Hypoxysordarin, a New Sordarin Derivative from *Hypoxylon croceum*

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Dedicated to Professor Hans Zähner on the occasion of his 70th birthday

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Hypoxysordarin (**1**), a new sordarin derivative, was isolated from the fermentation broth of the facultative marine *Hypoxylon croceum* together with a new γ-lactone, hypoxylactone (**2**) and sordarin (**3**). The structures were determined by spectroscopic methods. Sordarin (**3**) has previously been isolated from the terrestrial *Sordaria araneosa* (Sordariaceae). Like the parent compound hypoxysordarin exhibits high antifungal activities due to a specific inhibition of protein biosynthesis.

Introduction

Terrestrial fungi constitute a rich source for new antibiotics. In comparison, reports on the isolation of novel antimicrobial compounds from marine fungi are scarce. The field has been reviewed recently (Cuomo *et al.*, 1995; Faulkner, 1998; Biabani and Laatsch, 1998). In many cases the same metabolites have been isolated from fungal species which usually occur in terrestrial habitats.

In our search for bioactive metabolites from marine fungi we have isolated siccayne (**4**) (see Fig. 1) from the basidiomycete *Halocyphina villosa* (Kupka *et al.*, 1981) and trapoxin A (**5**) from *Corollospora intermedia* (Daferner *et al.*, 1999). Both compounds had been isolated from terrestrial fungi before, siccayne from *Helminthosporium siccans* (Ishibashi *et al.*, 1968) and trapoxin A from *Helicoma ambiens* (Itazaki *et al.*, 1990). While siccayne is an antimicrobial and cytotoxic compound, trapoxin A is a potent inhibitor of histone deacetylase (M. Kijima *et al.*, 1993). Furthermore a β-lactone, antibiotic 1233A, (**6**) (Aldridge *et al.*, 1971), a potent inhibitor of cholesterol biosynthesis (Omura *et al.*, 1987) from terrestrial fungi was detected in cultures of the obligate marine *Corollospora maritima* CBS 214.60 (Daferner *et al.*, 1999).

In the following, we wish to describe the production, isolation, biological properties and structure elucidation of hypoxysordarin (**1**), hypoxylactone (**2**) and of sordarin (**3**) from a facultative marine strain of *Hypoxylon croceum*.

Experimental

Hypoxylon croceum strain M97-25

Fruiting bodies of *Hypoxylon croceum* were found on driftwood in a mangrove estuary in the Everglades/Florida. The dark brown ostiolate and conspicuously papillate perithecia are 0.3–0.5 mm in diameter and partially immersed into the substrate. The thin-walled asci (100–110×7–8 μm) contain eight one-celled spores and deliquesce at maturity. The brown, non-septate ascospores measure 10–12×5 μm. The germ slit, typical for *Hypoxylon* is very short. Genus and species fit the description of Miller (1961) for *H. croceum* which, however, had not been described as a facultative marine species. Mycelial cultures from single ascospore isolates were obtained using a modification of the method described by Johnson and Sparrow (1961). The spores germinated at 22 °C on GPYS agar (modified after Schaumann, personal communication) composed of (g/liters): Glucose 1, Peptone from soybean 0.5, yeast extract 0.1, synthetic sea salts 30, pH 7.2. For maintenance on agar slants the fungus was grown on YMG medium (g/liters): Yeast extract 4, malt extract 10,

