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Zopfiellamides A and B, antimicrobial pyrrolidinone derivatives from the marine fungus *Zopfiella latipes*

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Abstract—Two new antimicrobial metabolites, zopfiellamides A (**1**) and B (**2**) were isolated from fermentations of the facultative marine fungus *Zopfiella latipes* CBS 611.97. Their structures were elucidated by spectroscopic techniques, and they are pyrrolidinone derivatives.

1. Introduction

Marine fungi have been predicted to become an important source of novel secondary metabolites, and are expected to compete with terrestrial fungi in this respect in the near future.[1] However, compared with other marine organisms, only a few investigations of the secondary metabolites of marine fungi have been reported,[2] and most of the metabolites isolated are actually identical or very similar to those obtained from terrestrial fungi. As an example, the obligate marine fungus *Zopfiella marina* has been reported to produce the potent antifungal metabolite zofimarin which is a sordarin derivative,[3] but a number of terrestrial producers of this and other sordarin derivatives are already known.[4]

In the present study, another member of the same genus, the facultative marine ascomycete *Z. latipes*, was grown in submerged cultures, and two novel antimicrobial compounds, zopfiellamide A (**1**) and zopfiellamide B (**2**) were isolated from both the culture fluids and from the mycelia. The isolation, the structure elucidation and an evaluation of the antimicrobial activity of the zopfiellamides are reported in this paper (Fig. 1).

2. Results and discussion

The producing strain *Z. latipes* was obtained from CBS and had originally been isolated from a soil sample from the Indian Ocean near New Delhi. Extracts of the culture fluids as well as the mycelia showed activity against several Gram-positive bacteria and against yeasts, and bioassay-guided fractionation of the extracts (as described in Section 4) eventually yielded two pure compounds possessing antimicrobial activity.

In the LC–MS (negative mode) spectrum of **1**, the molecular ion appeared at 444 *m/z*, suggesting that the molecular weight of the compound is 445. However, this could not be confirmed by CIMS measurements with CH_4 as ionising gas, and the EI spectrum suggested that the molecular weight was 427. High-resolution measurements showed that this ion corresponded to the composition $C_{25}H_{33}O_5N$. The second compound also gave an apparently straight-forward LC–MS spectrum with a strong signal for *m/z* 458, and somewhat surprising but not unexpectedly this compound gave a strong M^+ signal for *m/z* 459 in the EI spectrum. High resolution measurements gave the composition $C_{26}H_{37}O_6N$ for **2**, and the similarities of the 1D NMR data of the two compounds led to the conclusion that the molecular weight of **1** is 445 and that *m/z* 427 is the ion for M^+-H_2O. Although the structural differences between **1** and **2** are minute (vide infra), their EI mass spectra differ substantially. The base peak of **1** is *m/z* 383, corresponding to $M^+-H_2O-CO_2$, and the apparent $M+H^+$ ion appears at *m/z* 384 in the CI spectrum. For **2**, no signal for $M^+-H_2O-CO_2$ is observed at all.

The 1D NMR data are given in Table 1 and according to the elemental compositions both molecules should possess 9 degrees of unsaturation. The structures were determined from 2D NMR data, COSY, HMQC and HMBC spectra, and the pertinent HMBC correlations observed with zopfiellamide B (**2**) are shown in Fig. 2. Two C–C double bonds, one *cis* (C-13/C-14) and one *trans* (C-4/C-5), one keto function (C-1′), and at least two additional carbonyl carbons (C-1, C-3 and/or C-5′) were suggested by the ^{13}C

