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Neosordarin and Hydroxysordarin, Two New Antifungal Agents from *Sordaria araneosa*

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Two novel antifungal agents belonging to the sordarin family have been isolated from fermentations of *Sordaria araneosa* by bioassay-guided purification and their structures elucidated by NMR techniques. Neosordarin (**1**) is closely related to the recently discovered hypoxysordarin (**2**), with only small differences on the aliphatic side chain acylating the hydroxyl in the 3′-position of the sordarose moiety. Hydroxysordarin (**3**) closely resembles sordarin (**4**), the only slight difference being the replacement of sordarose with altrose as the sugar unit.

Sordarin (**4**) is an antifungal metabolite isolated from fermentations of the ascomycete *Sordaria araneosa* Cain[1)], possessing an interesting tetracyclic diterpene glycoside structure. Its mode of action has only recently been established as a potent and selective inhibition of elongation factor 2 (EF2) in fungi, during the elongation cycle in the fungal protein synthesis[2)].

All natural sordarin analogues bear as key groups in the putative pharmacophore a carboxylic function vicinal to a formyl group, not forming a cyclic hydroxylactone because of their high dihedral angle. In addition, the sugar moiety seems to play some role in enhancing the binding of sordarins in the active site, and a possible function of this "appendage" as cell uptake modulator has been proposed[3,4)]. Recently, the importance of the lipophilicity of such an appendage for the activity has been shown[4)]. In this respect, it is noteworthy that most of the natural sordarins isolated up to now only differ in the sordarose substitution[5~8)]; in particular, the hydroxyl in the 3′-position can vary its substitution without loss of the antifungal activity, as also confirmed by the recently discovered hypoxysordarin (**2**), isolated from *Hypoxylon croceum* and from *Sordaria araneosa* as well[5)]. The only exceptions are SCH57404[9)] and xylarin[10)], which bear an unusual tricyclic sugar moiety.

In the course of large scale fermentations of *Sordaria araneosa*, HPLC-MS analysis of active fractions revealed the presence of new compounds bearing the sordaricin skeleton. Bioassay-guided purification of extracts led us to the isolation of two novel antifungal agents belonging to the sordarin family, neosordarin (**1**) and hydroxysordarin (**3**), whose chemical and biological characterisation we wish to describe herein.

Materials and Methods

Fermentation and Isolation of Neosordarin (1) and Hydroxysordarin (3)

Sordaria araneosa Cain (ATCC 36386) was fermented in 100 litres of a medium composed of (g/litre): glucose 20, malt extract 2, peptone from casein 2, Bacto-Yeast-Extract 2, KH_2PO_4 2 and $MgSO_4 \cdot 7H_2O$ 2 in a Biostat U-100 fermenter (Braun+Diessel GmbH, Melsungen) at 27°C with an aeration rate of 15 litres/minute and agitation (120 rpm). A well-grown culture of *S. araneosa* in the same medium (10 litres) was used as inoculum. After 5 days the mycelium was removed by filtration, and the culture filtrate was passed through a column (30×10 cm) packed with Mitsubishi Diaion HP 21 adsorbing resin. The column was

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washed with acetone/water 1 : 1 (8 litres) and then acetone (5.5 litres). The pooled eluates were concentrated *in vacuo*. The resulting brown slurry was diluted with water and the sordarins extracted with ethyl acetate (3×2 litres). The combined organic phases were dried on Na_2SO_4 and then evaporated, affording 16 g of crude extract.

An aliquot of the extract (around 5 g) was then subjected to flash chromatography on a silica gel (Merck 60, 0.063~0.2 mm particle size) column (32×5.5 cm). Sordarin-containing fractions were eluted with cyclohexane/ethyl acetate 30 : 70 (2 litres) and 10 : 90 (1 litre). The fractions were analyzed by analytical HPLC on a LiChrospher® 100 RP-18 (5 μm) column [water/acetonitrile gradient: 1 minutes, 0% CH_3CN; 15 minutes, 0~50%; 20 minutes, 50~100%; flow rate 1.5 ml/minute], confirming the presence of sordarin (4, Rt=14.8 minutes) together with the new antifungal derivative neosordarin (1, Rt=15.9 minutes) as the major compounds. Final purification of the pooled fractions containing **1** and **4** (777 mg) was performed by preparative HPLC on Nucleosil C18 [7 μm, 250×21.2 mm column (Macherey-Nagel), flow rate 5 ml/minute] using a water/acetonitrile gradient as the eluant (70 minutes, 0~55% CH_3CN; 110 minutes, 55~100%; 120 minutes, 100%). Elution with acetonitrile/water 60 : 40 v/v afforded sordarin (**4**, 228 mg) while neosordarin (**1**, 27 mg) was eluted with 75 : 25 v/v.