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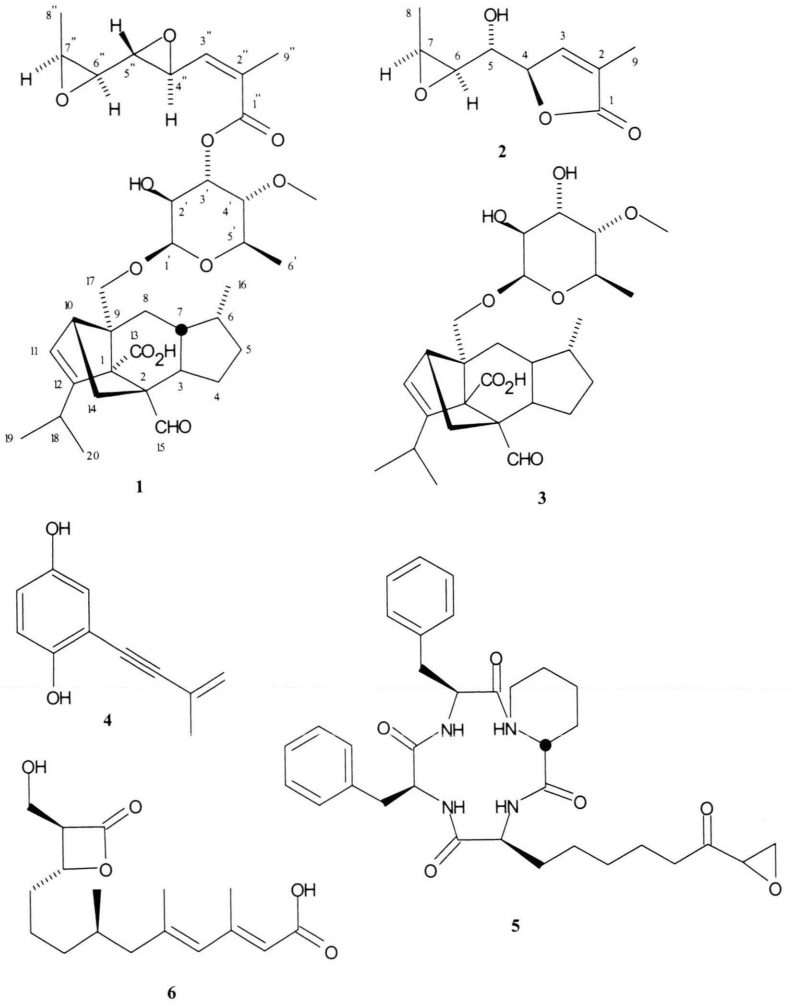

Fig. 1. Chemical structures of hypoxysordarin (**1**), hypoxylactone (**2**), sordarin (**3**), siccayne (**4**), trapoxin A (**5**) and antibiotic 1233 A (**6**).

glucose 4, pH 7.0. The strain is deposited in the culture collection of the LB Biotechnologie, Universität Kaiserslautern.

Fermentation and isolation of hypoxysordarin (**1**), *hypoxylactone* (**2**) *and sordarin* (**3**)

Fermentations were carried out in 20 liters of a cornmeal medium composed of (g/liter): cornmeal 10, glucose 10, KH_2PO_4 1.5, KCl 0.5, $NaNO_3$ 0.5, $MgSO_4 \times 7H_2O$ 0.5, pH 5.8, in a Biolafitte C6 fermenter at 22 °C with an aeration rate of 3 liters/minute and agitation (120 rpm). A well grown culture of *H. croceum* in the same medium (250 ml) was used as inoculum. During fermentation 100 ml samples were taken. The culture fluid was separated by filtration from the mycelia and the insoluble ingredients and then extracted with 100 ml of ethyl acetate. The residue obtained after evaporation of the organic solvent was taken up in 0.5 ml of methanol. 25 µl of the concentrated solutions were assayed for antifungal activity in the agar plate-paper disc diffusion assay using *Nematospora coryli* as test organism. After 14 days of fermentation the culture broth (17 liters) was separated from the mycelia and passed through a column (18×11 cm) containing Mitsubishi Diaion HP 21 adsorber resin. The column was washed with water and the compounds were eluted with 2 liters of acetone. The acetone eluate was concen-

trated and the crude product (1.2 g) was applied onto a silica gel column (Merck 60, 0.063–0.2 mm; 14×6.5 cm). Elution with cyclohexane–ethyl acetate 3:1 yielded 104 mg of an enriched product containing the antifungal compounds **1** and **3**. Another enriched product (130 mg), containing the γ-lactone **2** was eluted with cyclohexane–ethyl acetate 1:3. Final purification was achieved by preparative HPLC on Nucleosil C18 [7 µm; column 250×21.2 mm; flow rate 5 ml/minute]. Elution with water–methanol 65:35 v/v yielded 18 mg of **2**, 35:65 v/v yielded 41 mg of **3** and 15:85 v/v yielded 36 mg of **1**.

For comparison, *Sordaria araneosa* ATCC 36386 was grown in a medium composed of (g/l): glucose 50, malt extract 2, pepton from casein 2, Bacto yeast extract 2, KH_2PO_4 2, $MgSO_4 \times 7\,H_2O$, pH 5.5 for 5 days and sordarin was isolated as described above. Surprisingly, *S. araneosa* also afforded, besides sordarin, the new hypoxysordarin and hypoxylactone.