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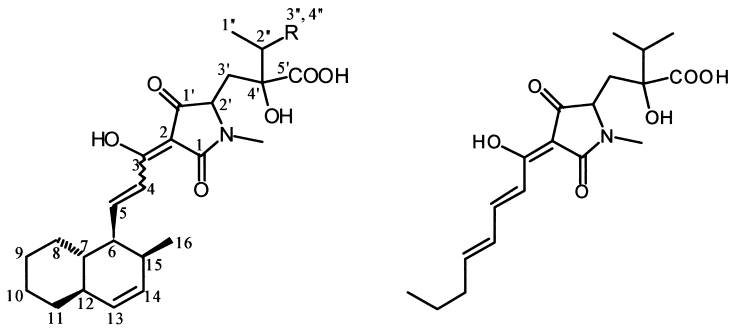

1 R = CH_3
2 R = CH_2CH_3

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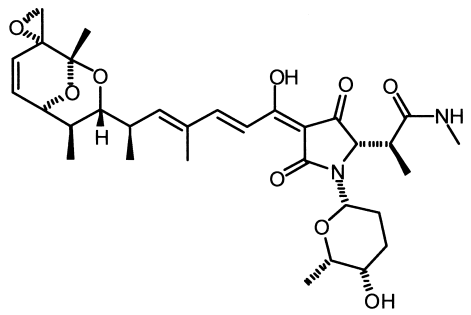

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Figure 1.

chemical shifts. The UV spectrum as well as the shift difference between C-4 and C-5 suggests that the molecule contains a strongly conjugated system, in which C-3 should be part (due to the HMBC correlations between 4-H and 5-H and C-3). No HMBC correlations were observed to C-2, but by placing it between C-3 and the carbonyl groups C-1 and C-1′, and attaching a hydroxyl group to C-3, the extreme ^{13}C shifts for C-2 (98.4 ppm) and C-3 (175.9 ppm) become reasonable. On the other side of the pyrrolidinone ring a quaternary carbon substituted with a hydroxyl group, a carboxylic acid and an isopropyl group (**1**) or a isobutyl group (**2**) is linked via a methylene group (C-3′). The HMBC correlations from 3′-H_2 to C-1′, C-2′, C-4′ and C-2″, as well as the COSY correlation between 2′-H and 3′-H_2 establish this. For this part of the molecule, the chemical shifts are very similar to those of harzianic acid (**3**) another pyrrolidinone derivative isolated from a fungus.[5] With this, 7 of the 9 degrees of unsaturation have been covered, and the remaining two should correspond to rings. In the suggested decalin system, the unsaturated ring is easy to close while the correlations in the saturated ring finish at C-9 and C-10. Due to the high degree of symmetry, the shifts for C-9/C-10 and 9-H_2/10-H_2 are very close and it is not possible to follow the correlation from C-9 to C-10 (or vice versa). However, this is the only alternative that remains. The configuration of the C-2/C-3 double bond could not be determined with the spectroscopic data obtained here, but as they, for the pyrrolidinone part, are very similar to those of other pyrrolidinone derivatives, streptolydigin (**4**) and tirandamycic acid (characterised by X-ray crystallography[6]), we assume that it should be *E*. Unexpectedly, no signals for hydroxyl protons could be observed in the ^1H NMR spectrum. The relative configuration of the decalin part could be determined by NOESY correlations, 6-H correlates to 12-H and 8-Hα, while 7-H correlates to 16-H_3 and 11-Hβ.

Several natural products with a pyrrolidinone, or tetramic acid, moiety have been reported from a range of different organisms, and besides harzianic acid (**3**)[5] and streptolydigin (**4**)[6,7] shown in Fig. 1, equisetin[8] from a *Fusarium* species and the aurantosides[9] from sponges of the *Theonellidae* family could also be mentioned.

