Competition between Trichodermin and several other Sesquiterpene Antibiotics for Binding to their Receptor Site(s) on Eukaryotic Ribosomes

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(Received 12 March 1976)

1. Of the five sesquiterpene antibiotics tested and found to inhibit protein synthesis in yeast spheroplasts, trichothecin, trichodermin or trichodermin stabilized polyribosomes whereas, in contrast, verrucarin A or T-2 toxin induced 'run off' of polyribosomes with a corresponding increase in 80S monoribosomes. The effect of fusarenol X on the system could not be determined as the drug failed to enter the cells. 2. [*acetyl*-¹⁴C]Trichodermin bound to yeast polyribosomes with a dissociation constant of 2.10 μ M and to yeast 'run off' ribosomes with a dissociation constant of 0.72 μ M. 3. Trichothecin, trichodermin, fusarenol X, T-2 toxin and verrucarin A competed with [*acetyl*-¹⁴C]trichodermin for binding to its receptor site on 'run off' ribosomes. The observed competition was quantitatively similar for all the drugs tested. In contrast, the five drugs competed to different extents with trichodermin for binding to its receptor site on polyribosomes. Thus trichothecin competed with relative efficiency, whereas verrucarin A competed poorly, and the other drugs occupied intermediate positions between these two extremes. 4. Studies were also carried out with yeast 'run off' ribosomes prepared from both a wild-type strain and a strain resistant to trichodermin. Competition experiments between verrucarin A and [³H]anisomycin indicated that verrucarin A bound to 'run off' ribosomes from the mutant strain less efficiently than to those from the wild-type.

The sesquiterpene antibiotics trichothecin, trichodermin, trichodermin, fusarenol X, verrucarin A and T-2 toxin are effective inhibitors of eukaryotic protein synthesis assayed under a variety of experimental conditions (Ueno, 1970; Bamberg & Strong, 1971; Ohtsubo *et al.*, 1972; Cundliffe *et al.*, 1974). Although these compounds can inhibit the activity of the peptidyl transferase centre on eukaryotic ribosomes as assayed by the 'fragment reaction' (Carrasco *et al.*, 1973), the drugs behave very differently when added to systems such as yeast spheroplasts synthesizing protein or to HeLa cells growing in culture (Cundliffe *et al.*, 1974). In these systems trichodermin, for example, stabilizes polyribosome profiles, whereas, in contrast, verrucarin A induces extensive 'run off' of polyribosomes. It has also been claimed that fusarenol X induces complete or partial 'run off' of polyribosomes in HeLa cells (Ohtsubo *et al.*, 1972) or whole reticulocytes (Ueno *et al.*, 1973) respectively and the possibility is therefore raised that verrucarin A and fusarenol X can only inhibit protein synthesis on eukaryotic ribosomes at early stages of mRNA translation.

Several studies have been carried out involving quantitative binding of radioactively labelled antibiotics to ribosomes from *Escherichia coli* (Mao & Putterman, 1969; Fernandez-Muñoz & Vazquez, 1973; Contreras *et al.*, 1974) and similar methods have been applied successfully to eukaryotic ribosomes (Barbacid & Vazquez, 1974a, 1975). Such binding studies have provided information on the location of ribosomal target sites for several antibiotics and have given useful knowledge about ribosomal structure. Considerable attention has been focused on antibiotics acting on the ribosomal peptidyl transferase catalytic centre. Antibiotics in this group include gougerotin, which associates with the centre on both prokaryotic and eukaryotic ribosomes, and the two drugs anisomycin and trichodermin, which associate with the centre specifically on eukaryotic ribosomes.

In the present work we have attempted to ascertain why the various drugs in the sesquiterpene group can affect polyribosome profiles differently when added to systems synthesizing protein despite the fact that the compounds studied here have all been shown to

inhibit the peptidyl transferase catalytic centre of eukaryotic ribosomes under selected conditions *in vitro* (Carrasco *et al.*, 1973). We have bound [*acetyl*-¹⁴C]trichodermin to each of two types of ribosome preparations, yeast polyribosomes and 'run off' ribosomes obtained by treating yeast with NaN₃. We observed that trichodermin bound to 'run off' ribosomes with an affinity that was approximately threefold higher than that observed for binding of the drug to polyribosomes. We then studied the abilities of trichothecin, fusarenon X, verrucarín A, T-2 toxin and trichodermol to compete with [*acetyl*-¹⁴C]trichodermin for binding to various ribosomal preparations. All the drugs tested competed, to approximately equal extents, with trichodermin for binding to its receptor site on 'run off' ribosomes. In contrast, however, verrucarín A competed poorly with trichodermin for its binding site on either polyribosomes or polyribosomes previously degraded to monoribosomes with ribonuclease. The other four sesquiterpene antibiotics tested competed to different extents with trichodermin for binding to its receptor site on polyribosomes. Thus T-2 toxin competed only slightly more efficiently than did verrucarín A, whereas trichothecin was very efficient in this respect. The other drugs occupied intermediate positions between trichothecin and T-2 toxin.