Analytical methods

For analytical HPLC a Hewlett Packard 1090 series II instrument and for preparative HPLC a Jasco model PU-980 instrument was used. TLC analyses were performed on Macherey-Nagel Alugram Sil G/UV_{254} precoated plates and visualised 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 standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol SX102 spectrometer. The UV and the IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer, and the optical rotation measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Hypoxysordarin (**1**) was obtained as a colourless oil. $[\alpha]_D$ +17° (*c* 0.35 in $CHCl_3$). UV (MeOH), λ_{max} (ε): 222 nm (11,700) IR (KBr): 3435, 2955, 1720, 1645, 1455, 1380, 1225, 1160, 1100, 1070, 1015 and 895 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm), mult., *J* (Hz): 9.70, s, 15-H; 6.08, dd, J_{10-11} = 3.3, J_{11-18} = 1.1, 11-H; 5.58, dq, $J_{3''-4''}$ = 8.0, $J_{3''-9''}$ = 1.5, 3″-H; 5.54, dd, $J_{2'-3'}$ = 4.3, $J_{3'-4'}$ = 3.2, 3′-H; 4.57, d, $J_{1'-2'}$ = 1.3, 1′-H; 4.29, dd, $J_{3''-4''}$ = 8.0, $J_{4''-5''}$ = 2.1, 4″-H; 4.07, d, $J_{17a-17b}$ = 9.3, 17-Ha; 3.85, dd, $J_{1'-2'}$ = 1, $J_{2'-3'}$ = 4.3, 2′-H; 3.73, dq, $J_{4'-5'}$ = 8.7, $J_{5'-6'}$ = 6.3, 5′-H; 3.68, d, $J_{17a-17b}$ = 9.3, 17-Hb; 3.35, s, 4′-CH_3; 3.32, dd, $J_{3'-4'}$ = 3.2, $J_{4'-5'}$ = 8.7, 4′-H; 3.15, dq, $J_{6''-7''}$ = 4.3, $J_{7''-8''}$ = 5.6, 7″-H; 2.86, dd, $J_{4''-5''}$ = 2.1, $J_{5''-6''}$ = 6.1, 5″-H; 2.77, dd, $J_{5''-6''}$ = 6.1, $J_{6''-7''}$ = 4.3, 6″-H; 2.68, dd, J_{10-11} = 3, J_{10-14a} = 4, 10-H; 2.34, qq, J_{18-19} = 7, J_{18-20} = 7, 18-H; 2.08, m, 6-H; 2.05, m, 5-Ha; 2.00, m, 3-H; 1.98, d, $J_{3''-9''}$ = 1.5, 9″-H_3; 1.96, m, 14-Ha; 1.94, m, 8-Ha; 1.86, m, 4-Ha; 1.83, m, 8-Hb; 1.76, m, 7-H, 1.38, d, $J_{7''-8''}$ = 5.6, 8″-H_3; 1.30, d, $J_{5'-6'}$ = 6.3, 6′-H_3; 1.22, m, 5-Hb; 1.03, d, J_{18-19} = 6.7, 19-H_3; 1.02, m, 4-Hb; 0.98, d, J_{18-20} = 6.7, 20-H_3; 0.79, d, J_{6-16} = 6.8, 16-H_3. ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 202.1 C-15; 174.9 C-13; 165.6 C-1″; 148.4 C-12; 139.8 C-3″; 132.6 C-2″; 130.8 C-11; 98.3 C-1′; 78.3 C-4′; 74.3 C-17; 72.2 C-1; 69.8 C-5′; 69.1 C-2′; 68.3 C-3′; 65.8 C-9; 59.0 C-2; 57.7 4′-OCH_3; 57.0 C-5″; 56.0 C-6″; 52.3 C-7″; 52.0 C-4″; 46.2 C-10; 41.9 C-3; 41.4 C-7; 32.1 C-5; 30.9 C-6; 29.5 C-14; 29.2 C-8; 27.7 C-18; 26.1 C-4; 22.6 C-19; 21.2 C-20; 20.3 C-9″; 18.1 C-6′; 17.4 C-16; 14.0 C-8″. FABMS, *m/z* (rel. int.): 703.3068 (M – H^+ + 2 Na^+, $C_{36}H_{49}O_{11}Na_2$ requires 703.3070), 681 (M + Na^+), 659 (M + H^+).