For the purification of hydroxysordarin (**3**), 10 g of the remaining crude extract was applied to a flash silica gel 60 column (80×7 cm) and flash chromatographed with a gradient of cyclohexane/ethyl acetate as the eluant. Hydroxysordarin-containing fractions were eluted with cyclohexane/ethyl acetate 30 : 70, affording 4.9 g of enriched product. This was applied to a silica gel 60 column (20×7 cm) and eluted with 100% ethyl acetate yielding 80 mg of enriched **3**. A 40 mg portion of this product was subjected to preparative HPLC on Nucleosil C18 [7 μm, 250×21.2 mm column (Macherey-Nagel), flow rate 5 ml/minute] using a water/methanol gradient (80 minutes, 0~80% MeOH; 90 minutes, 80%; 110 minutes, 80~100%). Elution with methanol/water 80 : 20 v/v yielded 16 mg of hydroxysordarin (**3**).

Analytical Methods

For analytical and preparative HPLC a Hewlett Packard 1090 Series II liquid chromatograph and a Jasco PU-980 instrument were used, respectively. TLC analyses were performed on Macherey-Nagel Alugram Sil G/UV_{254} precoated plates and visualized by spraying with anisaldehyde/sulphuric acid and heating. ^{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 $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used 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 $^1J_{CH}=145$ Hz and $^nJ_{CH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the

Fig. 1. Chemical structures of the two novel sordarin derivatives isolated from fermentations of *Sordaria araneosa*: neosordarin (**1**) and hydroxysordarin (**3**), compared with the known hypoxysordarin (**2**) and sordarin (**4**).

1 **2** **3** R = OH **4** R = H

standard Bruker UXNMR software (rev. 941001). For mass spectral determinations, an HPLC-coupled APCI mass spectrometer was used (Hewlett Packard Series 1100LC-MSD), either in the positive (PI) or negative (NI) ionisation mode.

Neosordarin (**1**, IUPAC name: [1*R*-(1α,3aβ,4β,4aβ,7β,7aα,8aβ)]8a[[6-deoxy-3-*O*-[(*Z*,*E*)-1,4-dioxo-7-hydroxy-2-methylocta-2,5-dienyl]-4-*O*-methyl-β-D-mannopyranosyloxy]methyl]-4-formyl-4,4a,5,6,7,7a,8,8a-octahydro-7-methyl-3-(1-methylethyl)-1,4-methano-*s*-indacene-3a(1*H*) carboxylic acid) was obtained as a brownish solid, m.p. 53~57°C; $[\alpha]_D^{22}$ −44° (*c* 0.8 in $CHCl_3$); UV λ_{max}^{MeOH} 243 nm log ε 3,61. IR (KBr): 3493, 2932, 2869, 1719, 1665, 1637, 1448, 1382, 1148, 1103, 1070 and 911 cm^{-1}; MS-APCI (NI), *m/z*: 658 $(M)^-$, 657 (base peak), 491, 165; MS-APCI (PI), *m/z*: 333 (sordaricin+H)$^+$, 315, 297, 271 (base peak),

Table 1. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data for neosordarin (**1**) and hydroxysordarin (**3**) in $CDCl_3$.

