RESTRICTICIN, A NOVEL GLYCINE-CONTAINING ANTIFUNGAL AGENT

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Restricticin (1) is a naturally-occurring antifungal agent which contains triene, pyran and glycine ester functionalities and is unrelated to any previously known family of natural products. This unstable compound, as well as its corresponding *N*,*N*-dimethyl derivative (2), have been produced and isolated from both solid and liquid fermentations of *Penicillium restrictum*. The desglycyl hydrolysis product, restrictinol (3), was produced *via* the hydrolysis of pure restricticin and as an artifact of the isolation of restricticin.

Naturally-occurring antibiotics are often notoriously unstable, as illustrated by the antibacterial agent thienamycin[1] and the antifungal triyne carbonate[2~5], which literally self-destruct upon concentration. Although steps to minimize the decomposition of unstable compounds may be determined empirically before isolation and structure determination, often times pure material generated *via* a "brute force" isolation is used to determine the structure. This structure can then suggest possible points of instability, which in turn suggest modifications in the isolation. The isolation that evolves based on structure/stability information can be radically different from the originally developed method as is well illustrated by the isolation procedures of restricticin (1)[6], described in the following paper. This paper also describes the producing organism, as well as fermentation studies in solid and submerged fermentation media. A full description of the structural elucidation of these compounds will be described elsewhere[7].

Materials and Methods

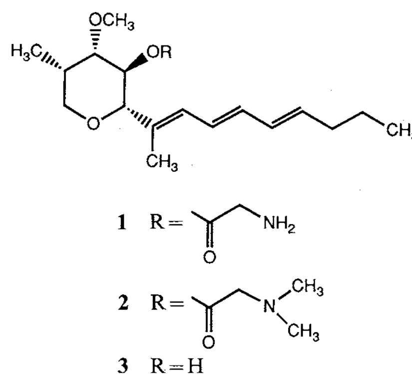
Identification of the Producing Strain

The strain of *Penicillium restrictum* Gilman & Abbott (ATCC 20927, MF 5261) was recovered by conventional dilution plating of a soil sample collected in Tamil Nadu, India. The strain was identified using the procedure of PITT[8]; in addition, its morphological features conformed well with descriptions provided by RAMIREZ[9] and DOMSCH *et al.*[10]. Capitalized color in the description names are from RIDGWAY[11].

Solid Fermentation

The contents of one vial (containing approximately 2 ml frozen vegetative mycelial cells of

Fig. 1. The structures of restricticin (1), *N*,*N*-dimethylrestricticin (2) and restrictinol (3).



ATCC 20927, MF 5261) were inoculated into a 250-ml Erlenmeyer flask containing 50 ml of the following medium (I): corn steep liquor, 5 g; tomato paste, 40 g; oat flour, 10 g; glucose, 10 g; and trace elements mix, 10 ml in 1 liter of distilled water. The trace element mix contained $FeSO_4 \cdot 7H_2O$ 1.0 g, $MnSO_4 \cdot 4H_2O$ 1 g, $CuCl_2 \cdot 2H_2O$ 0.025 g, $ZnSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, H_3BO_3 0.06 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.019 g in 1 liter distilled water. The pH of the medium was adjusted to 6.8 and autoclaved for 20 minutes at 121°C and allowed to cool to room temperature prior to inoculation. The culture was incubated at 27°C on a rotary shaker, 220 rpm for 72 hours. Ten ml of this culture was used to inoculate a second stage seed flask containing 500 ml of the same seed medium in a 2-liter Erlenmeyer flask. The second stage seed was cultivated at 27°C, 200 rpm for 48 hours.

Fermentation was performed in a solid production medium (contained in 6.5 cm × 13 cm × 43 cm plastic trays with lids) and consisted of the following: cracked corn 480 g, and nutrient solution 720 ml (containing yeast extract 33.3 g, sodium tartrate 6.7 g, $FeSO_4 \cdot 7H_2O$ 0.67 g in 1 liter distilled water). The medium was autoclaved for 20 minutes at 121°C, followed by the addition of 500 ml H_2O and a second autoclaving for 20 minutes at 121°C.

After cooling, each tray was inoculated with 50 ml of the seed culture, which was distributed throughout the solid matrix by mixing the contents mixed with a sterile spatula. Fermentation trays were incubated (covered) at 26°C for 14 days without agitation.

Submerged Fermentation

The contents of one vial (containing approximately 2 ml frozen vegetative mycelial cells of ATCC 20927, MF 5261) were inoculated into a 250-ml Erlenmeyer flask containing 50 ml of medium I. This culture was incubated at 26°C on a gyrotory shaker at 220 rpm for 48 hours.

