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The Production of Novel Sordarin Analogues by Biotransformation

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The biotransformation of the fungal protein synthesis inhibitor sordarin is reported. Nine taxonomically diverse organisms supported the isolation and identification of twelve modified products. The structural diversity of the biotransformation products observed and their value in supporting further chemistry is discussed.

Sordarin (**1**), a diterpene glycoside produced by the fungus *Sordaria araneosa*, is a key intermediate in the synthesis of novel anti-fungal agents. Using a whole cell microbial biotransformation approach, both sordarin (**1**), and the closely related aglycone sordaricin (**2**), were modified by a range of organisms yielding a number of novel derivatives that were subsequently isolated and identified. Biotransformations ranged from simple esterifications to reduction, oxidation and demethylation. Selected biotransformations were scaled up to support both structure-activity studies and further chemical derivatisation.

Opportunistic fungal infections are increasingly common in severely immunocomprimised patients especially following cancer chemotherapy, bone marrow or organ transplantation and HIV infection[1]. The prognosis for these patients is typically very poor. Consequently, the search for novel anti-fungal compounds displaying new modes of action and improved pharmacological characteristics has intensified across the pharmaceutical industry[2].

Over the last few years, several companies including GlaxoSmithKline and Merck have shown considerable interest in a group of anti-fungal compounds known as the sordarins. These compounds were originally isolated from the fungus *Sordaria araneosa* by workers at Sandoz over thirty years ago[3]. However, they showed relatively modest anti-fungal activity and appear not to have been progressed further. A closely related compound (GR135402 (**3**) differing from sordarin only in the presence of a 2-methyl-hexa-2,4-dienoate side chain) was identified as part of our natural products screening programme (Fig. 1).

This compound, produced by *Graphium* sp. (F13302), was found to be a potent inhibitor of protein synthesis in *Candida albicans* (IC_{50} Candida protein synthesis (CPS) 0.03 μg/ml)[4]. Unlike sordarin (**1**), GR135402 (**3**) also showed very good whole cell activity against several species of fungi (MIC *C. albicans* 0.03 μg/ml; *Cryptococcus neoformans* 1 μg/ml)[5].

In order to support a rapidly expanding chemical programme, it became essential that production levels of GR135402 (**3**) be substantially increased. However, fermentation titres remained modest compared with levels of the related product sordarin observed in cultures of *Sordaria araneosa*. Consequently, it was decided to concentrate on sordarin as the basis for further work.

A biotransformation programme was initiated in order to provide novel sordarin analogues to support further chemistry and also add to the growing SAR profile. Sordarins are known to be highly selective inhibitors of fungal protein biosynthesis with elongation factor 2 (EF-2) and the ribosomal P-protein stalk as their target[6~8]. Sordarin derivatives have been shown to inhibit most fungal pathogens known to affect immunocompromised patients. These include *Candida albicans* and a number of non-albicans species[9]. Excellent activity has also been observed against the opportunistic pathogen *Cryptococcus neoformans* and also *Pneumocystis carinii* the causal agent

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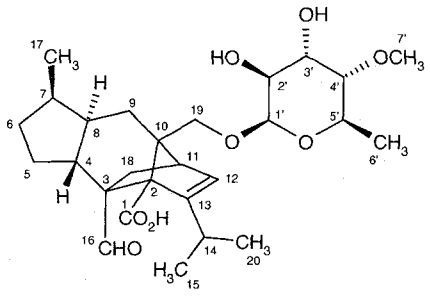
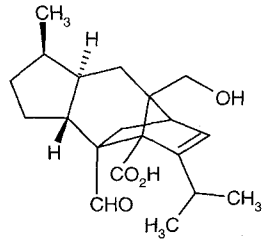
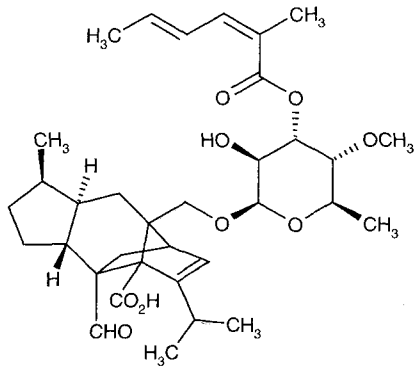
Fig. 1. Structures of sordarin (**1**), sordaricin (**2**) and GR135402 (**3**).



Sordarin (1)



Sordaricin (2)



GR135402 (3)

of pneumonia[9]. It has also been demonstrated that sordarin derivatives have potent fungicidal activity against important dimorphic endemic fungal pathogens such as *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis* and *Coccidioides immitis*[2]. However, two important pathogens namely *Aspergillus fumigatus* and *Candida krusei* are both essentially resistant to these compounds[2]. A recent comprehensive review by ODDS[10] highlights the promise of sordarins as novel antifungal agents.

This paper describes the biotransformation approaches used and includes details of the fermentation conditions, the isolation procedures and also the biological activities of the compounds produced.

Materials and Methods

Micro-organisms Used for Biotransformation Reactions

A broad range of fungi and bacteria (>220 taxonomically diverse organisms) were selected from the GlaxoSmithKline culture collection and screened for their ability to biotransform sordarin (**1**) and sordaricin (**2**). Organisms reported to hydroxylate or degrade diterpenes were particularly targeted[11~13].