Experimental

Growth of yeast and preparation of yeast spheroplasts

Cells of *Saccharomyces cerevisiae* strain Y166 (wild-type) and strain TR₁ (trichodermin-resistant mutant derived from strain Y166) were grown and converted into spheroplasts (Cannon *et al.*, 1973). Analysis of polyribosomes from spheroplasts before and after exposure to the various sesquiterpene antibiotics tested was carried out exactly as described previously (Cannon *et al.*, 1973). The drugs were present for 2 min each at a final concentration of 0.1 mM before analysis of polyribosomes.

Preparation of yeast polyribosomes and 'run off' ribosomes for binding studies

Polyribosomes from *S. cerevisiae* were obtained from spheroplasts inhibited by cycloheximide (10 µg/ml) exactly as described by Jimenez *et al.* (1975). 'Run off' ribosomes were prepared from protein-synthesizing spheroplasts that had been treated with 1 mM-NaN₃ for 5 min (Barbacid & Vazquez, 1975). For estimations of concentration it was assumed that a monoribosome unit had a mol.wt. of 4×10^6 , and a solution of 1 mg of either polyribosomes or monoribosomes/ml had an E_{260} of 12 or 14 respectively.

Preparation of ribonuclease-treated polyribosomes

Polyribosomes prepared as above were incubated at 0°C for 10 min with pancreatic ribonuclease [final concentration 10 µg/ml; Sigma (London) Chemicals Ltd., London S.W.6, U.K.]. This treatment was just sufficient to degrade polyribosome preparations entirely to monoribosomes.

Preparation of high-salt-washed ribosomes

Ribosomes from *S. cerevisiae* were prepared and then washed extensively in high-salt solution as described by Battaner & Vazquez (1971).

Binding of radioactively labelled antibiotics to ribosomes

Binding of [*acetyl*-¹⁴C]trichodermin or [³H]-anisomycin to ribosomes was carried out by following the method of sedimentation in the ultracentrifuge essentially as described by Jimenez & Vazquez (1975). Reactions (100 or 80 µl final vol.) were carried out at 0°C in a buffer containing (at final concn.) 50 mM-Tris/HCl, pH 7.4 at 20°C, 12.5 mM-MgCl₂ and 80 mM-KCl. Polyribosomes or 'run off' ribosomes along with the relevant radioactively labelled antibiotic were present at the concentrations indicated in the legends to the corresponding Table or Figure. The data for binding of [*acetyl*-¹⁴C]trichodermin were plotted as described by Scatchard (1949), and the dissociation constant K_d is presented as a measure of the affinity of [*acetyl*-¹⁴C]trichodermin for ribosomes.

Studies on the competition between trichodermin and other sesquiterpene antibiotics for binding to yeast ribosomes

Polyribosomes, 'run off' ribosomes and, where indicated, ribonuclease-treated polyribosomes were each present at a concentration of 3 µM. [*acetyl*-¹⁴C]Trichodermin was present at a concentration of 4.5 µM. The other relevant sesquiterpene antibiotics were added at concentrations between 0 and 1 mM. Amounts of [*acetyl*-¹⁴C]trichodermin bound to the various ribosome preparations in the presence and absence of the other relevant drugs were determined as described above. In all cases [*acetyl*-¹⁴C]trichodermin was added to ribosomes 1 min before the addition of the other sesquiterpene antibiotic. All reaction mixtures were held at 0°C.

Counting of radioactivity

Samples (20 or 30 µl) were processed exactly as described by Fernandez-Muñoz & Vazquez (1973) and counted for radioactivity in a Beckman liquid-

scintillation counter with an efficiency of 90 and 37% for ^{14}C and ^3H respectively.

Antibiotic samples

[acetyl- ^{14}C]Trichodermin and [^3H]anisomycin, prepared as described by Barbacid & Vazquez (1974a), had specific radioactivities of 15.4 and 236 mCi/mmol respectively. Verrucaric acid was kindly supplied by Dr. A. von Wartburg (Sandoz Institute, Basel, Switzerland). It was dissolved in dimethyl sulphoxide, and the final concentration of the dimethyl sulphoxide in binding experiments involving verrucaric acid was 2% (v/v). The origin of other antibiotics is given elsewhere (Carrasco *et al.*, 1973).