An aliquot (7.5 ml) of this fermentation was used to inoculate a 2-liter Erlenmeyer flask containing 500 ml of meidum I. This second stage seed fermentation was incubated at 26°C, 220 rpm, for 48 hours.

Four 2 liters seed flasks were used to inoculate a 300-liter agitated fermentor charged with 180 liters of medium I. The medium had a presterile pH of 7 and was sterilized for 25 minutes at 122°C. This third stage seed fermentation was incubated for 36 hours at 26°C with air flow 0.3 v/v/m, back pressure 0.6 kg/cm^2 and agitation speed 200 rpm.

The production medium contained Cerelose 50 g, yellow corn meal 10 g, pectin 10 g, glycerol 10 g, tomato paste 5 g, ammonium sulfate 2 g, sodium citrate 2 g, glycine 2 g, cod liver oil 2 g, $CoCl_2 \cdot 6H_2O$ 0.01 g, and P-2000 polyglycol 3 ml in 1-liter of distilled water. The presterile pH was 7. The medium was batch sterilized for 35 minutes at 122°C, except for the Cerelose, which was sterilized separately and added aseptically to the sterilized production medium prior to inoculation. A 5% inoculum from the third stage seed was employed to inoculate three 1,400-liter-scale production fermentations (pH 4.0~4.5). The culture was incubated at 26°C for 138 hours, air flow 0.3 v/v/m, back pressure 0.6 kg/cm^2 and agitation speed 180 rpm.

Antifungal, TLC and HPLC Assays

Disk diffusion assays on MY092 (*Candida albicans*) were utilized, along with TLC and HPLC, to follow the original isolation of restricticin (**1**).

TLC chromatograms were performed on E. Merck Silica gel $60F_{254}$ pre-coated 5 cm × 10 cm plates and were visualized *via* UV, iodine vapors or charring with phosphomolybdate.

HPLC analysis was performed on a system consisting of a Spectraphysics 8700 pump, a Spectraphysics 8780 autoinjector or a Waters U6K manual injector, a DuPont Zorbax ODS, DuPont Zorbax RX or Whatman ODS3 4.6 mm i.d. × 25 cm column, an LKB 2151 variable wavelength UV detector equipped with a 10 mm pathlength cell and a Spectraphysics 4200 integrator. Column temperature was maintained constant at 40°C *via* jacketing and a constant temperature water bath. The flow rate was 1 ml/minute and the UV of the effluent was monitored at 276 nm and an attenuation of 0.64 aufs.

Spectroscopic Methods

NMR spectra were recorded on Varian XL-400 or XL-300 NMR spectrometers. ^1H NMR were recorded in CD_2Cl_2 at ambient temperature using the solvent peak at δ 5.32 as an internal reference downfield of TMS at 0 ppm. ^{13}C NMR were recorded in CD_2Cl_2 at ambient temperature where chemical

shifts are given in ppm downfield of TMS using the solvent peak at 53.8 ppm as an internal reference. EI-MS were obtained on a Finnigan MAT-212 mass spectrometer. IR absorption spectra were obtained with a Perkin-Elmer model 1750 IR FT spectrophotometer using a multiple internal reflectance cell (MIR, ZnSe) on neat 10~20 μg samples. The UV absorption spectra were measured with a Beckman DU-70 spectrophotometer.

Isolation and Physical Characterization of Restricticin (**1**) from Solid Fermentation

A solid fermentation consisting of 10 trays was first wetted with 300 ml MeOH/tray, to facilitate removal from the tray, and an additional 17 liters of MeOH was added after removal for a total of 20 liters. This suspension was stirred for 10~15 minutes and then vacuum filtered to produce 14 liters of MeOH extract filtrate (237 g total solids).

The extract filtrate was diluted with 4.2 liters of water and partitioned with 14 liters CH_2Cl_2. The 12-liter lower layer was removed, dried over Na_2SO_4, filtered and concentrated under vacuum to a volume of 1.44 liters (4.7 g total solids). This concentrate was stored at $-80°C$ to slow decomposition.

Five hundred ml of this CH_2Cl_2 concentrate was taken to dryness and reconstituted with 40 ml EtOAc-hexane (75:25). This preparation was chromatographed on 1-liter silica gel (E. Merck, 60~200 mesh) using a step gradient (EtOAc-hexane (75:25), EtOAc, EtOAc-MeOH (90:10), EtOAc-MeOH (80:20), MeOH). The major active component was found in the EtOAc-MeOH (90:10) eluate. This rich cut was again chromatographed on silica gel *via* a step gradient (500 ml, Kieselgel 60, 230~400 mesh, EtOAc, EtOAc-MeOH (95:5), EtOAc-MeOH (90:10)). The active component eluted with EtOAc-MeOH (95:5).