Fungal strains were maintained as 6 mm agar plugs stored at room temperature in sterile water. Actinomycete strains were stored as frozen (−20°C) spore suspensions in 15% (v/v) glycerol.

Growth of Seed Cultures

Fungal strains were grown in 250 ml Erlenmeyer flasks containing 50 ml of FS medium with the following

composition (g/litre): peptone (Oxoid L34), 10; malt extract (Oxoid L39), 21; glycerol, 40; Junlon PW110, 1 (Honeywill and Stein Ltd., Wellington, Surrey, U.K) prepared in distilled water. The culture medium was adjusted to pH 6.5 by the addition of aqueous NaOH prior to both sterilisation (121°C, 15 minutes) and Junlon addition. Junlon was homogenised in a Waring blender prior to incorporation into the medium. Fungal cultures were inoculated using two agar plugs and incubated on a rotary shaker (250 rpm; 50 mm throw) at 25°C for 5 days.

For actinomycete cultures, SB1 seed medium was employed dispensed as shown above. SB1 medium had the following composition (g/litre): Arkasoy, 25; yeast extract, 5; KH_2PO_4, 5 in distilled water. The pH of the medium was adjusted to 7 prior to sterilisation (121°C, 15 minutes). Glucose was added post sterilisation (5 ml of a 20% (v/v) solution sterilised 121°C, 15 minutes) to give a final concentration of 20 g/litre. Streptomycete cultures were inoculated using 0.1 ml of a frozen spore suspension. Cultures were incubated on a rotary shaker (250 rpm; 50 mm throw) at 28°C for 5 days.

Conditions Used for Biotransformation

For initial biotransformation screening studies, organisms were cultured either in shake tubes or 250 ml Erlenmeyer flasks, containing 5 ml or 50 ml aliquots of appropriate growth media (FB1 for fungal strains and SB1 for actinomycetes). Incubation conditions used were as detailed above. Inoculum was added at a final concentration of 1% (v/v) to flasks or 3% (v/v) to shake tubes. For biotransformation studies, fungal strains were grown in FB1 medium having the following composition (g/litre): Soya oil, 30; Arkasoy, 10; yeast extract, 5; K_2HPO_4, 5. The medium was adjusted to pH 5.5 pre-sterilisation (121°C 15 minutes). Glucose was added post sterilisation (5 ml of a 20% (v/v) solution sterilised 121°C, 15 minutes) to give a final concentration of 20 g/litre. Biotransformation using actinomycete cultures employed SB1 medium prepared as detailed above.

The substrate (sordarin (**1**) or sordaricin (**2**)) was typically added in 1ml of aqueous ethanol (80% (v/v)) after 2 days growth to a final concentration of 500 μg/ml. Cultures were then reincubated for a further 3 days prior to analysis for biotransformation products (see below). Suitable control cultures (plus/minus inoculum or substrate) were also prepared and appropriate samples analysed to monitor substrate utilisation and also support the identification of biotransformation products.

Sample Analysis

Samples were prepared for analysis by HPLC and HPLC/MS by taking aliquots of the whole biotransformation broth and diluting them 1 : 1 with acidified acetonitrile (0.1% TFA). Samples were then centrifuged and the supernatant transferred to HPLC vials. Primary analysis was performed using a HPLC with diode array detection and samples showing substrate utilisation or the presence of novel peaks were progressed to secondary analysis by HPLC/MS. The MS spectra were acquired on a Finnigan Mat TSQ700 mass spectrometer with a thermospray interface operating in positive ion mode (TSP+ve). Separation was carried out on a Spherisorb C6 reverse phase column (15 cm×4.6 mm, 5 μm) in isocratic mode using a mobile phase of acetonitrile - water - TFA, 700 : 300 : 1. The TSP+ve full scan spectrum for sordarin (**1**) showed characteristic ions at *m/z* 493 (M+H), *m/z* 333 (M−sordarose+2H) and *m/z* 315 (M−sordarose−H_2O+2H). This fragmentation pattern supported the rapid identification of sordarin related biotransformation products and also gave some indication as to the possible site of the modification (*i.e.* diterpene or sordarose moieties). Based on this data, micro-organisms were selected for scale-up supporting product isolation and identification. Purified components following preparative HPLC were flow injected on the above system to confirm molecular weight and their relationship to sordarin prior to their full structural elucidation by NMR.

NMR spectra were recorded on a Bruker AM500 or a Varian VXR-400 using standard pulse sequences.

Scaled-up Production of Biotransformation Products

Micro-organisms providing modifications of potential interest were subsequently scaled-up to support product isolation and identification. Where only a relatively small amount of compound was needed, typically a few milligrams, conditions described above (50 ml of appropriate culture aliquoted into 250 ml Erlenmeyer flasks) were used employing multiple flasks. However, for gram quantities of material, scale-up into fermenters was essential and the following conditions were employed. A spore suspension (5 ml aliquots stored −20°C) was used to inoculate a 50 ml aliquot of SB1 medium dispensed into a 250 ml Erlenmeyer flask. The culture was incubated for 4 days at 28°C on a rotary shaker (250 rpm; 50 mm throw). A 1% (v/v) transfer of inoculum was then carried out into eight similar flasks and these were incubated for 5 days (as above) prior to bulking and transfer to the fermenter. Biotransformation in fermenters was undertaken at 40-litre scale using Biolafitte vessels (nominal volume 70 litres).