Results and Discussion

The various antibiotics tested were all potent inhibitors of protein synthesis as assayed *in vivo* or *in vitro* (results not shown), and we have studied the effect of these compounds on profiles of polyribosomes from spheroplasts of the yeast *S. cerevisiae*. Fig. 1(a) illustrates a control polyribosome profile obtained after lysis of yeast spheroplasts. The addition of trichothecin to spheroplasts in suspension and synthesizing protein stabilized this profile (Fig. 1b). In contrast, verrucaric acid induced extensive breakdown of polyribosomes with a corresponding accumulation of 80S monoribosomes (Fig. 1c). These results are essentially similar to those of Cundliffe *et al.* (1974). These authors showed that trichodermin stabilized polyribosome profiles in HeLa cells and

Fig. 1. Ribosome profiles from yeast spheroplasts and the effect of incubation with trichothecin, verrucaric acid and NaN_3 .

Conditions for preparation of yeast spheroplasts are described in the Experimental section. Samples (10 ml) were incubated at 30°C for 2 min in the absence or presence of 0.1 mM-trichothecin or 0.1 mM-verrucarin A. Protein synthesis was stopped by the addition of cycloheximide (100 µg/ml) to 'freeze' polyribosomes. Spheroplasts were then treated and analysed for their polyribosome content as described by Cannon *et al.* (1973). (a) Control lysate; (b) effect of trichothecin; (c) effect of verrucaric acid. For binding studies, polyribosomes and 'run off' ribosomes were prepared and analysed on sucrose gradients as described by Cannon *et al.* (1973). The sedimentation characteristics of the preparations are illustrated: (d) polyribosomes; (e) 'run off' ribosomes. In all cases the position of the 80S peak of ribosomes is marked with a vertical arrow and the left-hand side of each tracing represents the bottom of the gradient. The horizontal arrow under the abscissa represents the direction of sedimentation of polyribosomes.

yeast spheroplasts. We have confirmed this result for the latter system and have also shown that trichodermin behaves similarly (results not shown). In addition, T-2 toxin behaves like verrucarins A in causing 'run off' of polyribosomes, although this drug enters yeast spheroplasts rather slowly (M. Cannon, unpublished work). We were unable to check the effect of fusarenon X on the spheroplast system, since the drug fails to enter the cells (Jimenez *et al.*, 1975). However, it has been reported that fusarenon X induced 'run off' of polyribosomes in both HeLa cells and whole reticulocytes (Ohtsubo *et al.*, 1972; Ueno *et al.*, 1973), although in the latter system the 'run off' effect was incomplete. From our observations and those of the other authors cited above we may deduce that trichothecin, trichodermin and trichodermin inhibit protein synthesis at some stage during elongation, whereas verrucarins A, T-2 toxin and possibly fusarenon X inhibit at or shortly after the initiation stage of protein synthesis. It should be emphasized, however, that from the above results it is impossible to decide whether verrucarins A, T-2 toxin and fusarenon X inhibit some reaction specific to the initiation process of protein synthesis or alternatively are somehow excluded from functional interaction with polyribosomes. Accordingly, we decided to approach the problem by studying the effect of the various sesquiterpene antibiotics on the binding of [*acetyl*- ^{14}C]trichodermin to its ribosomal receptor site.

We first studied the binding of [*acetyl*- ^{14}C]trichodermin to both polyribosomes and 'run off' ribosomes. The absorbance profiles of the two preparations are illustrated in Figs. 1(d) and 1(e) respectively. The polyribosome preparation contained very few monoribosomes (approx. 8% contamination), but, in contrast, the preparation of 'run off' ribosomes, obtained by treating protein-synthesizing yeast spheroplasts with NaN_3 , contained 80S ribosomes only. Samples from each ribosome preparation were mixed with [*acetyl*- ^{14}C]trichodermin at 0°C and ribosomes were sedimented. Ribosome amounts were constant for each tube and the concentration of trichodermin was varied as indicated in Fig. 2, which illustrates a Scatchard (1949) plot of the data. Both polyribosomes and 'run off' ribosomes display a single type of trichodermin binding, and one molecule of antibiotic is bound per ribosome. However, the values of the dissociation constants differ very markedly for the two ribosome preparations. Thus for 'run off' ribosomes $K_d = 0.72 \times 10^{-6}\text{ M}$, indicating that the affinity of trichodermin for these ribosomes is approximately threefold higher than that for polyribosomes, where the dissociation constant $K_d = 2.10 \times 10^{-6}\text{ M}$.

