Ribosomal resistance to the 12,13-epoxytrichothecene antibiotics in the producing organism *Myrothecium verrucaria*

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An extract of *Myrothecium verrucaria*, a fungus which produces a range of 12,13-epoxytrichothecene toxins, was found to be resistant to T-2 toxin, one of its products. The epoxytrichothecenes are inhibitors of eukaryotic protein synthesis and normally bind to the 60S ribosomal subunit so as to inhibit peptidyltransferase activity. Ribosomes from *M. verrucaria* contain 60S subunits which are not subject to inhibition by T-2 toxin and are also resistant to certain other drugs such as anisomycin and homoharringtonine, but not sparsomycin or cycloheximide.

The 12,13-epoxytrichothecenes, a group of closely related sesquiterpenoids, are toxic metabolites of various imperfect fungi of the genera *Fusarium*, *Myrothecium*, *Trichoderma*, *Stachybotrys*, *Cephalosporium* and *Verticimonosporium*. These toxins are believed to have been the causative agents of several mycotoxicoses in both humans and animals, including akakabi (red-mould) toxicosis in Japan (Ueno et al., 1971), mouldy-corn toxicosis in the U.S.A. (Hsu et al., 1972), alimentary toxic aleukia in the U.S.S.R. (Bilai, 1970) and stachybotryotoxicosis in Northern Europe (Roddicks & Eppley, 1973).

The trichothecenes display anti-fungal and phytotoxic activity when tested *in vivo* (Nespiak et al., 1961; Brian et al., 1961; Härri et al., 1962), an effect that is due to inhibition of protein synthesis (Ueno et al., 1968; Carrasco et al., 1973; Cundliffe et al., 1974; Schindler, 1974). Several of the 12,13-epoxytrichothecenes compete with one of their number, [14C]trichodermin, for binding to yeast ribosomes (Barbadic & Vazquez, 1974a; Cannon et al., 1976), and appear therefore to have common or overlapping binding sites on the 60S subunit of the eukaryotic ribosome.

The organism used in these experiments, the fungus *Myrothecium verrucaria*, is widespread in Nature, and various toxicoses have been ascribed to the intake of food contaminated by it (Mortimer et al., 1971). Such toxicoses are probably caused by the wide range of 12,13-epoxytrichothecenes (of the verrucarin and roridin sub-groups), which have been shown to be produced by strains of *M. verrucaria* (Härri et al., 1962; Bohner et al., 1965; Traxler et al., 1970).

Here we describe experiments with *M. verrucaria*, which was found to be insensitive to nine members of the 12,13-epoxytrichothecene group of toxins *in vivo*. Furthermore, the ribosomes of this fungus are resistant to the toxins *in vitro* by virtue of some property of their 60S ribosomal subunits. We also describe techniques for the preparation of extracts of yeast and fungi which are highly active in cell-free protein synthesis when programmed by poly(U). Routinely, extracts of yeast or fungi were capable of polymerizing between 10 and 20 molecules of phenylalanine per ribosome (Fig. 1).

**Experimental**

*Yeast and fungal strains*

*Saccharomyces cerevisiae* strain Y116 was obtained from Dr. M. Cannon, Department of Biochemistry, King's College, London, and *Myrothecium verrucaria* N.R.R.L. 3001 from the Northern Regional Research Center, Peoria, IL, U.S.A.

**Growth of cells**

(a) *Saccharomyces cerevisiae*. Yeast were grown at 30°C in PYG pH 5.9 medium, which contained (per litre) 5 g of peptone (Difco), 3 g of yeast extract (Difco), 5 g of glucose and 0.1 M potassium phosphate buffer, pH 5.9. Growth was halted in middle to late exponential growth phase, when the cell density was approx. 10⁷/ml (A₆₅₀ = 1.2) by removing the flasks to ice. Cells were then harvested by centrifugation in a 6 x 1-litre rotor at 2000 rev./min for 10 min in an M.S.E. Mistral 4L centrifuge.
Cell pellets were washed by resuspension in, and centrifugation through, ice-cold deionized water (as described above) and finally washed three times with ice-cold 'grinding buffer' (10 mM-Tris/acetate, pH 7.6 at 20°C, containing 90 mM-potassium acetate, 1 mM-magnesium acetate, 2 mM-calcium acetate and 3 mM-2-mercaptoethanol). If not required for immediate use, cells were stored frozen at −70°C.