SB1 medium was used containing Tween 80 (0.1% (v/v) added post sterilisation) to minimise foaming (sterilised 121°C, 30 minutes). Following addition of the inoculum, cultures were maintained at 28°C with aeration at 30 litres/minute and agitation at 400 rpm. Antifoam (PPG2000) was added directly to each fermentation (0.25 ml/litre) and was also provided on demand. An ethanolic stock solution of the substrate (sordarin (**1**)) was prepared as previously shown and added 2 days post inoculation to a final concentration of 500 μg/ml. The fermentation was typically stopped some 5 days post substrate addition. Preparative isolations were undertaken when significant products were evident by HPLC-MS analysis of biotransformation cultures, and where sordarin utilisation was >60%.

Product Isolation

Whole fermentation broth was pH adjusted with NaOH (to 0.1 N final concentration), then cells were removed by centrifugation. Supernatant was acidified (1 N HCl) to pH 3~4, then sordarins were adsorbed onto reverse phase silica (Spherisorb C18 Bond-elute column). The adsorbent was washed with water, and sordarins were eluted with acetonitrile. Eluates were processed by preparative reverse phase HPLC (Spherisorb 5 μm C6, 25 cm×2 cm i.d. column). Mobile phase A was water - TFA (1000 : 1) and mobile phase B was acetonitrile - water - TFA (800 : 200 : 1). A 40 minutes linear gradient from A to 80%B was used with a flow rate of 25ml/minute. Both substrate fed and unfed control cultures were compared in this way, and putative biotransformation products were collected. Following removal of acetonitrile from product-containing fractions, products were recovered by adsorption onto reverse phase silica, then elution with acetonitrile and rotary evaporation to dryness.

Characterising Data

Compound **4**

MS (TSP +ve) *m/z* 509 (M+H), 349 (M−sordarose+2H). ^{1}H NMR (500 MHz, CD_3OD) δ 0.87 (3H, d, J=7.5 Hz, H-17), 0.95 (1H, m, 1/2 H-5), 0.98 (3H, d, J=6.5 Hz, H-15), 1.05 (3H, d, J=6.5 Hz, H-20), 1.25 (3H, d, J=6.5 Hz, H-6'), 1.27 (1H, d, J=12.5 Hz, 1/2 H-18), 1.75 (1H, t, J=13 Hz, 1/2 H-9), 1.85 (1H, m, H-7), 2.00 (1H, dd, J=12.5 and 4.5 Hz, 1/2 H-18), 2.04~2.13 (3H, m, H-4, H-8 and 1/2 H-9), 2.33 (1H, m, H-14), 2.39 (1H, m, 1/2 H-5), 2.84 (1H, m, H-11), 3.13 (1H, dd, J=9.5 and 3 Hz, H-4'), 3.37 (3H, s, H-7'), 3.69~3.76 (3H, m, H-2', H-5' and 1/2 H-19), 3.84 (1H, t, J=6.5 Hz, H-6) 3.91 (1H, d, J=9.5 Hz, 1/2 H-19), 4.13 (1H, dd, J=4 and 3 Hz, H-3'), 4.58 (1H, d, J=1 Hz, H-1'), 6.14 (1H, d, J=3.5 Hz, H-12), 9.63 (1H, s, H-16).

Compound **5**

MS (TSP +ve) *m/z* 349 (M+H). ^{1}H NMR (500 MHz, CD_3OD) δ 0.87 (3H, d, J=7.5 Hz, H-17), 0.97 (1H, m, 1/2 H-5), 0.98 (3H, d, J=6.5 Hz, H-15), 1.06 (3H, d, J=6.5 Hz, H-20), 1.26 (1H, d, J=12.5 Hz, 1/2 H-18), 1.82~1.91 (3H, m, H-7 and H-9), 2.02 (1H, dd, J=12.5 and 4.5 Hz, 1/2 H-18), 2.09 (1H, m, H-4) 2.13 (1H, m, H-8), 2.34 (1H, m, H-14), 2.42 (1H, ddd, J=13, 7 and 6 Hz, 1/2 H-5), 3.69 (1H, d, J=11 Hz, 1/2 H-19), 3.73 (1H, d, J=11 Hz, 1/2 H-19), 3.85 (1H, ddd, J=7, 5.5 and 1 Hz, H-6), 6.13 (1H, dd, J=3.5 and 1 Hz, H-12), 9.63 (1H, s, H-16).

^{13}C NMR (500 MHz, CD_3OD, From HMQC) δ 13.9 (C-17), 21.0 (C-20), 22.6(C-15), 28.5 (C-14), 28.8 (C-9), 29.9 (C-18), 37.5 (C-5), 39.6 (C-8), 41.6 (C-4), 43.3 (C-7), 47.4 (C-11), 67.2 (C-19), 80.6 (C-6), 131.6 (C-12).