	1		**3**	
C	^1H δ; mult.; *J*	^{13}C δ; mult.	^1H δ; mult.; *J*	^{13}C δ; mult.
1	-	72.5, s	-	72.3, s
2	-	59.0, s	-	58.9, s
3	2.02, m	41.7, d	2.02, m	41.7, d
4	1.01, m	26.2, t	1.02, m	26.2, t
	1.88, m		1.87, m	
5	1.22, m	32.1, t	1.24, m	32.0, t
	2.06, m		2.06, m	
6	2.04, m	31.0, d	2.09, m	30.9, d
7	1.75, m	41.4, d	1.77, m	41.3, d
8	1.83, m	29.2, t	1.86, m	29.0, t
	1.95, m		1.89, m	
9	-	65.8, s	-	65.6, s
10	2.72, dd, 3, 4	46.3, d	2.67, dd, 3, 4	46.3, d
11	6.12, d, 3.1	130.9, d	6.08, dd, 1.1, 3.3	130.7, d
12	-	148.4, s		148.5, s
13	-	175.2, s		174.5, s
14	1.29, m	29.4, t	1.30, m	29.4, t
	1.93, m		1.93, m	
15	9.72, s	205.2, d	9.72, s	205.1, d
16	0.79, d, 6.6	17.4, q	0.81, d, 6.7	17.4, q
17	3.98, d, 9.5	74.4, t	4.10, d, 9.2	74.6, t
	3.75, d, 9.5		3.68, m	
18	2.34, hept., 6.5	27.7, d	2.36, hept., 6.5	27.7, d
19	1.00, d, 6.7	22.6, q	0.98, d, 6.7	22.6, q
20	1.04, d, 6.7	21.2, q	1.04, d, 6.7	21.1, q
1'	4.50, d, 0.9	98.5, d	4.73, s	98.3, d
2'	4.00, dd, 0.9, 4.5	68.0, d	3.92, d, 4.0	70.0, d
3'	5.54, d, 3, 4	68.6, d	4.28, dd, 3, 4	67.0, d
4'	3.33, dd, 3.1, 8.7	78.2, d	3.59, dd, 3.0, 9.5	74.0, d
5'	3.68, dd, 6.3, 8.7	69.7, d	3.69, m	72.4, d
6'	1.30, d, 6.3	18.1, q	3.86, dd, 2.8, 11.7	62.4, t
			3.73, dd, 4.6, 11.7	
1"	-	167.3, s		
2"	-	140.4, s		
3"	6.40, d, 1.4	130.0, d		
4"	-	190.5, s		
5"	6.29, dd, 1.4, 15.9	128.1, d		
6"	6.80, dd, 5.0, 15.9	151.4, d		
7"	4.50, m	67.3, d		
8"	1.35, d, 6.6	22.5, q		
9"	2.10, s	20.5, q		
4'-OMe	3.36, s	57.5, q	3.43, s	57.4, q

The chemical shifts are given in ppm relative to the solvent signals (7.26 and 77.0 ppm, respectively). The coupling constants (*J*) in Hz, the ^{13}C multiplicity was determined indirectly from HMQC spectra.

259, 253, 241, 167, 149, 121. See Table 1 for ^{1}H and ^{13}C NMR data.

Hydroxysordarin (**3**) was obtained as white powder, m.p. 135~137°C. $[\alpha]_D^{22}$ −59° (*c* 0.2 in $CHCl_3$). IR (KBr): 3430, 2956, 2870, 1715, 1463, 1383, 1236, 1094, 911 cm^{-1}. See Table 1 for ^{1}H and ^{13}C NMR data. MS-APCI (NI), *m/z*: 507 $(M-H)^-$ (base peak); MS-APCI (PI), *m/z*: 333 $(sordaricin+H)^+$, 315 (base peak), 297, 271, 269, 253, 243.

Biological Assays

Antifungal activities were determined by agar plate diffusion assay as described previously[5].

In Vitro Translation Assay

The cells from five 400 ml cultures of *Saccharomyces cerevisiae* BY4742 in YPD medium (g/litre: yeast extract 10; peptone from casein 20; glucose 20; pH 6.3) grown to an OD_{650} of 1.9 to 2.0 (*ca.* 10 g) were harvested by centrifugation (4 minutes at 6500×*g* and 4°C). The pellet was washed twice with an equal volume of deionized water and resuspended in about 30 ml of H_2O. After centrifugation (4 minutes at 6000×*g* and 4°C) the pellet was rinsed once with water and resuspended in 30 mM HEPES-KOH buffer, pH 7.4, containing 8.5% mannitol, 100 mM potassium acetate, 2 mM magnesium acetate and 2 mM dithiothreitol, using 2.5 ml per g of pellet. Then 20 μl of complete-protease-inhibitor (Roche, 1 tablet per ml of water) and 20 μl of lyticase (Sigma, 10 mg/ml) were added per g of suspended cells. After 1 hour at 37°C the suspension was frozen at −20°C and passed five to six times through an X-press (AB Biox, Nacka, Sweden, type X25). The broken cells were centrifuged (15 minutes at 18000×*g*), the supernatant decanted and centrifuged 30 minutes at 100000×*g* at 4°C. The polysome-free supernatant was removed, partitioned in 1~2 ml batches and the cell extract kept at −70°C.

To 30 μl of thawed cell extract 5 μl of a suitable solution of the test compound in methanol was added. After preincubation for 10 minutes at room temperature 15 μl of reaction mix (see below) was added. After 60 minutes at 25°C the *in vitro* translation was stopped by the addition of 50 μl of 1 M NaOH. After 10 minutes at room temperature 50 μl of ice cold 25% trichloroacetic acid (TCA) was added and the mixture kept for 30 minutes at 4°C. The precipitated peptides were collected on nitrocellulose filters (pore size 0.45 μ) and washed twice with 1 ml of 5% TCA. The radioactivity of the dried filters was measured in a liquid scintillation counter (Wallac 1410) after addition of 5 ml of Quickscint 501 (Zinsser).