Hypoxylactone (**2**) was obtained as a colourless oil. $[\alpha]_D$ + 153° (*c* 0.7 in $CHCl_3$). UV (MeOH), λ_{max} (ε): No absorbation above 215 nm. IR (KBr): 3430, 2925, 1760, 1655, 1435, 1210, 1100, 1060 and 835 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm), mult., *J* (Hz): 7.28, dq, J_{3-4} = J_{3-9} = 2, 3-H; 4.86, ddq, J_{3-4} = 1.8, J_{4-5} = 7.6, J_{4-9} = 1.8, 4-H; 3.48, dd, J_{4-5} = 7.6, J_{5-6} = 6.4, 5-H; 3.26, dq, J_{6-7} = 4.3, J_{7-8} = 5.6, 7-H; 3.15, dd, J_{5-6} = 6.4, J_{6-7} = 4.3, 6-H; 1.95, dd, J_{3-9} = J_{4-9} = 1.8, 9-H_3; 1.35, d, J_{7-8} = 5.6, 8-H_3. ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 173.6 C-1; 146.5 C-3; 131.2 C-2; 81.0 C-4; 70.2 C-5; 57.6 C-6; 53.9 C-7; 13.7 C-8; 10.7 C-9. CIMS (CH_4), *m/z* (rel. int.): 369 (10%, 2M + H^+), 225 (8%, M + C_3H_5+), 213 (23%, M + C_2H_5+),

201 (55%, M + CH_5+), 185 (89%, M + H^+), 167 (98%, M – H_2O + H^+), 111 (100%).

Biological assays

The assays for antimicrobial (Anke *et al.*, 1989) and cytotoxic activities (Zapf *et al.*, 1995) were carried out as described previously. The incorporation of the precursors [2-^{14}C]thymidine into DNA (HL-60), [8-^{14}C]adenine into fungal DNA, [2-^{14}C]uridine into RNA and [1-^{14}C]leucine into proteins was assayed with HL-60 cells (Becker and Anke, 1994) and with *Nematospora coryli*. The incorporation of N-acetyl-[1-^{14}C]glucosamin into chitin of *N. coryli* cells was measured as described by Pfefferle *et al.*, 1990.

Results and Discussion

Structure elucidation of hypoxysordarin and hypoxylactone, and identification of sordarin

The structure of sordarin (**3**) isolated in this investigation could be established by comparing the spectroscopic data with those originally published (Hauser and Sigg, 1971) and those of sordarin derivatives (e.g. Schneider *et al.*, 1995). For hypoxysordarin (**1**), the NMR data for the diterpene and carbohydrate parts are almost identical, with the exception for the upshifting of the signal for 3′-H indicating that the 3′-OH is acylated, and the HMBC and NOESY correlations shown in Fig. 2 confirm the structure and the relative stereochemistry of those parts of **1**. The MS data indicate that the 3′-O acyl group has the elemental composition $C_9H_{11}O_3$, and the ^{1}H NMR data show that the 11 protons are part of a spin system that has methyl groups in both ends. The presence of two epoxide functions in this is suggested by the chemical shifts for protons 4″-H to 7″-H and C-4″ to C-7″, as well as by the relatively small ^{1}H–^{1}H coupling constants. As $J_{4''-5''}$ is only 2.1 Hz and a strong NOESY correlation between 3″-H and 5″-H can be observed, the C-4″/C-5″ epoxide ring should be cis substituted, and a $J_{6''-7''}$ of 4.3 Hz together with a NOESY correlation between 6″-H and 7″-H indicate that the C-6″/C-7″ epoxide is trans. Strong NOESY correlations between 4″-H and 6″-H, and 5″-H and 8″-H_3, together with a fairly large $J_{5''-6''}$ (6.1 Hz), suggest that the two epoxide rings are parallel, and the most stable conformation would be with the epoxide oxygens as far apart as possible as indicated in the structure of **1**. It was not possible to correlate the absolute stereochemistry of the acyl moiety to that of sordanin based on NMR data, so the configurations of C-4″, C-5″, C-6″ and C-7″ (all carbons) could also be inverted. The corresponding 2D NMR correlations were also observed with hypoxylactone (**2**), the presence of a lactone ring (instead of a free hydroxy acid) is demonstrated by the MS data while its nature as a γ-lactone is shown by the ^{1}H NMR shift for 4-H.