Final purification was accomplished *via* preparative HPLC on a DuPont Zorbax ODS 21.2 mm i.d. × 25 cm column. An isocratic solvent system (MeOH-0.01 M potassium phosphate buffer pH 7, 70:30) was utilized with a flow rate of 20 ml/minute and the column temperature was maintained at 40°C *via* jacketing and a constant temperature bath. Column effluent was monitored at 276 nm with a Gilson Model 116 UV detector equipped with a 0.05-mm pathlength cell. TLC (Rf 0.53, CH_2Cl_2-MeOH (95:5)) and HPLC analyses of the individual fractions were carried out as described in the "Antifungal, TLC and HPLC Assays" section. Fractions containing **1** were combined and extracted with an equal volume of CH_2Cl_2. The CH_2Cl_2 layer was concentrated to yield approximately 100 mg of pure **1** as a colorless oil.

Some decomposition of the pure material, however, did occur with time and 40 mg of this preparation had to be further purified *via* a step gradient on silica gel (EtOAc-hexane-NH_4OH (50:50:2), EtOAc-hexane-NH_4OH (75:25:2), EtOAc-NH_4OH (100:2)). The active compound eluted with EtOAc-NH_4OH (100:2).

HREI-MS of **1** yielded the empirical formula $C_{19}H_{31}NO_4$ (Calcd *m/z* 337.2253, Found *m/z* 337.2251); **1** forms a mono-TMS derivative upon silylation. ^1H NMR (400 MHz, CD_2Cl_2) δ_{ppm} (J_{HH} Hz) 0.90 3H t (7.3), 1.07 3H d (7.2), 1.41 2H tq (7.3 and 7.3), 1.74 3H d (1.2), 2.08 2H ddt (~1, 7.5 and 7.5), 2.25 1H m, 3.20 1H d (18.0), 3.30 3H s, 3.31 1H d (18.0), 3.37 1H dd (5.2 and 9.5), 3.51 1H d (9.6), 3.56 1H dd (2.4 and 11.8), 3.78 1H dd (1.8 and 11.7), 4.96 1H dd (9.6 and 9.6), 5.73 1H dt (14.5 and 7.0), 5.94 1H dd (1.4 and 10.8), 6.10 1H ddt (10.3, 14.6 and 1.5), 6.17 1H dd (10.3 and 14.2), 6.28 1H, dd, (10.8 and 14.3). ^{13}C NMR (100 MHz, CD_2Cl_2) δ_{ppm} (multiplicity) 10.9 (q), 11.9 (q), 13.8 (q), 22.8 (t), 32.8 (d), 35.3 (t), 44.3 (t) 56.5 (q), 69.5 (d), 71.1 (t), 81.9 (d), 85.7 (d), 126.2 (d), 129.6 (d), 131.0 (d), 133.7 (s), 134.3 (d), 136.1 (d), 173.9 (s). The UV spectrum (MeOH) of **1** showed maxima at 286 (ε 28,682), 275 (ε 36,453) and 265 nm (ε 28,007), while the IR spectrum indicated a strong absorbance at 1747 cm^{-1}.

Isolation of Restricticin (**1**) from Submerged Fermentation

Three 1,400-liter scale fermentations yielded 2,600 liters of whole broth to which 1,300 liters of EtOAc was added. A 2-M potassium mono-hydrogen phosphate solution was then added until the pH of the aqueous phase was *ca.* 6.8 and the mixture was stirred for 1 hour. The EtOAc phase was then decanted *via* centrifugation and the spent broth reextracted with a second portion of EtOAc (1,060 liters). The two EtOAc extracts were combined and concentrated under reduced pressure to a volume of 480 liters. Hexane (475 liters) was added to the concentrated EtOAc extract, followed by 189 liters of dilute sulfuric acid in an aqueous MeOH solution (13 ml concentrated sulfuric acid per 100-liter methanol-water (1:1)). After 30 minutes stirring, the aqueous phase was collected. The acid extraction was repeated twice with two

additional portions of dilute sulfuric acid in MeOH solution (265 and 295 liters, respectively). All three aqueous extracts were combined to give 749 liters. The pH of the aqueous extract was adjusted to 6.8 by adding 18.9 liters of 1 M pH 7 potassium phosphate buffer. This aqueous extract was then back-extracted with three 235 liters portions of hexane. The combined hexane extracts were concentrated under reduced pressure to a volume of 66 liters, containing 15.5 g of **1**.