(b) *Myrothecium verrucaria*. Lawns of *M. verrucaria* were grown at 30°C on plates containing 2 g of malt extract, 0.5 g of sucrose and 1.5 g of agar per 100 ml, from which spores were collected by scraping the surface of the plates under sterile water. These were used to inoculate 1 litre of PYG pH 5.9 medium in a 2-litre Erlenmeyer flask. After incubation for 24–30 h on an orbital shaker at 30°C, growth was stopped by removal of the flask to ice. The cell density at this stage was judged to be approximately half that seen when cells were allowed to grow to stationary phase. Because of the growth habit of this organism, it was not possible to measure the absorbance of cultures with any degree of accuracy.

Cells were harvested by filtration under suction through Whatman no. 1 paper on a Buchner funnel and were washed thoroughly with ice-cold water, followed by three changes of cold 'dialysis buffer' (10 mM-Tris/acetate, pH 7.6 at 20°C, containing 90 mM-potassium acetate, 1 mM-magnesium acetate and 3 mM-2-mercaptoethanol). Finally, the cells were gently scraped from the filter paper and stored at −70°C.

**Preparation of cell extracts**

(a) *Saccharomyces cerevisiae*. Cell pellets (either freshly prepared or previously stored frozen) were placed in a mortar that had been pre-cooled to −20°C. An equal weight of levigated alumina (Norton Abrasives, Welwyn Garden City, Herts., U.K.) was added and the mixture ground vigorously in the cold (4°C) until the pellet had thawed. More alumina (approximately half the original quantity) was then added and the grinding continued until the mixture was viscous. Further grinding beyond this point did not contribute significantly to the yield of broken cells, and often yielded less-active extracts.

The ground paste was washed from the mortar with 2 vol. of 'grinding buffer' and centrifuged at 30000 g for 20 min. The supernatant ('S30') was then made 10 mM in Tris/acetate, pH 7.6, by addition of the appropriate volume of 1 M-Tris/acetate acid, pH 7.6 at 20°C, and 1 mM in magnesium acetate. Finally, the supernatant was dialysed overnight at 4°C against 'dialysis buffer' and stored as 0.2 ml batches at −70°C.

(b) *Myrothecium verrucaria*. A pellet of *M. verrucaria* cells was disrupted by two passages through a French pressure cell at 0°C, first at 5000 lb/in² (35 MPa) and then at 10000 lb/in² (70 MPa). It was not necessary to add buffer to the cells at this stage. A 30000 g supernatant ('S30') was derived from the preparation of broken cells and then dialysed exactly as described above and was stored as batches at −70°C.

**Preparation of ribosomes and postribosomal supernatant**

The dialysed 30000 g supernatant (8 ml) prepared from either organism was layered on 2 ml of 30% (w/v) sucrose containing 'high-salt buffer' (10 mM-Tris/acetate, pH 7.6 at 20°C, 1 mM-ammonium acetate, 100 mM-magnesium acetate, 3 mM-2-mercaptoethanol) and centrifuged in a Beckman Ti 75 rotor for 4 h at 50000 rev./min and 2°C. The supernatant, designated 'S100', was dialysed against 'dialysis buffer' at 0°C and stored in batches at −70°C. The ribosome pellet was resuspended in 'high-salt buffer' and re-centrifuged as described above, the process being repeated three times. Finally, the ribosomes were dialysed into 'dialysis buffer' and stored in batches at −70°C.

Ribosomes that were required for the preparation of ribosomal subunits were obtained by layering 5 ml of supernatant S30 over 2 ml of 'dialysis buffer' containing 30% (w/v) sucrose and centrifuging as described above. Such 'sucrose-washed' ribosomes were resuspended in 'dialysis buffer'. Ribosome concentrations were estimated from their u.v. absorption, by assuming that 13A_{260} units represented 1 mg of ribosomes/ml (Sissons, 1974).