Having established the kinetics for interaction of trichodermin with the two ribosome types, we then studied the ability of the other sesquiterpene drugs

used in this study to compete with [*acetyl*- ^{14}C]trichodermin for binding to its ribosomal receptor site (Fig. 3). With the concentration of trichodermin fixed at $4.5\text{ }\mu\text{M}$, binding of this drug to polyribosomes was not affected by the presence of verrucarins A at the same concentration, and even at very high verrucarins A concentrations ($100\text{ }\mu\text{M}$) there was only

Fig. 2. Scatchard (1949) plots for binding of trichodermin to yeast polyribosomes and 'run off' ribosomes

Final incubation volumes for binding of [*acetyl*- ^{14}C]trichodermin were either 100 or $80\text{ }\mu\text{l}$, and $30\text{ }\mu\text{l}$ and $20\text{ }\mu\text{l}$ samples were taken from each incubation mixture respectively for determination of radioactivity. The polyribosome concentration was $3\text{ }\mu\text{M}$ for [*acetyl*- ^{14}C]trichodermin concentrations ranging from 0.6 to $6\text{ }\mu\text{M}$, and $4\text{ }\mu\text{M}$ for [*acetyl*- ^{14}C]trichodermin concentrations ranging from 6 to $15\text{ }\mu\text{M}$. Final incubation volume for binding of [*acetyl*- ^{14}C]trichodermin to 'run off' ribosomes was $100\text{ }\mu\text{l}$, and $30\text{ }\mu\text{l}$ samples were removed for determination of radioactivity. The concentration of 'run off' ribosomes was $3\text{ }\mu\text{M}$ for [*acetyl*- ^{14}C]trichodermin concentrations ranging from 0.2 to $6\text{ }\mu\text{M}$. Other conditions were as specified in the Experimental section. $\bar{v}$ is the number of antibiotic molecules bound per ribosome. ■, 'Run off' ribosomes; ●, polyribosomes.

Fig. 3. Effects of verrucarins A, T-2 toxin, fusarenon X and trichothecin on the binding of trichodermin to its ribosomal receptor site

Binding of [acetyl- ^{14}C]trichodermin to polyribosomes and 'run off' ribosomes (and where specified to ribonuclease-degraded polyribosomes) was carried out as described in the Experimental section in the presence or the absence of verrucarins A, T-2 toxin, fusarenon X or trichothecin at the concentrations indicated. Reaction mixtures containing verrucarins A or T-2 toxin had a final volume of 100 μl , and 30 μl samples were removed before and after centrifugation for determination of radioactivity. Those reaction mixtures containing fusarenon X and trichothecin had a final volume of 80 μl and the samples removed for determination of radioactivity had a volume of 20 μl . In all reaction mixtures containing verrucarins A or T-2 toxin dimethyl sulphoxide (2%, v/v) was present. Similarly, ethanol was present at final concentrations (v/v) of 2% or 5% in all the reaction mixtures containing fusarenon X or trichothecin respectively. ●, Binding to polyribosomes; ■, ribonuclease-treated polyribosomes; ○, 'run off' ribosomes. (a) Verrucarins A; (b) fusarenon X; (c) T-2 toxin; (d) trichothecin.

a 21% decrease in the binding of [acetyl- ^{14}C]trichodermin (Fig. 3a). The same graph indicates that, in complete contrast with this result, verrucarins A at a concentration of 4.5 μM inhibited binding of [acetyl- ^{14}C]trichodermin to 'run off' ribosomes by 55% and at a concentration of 100 μM by 84%. We also carried out identical competition experiments using a preparation of polyribosomes that had been completely degraded to monoribosomes by incubation with pancreatic ribonuclease (absorbance profile not shown) and again the results are illustrated in Fig. 3(a). The data were essentially identical with those obtained when [acetyl- ^{14}C]trichodermin was bound to polyribosomes, and again verrucarins A failed to inhibit significantly the binding of the drug, except when verrucarins A was present at very high concentrations. This last result indicates that the physical presence of the polyribosome aggregate is not in itself responsible for the lack of competition between verrucarins A and trichodermin, and suggests that the exclusion of verrucarins A is a property of each individual monoribosome engaged in protein synthesis.

We then repeated the same binding experiments with polyribosomes and 'run off' ribosomes using [acetyl- ^{14}C]trichodermin in the presence of either fusarenon X or T-2 toxin; our results with fusarenon X are shown in Fig. 3(b). Fusarenon X competed

with trichodermin for binding to 'run off' ribosomes in a quantitatively similar way to that observed for verrucarins A. However, in addition fusarenon X competed with trichodermin, more efficiently than had verrucarins A, for binding to polyribosomes. Thus fusarenon X at a concentration tenfold in excess of that of trichodermin competed with the latter by 40%. This value is to be compared with that for verrucarins A (10%) added at the same relative concentration (see Fig. 3a). Our results for the corresponding experiments using T-2 toxin are in Fig. 3(c). Binding experiments using 'run off' ribosomes gave results almost identical with those obtained for the other two drugs (fusarenon X and verrucarins A), and the data obtained by using polyribosomes were very similar to those obtained with verrucarins A, although T-2 toxin had a slightly higher affinity for such preparations.