The concentrated hexane extracts were then extracted with 0.1 N hydrochloric acid in three portions (7.6 liters dilute HCl per 22.7-liter portion of concentrated hexane extract). The three acid extracts were combined and washed with 7.6 liters of hexane. The pH of the aqueous phase was then adjusted to 6.8 using 1 M pH 7 potassium phosphate buffer as described above. The neutralized aqueous phase was extracted with hexane (7.6 liters). MeOH (3.8 liters) was then added to the hexane extract. The MeOH layer, containing *ca.* 10 g of **1**, was collected and stored at −20°C.

^{1}H NMR of this preparation indicated **1** with no obvious impurities, while HPLC indicated a few low-level impurities.

Isolation and Physical Characterization of *N,N*-Dimethylrestricticin (2) from Solid Fermentation

A series of CH_2Cl_2 partitions were prepared from a variety of MeOH extracts of solid fermentations of *P. restrictum* prepared as previously described in the "Solid Fermentation" and "Isolation and Physical Characterization of Restricticin (1) from Solid Fermentation" sections. These partitions were combined and concentrated to yield 10.2 g of an oil.

This concentrate was taken up in 300 ml of EtOAc - MeOH (75 : 25) and chromatographed on a 6-liter silica gel column (E. Merck, 60~200 mesh) at 150 ml/minute using a step gradient of EtOAc - hexane (75 : 25), EtOAc, EtOAc - MeOH (98 : 2), EtOAc - MeOH (95 : 5), EtOAc - MeOH (90 : 10) and MeOH. The EtOAc - MeOH (90 : 10) eluants contained **1**, while the EtOAc, EtOAc - MeOH (98 : 2) and EtOAc - MeOH (95 : 5) eluants contained **2**. These latter eluants were combined and concentrated to yield 3.1 g of an oil.

This oil was taken up in 20 ml of EtOAc - hexane (1 : 1) to yield 15 ml of supernatant and 5 ml of precipitate. The supernatant was chromatographed on a 500-ml silica gel column using a step gradient of EtOAc - hexane (50 : 50), EtOAc, EtOAc - MeOH (90 : 10) and EtOAc - MeOH (75 : 25). The later fractions from the EtOAc elution were combined based on biological activity and TLC (Rf 0.79, CH_2Cl_2 - MeOH (95 : 5)) and concentrated to yield 24 mg of an oil. This concentrate was further purified *via* an additional step gradient on a 50-ml silica gel column. The active constituent again eluted with EtOAc and was concentrated to yield 1.5 mg of pure **2**.

HREI-MS of **2** yielded the empirical formula $C_{21}H_{35}NO_4$ (Calcd *m/z* 365.2566, Found *m/z* 365.2558); **2** did not silylate. Intense ions were seen at *m/z* 58 ($CH_2{=}N(CH_3)_2$) and *m/z* 262 ($C_{17}H_{26}O_2$; Calcd *m/z* 262.1933, Found *m/z* 262.1917). Comparison of the NMR data for **2** with **1** indicated an additional 6-proton singlet at δ 2.22 in the ^{1}H NMR spectrum and a methyl carbon at 60.6 ppm in the ^{13}C NMR spectrum. ^{1}H NMR (300 MHz, CD_2Cl_2) δ_{ppm} (J_{HH} Hz) 0.90 3H t (7.3), 1.07 3H d (7.1), 1.41 2H q (~7.3), 1.75 3H br s, 2.07 2H br dt (7.3 and 7.3), 2.22 6H s, 2.25 1H m, 2.97 1H d (16.4), 3.11 1H d (16.4), 3.31 3H s, 3.38 1H dd (5.1 and 9.6), 3.53 1H d (9.6), 3.57 1H dd (2.5 and ~12), 3.78 1H dd (2.0 and 11.7), 4.97 1H t (~9.5), 5.72 1H dt (14.5 and 7.0), 5.96 1H br d (11.1), 6.10 1H m, 6.18 1H m, 6.27 1H dd (10.6 and 14.1). ^{13}C NMR (CD_2Cl_2, 75 MHz) δ_{ppm} (mutiplicity) 10.9 (q), 11.9 (q), 13.8 (q), 22.8 (t), 32.8 (d), 35.2 (t), 45.0 (t), 56.5 (q), 60.6 (q), 60.6 (q), 69.1 (d), 71.1 (t), 81.9 (d), 85.7 (d), 126.3 (d), 129.8 (d), 131.0 (d), 133.6 (s), 134.3 (d), 135.9 (d), 169.9 (s). The IR spectrum of this compound contained an absorbance at 1750 cm^{-1} and the UV spectrum in MeOH contained maxima at 287 (ε 39,890), 275 (ε 50,842), 266 (ε 38,938) and 205 nm (ε 62,124).