**Preparation of ribosomal subunits**

Sucrose-washed ribosomes were dialysed against 10 mM-Tris/HCl, pH 7.6 at 20°C, containing 500 mM-KCl, 12.5 mM-MgCl₂ and 3 mM-2-mercaptoethanol at 0°C for 4 h, and then 5 mg of ribosomes (in 0.5 ml of the buffer) was layered on 34 ml 10–30% (w/v) linear sucrose density gradients made up in the same buffer. Centrifugation was for 6 h at 26000 rev./min and 2°C in the Beckman SW27 rotor, and ribosomal subunits were located by pumping gradient material through an ISCO UA5 monitor and following the absorbance at 254 nm continuously. The two bands of u.v.-absorbing material that were detected in the gradients were assumed to be the 40S and 60S ribosomal subunits. Portions of the gradient containing 40S or 60S ribosomal subunits were collected, pooled and dialysed against 'dialysis buffer' at 0°C. The subunits were then centrifuged at 50000 rev./min for 6 h in a Beckman Ti 75 rotor and resuspended in a small quantity of the same buffer.

In estimating concentrations of 40S and 60S ribosomal subunits, 250 nm suspensions were
assumed to represent 3.7 and 9.3 $A_{260}$ units respectively (Loening, 1968).

**Incubation conditions for protein synthesis**

An incubation mixture for the poly(U)-directed synthesis of polyphenylalanine contained, in 100$\mu$l total volume: 20 mm-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH, pH 7.5 at 20°C, 50 mm-potassium acetate, 13.5 mm-magnesium acetate, 0.375 mm-GTP, 2.5 mm-ATP, 5 mm-phosphoenolpyruvate, 0.038 mm in each amino acid (19 in total, phenylalanine omitted), 0.5 $\mu$Ci of L-[U-14C]phenylalanine (530 mCi/mmol), 10 $\mu$g of yeast phenylalanine-specific tRNA, together with either supernatant S30 or ribosomes and supernatant S100. Amounts of ribosomes used are indicated in Figure legends; usually supernatant S30 or S100 accounted for 50% by volume of the incubation mixture. Incubation was at 25°C, and at intervals samples (5 or 10 $\mu$l) were taken into 10% (w/v) trichloroacetic acid. These were heated at 90°C for 30 min to hydrolyze aminoacyl-tRNA and then filtered on Whatman GFC glass-fibre discs. After washing three times with 5% (w/v) trichloroacetic acid, filters were dried and their radioactivity measured by liquid-scintillation spectrometry in a 0.4% (w/v) solution of 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole in toluene. The estimated counting efficiency for 14C under these conditions was 90%.

The concentrations of inhibitors of protein synthesis were as indicated in the Figure legends.

**Assay of peptidyltransferase activity**

This was carried out by the method of Monro & Marcker (1967), whereby acetyl-leucyl-oligonucleotide 'fragments', derived from acetyl-leucyl-tRNA by digestion with T1 ribonuclease, react with puromycin to produce acetyl-leucyl-puromycin in a process catalysed by the ribosome. Fragments acetyl[3H]-leucyl-ACCAC and acetyl[3H]leucyl-ACCAU were prepared as described by Monro (1971), by using L-[4,5-3H]leucine at 10.2 Ci/mmol.

The incubation mixtures contained, in a final volume of 150$\mu$l: 33 mm-Tris/HCl, pH 7.6 at 20°C, 270 mm-KCl, 13.5 mm-MgCl$_2$, 25 $\mu$g of puromycin, 20 pmol of ribosomes, 4000 c.p.m. of acetyl-leucyl-oligonucleotide fragment and 33% (v/v) methanol.

Progress of the peptidyltransferase (‘fragment’) reaction was followed by stopping the reaction at various intervals by addition of 0.1 ml of 0.3 M-sodium acetate buffer, pH 5.5, saturated with MgSO$_4$ and subsequent extraction with 1.5 ml of ethyl acetate. Samples (1 ml) of the ethyl acetate extract were taken into water-miscible scintillation fluid and their radioactivity was measured by liquid-scintillation spectrometry. The counting efficiency of 3H under these conditions was approx. 13%.

**Materials**

Commercial sources of materials were as follows: L-[4,5-3H]leucine and L-[U-14C] phenylalanine were from The Radiochemical Centre, Amersham, Bucks., U.K.; phosphoenolpyruvate, ATP (disodium salt), GTP (trilithium salt) and yeast tRNA (phenylalanine specific) were from Boehringer and Soehne, Mannheim, Germany; L-amino acids, Heps, poly(U), ribonuclease T1, and *Escherichia coli* tRNA were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The 12,13-epoxytrichotheccenes were a gift from Dr. F. Strong, Department of Biochemistry, University of Wisconsin, Madison, WI, U.S.A. Anisomycin was obtained from the Upjohn Co., Kalamazoo, MI, U.S.A., and sparsomycin was from the Drug Development and Chemotherapy Board, National Cancer Institute, Bethesda, MD, U.S.A. All other chemicals were of the highest grade available.