Reaction mix: 0.91 ml 1 M dithiothreitol; 4.5 ml 1 M potassium acetate; 0.44 ml 1 M magnesium acetate; 0.5 ml creatine kinase (7 mg/ml, Roche); 1 ml creatine phosphate (400 mg/ml, Roche); 0.5 ml 10 mM GTP; 0.225 ml 100 mM ATP; 1 ml RNasIn (20~40 u/μl RNase-Inhibitor, Promega); 5.5 ml polyU-RNA (5 μg/μl, Pharmacia); 0.425 ml (786 kBq) L-[^{14}C (U)]-phenylalanine (2.06 GBq/mM, ICN)

Results and Discussion

Structure Elucidation of Neosordarin and Hydroxysordarin

Neosordarin (**1**) is closely related to the recently discovered hypoxysordarin (**2**)[5], with only small differences on the aliphatic side chain acylating the hydroxyl in the 3′-position of the sordarose moiety. Its elemental composition, $C_{36}H_{50}O_{11}$, as suggested by NMR and MS data, is identical to that of hypoxysordarin (**2**), and comparison of the NMR data show that the only differences are present in the acyl moiety. The two adjacent epoxides in hypoxysordarin (**2**) have been exchanged for a 4-hydroxy-but-2-enone moiety in neosordarin (**1**), a process that not is likely to take place by a simple chemical reaction (**1** was never observed as a transformation product of **2** during isolation and recording of NMR spectra in various solvents at room temperature for several hours and days). The configurations of the two double bonds were determined by a NOESY experiment to be (2″*Z*) and (5″*E*). The relative configuration of C-7″ was not determined. Sordarin derivatives similar to neosordarin have also been isolated from the ascomycete *Zopfiella marina*[8] and from the deuteromycete *Graphium putredinis*[6,7].

Hydroxysordarin (**3**) on the other hand closely resembles sordarin (**4**), and the difference in the elemental composition, according to MS and NMR data, is one oxygen. This variation is situated in the sugar moiety, which is 4-*O*-methyl-altrose in **3** instead of sordarose in **4**, a relationship confirmed by a full set of 2D NMR experiments. Amongst all natural sordarin derivatives, such a replacement is reported only for one compound isolated from fermentations of *Graphium putredinis*[7]. In this case, however, the hydroxyl in the 3′-position is acylated, whereas hydroxysordarin bears no substitution at this group. As far as sugar modifications on the sordaricin skeleton are concerned, the literature reports two other members of the sordarin family which totally differ in the sugar moiety, namely xylarin, isolated from the ascomycete *Xylaria longipes*[10], and SCH57404 from an unidentified fungus[9], both bearing an unusual tricyclic sugar.

Table 2. Antifungal activity and inhibition of *in vitro* translation (IVT) by neosordarin (**1**), hypoxysordarin (**2**), and hydroxysordarin (**3**) in comparison to sordarin (**4**).

Compound	Activity in IVT *S. cerevisiae* BY4742 IC_{50} (μg/ml)	Agar diffusion assay, 10 μg/disc Diameter inhibition zone (mm)			
		Nematospora coryli	*Mucor miehei*	*Penicillium notatum*	*Paecilomyces variotii*
Neosordarin (**1**)	0.2-0.3	40	14	-	-
Hypoxysordarin (**2**)	0.25-0.5	32	32	30	26
Hydroxysordarin (**3**)	0.2-0.25	19	–*	-	-
Sordarin (**4**)	0.15-0.2	38	23	-	-

* –: no inhibition zone

Biological Properties of Neosordarin and Hydroxysordarin

The biological activities of neosordarin (**1**), hypoxysordarin (**2**), hydroxysordarin (**3**) and sordarin (**4**) are shown in Table 2.

While the *in vitro* inhibitory activity on fungal protein biosynthesis is comparable for all four sordarin-dervatives, their *in vivo* activities against *Nematospora coryli* and *Mucor miehei* differ to some extent. Surprisingly, hydroxysordarin is not active againt *M. miehei*. In contrast to hypoxysordarin (**2**) the derivatives **1**, **3** and **4** did not exhibit activity against *Paecilomyces variotii* and *Penicillium notatum* at 10 μg/disc. Therefore, the nature of the side chain seems to be decisive for the antifungal activity on filametous ascomycetes. A comparison of the activities of sordarin and hydroxysordarin shows that the hydroxymethyl group in the sugar moiety decisively decreases the *in vivo* activity although the *in vitro* activity is comparable. This is in accordance with results of KENNEDY *et al.* (1998)[7] obtained with zofimarin derivatives.

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