Hydrolysis of Restricticin (1) to Restrictinol (3)

Restrictinol (**3**) was produced *via* hydrolysis of **1** as described below. Fifty mg of **1** was dissolved in 5 ml MeOH to which 500 μl of a 1.0-M solution of NaOH was added at room temperature with stirring. After stirring for 2 hours, 80 ml of EtOAc and 40 ml of water were added to the reaction mixture. The resulting layers were separated and the EtOAc layer was washed with another 40 ml of water. The organic layer was dried over Na_2SO_4 and the solvents removed *in vacuo*. A slightly yellow oil (34 mg, 78% yield) was obtained, which exhibited a single spot by TLC (Rf 0.47, hexane - EtOAc (1 : 1)).

^{1}H NMR of **3** (300 MHz, CD_2Cl_2) δ_{ppm} (J_{HH} Hz) 0.90 3H t (7.3), 1.01 3H d (7.1), 1.42 2H tq (7.3 and 7.3), 1.79 3H d (1.4), 2.08 2H ddt (1.4, 7.2 and 7.2), 2.19 1H m, 3.22 1H dd (5.3 and 9.0), 3.37 3H s, 3.43 1H d (9.3), 3.56 1H dd (9.2 and 9.2), 3.58 1H dd (2.6 and 11.7), 3.77 1H dd (1.6 and 11.7), 5.74 1H dt (14.6 and 7.2), 6.09 1H dd (1.3 and ~10.6), 6.12 1H ddt (10.4, 14.8 and 1.4), 6.22 1H dd (10.5 and 14.5), 6.36 1H dd (11.0 and 14.5). ^{13}C NMR (CD_2Cl_2, 75 MHz) δ_{ppm} (multiplicity) 11.0 (q), 12.4 (q), 13.8 (q), 22.8 (t), 32.2 (d), 35.3 (t), 56.2 (q), 68.1 (d), 71.3 (t), 84.7 (d), 87.0 (d), 126.3 (d), 129.5 (d), 131.0 (d), 134.3 (d), 134.6 (s), 135.9 (d). MS *m/z* 280 $(M)^+$. The UV spectrum of this compound exhibited maxima at 284 (ε 35,854), 275 (ε 45,658), 265 (ε 32,294) and 201 nm (ε 7,983). The IR spectrum showed an absorbance at 3420 cm^{-1} for the alcohol that was generated and an absence of carbonyl absorbances.

Restrictinol was often isolated from these fermentations as a decomposition product of restricticin and, in fact, in many cases whole batches decomposed to restrictinol with little or no restricticin remaining.

Antifungal Activity: Agar Dilution Assay

Ketoconazole and **1** were solubilized in 10% aqueous DMSO and diluted 2-fold in sterile distilled water. The two agars used for this study were yeast nitrogen base glucose agar and Kimmig agar. Each diluted drug was added to cooled, molten agar (1.0 ml of drug plus 9.0 ml of agar). Appropriate solvent and media controls (drug free) also were prepared. Prepared plates were stored in the dark at room temperature 18 hours prior to use. Drug concentrations tested ranged from 128~0.06 μg/ml.

All fungi used in the assay were from the Merck Culture Collection. The yeast cultures, maintained in yeast-maltose (YM) broth, were transferred to fresh YM medium and incubated for 18 hours at 35°C with shaking (250 rpm). After incubation, each culture was diluted in sterile saline to yield final concentrations of 3×10^5 to 3×10^6 cfu/ml. The filamentous fungi (*Aspergillus* and *Penicillium*) were maintained on potato-glucose agar slants and spore suspensions perpared by washing spores from the agar surface with sterile distilled water. The spore preparations were used as the inocula for the filamentous fungi tested.

Each prepared plate was inoculated with the test organisms (18 yeasts and three filamentous fungi) using a Denley Multipoint Inoculator (Denley, Sussex, England), which delivers approximately 0.001 ml to the agar surface resulting in an inoculum of from 3×10^2 to 3×10^3 cfu. The plates were incubated at 30°C for 48 hours. The MICs were recorded as the lowest concentration of drug showing no growth or less than 3 cfu/spot.

