 8.8	7.54, d, 8.7	7.52, d, 8.8	6.80, d, 8.4
3′	6.97, d, 8.8	6.99, d, 8.7	6.95, d, 8.8	
			6.74, d, 8.4	
5′	4.56, d, 6.9	4.19, dd, 2.9, 9.6	4.55, d, 6.8	4.41, 6.6
		4.07, dd, 7.7, 9.6		
6′	5.50, t, 6.9	3.84, dd, 2.9, 7.7	5.49, t, 6.8	5.44, t. 6.6
8′	1.82, s	1.34, s	1.81, s	1.79, s
9′	1.76, s	1.29, s	1.76, s	1.71, s
1″	3.96, s	3.94, s	3.94, s	3.18, dd, 4.3, 13.6
				3.03, dd. 7.7, 13.6
3″	7.22, d, 7.2	7.20, d, 7.5	7.18, d, 7	7.10, d, 7
4″	7.31, t, 7	7.30, t, 7.5	7.27, t, 7	7.24, t, 7
5″	7.24, t, 7	7.23, t, 7	7.21, t, 7	7.21, t, 7
NH	7.39, brs	7.62, brs	–	–

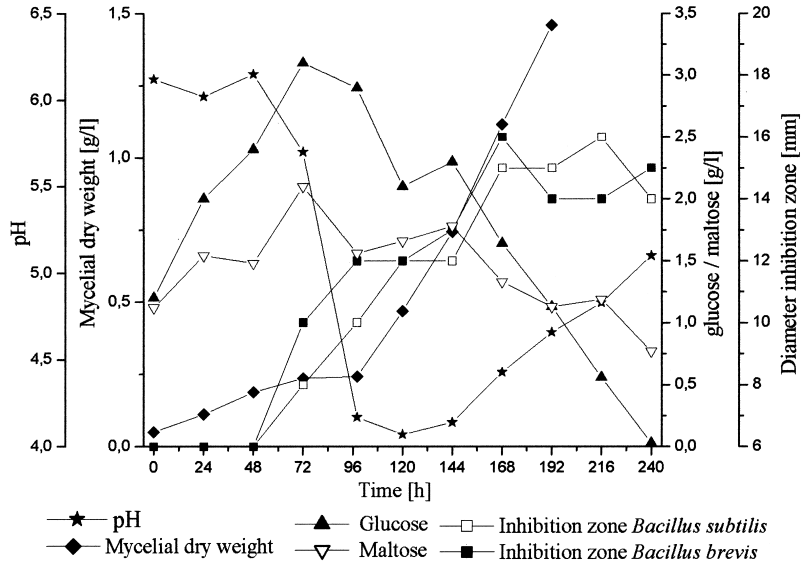

Fig. 2. Fermentation of *Serpula himantoides* and production of himanimides.

Table II. ^{13}C (125 MHz) NMR data (δ; multiplicity) for himanimide A (**1**), B (**2**), C (**3**) and D (**4**). The spectra were recorded in $CDCl_3$, and the solvent signal for $CDCl_3$ (77.0 ppm) was used as reference. The multiplicities of the carbon signals were determined indirectly from HMQC experiments.

C	**1**	**2**	**3**	**4**
1	170.8; s	170.8; s	167.8; s	172.8; s
2	139.2; s	138.9; s	136.2; s	47.5; d
3	136.9; s	137.4; s	133.5; s	48.2; d
4	171.3; s	171.3; s	168.4; s	173.8; s
1′	120.8; s	121.6; s	120.5; s	127.9; s
2′	131.1; d	131.2; d	131.2; d	128.9; d
3′	115.0; d	114.9; d	115.0; d	115.2; d
4′	160.5; s	159.9; s	160.6; s	158.5; s
5′	65.0; t	69.3; t	65.0; t	64.8; t
6′	119.2; d	75.7; d	119.1; d	119.5; d
7′	138.7; s	71.7; s	138.7; s	138.2; s
8′	25.8; q	26.6; q	25.8; q	25.8; q
9′	18.2; q	25.0; q	18.2; q	18.2; q
1″	29.8; t	29.8; t	29.9; t	35.3; t
2″	137.2; s	137.1; s	136.8; s	136.4; s
3″	128.4; d	128.3; d	128.3; d	129.4; d
4″	128.9; d	128.9; d	128.9; d	128.8; d
5″	126.8; d	126.9; d	126.9; d	127.2; d