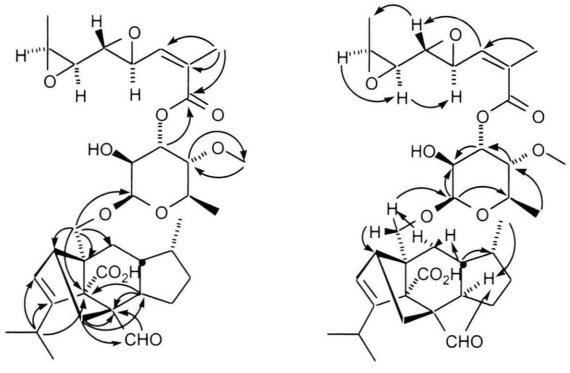

Fig. 2. Pertinent HMBC (left) and NOESY (right) correlations observed with hypoxysordarin (**1**).

Sordarin (**3**) was originally isolated from the terrestrial ascomycete *Sordaria araneosa* (Hauser and Sigg, 1971) as an antifungal compound. Its aglycon, sordaricin, was found to exhibit similar antifungal activity. Hypoxysordarin (**1**) differs from sordarin in the substitution with an unusual side chain. Hypoxylactone (**2**), a presumed hydrolysis product of this side chain could be detected in the culture broth. Sordarin derivatives similar to **1** have been isolated from the obligate marine species *Zopfiella marina* (Sordariaceae) (Ogita *et al.*, 1987), from the terrestrial deuteromycete *Graphium putredinis* (Kinsman *et al.*, 1998) and from the terrestrial *Xylaria longipes* (Schneider *et al.*, 1996).

Besides hypoxysordarin, hypoxylactone and sordarin, 5-methylmellein and 16-hydroxy-7-isopimaren-19-oic acid were also identified in cultures of *Hypoxylon croceum* M97-25. The latter two compounds have been reported from other *Hypoxylon* species (Borgschulte *et al.*, 1991). Whalley and Edwards (1986) concluded that the formation of dihydroisocoumarin derivatives, especially 5-

methylmellein, is widely distributed throughout the genus *Hypoxylon* and other members of the *Xylariaceae*.

The only known obligate marine *Hypoxylon* species, *H. oceanicum* produces an antifungal cyclic depsipeptide which inhibits the cell wall biosynthesis (Abbanat *et al.*, 1998).

Table I. Antifungal activity of **1** and **3** in the serial dilution assay.

Test organism	MIC [μg/ml] Hypoxy. sordarin	Sordarin
Yeasts		
Nadsonia fulvescens	>50	>50
Nematospora coryli	0.5	0.2
Rhodotorula glutinis	>50	>50
Saccharomyces cerevisiae α 288c[a]	>50	50s
Saccharomyces cerevisiae is1	2s	2s
Filamentous fungi		
Absidia glauca (+)	20s	50s
Absidia glauca (−)	10s	20s
Alternaria porri	>50	>50
Aspergillus ochraceus	10s	>50
Botrytis cinerea	>50	>50
Cladosporium cladosporioides	>50	>50
Curvularia lunata	>50	>50
Fusarium fujikuroi	>50	>50
Fusarium oxysporum	>50	>50
Mucor miehei	1s	10s
Paecilomyces variotii	2s	50s
Penicillium islandicum	10s	>50
Penicillium notatum	2s	>50
Zygorhynchus moelleri	20s	20s
Ustilago nuda	>50	>50

[a] Gift from Prof. F. Lacroute, Strasbourg.
s Fungistatic, the growth restarted after removal of the compound.

Table II. Cytotoxic activity of the isolated compounds.

Cell line	IC_{50} caused by Hypoxysordarin [μg/ml]	Sordarin [μg/ml]
HL-60[1]	50	50
L1210[2]	>100	100
HeLa S3[3]	>100	>100
COS-7[4]	>100	100
Colo-320[5]	>100	>100
HepG2[6]	>100	>100

[1] Promyelocytic leukemia, human; ATCC CCL 240.
[2] Lymphocytic leukemia, mouse; ATCC CCL 219.
[3] Epitheloid carcinoma, cervix, human; ATCC CCL 2.2.
[4] Kidney fibroblast, SV 40 transformed, African Green monkey; ATCC CRC-1651.
[5] Colon adenocarcinoma, human; DSMZ ACC 144.
[6] Hepatocellular carcinoma, human; ATCC HB-8065.