Results and Discussion

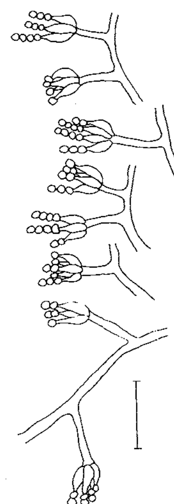
Description of Producing Strain

The diagnostic microscopic features of the producing organism, *P. restrictum*, are illustrated in Fig. 2. Colonies grown according to the method of PITT[8], exhibited the characteristics described in the following paragraphs.

Colonies (one week old) on CZAPEK-yeast extract agar, effuse, 20 mm i.d., with predominately floccose aerial mycelium, up to 1 mm deep, plane to sulcate in side view, with some medium buckling in reverse, surface white to pale grayish buff, Cream Color, Light Buff, Warm Buff, buff to pale cream at the margin, with reverse tan, to pinkish or grayish tan, Warm Buff, Apricot Buff, Cinnamon-Buff, Cinnamon, without soluble pigments, exudates, or odors.

Fig. 2. Conidiophores and conidia of *Penicillium restrictum* (ATCC 20297, MF 5261).

Standard bar = 10 μm.



Conidiophores micronematous to semi-macronematous, monoverticilliate, without rami or metulae, unbranched, aseptate, straight to slightly curved, $15\sim28\times2\sim4\,\mu m$, with non-inflated to slightly inflated apices, arising directly from aerial mycelium. Phialides arising directly from conidiophore apices, ampulliform to cylindrical, tapered to a truncate apex, $5.5\sim8\times2.5\sim3\,\mu m$, usually $3\sim5$/conidiophore, but ranging $1\sim8$/conidiophore.

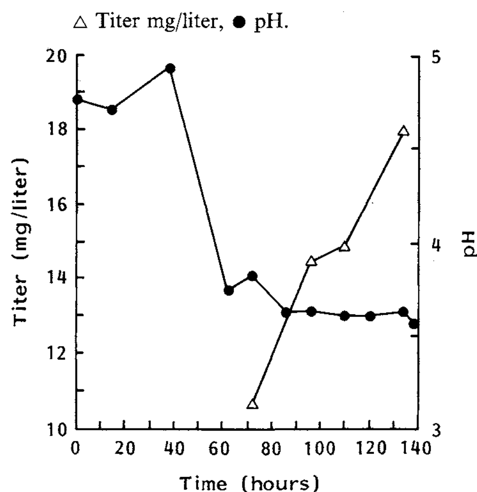
Conidia globose to subglobose, $3\sim3.5\,\mu m$ i.d., hyaline, smooth to faintly punctate, occasionally with faint traces of disjunctors, adhering to conidiophores in dry chains.

Fermentation

Original fermentations of culture MF 5261 (ATCC 20927) were performed on solid substrate medium contained in 250-ml Erlenmeyer flasks. Assay results on extracts of these flasks indicated the presence of a novel antifungal agent. Larger fermentations were required to obtain sufficient material for isolation and identification of the active constituent. Initial isolation of the active compound **1** was achieved from solid fermentations in trays. Sufficient material was isolated from solid fermentation for structural elucidation of **1**, however, these fermentations proved to be both cumbersome and inappropriate for large scale isolation. Larger scale fermentations required the development of a liquid fermentation process. Several different media formulations as well as fermentation conditions were tested with *P. restrictum* (ATCC 20927, MF 5261). One formulation in particular (described in the Materials and Methods section) supported production of approximately 5 mg/liter **1** after $4\sim5$ days incubation. This titer and short fermentation time provided sufficient material for additional chemical and biological studies.

Fig. 3. Production of restricticin (**1**)[a].

△ Titer mg/liter, ● pH.



[a] Titer and pH values represent the average of three fermentations.

Analysis

The original HPLC system I (Table 1), consisting of CH_3CN - 0.01 M potassium phosphate pH 7 (70 : 30) on a RP-C18 column, did not

Table 1. Rt's of restricticin (**1**) and restrictinol (**3**) in a variety of HPLC systems.