**Results and discussion**

The fungus *Myrothecium verrucaria* produces toxins of the 12,13-epoxytrichotheccene family (Härri et al., 1962; Bohner et al., 1965; Traxler et al., 1970), to which it is resistant *in vivo* (results not shown). To investigate the basis of this resistance, a cell-free preparation (S30) from *M. verrucaria* was used to translate poly(U) with the production of polyphenylalanine. This extract, which was highly active in protein synthesis, was virtually unaffected by addition of the 12,13-epoxytrichotheccene, T-2 toxin (Table 1). In contrast, protein synthesis in a similar extract of *S. cerevisiae* was potently inhibited by the toxin (Table 1).

These data suggested either that the ribosomes of *M. verrucaria* were resistant to T-2 toxin or that the cytosol contained an enzyme capable of inactivating the drug. This question was resolved when ribosomes were prepared from *M. verrucaria* and *S. cerevisiae*, combined with a ribosome-free supernatant (S100) derived in each case from *S. cerevisiae*, and their response to T-2 toxin was re-examined *in vitro*. Again, T-2 toxin failed to inhibit protein synthesis carried out on the ribosomes of *M. verrucaria*, but retained its inhibitory effect on the activity of yeast ribosomes functioning in a similar supernatant (Fig. 1).

Evidently the ribosomes of *M. verrucaria* were resistant to the action of T-2 toxin, and we therefore wished to determine which ribosomal subunit was responsible for such resistance. Accordingly, ribosomal subunits were prepared from *M. verrucaria* and *S. cerevisiae* and were recombined in both homologous and heterologous fashion so that their sensitivity to T-2 toxin could be examined in
Table 1. Inhibition of incubation mixtures containing either S. cerevisiae or M. verrucaria S-30 extracts
Mixtures for poly(U)-directed protein synthesis (see the Experimental section) containing either S. cerevisiae or M. verrucaria S-30 extract (final concn. 36 A$_{260}$ units) were incubated in the presence of various concentrations of antibiotics at 25°C. Samples (5 µl) were removed at 10, 20, 30, 40 and 60 min, precipitated with trichloroacetic acid and radioactivity in the precipitates was measured by liquid-scintillation counting. The percentage inhibition of protein synthesis given here was calculated from the initial reaction rates.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Final concn. (µg/ml)</th>
<th>Yeast (Y166)</th>
<th>M. verrucaria</th>
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<td>Alpha sarcin</td>
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<td>Sparosmycin</td>
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<td>95</td>
<td>95</td>
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<tr>
<td>T-2 toxin</td>
<td>10</td>
<td>73</td>
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Fig. 1. Inhibition of poly(U)-directed protein synthesis by T-2 toxin
Mixtures for poly(U)-directed protein synthesis (see the Experimental section) containing either S. cerevisiae (final concn. 19 A$_{260}$ units) or M. verrucaria (final concn. 22 A$_{260}$ units) ribosomes and S. cerevisiae S100 fraction were incubated at 25°C. T-2 toxin (10 µg/ml final concn.) was mixed with the ribosomes before the addition of S100 fraction. Samples (5 µl) were precipitated with trichloroacetic acid and radioactivity in the precipitates was measured by liquid-scintillation counting. O——O, S. cerevisiae ribosomes; O——O, S. cerevisiae ribosomes plus T-2 toxin; ———, M. verrucaria ribosomes; ———, M. verrucaria ribosomes plus T-2 toxin.

Cell-free protein synthesis. As shown in Fig. 2, hybrid ribosomes composed of 40S subunits from M. verrucaria and 60S subunits from S. cerevisiae were active in vitro, and, moreover, were sensitive to the action of T-2 toxin. This result suggested that 40S ribosomal subunits of M. verrucaria are not responsible for the resistance of this organism to T-2 toxin and, by elimination, implicated the 60S subunits in determining such resistance. Unfortunately, however, despite the fact that M. verrucaria 60S and S. cerevisiae 40S ribosomal subunits were each able to complement homologous partners in vitro so as to yield active ribosomes (Fig. 2), they did not yield active hybrid ribosomes when combined with each other. Thus we were not able to establish our hypothesis in the ideal manner, but were forced instead to use an alternative assay system.