Biological properties of hypoxysordarin (**1**) *and sordarin* (**3**)

The antifungal activities of **1** and **3** are compared in Table I. **1** exhibits higher antifungal activities towards *Absidia glauca, Mucor miehei, Paecilomyces variotii, Penicillium notatum* and *Penicillium islandicum* in the serial dilution assay. No antibacterial activity could be observed with both compounds. The cytotoxic effects on several cell lines are very weak (Table II). Former studies

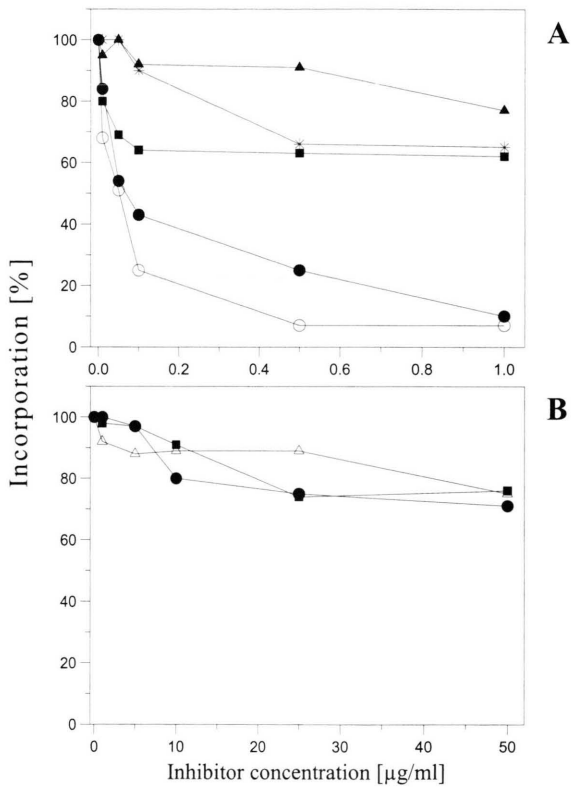

Fig. 3. Incorporation of radiolabelled precursors into macromolecules of *Nematospora coryli* and HL-60 cells. **A:** *Nematospora coryli* with hypoxysordarin as inhibitor: [^{14}C]adenine into DNA (▲), control 12686 cpm; [^{14}C]uridine into RNA (■), control 46315 cpm; [^{14}C]N-acetylglucosamine into chitin (⨯), control 2030 cpm and [^{14}C]leucine into proteins (●), control 5014 cpm. With sordarin as inhibitor: [^{14}C]leucine into proteins (○), control 5014 cpm. – **B:** HL-60 cells with hypoxysordarin as inhibitor: [^{14}C]thymidine into DNA (△), control 5020 cpm; [^{14}C]uridine into RNA (■), control 19667 cpm and [^{14}C]leucine into proteins (●), control 4845 cpm.

have identified fungal protein biosynthesis with the elongation factor 2 (EF2) and the ribosomal P-protein stalk function as the target for sordarins (Justice *et al.*, 1998; Gomez-Lorenzo and García-Bustos, 1998). Incorporation of ^{14}C-labelled precursors into macromolecules in *Nematospora coryli* showed a preferential inhibition of protein biosynthesis starting at 0.01 µg sordarin/ml and 0.05 µg hypoxysordarin/ml, respectively (Fig. 3A). 0.5 µg **3**/ml or 1 µg **1**/ml resulted in a complete arrest of translation. Higher concentrations of **1** and **3** partially inhibited the synthesis of DNA, RNA and chitin. Incorporation of precursors into macromolecules of HL-60 cells was very weakly and not selectively inhibited starting from 5–10 µg/ml **1** or **3** (Fig. 3B) as has been described for other sordarin derivatives (Schneider *et al.*, 1995; Justice *et al.*, 1998).

The side chain of hypoxysordarin apparently confers a higher antifungal activity (with the exception of *Nematospora coryli*) and an extended spectrum of sensitive fungal strains. The precursor of the side chain (**2**) itself does not exhibit antibiotic activities.

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