Compound **6**

MS (FAB +ve) *m/z* 509 (M+H), 349 (M−sordarose+2H). ^{1}H NMR (500 MHz, CD_3OD) δ 0.98 (3H, d, J=6.5 Hz, H-15), 1.04 (3H, d, J=6.5 Hz, H-20), 1.15 (3H, s, H-17), 1.24 (1H, m, 1/2 H-5), 1.25 (3H, d, J=6.5 Hz, H-6'), 1.30 (1H, d, J=12.5 Hz, 1/2 H-18), 1.71~1.80 (2H, m), 1.80~1.89 (3H, m), 1.96 (1H, dd, J=12.5 and 4.5 Hz, 1/2 H-18), 2.02 (1H, m, H-4), 2.13 (1H, dd, 14 and 6 Hz, 1/2 H-6), 2.33 (1H, m, H-14), 2.81 (1H, dd, J=4.5 and 3.5 Hz, H-11), 3.13 (1H, dd, J=9.5 and 3 Hz, H-4'), 3.38 (3H, s, H-7'), 3.69~3.76 (3H, m), 3.93 (1H, d, J=9.5 Hz, 1/2H-19), 4.12 (1H, dd, J=3 and 4 Hz, H-3') 4.58 (1H, d, J=1 Hz, H-1'), 6.12 (1H, dd, J=3.5 and 1 Hz, H-12) 9.67 (1H, s, H-16).

Compound **7**

MS (FAB +ve) *m/z* 479 (M+H), 333 (M−demethyl sordarose+2H). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.80 (3H, d, J=6.5 Hz, H-17), 0.99 (3H, d, J=7 Hz, H-15), 1.02 (1H, m, 1/2 H-5), 1.04 (3H, d, J=7 Hz, H-20), 1.23 (1H, m, 1/2 H-6), 1.30 (1H, d, J=12.5 Hz, 1/2 H-18), 1.31 (3H, d, J=6.5 Hz, H-6'), 1.78 (1H, m), 1.83~2.13 (7H, m), 2.34 (1H, m, H-14), 2.65 (1H, dd, J=4.5 and 3.5 Hz, H-11), 3.61 (1H, d, J=9.5 Hz, 1/2H-19), 3.67 (1H, dd, J=8.5 and 3.5 Hz, H-4'), 3.74 (1H, dq, J=8.5 and 6.5 Hz, H-5'), 3.88 (1H, dd, J=4.5 and 1 Hz, H-2'), 4.08 (1H, dd, J=4.5 and 3.5 Hz, H-3'), 4.15 (1H, d, J=9.5 Hz, 1/2H-19), 4.70 (1H, d, J=1.5 Hz, H-1'), 6.08 (1H, dd, J=3.5 and 1 Hz, H-12), 9.73 (1H, s, H-16).

Compound **8**

MS (TSP +ve) *m/z* 535 (M+H), 333 (M−acetyl sordarose+2H). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.82 (3H, d, *J*=7 Hz, H-17), 0.99 (1H, m, 1/2 H-5), 1.00 (3H, d, *J*=6.5 Hz, H-15), 1.04 (3H, d, *J*=6.5 Hz, H-20), 1.23 (1H, m, 1/2 H-6), 1.27 (3H, d, *J*=6.5 Hz, H-6′), 1.27 (1H, d, *J*=12.5 Hz, 1/2 H-18), 1.63 (1H, dd, *J*=14.5 and 6 Hz, 1/2 H-9), 1.81 (1H, m, H-7), 1.85~1.97 (3H, m), 2.01~2.13 (3H, m), 2.17 (3H, s, Ac), 2.32 (1H, m, H-14), 2.44 (1H, dd, *J*=4.5 and 3.5 Hz, H-11), 3.10 (1H, dd, *J*=9.5 and 3 Hz, H-4′), 3.38 (1H, d, *J*=9 Hz, 1/2 H-19), 3.41 (1H, s, H-7′) 3.72 (1H, dq, *J*=9.5 and 6.5 Hz, H-5′), 4.10 (1H, dd, *J*=4 and 3 Hz, H-3′), 4.42 (1H, d, *J*=9 Hz, 1/2H-19), 4.79 (1H, d, *J*=1.5 Hz, H-1′), 5.20 (1H, dd, *J*=4 and 1.5 Hz, H-2′), 6.04 (1H, dd, *J*=3.5 and 1 Hz, H-12), 9.86 (1H, s, H-16).