The antimicrobial activity of zopfiellamides A and B towards various bacteria and yeasts was assayed in the serial dilution assay. Moderate antibacterial effects towards the Gram-positive *Arthrobacter citreus*, *Bacillus brevis*, *B. subtilis*, *B. licheniformis*, *Corynebacterium insidiosum*, *Micrococcus luteus*, *Mycobacterium phlei*, and *Streptomyces* sp. and towards the Gram-negative *Acinetobacter calcoaceticus* were detected, with minimal inhibitory concentrations between 2 and 10 μg/ml for zopfiellamide A (**1**). Zopfiellamide B (**2**) was approximately 5 times less active, and again it is interesting to note how the extra methyl group in **2** affects its properties. Among the fungi tested, only the yeasts *Nematospora coryli* and *Saccharomyces cerevisiae* were affected by the two metabolites, with minimal inhibitory concentrations starting at 2 μg/ml for both compounds. No cytotoxic effects towards several mammalian cell lines were observed for either compound, at concentrations up to 100 μg/ml.

Table 1. ^{1}H (500 MHz) (δ; multiplicity; J) and ^{13}C (125 MHz) NMR data (δ; multiplicity) for zopfiellamides A (**1**) and B (**1**)

C	**1**		C	**1**	
	^{1}H	^{13}C		^{1}H	^{13}C
1	–	173.3, s	1	–	173.3, s
2	–	98.4, s	2	–	98.4, s
3	–	175.8, s	3	–	175.9, s
4	7.05; d, 15.5	122.0, d	4	7.04; d, 15.6	121.9, d
5	7.26; dd, 10.4, 15.5	156.1, d	5	7.30; dd, 10.8, 15.6	156.5, d
6	2.50; ddd, 5, 10, 10	48.3, d	6	2.51; ddd, 5, 11, 10	48.4, d
7	1.31; m	38.7, d	7	1.32; m	38.7, d
8	1.52; m	31.1, t	8	1.52; m	31.1, t
	0.87; m			0.88; m	
9	1.74; m	26.6, t	9	1.75; m	26.6, t
	1.27; m			1.28; m	
10	1.74; m	26.6, t	10	1.74; m	26.6, t
	1.27; m			1.26; m	
11	1.74; m	33.0, t	11	1.74; m	33.1, t
	1.06; m			1.06; m	
12	1.71; m	42.7, d	12	1.71; m	42.7, d
13	5.42; d, 9.9	131.2, d	13	5.43; d, 9.8	131.2, d
14	5.56; ddd, 2.6, 4.3, 9.9	131.5, d	14	5.57; ddd, 2.5, 4.3, 9.8	131.5, d
15	2.32; m	35.9, d	15	2.34; m	35.9, d
16	0.95; d, 7	16.5, q	16	0.97; d, 7.1	16.5, q
1′	–	197.3, s	1′	–	197.4, s
2′	3.63; d, 10.0	64.1, d	2′	3.63; d, 10.5	64.1, d
3′	2.44; d, 14.3	34.0, t	3′	2.47; d, 15.0	33.8, t
	1.86; dd, 10.8, 13.9			1.90; dd, 10.5, 15.0	
4′	–	79.8, s	4′	–	80.5, s
5′	–	176.8, s	5′	–	176.2, s
1″	0.94; d, 7	17.5, q	1″	0.96; d, 6.5	12.3, q
2″	2.01; hept., 7	35.8, d	2″	1.74; m	48.3, d
3″	0.94; d, 7	16.2, q	3″	1.47; m	24.2, t
N–CH_3	2.94; s	26.6, q		1.24; m	
			4″	0.91; t, 7.4	12.2, q
			N–CH_3	2.96; s	26.5, q

The spectra were recorded in $CDCl_3$, and the solvent signals (7.25 and 77.0 ppm, respectively) were used as reference. The coupling constants J are given in Hz. The multiplicities of the carbon signals were determined indirectly from HMQC experiments.

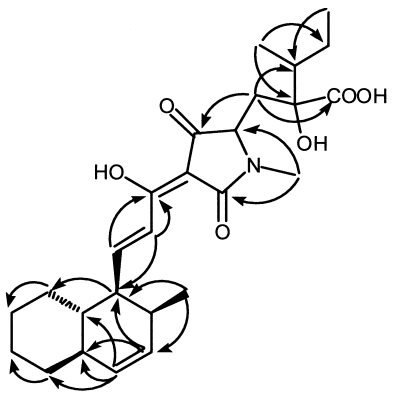

Figure 2. Pertinent HMBC correlations observed with zopfiellamide B (**2**).