With equimolar amounts of [acetyl- ^{14}C]trichodermin and trichodermin, the binding of the former is predictably decreased to approx. 50% relative to controls containing only [acetyl- ^{14}C]trichodermin, and this effect, as expected, is identical when either polyribosomes or 'run off' ribosomes are used (results not shown). From the above results it is clear that fusarenon X, verrucarins A and T-2 toxin can compete fully with trichodermin for its binding site only when 'run off' ribosomes are used. We turned

our attention therefore to the drug trichothecin, which, as we have shown, stabilized polyribosome profiles in a manner characteristic also of trichodermin. This similarity indicates that both drugs can gain access to their target sites on polyribosomes and we should predict that the drugs might compete for this same receptor site(s) on isolated polyribosomes. We therefore carried out competition experiments with both polyribosomes and 'run off' ribosomes (Fig. 3*d*). Clearly, trichothecin can compete with trichodermin for binding to both ribosome types. The extent of this competition varies between the two ribosome preparations, although this variation is considerably less than we have shown above for verrucaric acid, T-2 toxin or fusarenon X. Nevertheless, trichothecin binds to polyribosomes with a twofold lower affinity (50% inhibition of trichodermin binding at a trichothecin concentration of $13\ \mu\text{M}$) than to 'run off' ribosomes (50% inhibition of trichodermin binding at a trichothecin concentration of $6\ \mu\text{M}$). We have also carried out preliminary experiments involving competition between trichodermin and trichodermol. As with the other sesquiterpene drugs studied here in more detail, trichodermol competes efficiently with trichodermin for binding to 'run off' ribosomes. With polyribosomes the effect appears to be intermediate between those observed for the two drugs trichothecin and fusarenon X (results not shown).

The sesquiterpene antibiotics studied here appear to inhibit protein synthesis in eukaryotes specifically and efficiently. These compounds are of particular interest since their chemical structures are known and their modes of action as inhibitors of protein synthesis apparently vary subtly for compounds within the group. The work of Cundliffe *et al.* (1974) had suggested that the compounds could be divided into two general classes. Thus E-type inhibitors, such as trichodermin, were inhibitors of elongation, whereas I-type inhibitors, such as verrucaric acid, were thought of as highly selective inhibitors of the initiation of protein synthesis. By the criteria of Cundliffe *et al.* (1974), trichothecin and trichodermol would qualify as E-type inhibitors, whereas T-2 toxin and fusarenon X would qualify as I-type inhibitors. A somewhat different interpretation for the mode of action of the I-type inhibitor T-2 toxin has been invoked (Smith *et al.*, 1975; Cannon *et al.*, 1976), and this interpretation is supported by the work reported here. Although Smith *et al.* (1975) reported that T-2 toxin was capable of inhibiting polypeptide-chain initiation by preventing the formation of the first peptide bond in the protein-synthesizing cycle, this reaction is not uniquely susceptible. Thus Cannon *et al.* (1976) have claimed that T-2 toxin can inhibit early peptide-bond-forming steps during poly(U)-directed polyphenylalanine synthesis in reticulocyte lysates, but that nascent polyphenylalanine chains

above a certain critical chain length (and bound to ribosomes through tRNA) exclude the drug from functional interaction with its target site. Our present work with both T-2 toxin and verrucaric acid shows that these inhibitors also fail to associate with their ribosomal receptor site(s), presumably the peptidyl transferase catalytic centre, under certain conditions. Thus both drugs bind to polyribosomes rather inefficiently, but nevertheless associate in specific fashion with 'run off' ribosomes. One obvious difference between these ribosome types is the absence of nascent peptides on the 'run off' ribosomes. From these considerations it is clear that division of the sesquiterpene drugs into I types and E types is arbitrary, since both T-2 toxin and verrucaric acid can now be thought of as inhibitors of peptide-chain elongation (E-type inhibitors) under conditions where the chain length of the ribosome-bound nascent peptide still allows the drugs to react with their ribosomal target site(s).