Compound **9**

MS (TSP +ve) *m/z* 507 (M+H), 347 (M−sordarose+2H). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.76 (3H, d, *J*=6.5 Hz, H-17), 0.86 (3H, d, *J*=6.5 Hz, H-15), 0.96 (1H, m, 1/2 H-5), 1.03 (3H, d, *J*=6.5 Hz, H-20), 1.21 (1H, m, 1/2 H-6), 1.29 (1H, d, *J*=12.5 Hz, 1/2 H-18), 1.30 (3H, d, *J*=6.5 Hz, H-6′), 1.67 (1H, m, H-8), 1.74 (1H, t, *J*=13 Hz, 1/2 H-9) 1.81~1.92 (3H, m, H-4, 1/2 H-5 and 1/2 H-18), 1.95~2.08 (3H, m, 1/2 H-6, H-7 and 1/2 H-9), 2.23 (1H, m, H-14), 2.74 (1H, dd, *J*=4.5 and 3.5 Hz, H-11), 3.21 (1H, dd, *J*=9 and 3.5 Hz, H-4′), 3.42 (3H, s, H-7′), 3.70 (1H, dq, *J*=9 and 6.5 Hz, H-5′), 3.72 (1H, d, *J*=9.5 Hz, 1/2 H-19), 3.76 (3H, s, OMe), 3.88 (1H, dd, *J*=3.5 and 1 Hz, H-2′), 3.94 (1H, d, *J*=9.5 Hz, 1/2 H-19), 4.22 (1H, t, *J*=3.5 Hz, H-3′), 4.64 (1H, d, *J*=1 Hz, H-1′), 6.05 (1H, d, *J*=3.5 Hz, H-12), 9.72 (1H, s, H-16).

Compound **10**

MS (TSP +ve) *m/z* 672 (M+NH_4), 677 (M+Na), 493 (M−glucose+2H), 333 (M−glucose−sordarose+2H). ^{1}H NMR (500 MHz, CDO_3D) δ 0.82 (3H, d, *J*=6.5 Hz, H-17), 0.93 (1H, m, 1/2 H-5), 0.95 (3H, d, *J*=6.5 Hz, H-15), 1.03 (3H, d, *J*=6.5 Hz, H-20), 1.24 (1H, m, 1/2 H-6), 1.26 (3H, d, *J*=6.5 Hz, H-6′), 1.30 (1H, d, *J*=12.5 Hz, 1/2 H-18), 1.76 (1H, m, H-8), 1.83 (1H, t, *J*=14 Hz, 1/2 H-9), 1.89 (1H, m, 1/2 H-5), 1.93 (1H, dd, *J*=12.5 and 4.5 Hz, 1/2 H-18), 2.00~2.10 (3H, m, 1/2 H-6, H-7 and 1/2 H-9), 2.14 (1H, td, *J*=12.5 and 6 Hz, H-4), 2.35 (1H, m, H-14), 2.84 (1H, dd, *J*=4.5 and 3.5 Hz, H-11), 3.14 (1H, dd, *J*=9 and 3 Hz, H-4′), 3.34 (1H, t, *J*=8.5 Hz, H-2″), 3.36 (1H, dd, *J*=9.5 and 8.5 Hz, H-4″), 3.38 (3H, s, OMe), 3.41 (1H, m, H-5″), 3.44 (1H, t, *J*=8.5 Hz, H-3″), 3.69 (1H, dd, *J*=12.0 and 5 Hz, 1/2 H-6″), 3.71 (1H, dd, *J*=4 and 1.5 Hz, H-2′), 3.72 (1H, m, H-5′), 3.75 (1H, d, *J*=9.5 Hz, 1/2 H-19), 3.83 (1H, dd, *J*=12.0 and 2.5 Hz, 1/2 H-6″), 3.95 (1H, d, *J*=9.5 Hz, 1/2 H-19), 4.12 (1H, dd, *J*=4 and 3 Hz, H-3′), 4.59 (1H, d, *J*=1.5 Hz, H-1′), 5.59 (1H, d, *J*=8.5 Hz, H-1″), 6.11 (1H, dd, *J*=3.5 and 1 Hz, H-12), 9.76 (1H, s, H-16).

Compound **11**

MS (FAB +ve) *m/z* 495 (M+H), 333 (M−glucose+2H). ^{1}H NMR (500 MHz, CDO_3D) δ 0.81 (3H, d, *J*=6.5 Hz, H-17), 0.94 (3H, d, *J*=6.5 Hz, H-15), 0.97 (1H, m, 1/2 H-5), 1.04 (3H, d, *J*=6.5 Hz, H-20), 1.26 (1H, m, 1/2 H-6), 1.29 (1H, d, *J*=12.5 Hz, 1/2 H-18), 1.76 (1H, m, H-8), 1.82 (1H, t, *J*=13 Hz, 1/2 H-9), 1.86~2.18 (6H, m, H-4, 1/2 H-5, 1/2 H-6, H-7, 1/2 H-9 and 1/2 H-18), 2.34 (1H, m, H-14), 2.75 (1H, dd, *J*=4.5 and 3.5 Hz, H-11), 3.33 (1H, t, *J*=9 Hz, H-2″), 3.36 (1H, dd, *J*=10 and 9 Hz, H-4″), 3.41 (1H, m, H-5″), 3.44 (1H, t, *J*=9 Hz, H-3″), 3.66 (1H, d, *J*=11.5 Hz, 1/2 H-19), 3.69 (1H, dd, *J*=12.5 and 5 Hz, H-6″), 3.82 (1H, dd, *J*=12.5 and 2.5 Hz, H-6″), 3.83 (1H, d, *J*=11.5 Hz, 1/2 H-19), 5.59 (1H, d, *J*=9 Hz, H-1″), 6.11 (1H, m, H-12), 9.74 (1H, s, H-16).