HPLC system	Rt (minutes)	
	Restricticin (**1**)	Restrictinol (**3**)
I. DuPont Zorbax ODS (70 : 30) CH_3CN - potassium phosphate buffer pH 7.0	7.8	7.4
II. DuPont Zorbax RX (70 : 30) CH_3CN - potassium phosphate buffer pH 7.0	8.2	6.1
III. DuPont Zorbax ODS CH_3CN - aq H_3PO_4 pH 2.5 (60 : 40)	>30	13.8
IV. Whatman ODS3 CH_3CN - aq H_3PO_4 pH 2.5 (60 : 40)	16.0 (Tails)	7.2
V. DuPont Zorbax RX CH_3CN - aq H_3PO_4 pH 2.5 (60 : 40)	2.8	9.1

sufficiently separate **1** from its desglycyl decomposition product, **3**, for quantitation of the individual components. Adjusting the pH of the aqueous portion of the mobile phase to 2.5 using H_3PO_4 in system III, in order to protonate the amine of **1**, differentiated it from **3** (Rt 13.8 minutes), however, **1** did not elute after 30 minutes from the Zorbax ODS column. In its protonated form, **1** should have been significantly more polar than **3** and had a shorter Rt in the absence of interactions with the silica backbone. On a Whatman ODS3 column, using an acidic mobile phase (system IV), **1** also had a longer Rt than **3**, and a very poor peak shape, again suggesting interaction with exposed silica sites. On the other hand, **1** behaved radically different on a Zorbax RX column, under acidic conditions (system V), with a significantly shorter Rt than **3**, indicating little interaction with the silica sites. The most useful HPLC assay, from a practical point of view, was the DuPont Zorbax RX column CH_3CN - 0.01 M potassium phosphate pH 7, 70 : 30 (system II), since both compounds of interest had reasonable Rt's but were well resolved.

Stability of Restricticin

The lack of stability of **1** severely complicated the isolation, storage and chemical manipulation of this molecule. Two modes of instability were noted, *i.e.*, the lability of the glycine ester towards base hydrolysis and the tendency of the triene functionality to decompose. The hydrolytic instability could be minimized by maintaining solutions of **1** at either neutral or acidic pH, while the decomposition of the side chain could be minimized by storing **1** in polar solvents like methanol and avoiding long-term storage in non-polar solvents like methylene chloride. It seems likely, in retrospect, that **3** is not a natural product, but results from the decomposition of **1**.

Isolation

The original isolation from solid fermentation was essentially a race against the instability of **1**. Several previous isolation attempts resulted in the loss of biological activity and the isolation of **3**. Extraction of the solid fermentation, followed by partition into methylene chloride, repeated silica gel chromatographies and a final preparative reverse phase chromatography yielded sufficient material for structure determination, some chemical modification and biological evaluation.

A more rational isolation scheme was developed for the large scale liquid fermentation based on knowledge of the structure and instability of **1**. The basic isolation strategy was to make use of the amine functionality to extract and purify **1** from the fermentation broth, while avoiding conditions which favored its decomposition. An acid/base extraction scheme appeared to be the easiest to implement on a pilot plant scale.

The whole broth (2,600 liters) was adjusted to pH 7 and extracted with ethyl acetate. A four solvent system was developed for optimal partitioning of **1** into the aqueous phase from the organic phase. The concentrated extract was thus diluted with hexane, and extracted with dilute sulfuric acid in aqueous methanol. The aqueous extract was then adjusted to pH 7 using potassium phosphate buffer, to prevent hydrolysis of the base labile glycyl ester, and extracted with hexane. After concentration of the hexane extract, the acid extraction - hexane back-extraction process was repeated. After a final partition into methanol, *ca.* 10 g purified **1** was obtained. No chromatography steps were necessary.

Antifungal Activity

The antifungal activities of **1** and ketoconazole in both yeast nitrogen base agar and Kimmig agar are shown in Table 2.

Table 2. Antifungal activity (MICs[a] in μg/ml) of restricticin (1), and ketoconazole determined *in vitro* using an agar dilution assay.

Culture name	Culture No.	Restricticin		Ketoconazole	
		YNBA	KA	YNBA	KA
Cryptococcus neoformans	MY1051	16.0	2.0	2.0	0.25
C. neoformans	MY1146	32.0	4.0	4.0	1.0
Candida albicans	MY1058	>128.0	0.5	128.0	≤0.06
C. albicans	MY1055	>128.0	>128.0	128.0	32.0
C. albicans	MY0992	64.0	4.0	8.0	2.0
C. albicans	MY1585	>128.0	>128.0	128.0	32.0
C. albicans	MY1029	>128.0	>128.0	128.0	32.0
C. parapsilosis	MY1009	32.0	2.0	1.0	≤0.06
C. parapsilosis	MY1010	64.0	2.0	0.5	≤0.06
C. tropicalis	MY1011	>128.0	128.0	64.0	32.0
C. tropicalis	MY1012	>128.0	128.0	64.0	64.0
C. pseudotropicalis	MY1040	32.0	32.0	64.0	16.0
C. krusei	MY1020	64.0	8.0	4.0	1.0
C. rugosa	MY1022	32.0	0.5	2.0	≤0.06
C. guilliermondii	MY1019	128.0	16.0	4.0	0.25
C. stellatoidea	MY1017	>128.0	>128.0	64.0	32.0
Torulopsis glabrata	MY1059	128.0	32.0	32.0	4.0
Saccharomyces cerevisiae	MY1027	>128.0	128.0	64.0	32.0
Aspergillus fumigatus	MF4839	>128.0	>128.0	32.0	4.0
A. flavus	MF0383	>128.0	>128.0	32.0	4.0
Penicillium italicum	MF2819	32.0	2.0	4.0	0.5