The peptidyltransferase activity of eukaryotic ribosomes is inhibited potently by 12,13-epoxytrichothecenes such as T-2 toxin (Carrasco et al., 1973; Jimenez et al., 1975). A convenient assay of this activity is the so-called 'fragment' reaction in which N-acetylaminoacyl-oligonucleotide (derived from aminoacyl-tRNA by the action of T$_4$ ribonuclease) acts as aminoacyl-donor substrate, together with puromycin as acceptor, in a reaction dependent on ribosomes in the presence of organic solvent. As a result of peptidyltransferase activity, N-acetylaminoacyl-puromycin is formed, and it has been convincingly demonstrated, by using both prokaryotic (Monro & Marcker, 1967; Monro, 1969) and eukaryotic (Neth et al., 1970) ribosomes, that the ability to catalyse this reaction is exclusive to the larger (50S or 60S respectively) ribosomal subunit. We therefore examined the peptidyltransferase activity of ribosomes from both M. verrucaria and S. cerevisiae in the presence and
Antibiotic resistance in *Myrothecium verrucaria*

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verrucaria. M. peptidyltransferase the N-acetyl-leucyl-pentanucleotide property of the catalysed by whether cerevisiae but absence of M. verrucaria. The substrates for this reaction were N-acetyl-leucyl-pentanucleotide and puromycin (Neth et al., 1970).

As shown in Fig. 3, T-2 toxin inhibited potently the peptidyltransferase reaction on ribosomes of *S. cerevisiae* but was without effect on this reaction catalysed by ribosomes of the producing organism *M. verrucaria*.

We therefore concluded that ribosomes of *M. verrucaria* are resistant to T-2 toxin via some property of the 60S ribosomal subunit and wondered whether they were also cross-resistant to any other inhibitors of eukaryotic protein synthesis. As seen in absence of T-2 toxin. The substrates for this reaction were N-acetyl-leucyl-pentanucleotide and puromycin (Neth et al., 1970).

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Table 1, when employed in cell-free protein synthesis, extracts of *M. verrucaria* were resistant not only to T-2 toxin but also to the alkaloid homoharringtonine and to the antibiotic anisomycin. In contrast, extracts of *S. cerevisiae* were sensitive to each of these compounds. The specificity of the observed resistances became clear when extracts from both organisms were shown to be sensitive to inhibition by cycloheximide or sparsomycin. The observations with sparsomycin are especially significant, since this antibiotic, like T-2 toxin, anisomycin and homoharringtonine, is an inhibitor of the ribosomal peptidyltransferase centre (Vazquez, 1974). Evidently, strategic modification of the eukaryotic ribosome can (as in ribosomes from *M. verrucaria*) result in selective resistance to drugs known to inhibit peptide transfer. Our data therefore support the hypothesis (Barbacid & Vazquez, 1974a,b; Jimenez et al., 1975; Fresno et al., 1977) that the 12,13-epoxytrichothecenes share with
anisomycin and homoharringtonine, but not with sparsomycin, common or overlapping binding sites on the 60S subunit of the eukaryotic ribosome.

We have not been able to characterize further the manner in which ribosomes of \textit{M. verrucaria} are resistant to T-2 toxin, anisomycin and homoharringtonine. However, when techniques become available for reconstitution of fungal ribosomes \textit{in vitro}, it should be possible to ascertain whether resistance to T-2 toxin etc. is determined by one or more species of ribosomal protein or rRNA.

There is only one documented example of the resistance of an antibiotic-producing organism to its own product being exerted at the level of the ribosome. This involves the prokaryote \textit{Streptomyces azureus}, which produces thiostrepton and which achieves resistance to this drug via the pattern of post-transcriptional methylation of rRNA (Cundliffe, 1978; Cundliffe & Thompson, 1979). It remains to be seen whether antibiotic-producing eukaryotes such as \textit{M. verrucaria} (the first relevant example) also resort to similar stratagems of ribosomal modification.

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References