Compound **12**

MS (TSP +ve) *m/z* 509 (M+H), 349 (M−sordarose+2H). ^{1}H NMR (500 MHz, CD_3OD) δ 0.80 (3H, s, H-17), 1.02 (3H, d, *J*=6.5 Hz, H-15), 1.05 (3H, d, *J*=6.5 Hz, H-20), 1.21 (2H, m, 1/2 H-5 and 1/2 H-6), 1.25 (3H, d, *J*=6.5 Hz, H-6′), 1.42 (1H, d, *J*=13 Hz, 1/2 H-18), 1.63 (1H, m, 1/2 H-5), 1.73 (1H, dd, *J*=14 and 13 Hz, 1/2 H-9), 1.86 (1H, m, H-8), 2.03 (1H, m, H-4), 2.07 (1H, dd, *J*=14 and 6 Hz, 1/2 H-9), 2.09 (1H, m, 1/2 H-6), 2.12 (1H, m, H-7) 2.20 (1H, d, *J*=13 Hz, 1/2 H-18), 2.41 (1H, m, H-14), 2.75 (1H, dd, *J*=4.5 and 3.5 Hz, H-11), 3.13 (1H, dd, *J*=9 and 3 Hz, H-4′), 3.38 (3H, s, H-7′), 3.71 (1H, dd, *J*=4 and 1 Hz, H-2′), 3.72 (1H, m, H-5′), 3.74 (1H, d, *J*=9.5 Hz, 1/2 H-19), 3.92 (1H, d, *J*=9.5 Hz, 1/2 H-19), 4.12 (1H, dd, *J*=4 and 3 Hz, H-3′), 4.57 (1H, d, *J*=1 Hz, H-1′), 6.13 (1H, dd, *J*=3.5 and 1 Hz, H-12).

Compound **13**

MS (TSP +ve) *m/z* 495 (M+H), 335 (M−sordarose+2H). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.18 (1H, d, *J*=12.5 Hz, 1/2 H-18), 0.80 (3H, d, *J*=7 Hz, H-17), 1.04 (3H, d, *J*=7 Hz, H-15), 1.10 (1H, m, 1/2 H-5), 1.12 (3H, d, *J*=7 Hz, H-20), 1.21 (1H, m, 1/2 H-6), 1.29 (3H, d, *J*=6.5 Hz, H-6′), 1.77~1.90 (5H, m), 1.99 (1H, dd, *J*=12.5 and 4.5 Hz, 1/2 H-18), 2.00~2.13 (2H, m), 2.46 (1H, m, H-14), 2.52 (1H, dd, *J*=4.5 and 3.5 Hz, H-11), 3.19 (1H, dd, *J*=9 and 3 Hz, H-4′), 3.36 (1H, d, *J*=11.5 Hz, 1/2 H-16), 3.42 (1H, d, *J*=11.5 Hz, 1/2 H-16), 3.42 (1H, s, H-7′), 3.63 (1H, d, *J*=9.5 Hz, 1/2 H-19), 3.70 (1H, dq, *J*=9 and 6.5 Hz,

H-5′), 3.90 (1H, dd, J=4 and 1 Hz, H-2′), 4.19 (1H, d, J=9.5 Hz, 1/2 H-19), 4.20 (1H, dd, J=4 and 3 Hz, H-3′), 4.69 (1H, d, J=1 Hz, H-1′), 5.96 (1H, dd, J=3.5 and 1 Hz, H-12).

Compound **14**

MS (TSP +ve) m/z 349 (M+H). ^{1}H NMR (500 MHz, CD_3OD) δ 0.97 (3H, d, J=6.5 Hz, H-15), 1.05 (3H, d, J=6.5 Hz, H-20), 1.16 (3H, s, H-17), 1.25 (1H, m, 1/2 H-5), 1.28 (1H, d, J=12.5 Hz, 1/2 H-18), 1.73~1.94 (5H, m), 1.94~2.05 (3H, m), 2.32 (1H, m, H-14), 2.68 (1H, m, H-11), 3.72 (2H, m, H-19), 6.12 (1H, dd, J=3.5 and 1 Hz, H-12), 9.66 (1H, s, H16).

Compound **15**

MS (TSP +ve) m/z 495 (M+H), 349 (M−demethyl sordarose+2H). ^{1}H NMR (500 MHz, CD_3OD) δ 0.97 (3H, d, J=6.5 Hz, H-15), 1.03 (3H, d, J=6.5 Hz, H-20), 1.14 (3H, s, H-17), 1.23 (1H, m, 1/2 H-5), 1.25 (3H, d, J=6.5 Hz, H-6′), 1.29 (1H, d, J=12.5 Hz, 1/2 H-18), 1.69~1.79 (2H, m), 1.79~1.89 (3H, m), 1.95 (1H, dd, J=12.5 and 4.5 Hz, 1/2 H-18), 2.01 (1H, m, H-4), 2.13 (1H, dd, J=14 and 6 Hz, 1/2 H-6), 2.31 (1H, m, H-14), 2.81 (1H, dd, J=4.5 and 3.5 Hz, H-11), 3.45 (1H, dd, J=9.5 and 3 Hz, H-4′), 3.67 (1H, dq, J=9.5 and 6.5 Hz, H-5′), 3.68 (1H, dd, J=4.5 and 1 Hz, H-2′), 3.72 (1H, d, J=9.5 Hz, 1/2 H-19), 3.87 (1H, dd, J=4.5 and 3.5 Hz, H-3′), 3.92 (1H, d, J=9.5 Hz, 1/2 H-19), 4.57 (1H, d, J=1 Hz, H-1′), 6.11 (1H, dd, J=3.5 and 1 Hz, H-12) 9.67 (1H, s, H-16).