We must now consider in more detail the binding of [*acetyl*- ^{14}C]trichodermin to polyribosomes and 'run off' ribosomes. Barbacid & Vazquez (1974*a*) have shown that trichodermin binds to the peptidyl transferase centre and that this binding is inhibited by anisomycin, a known inhibitor of peptidyl transferase reactions on eukaryotic ribosomes (Grollman, 1967). Similarly, trichodermin itself totally blocks binding of [^3H]anisomycin to either yeast or human tonsil ribosomes (Barbacid & Vazquez, 1974*a*). Barbacid *et al.* (1975) have shown that in polyribosome preparations derived from cycloheximide-treated yeast spheroplasts approx. 80% of the ribosomes have peptidyl-tRNA bound in the ribosomal A site, whereas the remainder have peptidyl-tRNA bound in the ribosomal P site. Such polyribosome populations show two types of affinity for binding of [^3H]anisomycin, indicating that yeast ribosomes have different structures depending on their functional state during the ribosome cycle (Barbacid & Vazquez, 1975). Polyribosomes have at least two different conformations associated with either a pre- or post-translocated state. Those with peptidyl-tRNA in the ribosomal P site (post-translocated polyribosomes) have a high affinity for anisomycin as compared with ribosomes in other states. In contrast with the results obtained with anisomycin, our data suggest that polyribosomes carrying peptidyl-tRNA bound either to the P or A sites of ribosomes have an equal affinity for trichodermin, since our Scatchard (1949) plot (Fig. 2) indicates that polyribosomes bind one molecule of trichodermin per molecule of ribosome with a single dissociation constant of $2.10 \times 10^{-6}\text{M}$. Thus ribosomes with peptidyl-tRNA in either the P or A sites presumably have a similar conformation at the trichodermin receptor site. However since 'run off' ribosomes have a greatly increased affinity for

trichodermin, relative to polyribosomes, they may have a particular conformation at the trichodermin receptor site that facilitates binding of the antibiotic. This conformation would be very different from the one associated with polyribosomes. However, although this hypothesis is extremely attractive, it may not be valid. Thus, as suggested above for T-2 toxin and verrucarín A, the physical presence of nascent polypeptide chains on polyribosomes may control the ability of trichodermin to interact with its receptor site. In this case, conformational changes may be less important for trichodermin binding than seems to be the case for anisomycin binding. In either instance, however, the difference in affinity shown by polyribosomes and 'run off' ribosomes for trichodermin binding has been suggested in other experiments. Thus Carter *et al.* (1976) have shown that, although trichodermin at high concentration completely stabilizes polyribosomes in reticulocyte lysates synthesizing protein, low concentrations of the drug allow an initial partial 'run off' of polyribosomes before stabilization of the profile occurs. This effect presumably results from the difference in affinity between polyribosomes and 'run off' ribosomes, since trichodermin would be expected to bind preferentially to the latter.

The various sesquiterpene antibiotics tested here all bind efficiently to their receptor site(s) on 'run off' ribosomes, but vary in their ability to interact with what we presume to be the same site(s) on polyribosomes. Further, we have implied that the presence of peptidyl-tRNA on polyribosomes might well influence the affinity of each particular drug for its receptor site. The ability of a given drug to associate with ribosomes will ultimately be determined by its chemical structure, and Cundliffe *et al.* (1974) suggested that with the sesquiterpenes the presence or absence of substituents on C-15 (a prominent position of the molecule as judged by space-filling models) could determine their mode(s) of action. Our results suggest that the chemical structure of a given sesquiterpene determines whether or not it is impeded by a ribosome-bound nascent peptide chain and this interpretation allows us to link together certain facts reported in the literature which have hitherto appeared to be contradictory. All the drugs studied here inhibit the reaction (the fragment reaction) between puromycin and CACCA-Leu-Ac as catalysed by the peptidyl transferase centre on yeast ribosomes (Carrasco *et al.*, 1973). Further, the drugs inhibit, albeit partially, the binding of CACCA-Leu-Ac to the donor site and of UACCA-Leu to the acceptor site of the yeast ribosomal peptidyl transferase catalytic centre, the latter reaction being inhibited preferentially. These observations support the idea that all the drugs have the peptidyl transferase centre as their target site, but it is noteworthy that both assays, the fragment