Comparison of activity on yeast nitrogen base glucose agar (YNBA) to Kimmig agar (KA).
[a] MIC, recorded as the lowest concentration of drug showing no growth or less than 3 cfu/spot.

The antifungal spectrum of **1** was similar to ketoconazole, as was the significant increase in potency observed in the complex Kimmig agar vs. the defined yeast nitrogen base glucose agar. The results of this evaluation indicated that the antifiungal spectrum of **1** is broad with activity against both yeast and filamentous fungi.

N,N-Dimethylrestricticin (**2**) had severely reduced antifungal activity compared to **1**, while restrictinol (**3**) had no antifungal activity, suggesting that the presence of the glycine ester was critical for the observation of antifungal activity.

References

1) KAHAN, J. S.; F. M. KAHAN, R. GOEGELMAN, S. A. CURRIE, M. JACKSON, E. O. STAPLEY, T. W. MILLER, A. K. MILLER, D. HENDLIN, S. MOCHALES, S. HERNANDEZ, H. B. WOODRUFF & J. BIRNBAUM: Thienamycin, a new β-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J. Antibiotics 32: 1~12, 1979
2) WRIGHT, J.; M. S. PUAR, B. PRAMANIK & A. FISHMAN: EV-22, a novel antifungal triacetylenic dioxolone. J. Chem. Soc. Chem. Commun. 1988: 413~414, 1988
3) PATEL, M.; M. CONOVER, A. HORAN, D. LOEBENBERG, J. MARQUEZ, R. MIERZWA, M. S. PUAR, R. YARBOROUGH & J. A. WAITZ: Sch 31828, a novel antibiotic from a *Microbispora* sp.: Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 41: 794~797, 1988
4) PATEL, M.; M. CONOVER, A. HORAN, D. LOEBENBERG, R. MIERZWA, J. A. WAITZ, R. YARBOROUGH & J. MARQUEZ: Novel antifungal antibiotics, Sch 31828, produced by *Microbispora* sp. SCC 1438: Taxonomy, fermentation and isolation. Program and Abstracts of the 27th Intersci. Conf. on Antimicrob. Agents Chemother, No. 983, p. 268, New York, Oct. 4~7, 1987
5) KEMPF, A. J.; O. HENSENS, R. SCHWARTZ, R. SYKES, C. WICHMANN, K. WILSON, D. ZINK & L. ZITANO: L-660,631 — a new antifungal agent. Abstracts of the New York Academy of Science 1st International Conference on Drug

Research in Immunological and Infectious Diseases; Antifungal Drugs: Synthesis, Preclinical and Clinical Evaluation. p. 7, New York, Oct. 8, 1987

6) HENSENS, O. D.; J. M. LIESCH, J. A. MILLIGAN, S. M. DEL VAL, R. E. SCHWARTZ & C. WICHMANN (Merck): Antifungal agent. U.S. 4,952,604, Aug. 28, 1990

7) HENSENS, O. D.; C. F. WICHMANN, J. M. LIESCH, F. L. VANMIDDLESWORTH, R. E. SCHWARTZ & K. E. WILSON: Structure determination of restricticin, a new antifungal agent. Tetrahedron in press

8) PITT, J. I. (*Ed.*): The Genus *Penicillium* and Its Teleomorphic States, *Eupenicillium* and *Talaromyces*. Academic Press, 1979

9) RAMIREZ, C. (*Ed.*): Manual and Atlas of the Penicillia. Elservier Biomedical Press, 1982

10) DOMSCH, K. H.; W. GAMS & T. H. ANDERSEN: Compendium of Soil Fungi. Vol. 1, *Ed.*, K. H. DOMSCH *et al.*, pp. 591 ~ 593, Academic Press, 1980

11) RIDGWAY, R.: Color Standards and Nomenclature. Published by the author, Washington, D. C., 1912