Structural Requirements for the Inhibitory Action of 12,13-Epoxytrichothecenes on Protein Synthesis in Eukaryotes

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1. The inhibitory actions of ten trichothecene antibiotics were investigated, in reticulocyte cell-free systems synthesizing protein *in vitro*, by studying polyribosome profiles and kinetics of amino acid incorporation in the presence or absence of the drugs. 2. The modes of action observed were critically dependent on the drug concentrations used, but the antibiotics tested could be divided into four distinct groups, each exerting a characteristic inhibitory response. 3. The inhibitory action observed in every case was controlled by the chemical structure of the individual trichothecene and in particular was closely related to the nature of the substituent groups present on C-3, C-4, C-8 and C-15 of the molecule.

The 12,13-epoxytrichothecenes, a structurally similar group of mycotoxins, are efficient inhibitors of protein synthesis in a variety of eukaryotic systems (Bamburg & Strong, 1971; Ueno *et al.*, 1973). The compounds appear to have a common binding site on the 80S ribosome (Wei *et al.*, 1974a; Cannon *et al.*, 1976b), and behave similarly in that they are potent inhibitors of the peptidyltransferase catalytic centre, as detected by the use of certain assays *in vitro*. Thus the compounds inhibit the fragment reaction (Carrasco *et al.*, 1973) and the reaction between puromycin and methionyl-tRNA^{Met} bound to 80S ribosomal initiation complexes (Smith *et al.*, 1975). However, the 12,13-epoxytrichothecenes do not appear to share a common mode of action when added to systems *in vivo* or *in vitro* containing polyribosomes, and on this basis they have been divided into two subgroups (Schindler, 1974). One subgroup, including trichodermin, stabilizes polyribosome profiles (E-type response), an effect that is consistent with inhibition of elongation (Cundliffe *et al.*, 1974). A similar stabilization would be observed if termination of nascent peptide chains was inhibited (Wei *et al.*, 1974b). The other subgroup, including T-2 toxin and diacetoxyscirpenol, causes rapid disaggregation of polyribosomes (I-type response), suggesting that the compounds affect either some stage in the initiation of protein synthesis (Cundliffe *et al.*, 1974) or at least are capable of binding only to recently initiated ribosomes (Cannon *et al.*, 1976a).

Carter *et al.* (1976) have claimed that trichodermin, although previously shown to give an E-type response (Cundliffe *et al.*, 1974), affects polyribosome profiles differently depending on the concentration of drug added. Thus at certain drug concentrations (4–25 µg/ml) instant and total stabilization of polyribosome

profiles was observed, an effect characteristic of total inhibition of elongation. In contrast, at lower drug concentrations (0.2–1 µg/ml), less inhibitory towards protein synthesis, a partial (30–35%) breakdown of polyribosomes was observed, an effect consistent with the drug having some preferential effect on initiation under the conditions used. Thus with trichodermin a concentration-dependent overlap of effects can occur and a true E-type response is observed only at high concentrations of the drug. The possibility exists, therefore, that a similar overlap might be observed with a drug originally shown to give an I-type response (Cundliffe *et al.*, 1974; Schindler, 1974) so that at most drug concentrations total polyribosome disaggregation is observed, whereas high drug concentrations may partially or even totally stabilize polyribosome profiles.

In the present work we have used the reticulocyte cell-free system to investigate the modes of action of ten trichothecene antibiotics, all closely related chemically. On the basis of studies on both polyribosome profiles and time-courses of inhibition of protein synthesis, we are able to classify the compounds into four distinct groups: (i) compounds stabilizing polyribosomes at most concentrations, but causing partial (30–35%), although never total, 'run off' at low concentrations (0.5–2 µg/ml); (ii) compounds causing total stabilization of polyribosomes at high concentrations and extensive 'run off' at low concentrations; (iii) compounds causing only partial stabilization of polyribosomes at high concentrations (up to 150 µg/ml), but total 'run off' at concentrations below 20 µg/ml; (iv) compounds causing total 'run off' in the concentration range 0.5–150 µg/ml. The above classification enables us to consider structure-function relationships within the