reaction and substrate binding, use ribosomes that are devoid of peptidyl-tRNA. Ohtsubo *et al.* (1972) found that fusarenon X induced polyribosome 'run off' in HeLa cells and subsequently Ueno *et al.* (1973) showed that the same drug (added at a concentration of 2 μ M) induced partial 'run off' of polyribosomes in whole reticulocytes. Thus fusarenon X would have been classified, along with verrucarín A and T-2 toxin, as an I-type inhibitor (Cundliffe *et al.*, 1974). However, although verrucarín A, T-2 toxin and fusarenon X all inhibit the fragment reaction (Carrasco *et al.*, 1973), fusarenon X alone of these three drugs is an effective inhibitor of both polypeptide synthesis and peptidyl-puromycin formation as catalysed by yeast polyribosomes, and this behaviour would not be expected from a drug that induces 'run off' of polyribosomes. This apparent paradox can now be explained by the results we have presented in this paper. The ability of a given sesquiterpene antibiotic to inhibit any function on polyribosomes depends critically on the affinity of that drug for its receptor site. Fusarenon X has a high enough affinity for polyribosomes to inhibit function(s) associated with these complexes, in contrast with both T-2 toxin and verrucarín A where affinity for polyribosomes is too low for any inhibition to be apparent. We emphasize, however, that the affinity of fusarenon X for polyribosomes is such that effective interaction is concentration-dependent and in the experiments of Ohtsubo *et al.* (1972) and Ueno *et al.* (1973) higher drug concentrations would perhaps have stabilized polyribosome profiles, although we stress that this is not the case for either verrucarín A or T-2 toxin (Cundliffe *et al.*, 1974). Similar considerations could also explain why trichodermin is more active than trichothecin in inhibiting peptidyl-puromycin formation as catalysed by yeast polyribosomes (Jimenez & Vazquez, 1975; Barbacid *et al.*, 1975) and why all the drugs studied here inhibit the fragment reaction where polyribosomes are not used (Carrasco *et al.*, 1973).

It has already been suggested by Jimenez & Vazquez (1975) that trichodermin, trichothecin and fusarenon X have a common binding site on 60S ribosomal subunits, which overlaps with or is closely linked to the binding site of anisomycin, and the results we have presented do not disagree with such an interpretation. Further, fusarenon X, trichodermin and trichothecin show a decrease in their binding efficiency to ribosomes from a yeast mutant resistant to trichodermin (Jimenez & Vazquez, 1975). Results with verrucarín A have, until now, been more difficult to interpret. Verrucarín A did not compete efficiently with either [*acetyl*- 14 C]trichodermin or [3 H]anisomycin for binding to yeast ribosomes that had been previously washed in high-salt solution (Barbacid & Vazquez, 1974a,b). Further, verrucarín A bound with equal affinity to high-salt-washed

Table 1. Competition between verrucarín A and [^3H]-anisomycin for binding to 'run off' ribosomes from a wild-type strain of yeast and a strain resistant to trichodermin

Binding of [^3H]anisomycin to 'run off' ribosomes was carried out as described in the Experimental section in the absence or presence of verrucarín A at the concentrations indicated. Verrucarín A was dissolved in dimethyl sulphoxide and this compound was present in all reaction mixtures (final vol. 80 μl) at a final concentration of 2% (v/v). For determination of radioactivity 20 μl samples were removed before and after centrifugation. [^3H]-Anisomycin was present at a final concentration of 1 μM . Controls containing no verrucarín A bound 27 and 32% of the total [^3H]anisomycin added for sensitive and resistant 'run off' ribosomes respectively. Ribosomes were at a concentration of 3 μM in all cases. [^3H]Anisomycin was added to ribosomes 1 min before the addition of verrucarín A. All reaction mixtures were kept at 0°C.

Additions (mM)	Binding of [^3H]anisomycin (% of control)	
	'Run off' ribosomes from strain Y166	'Run off' ribosomes from strain TR ₁
None	100	100
Verrucarín A (0.07)	29	44
Verrucarín A (7)	9	32

ribosomes from both a parent strain of yeast and a yeast mutant resistant to trichodermin (Jimenez & Vazquez, 1975). These results suggested strongly that verrucarín A had a different ribosomal receptor site from that for trichodermin and trichothecin (see also Schindler, 1974). It now appears, however, that ribosomes washed in high-salt solution contain a great deal of bound peptidyl-tRNA, in contrast with 'run off' ribosomes, which, as expected, do not (L. Carrasco & A. Jimenez, personal communication). Thus verrucarín A failed to produce an effect earlier (Barbacid & Vazquez, 1974a,b) because the drug was partially excluded from its ribosomal receptor site by nascent peptidyl-tRNA and hence only caused a small inhibition of binding of either trichodermin or anisomycin.

Finally, we extended the above conclusions about verrucarín A by carrying out further studies on the yeast mutant (TR₁) resistant to trichodermin (Jimenez *et al.*, 1975; Jimenez & Vazquez, 1975); this mutant is also resistant to anisomycin. However, although trichodermin and anisomycin bind to mutually exclusive sites, the ribosomal mutation leading to both trichodermin resistance and anisomycin resistance decreases the binding affinity for trichodermin alone. For anisomycin, therefore,