3. Conclusions

The production of pyrrolidinone derivatives is neither limited to a certain type of organism, nor to a habitat. Instead, this type of compound is widely spread in nature and can be isolated from bacteria, myxomycetes and sponges, as well as from terrestrial and marine fungi. In general, the pyrrolidinone derivatives isolated from natural sources have been reported to possess antimicrobial as well as cytotoxic activities. In contrast, the zopfiellamides are selectively antimicrobial as they exhibit no cytotoxicity.

4. Experimental

4.1. General

For analytical HPLC, a Hewlett Packard 1090 series II instrument (coloumn: Merck LiChrocart 125-4 filled with LiChrospher 100 RP-18) was used with H_3PO_4 (0.1%) and methanol as mobile phase. ^{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 probe head equipped with a shielded gradient coil in $CDCl_3$ with the solvent signals as reference. 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 $^{1}J_{CH}$=145 Hz and $^{n}J_{CH}$=10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). LC–MS mass spectra were recorded with a Hewlett Packard 1100 LC–MSD in negative mode, while CI, EI and HREI mass spectra were recorded with a Jeol SX102 spectrometer. UV and the IR spectra were recorded with a Perkin–Elmer λ 16 and a Bruker IFS 48 spectrometer. The melting point (uncorrected) were determined with a Reichert microscope, and the optical rotations were measured with a Perkin–Elmer 141 polarimeter at 22°C.

4.2. Producing organism and fermentation

The producing strain *Z. latipes* CBS 611.97 was obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and was originally isolated from a soil sample from the Indian Ocean near New Delhi. The genus *Zopfiella* comprises 19 species with only one obligate marine and with *Z. latipes* being a facultative marine species. Driftwood and submerged wood mainly serve as substrates. Besides from tropical estuarines, strains of *Z. latipes* could be isolated from subtropical and temperate coastlines. The fungus was cultivated and maintained on Emerson-YPsS agar (Difco 0739) or on YMG agar (yeast extract 0.4%, glucose 1%, malt extract 0.4% and 1.5% agar, pH 7.0). Fermentations were carried out in 20 l of a medium consisting of glucose 0.5%, yeast extract 0.1%, and peptone from soybean 0.1% at pH 7.0 in a Biolafitte C6 fermentation apparatus at 22°C with an aeration rate of 3 l/min and agitation (120 rpm). A well grown culture of the fungus in the same medium (250 ml) served as inoculum. After 264 h of fermentation, the culture fluid (16 l) was separated from the mycelia by filtration.

4.3. Isolation of zopfiellamides

The culture fluid was extracted twice with each 10 l of ethyl acetate and the mycelia (50 g dry weight) were extracted with 1 l of methanol–acetone 1:1. The obtained crude extracts were fractionated by chromatography on silica gel (Merck 60, 0.063–0.2 mm). Elution with ethyl acetate–methanol 1:1 yielded enriched products containing the compounds zopfiellamide A (**1**) and zopfiellamide B (**2**). Final purification was achieved by preparative HPLC Jasco model PU-980 with diode array detector; column: Macherey–Nagel Nucleosil 100 C18 (7 μm), 250×21.2 mm; flow rate 5 ml/min]. Elution with H_3PO_4 (0.1% v/v)–methanol 11:89 v/v yielded 25.7 mg of **1** and 9:91 v/v yielded 6.3 mg of **2**.