Generation of Biological Activity

Biotransformation products were tested for protein synthesis inhibition using cell-free preparations of *Candida albicans* C316. Inhibition was determined by assaying poly-uridine directed incorporation of L-[U-^{14}C] phenylalanine into trichloroacetic acid precipitatable material as previously described[4].

In vitro anti-fungal activity was determined against *Candida albicans* ATCC 10231 by the agar dilution method using Iso-Sensitest agar (pH 7.4; Oxoid Ltd.). The agar was inoculated with 10 μl of microbial suspension and incubated at 37°C. MIC's were determined after 18 hours.

Results

Structure Elucidation

Many of the micro-organisms tested were shown to readily modify or completely degrade sordarin (**1**) and sordaricin (**2**). However, initially a small subset of only nine organisms was selected for further work based on both potential product novelty and yield (Table 1). In total, twelve biotransformation products were isolated (Table 2). The structures of these compounds were determined by comparison of their NMR and MS data with those for substrates **1**[4] and **2**. The assignments of the ^{1}H NMR spectra was in some cases aided by acquisition of 2D COSY or 1D difference decoupled spectra.

Table 1. Strains used for biotransformation studies.

Species	Strain No.
[+]*Streptomyces capreolus*	ATCC31963
Streptomyces avermitilis	ATCC31272
Pithomyces graminicola	IMI 313510
Rhizopus japonicus	IMI 21600
Xylaria polymorpha	F5755*
Mortierella isabellina	ATCC38063
Mortierella ramanniana	IMI 035044
Streptomyces fradiae	C1281*
Aspergillus awamori	NRRL 3112

[+] Strain reclassified as *Saccharothrix mutabilis* subsp. *capreolus*. IMI-Cab International, BakehamLane, Egham, Surrey, U.K.
* GSK culture collection.

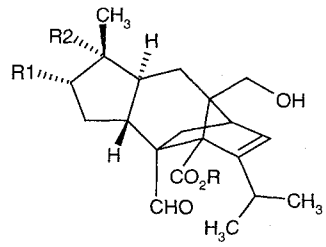
Biotransformations ranged from simple esterifications to reduction, oxidation (including hydroxylation) and demethylation as detailed below.

Modifications to the Sordarose Sugar Leading to **7** and **8**

Demethylation of the methyl ether of sordarin was very commonly observed. 4′-*O*-Demethylsordarin (**7**) was initially isolated from biotransformations using both *Streptomyces capreolus* (ATCC 31963) and *Streptomyces avermitilis* (ATCC 31272). The biotransformation using *Streptomyces capreolus* was carried out at 14 ml scale and yielded 1.5 mg of **7**. However, *Streptomyces avermitilis* was shown to be most efficient (6 mg from 50 ml) and this organism was subsequently scaled up further to 40 litres

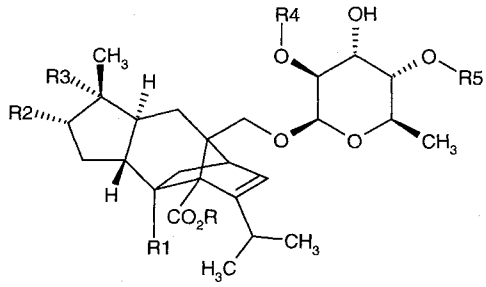
Table 2. Biotransformation products.

a) Sordaricin based products



Compound	R	R1	R2
(5)	H	OH	H
(11)	β-D-glucose	H	H
(14)	H	H	OH

b) Sordarin based products



Compound	R	R1	R2	R3	R4	R5
(4)	H	CHO	OH	H	H	CH_3
(6)	H	CHO	H	OH	H	CH_3
(7)	H	CHO	H	H	H	H
(8)	H	CHO	H	H	$COCH_3$	CH_3
(9)	CH_3	CHO	H	H	H	CH_3
(10)	β-D-glucose	CHO	H	H	H	CH_3
(12)	H	CO_2H	H	H	H	CH_3
(13)	H	CH_2OH	H	H	H	CH_3
(15)	H	CHO	H	OH	H	H

scale.

Aspergillus awamori (NRRL 3112) also modified the sordarose sugar moiety of sordarin by acetylation of the 2'-hydroxyl (**8**). 3.2 mg of **8** were obtained from a 100 ml-scale biotransformation.

Esterifications at C-1 Leading to **9**, **10** and **11**

Sordarin was metabolised to the methyl ester (**9**) by *Pithomyces graminicola* (IMI 313510). A 50 ml biotransformation yielded 2.6 mg of **9**. *Rhizopus japonicus* (IMI 21600) generated the glucose esters of both sordarin (**10**) (149 mg from 2.5 litres) and sordaricin (**11**) (22 mg from 100 ml) when fed the appropriate substrate. The ^1H-^1H coupling constants served to identify the sugar.