resistance does not result from a decreased binding affinity, but may be explained on the basis of a conformational and/or structural change at the peptidyl transferase catalytic centre, which allows anisomycin to bind but lowers the ability of the drug to inhibit peptide-bond formation. We prepared 'run off' ribosomes from both the parent yeast (strain Y166) and the mutant resistant to trichodermin (strain TR₁) and studied the ability of verrucarín A to compete with [^3H]anisomycin for binding to both ribosome preparations. The results are shown in Table 1. Clearly, 'run off' ribosomes from the trichodermin-resistant mutant bind verrucarín A with much less efficiency than do 'run off' ribosomes obtained from the yeast parent strain. This result is similar to those reported by Jimenez & Vazquez (1975) showing competition, between trichodermin, trichothecin and fusarenon X, for binding of [^3H]anisomycin to high-salt-washed ribosomes from both strains of yeast Y166 and TR₁. Our results indicate clearly that verrucarín A can now be included with trichothecin and fusarenon X in that all three drugs share with trichodermin a common, or at least overlapping, binding site on yeast ribosomes (see also Schindler, 1974). The suggestion by Jimenez & Vazquez (1975) that verrucarín A has a different ribosomal receptor site from the other three sesquiterpene drugs studied is apparently incorrect, since their results were obtained with ribosomes that were carrying nascent polypeptidyl-tRNA chains.

In the present work we have shown that at least four of the sesquiterpene antibiotics (trichodermin, trichothecin, fusarenon X and verrucarín A) share, at least in part, a common receptor site on eukaryotic ribosomes, and we suggest that this same site is also shared by T-2 toxin and trichoderminol. Barbacid & Vazquez (1974a,b) have claimed that trichoderminol shows good competition with [*acetyl*- ^{14}C]trichodermin for its ribosomal target site. We have suggested that the drugs can be impeded from interaction with the same (presumably) receptor site on polyribosomes by the presence of peptidyl-tRNA and that the degree of exclusion depends on the structure of each individual antibiotic. It would be instructive to extend our methodology towards other compounds within the sesquiterpene group with the intention of deciding whether or not all such compounds have the peptidyl transferase catalytic centre as their sole ribosomal receptor site.

M. C. thanks the Royal Society for providing him with a study visit award in the European Science Exchange Programme tenable in Spain. This work was also supported by institutional grants to the Centro de Biología Molecular from 'Comisión Administradora del Descuento Complementario (Instituto Nacional de Previsión)' and 'Dirección General de Sanidad' (Spain).

References

- Bamburg, J. R. & Strong, F. M. (1971) *Microb. Toxins* **7**, 207–292
- Barbacid, M. & Vazquez, D. (1974a) *Eur. J. Biochem.* **44**, 437–444
- Barbacid, M. & Vazquez, D. (1974b) *J. Mol. Biol.* **84**, 603–623
- Barbacid, M. & Vazquez, D. (1975) *J. Mol. Biol.* **93**, 449–463
- Barbacid, M., Fresno, M. & Vazquez, D. (1975) *J. Antibiot.* **28**, 433–462
- Battaner, E. & Vazquez, D. (1971) *Biochim. Biophys. Acta* **254**, 316–330
- Cannon, M., Davies, J. E. & Jimenez, A. (1973) *FEBS Lett.* **32**, 277–280
- Cannon, M., Smith, K. E. & Carter, C. J. (1976) *Biochem. J.* **156**, 289–294
- Carrasco, L., Barbacid, M. & Vazquez, D. (1973) *Biochim. Biophys. Acta* **312**, 368–376
- Carter, C. J., Cannon, M. & Smith, K. E. (1976) *Biochem. J.* **154**, 171–178
- Contreras, A., Barbacid, M. & Vazquez, D. (1974) *Biochim. Biophys. Acta* **349**, 376–388
- Cundliffe, E., Cannon, M. & Davies, J. E. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 30–34
- Fernandez-Muñoz, R. & Vazquez, D. (1973) *Mol. Biol. Rep.* **1**, 27–32
- Grollman, A. P. (1967) *Science* **157**, 84–85
- Jimenez, A. & Vazquez, D. (1975) *Eur. J. Biochem.* **54**, 483–492
- Jimenez, A., Sanchez, L. & Vazquez, D. (1975) *Biochim. Biophys. Acta* **383**, 427–434
- Mao, J. C. H. & Puttermann, M. (1969) *J. Mol. Biol.* **44**, 347–361
- Ohtsubo, K., Kaden, P. & Mittermayer, C. (1972) *Biochim. Biophys. Acta* **287**, 520–525
- Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672
- Schindler, D. (1974) *Nature (London)* **249**, 38–41
- Smith, K. E., Cannon, M. & Cundliffe, E. (1975) *FEBS Lett.* **50**, 8–12
- Ueno, Y. (1970) *Proc. U.S. Jpn. Conf. Toxic Micro-org.*, pp. 76–79
- Ueno, Y., Nakajima, M., Sakai, K., Ishii, J., Sato, N. & Shimada, N. (1973) *J. Biochem. (Tokyo)* **74**, 285–296