and C-4 in the HMBC spectrum. Taken together, these data are only compatible with the suggested 3,4-disubstituted maleimide derivative **1**. Himanimide B (**2**) is oxidised relative to **1**, its elemental composition is $C_{22}H_{24}O_5N$ and comparison of the NMR data reveals that the oxidation has taken place in the 3-methylbut-2-enoxy substituent. Both C-6′ and C-7′ are saturated but oxygenated in **2**, and the C-4′ substituent must therefore be 2,3-dihydroxy-3-methylbutoxy. Also himanimide C (**3**), with the composition $C_{22}H_{22}O_4N$, is oxidised compared to **1**, but the 1D NMR data of the two compounds are very similar. The only differences are observed in the maleimide part, where the ^{13}C signals are slightly upshifted, and no imide proton could be seen in the ^{1}H NMR spectrum. The conclusion is consequently that himanimide C (**3**) is a *N*-hydroxylated maleimide derivative, unusual in nature but previously suggested to be produced by the slime mould *Arcyria denudata* (as *N*-hydroxyarcyroxepin A and *N*-hydroxyarcyroxepin B, see Dictionary of Natural Products on CD, Chapman and Hall 2001). Finally, himanide D (**4**) with the composition $C_{22}H_{22}O_4N$, is reduced compared to **3**, and comparison of the 1D NMR data reveals that the change has taken place in the maleimide ring. COSY correlations from 1″-H_2 to 3-H and on to 2-H suggest that **4** is a succinimide derivative, the magnitude of the vicinal coupling constants are in agreement with this and the HMBC correlations both to and from the succinimide ring confirm the suggested structure. The configuration of C-2 and C-3 appear to be as shown in Figure 1, as no NOESY correlations can be observed between 1″-H_2 and 2-H. Himanide D (**4**)

is optically inactive, and it is possible that a racemisation takes place during the isolation procedure.

The antimicrobial spectrum and cytotoxic activity of the himanimides are presented in Table III and Table IV respectively. The himanimide C (**3**) exhibited fungicidal effects specially against *Alternaria porri, Aspergillus ochraceus* and *Pythium irregulare* from a concentration of 25 μg/ml (69.6 μM) on. Fungistatic effects were observed against *Absidia glauca*, *Cladosporium cladosporioides*, *Curvularia lunata*, *Zygorhynchus moelleri, Nadsonia fulvescens*, and *Saccharomyces cerevisiae* strain is 1. It also showed moderate or weak antibacterial activity against Gram-positive bacteria and yeast. No activity was observed against Gram-negative bacteria. **3** also showed strong cytotoxic activity on HL 60 with IC_{50}-values of 10 μg/ml (27.5 μM) against HL 60 and moderated against L1210 (25 μg/ml, 69.6 μM) whereas lower cytotoxic effects were observed with Colo 320. None of the other three compounds exhibited considerable antimicrobial effects against the bacteria, yeast, fungi and different cell lines tested, suggesting that the biological activity of himanide C (**3**) is linked to the *N*-hydroxylated maleimide moiety. On the other hand, none of the compounds exhibited phytotoxic activities up to concentration of 100 μg/disc

Table III. Antimicrobial activity of compounds isolated from *Serpula himantoides*.

Organism	MIC [μg/ml] Himanimide			
	A	B	C	D
Filamentous fungi				
Absidia glauca +	–	–	10s	–
A. glauca –	–	–	–	–
Alternaria porri	–	–	25†	–
Ascophyta pisi	–	–	–	–
Aspergillus ochraceus	–	–	10s 25†	–
Cladosporium clodosporioides	–	–	10s	–
Curvularia lunata	–	–	10s	–
Fusarium fujikuroi	–	–	–	–
F. oxysporum	–	–	–	–
Mucor miehei	–	–	100s	100†
Paecilomyces variotii	–	–	–	–
Penicillium islandicum	–	–	–	–
P. notatum	–	–	–	–
Pythium irregulare	25†	25†	10s 25†	–
Zygorhynchus moelleri	–	–	10s	–
Yeast				
Nadsonia fulvescens	–	–	10s	
Nematospora corily	–	–	–	–
Rhodotula glutinis	–	–	–	–
Saccharomyces cerevisiae is 1	–	–	10s	25s
S. cerevisiae α S 288 c	–	–	–	–
Bacteria				
Arthrobacter citreus	–	–	10s 100†	–
Bacillus brevis	–	50†	25s 50†	–
B. subtilis	–	–	25s 100†	–
B. licheniformis	–	–	50†	–
Corynebacterium insidiosum	–	–	–	–
Mycobacterium phlei	–	–	25s	–
Streptomyces sp. ATCC 23836	–	–	–	–
Escherichia coli K12	–	–	–	–
Salmonella typhimurium TA 98	–	–	25s	–

– no effects up 100 μg.
† = bacterio-/-fungicidal.
s = bacterio-/-fungistatic.

Table IV. Cytotoxicity of himanimides towards cell lines.

	IC_{50} [μg/ml] Himanimide			
Cell line	A	B	C	D
HL-60	50	25	10	50
Colo 320	100	100	100	>100
L1210	>100	100	25	100

in 150 μl water against *Setaria italica* and *Lepidium sativum.* Himanimide B (**2**) exhibited a weak inhibition of root-formation of *Lepidium sativum* at the concentration of 100 μg/disc.

Appressorium formation of *M. grisea* was inhibited *in vitro* by all compounds but not *in vivo* on leaves of *Oryza sativa.*

It is interesting to note the differences in biological activities between himanimide C and the other compounds. Probably, this activity is due to the presence of the a double-bond and the hydroxylated imino-nitrogen in the maleinimide part of the himanimides.

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