Modifications at C-3 Yielding **12** and **13**

The aldehyde group at C-3 was a common site for modification. *Xylaria polymorpha* (F5755) catalysed the oxidation of the aldehyde to the carboxylic acid, thus yielding 0.8 mg of **12** from a 50 ml biotransformation. The

Table 3. Biological activities of biotransformation products.

Compound	CPS (IC_{50})[a] (μg/ml)	MIC[b] (μg/ml)
Sordarin (**1**)	0.036	16
Sordaricin (**2**)	2.0	>125
GR135402 (**3**)	0.028	0.03
6-hydroxysordarin (**4**)	ND[c]	ND
6-hydroxysordaricin (**5**)	>40	ND
7-hydroxysordarin (**6**)	0.08	>125
4'-O-demethylsordarin (**7**)	0.035	>125
2'-O-acetylsordarin (**8**)	0.47	>125
Sordarin-1-methyl ester (**9**)	>10	62
Sordarin-1-glucose ester (**10**)	>10	>125
Sordaricin-1-glucose ester (**11**)	>10	>125
Sordarin-3-carboxylic acid (**12**)	>10	>125
3-deformyl-3-hydroxymethyl sordarin (**13**)	ND	>31[d]
7-hydroxysordaricin (**14**)	>40	>125
7-hydroxy-4'-O-demethylsordarin (**15**)	0.04	>125

a CPS - Protein synthesis inhib. vs. *C.albicans* ATCC10231

b MIC vs. *C.albicans* C316

c ND - not determined

d Highest concentration tested

aldehyde group of sordarin was reduced to the primary alcohol by *Mortierella isabellina* (ATCC 38063) to provide 3-deformyl-3-hydroxymethylsordarin (**13**) (7.2 mg from 100 ml). The shift to low field (δ 0.18) of one of the H-18's following this reduction is charasteristic[5].

Modifications at C-6 and C-7 Leading to **4**, **5**, **6**, **14**, **15**

Mortierella ramanniana (IMI 035044) provided the C-6 hydroxylated derivative (**4**) when fed sordarin (**1**) yielding 900 mg from a 2-litre scale biotransformation. The lack of coupling between H-6 and H-7 ($J_{67} \cong 0$) served to establish the stereochemistry of this hydroxylation. Workers at Merck have recently patented the use of a different organism (*Actinomyces* sp.) for the production of the same compound[14]. The reported spectroscopic data differs only slightly from that given here. The equivalent biotransformation was observed when sordaricin (**2**) was fed to the same organism yielding compound **5** (900 mg from 2 litres). *Mortierella ramanniana* (IMI 035044) also provided 7-hydroxysordaricin (**14**) when sordaricin (**2**) was fed (900 mg from 2 litres).

Streptomyces fradiae (C1281) proved to be especially versatile, generating a number of hydroxylated derivatives. Feeding sordarin to this organism yielded 6-hydroxysordaricin (**5**), 7-hydroxysordaricin (**14**), 7-hydroxysordarin (**6**) and 7-hydroxy-4'-*O*-demethylsordarin (**15**). The observation of a cross peak between H-4 and H-17 in the ROESY spectrum of **15** served to establish the stereochemistry of this and the other 7-hydroxy compounds. A 2-litre biotransformation provided 3.3, 26, 9 and 15 mg of each of these compounds respectively.

Biological Activity

Biological activities of the majority of these compounds are summarised in Table 3. In general, biotransformation products exhibited reduced anti-fungal activity compared

with either GR135402 (**3**) or sordarin (**1**). The aglycone sordaricin (**2**), in addition to several biotransformation products derived from it, showed poor anti-fungal activity compared with sordarin. This is exemplified when comparing 7-hydroxy sordaricin (**14**) and 7-hydroxysordarin (**6**) which gave CPS (IC_{50}) values of >40 and 0.08 μg/ml respectively. CPS activity of 7-hydroxy-4′-*O*-demethylsordarin (**15**) was also shown to be very good (0.04 μg/ml). CPS inhibition of 4′-*O*-demethylsordarin (**7**) proved to be closely comparable to that of sordarin.

Discussion

The twin aims of the biotransformation work were to generate novel compounds that might possess desirable biological activity in their own right, and to activate centres —particularly on the diterpene core— for subsequent chemical derivatisation. Both of those aims were accomplished in this work. For example, hydroxylations at C6 and C7 on the diterpene core represent biochemical modifications which would have been most difficult to achieve chemically, but which enable further chemical elaboration of that region of the sordarin molecule. In addition, the 4′-*O*-demethyl product was particularly significant, since it offered an attractive chemical template for synthesis of the drug candidate GM237354 subsequently progressed to pre-clinical development by GlaxoSmithKline[10,15,16]. As this biotransformation product was key to the chemistry programme, the reaction employing *Streptomyces avermitilis* was scaled up from 50 ml to 40 litres yielding 5.5 g of the desired compound **7**. Subsequently, a mutant was obtained that produced 4′-*O*-demethylsordarin as a single fermentation product and this organism became the preferred source of this compound.

This work can be viewed as contributing valuable structure-activity data to the chemical series as a whole, whilst opening new avenues within the medicinal chemistry programme for semi-synthetic derivatisation of these novel biotransformation products.

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