4.3.1. Zopfiellamide A (1). It was obtained as yellowish crystals, mp 225–230°C (EtOH). $[\alpha]_D^{22}=+5.2$ (*c* 1.1 in $CHCl_3$). UV (MeOH), λ_{max} (ε): 247 nm (11,000) and 320 nm (10,000). IR (KBr): 3435, 2925, 1640, 1580, 1450, 1250, 995, 885, 725 and 620 cm^{-1}. See Table 1 for NMR data. HREIMS: 445 (<1%), 427.2343 (10%, M^+-H_2O, $C_{25}H_{33}O_5N$ requires 427.2358, error−3.5 ppm), 409 (3%), 399 (4%), 383 (100%), 365 (22%), 340 (17%), 314 (87%), 224 (65%), 166 (44%), 160 (49%).

4.3.2. Zopfiellamide B (2). It was obtained as a yellowish oil. $[\alpha]_D^{22}=-24$ (*c* 0.3 in $CHCl_3$). UV (MeOH), λ_{max} (ε): 247 nm (3,800) and 321 nm (3,400). IR (KBr): 3420, 2925, 1685, 1640, 1580, 1455, 1365, 1255, 995, 880 and 725 cm^{-1}. See Table 1 for NMR data. HREIMS: 459.2628 (22%, M^+, $C_{26}H_{37}O_6N$ requires 459.2621, error+1.5 ppm), 441 (9%), 414 (16%), 328 (19%), 310 (25%), 266 (11%), 238 (13%), 168 (20%), 138 (26%), 43 (100%).

4.4. Biological assays

Assays for cytotoxic activitites[10] with HL60 (human promyelocytic leukemia, ATCC CCL 240), HeLa S3 (human epitheloid cervix carcinoma, ATCC CCL 2.2), L1210 (lymphocytic leukemia, mouse; ATCC CCL 219) and Colo-320 (human colorectal adenocarcinom, DSMZ ACC-144) cells and for antimicrobial activities[11] were performed as described previously.

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References

1. Cuomo, V.; Palomba, I.; Perretti, A.; Guerriero, A.; D'Ambrosio, M.; Pietra, F. *J. Mar. Biotechnol.* **1995**, *2*, 199–204.
2. Faulkner, D. J. *Nat. Prod. Rep.* **2001**, *18*, 1–49.
3. Ogita, T.; Hayashi, A.; Sato, S.; Furutani, W. Patent Japan 62, 040,292, February 21, 1987.
4. Kennedy, T. C.; Webb, G.; Cannell, R. J.; Kinsman, O. S.; Middleton, R. F.; Sidebottom, P. J.; Taylor, N. L.; Dawson, M. J.; Buss, A. D. *J. Antibiot.* **1998**, *51*, 1012–1018.
5. Sawa, R.; Mori, Y.; Iinuma, H.; Nganawa, H.; Hamada, M.; Yoshida, S.; Furutani, H.; Kajimura, Y.; Fuwa, T.; Takeuchi, T. *J. Antibiot.* **1994**, *47*, 731–732.
6. Duchamp, D. J.; Branfman, A. R.; Button, A. C.; Rinehart, Jr. K. L. *J. Am. Chem. Soc.* **1973**, *95*, 4077–4078.
7. Rinehart, Jr. K. L.; Beck, J. R.; Borders, D. B.; Kinstle, T. H.; Krauss, D. *J. Am. Chem. Soc.* **1963**, *85*, 4038–4041.
8. Vesonder, R. F.; Tjarks, L. W.; Rohwedder, W. K.; Burmeister, H. R.; Laugal, J. A. *J. Antibiot.* **1979**, *32*, 759–761.
9. Sata, N. U.; Matsunaga, S.; Fusetani, N.; van Soest, R. W. *J. Nat. Prod.* **1999**, *62*, 969–971.
10. Zapf, S.; Hossfeld, M.; Anke, H.; Velten, R.; Steglich, W. *J. Antibiot.* **1995**, *48*, 36–41.
11. Anke, H.; Bergendorff, O.; Sterner, O. *Food Chem. Toxic* **1989**, *27*, 393–397.