Fig. 1. Time-courses showing inhibition of protein synthesis by a group of ten trichothecenes. Incubation mixtures were prepared and incubated at 20°C as described in the Experimental section. All drugs were added at 2 min, except when T-2 toxin was added at 5 min in combination with either 3,15-didesacetylcalonecitrin or 15-desacetylcalonecitrin. Samples (50 μl) were removed from the control after 2 min incubation and then after 5, 10, 15 and 20 min incubation from all incubation mixtures. All samples were processed as described in the Experimental section. (a) Time-courses showing inhibition of protein synthesis by 3,15-didesacetylcalonecitrin at selected concentrations. Control with or without 1% dimethyl sulphoxide (●); 3,15-didesacetylcalonecitrin at 0.2 μg/ml (□); 0.5 μg/ml (▲); 1 μg/ml (○); 4 μg/ml (△); 20–150 μg/ml (■). One sample contained 3,15-didesacetylcalonecitrin (1 μg/ml) and T-2 toxin (20 μg/ml) (▼). (b) Time-courses showing inhibition of protein synthesis by either scirpentriol or 3-desacetyl-

group of trichothecene antibiotics studied and to ascertain how chemical modifications to certain areas of the molecule critically determine the observed inhibitory activity of these compounds.

Experimental

Preparation of lysates and cell-free protein-synthesizing systems from rabbit reticulocytes

Reticulocyte lysates were prepared from the blood of anaemic male New Zealand white rabbits, exactly as described previously (Cannon *et al.*, 1976a). Cell-free protein synthesis was assayed by adding 125 μ l of the 1:1 lysate (Smith *et al.*, 1975) prepared as described above to an equal volume of incubation mix (pH 7.2), so that the final concentrations of the components were 20 mM-Tris/HCl buffer (pH 7.2 at 20°C), 80 mM-KCl, 3 mM-magnesium acetate, 1 mM-dithiothreitol, 0.1 mM-EDTA, 20 mM-phosphocreatine, 1 mM-ATP, 1.6 mM-GTP, 0.02 mM each of 19 unlabelled amino acids without valine, 2 μ Ci of L-[2,3-³H]valine to measure protein synthesis, and 5 μ g of creatine kinase (Boehringer, Mannheim, W. Germany)/ml. The reaction mixture was incubated at 20°C, and 50 μ l samples were withdrawn at the times indicated, pipetted on to 2.5 cm discs of Whatman 3MM paper and then processed and assayed for radioactivity as described previously (Cannon *et al.*, 1976a). All samples containing radioactivity were then counted in a Packard liquid-scintillation counter at an efficiency, for ³H-labelled samples, of approx. 14%.

Sucrose-gradient analysis of polyribosome profiles

Samples (50 μ l) were removed from reaction mixtures at the times specified and diluted to 250 μ l with incubation buffer (20 mM-Tris/HCl, pH 7.2, 80 mM-KCl, 3 mM-magnesium acetate, 1 mM-dithiothreitol and 0.1 mM-EDTA final concentrations) containing cycloheximide (final concentration 250 μ g/ml) to prevent the polyribosomes undergoing further disaggregation (Carter *et al.*, 1976). The diluted reaction mixtures were then layered on linear 10–30% (w/v) sucrose gradients (volume 4.75 ml), prepared in the above buffer, and centrifuged at 42000 rev./min for 45 min, at 2°C, in a Spinco

SW50.1 rotor. Gradients were then analysed in an Isco gradient analyser as described previously (Carter *et al.*, 1976).

Antibiotics

Diacetoxyscirpenol, 15-acetoxyscirpendiol, scirpentriol, calonecetrin, 3-desacetylcalonecetrin, 15-desacetylcalonecetrin, 3,15-didesacetylcalonecetrin, 9,10-dihydrocalonecetrin and 3,15-diacetoxy-12-hydroxytrichothec-9-ene were synthesized as described by Gardner *et al.* (1972). T-2 toxin was kindly provided by Professor Julian Davies, Department of Biochemistry, University of Wisconsin, Madison, WI, U.S.A. Cycloheximide was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. All the above toxins were dissolved in 50% (v/v) dimethyl sulphoxide so that the final concentration of dimethyl sulphoxide in reaction mixtures never exceeded 1%. At this concentration dimethyl sulphoxide did not contribute to any of the effects observed in the experiments.

Radioactive chemicals

L-[2,3-³H]Valine (specific radioactivity 15.3 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

The effects of each of ten trichothecene antibiotics on time-courses of protein synthesis (Fig. 1) and on the extent of stabilization or disaggregation of polyribosomes (Figs. 2, 3 and 4) after a given period of incubation with the drug were investigated over a wide range of drug concentrations (0.2–150 μ g/ml). Polyribosome profiles from control incubations with or without dimethyl sulphoxide (1% final concentration), sampled at 0 min, 2 min (time of drug addition in subsequent experiments) or at 16 min (time of sampling for drug-treated reaction mixtures) were identical (Fig. 2a). This indicates that any changes observed in polyribosome profiles after drug treatment result neither from the presence of dimethyl sulphoxide nor from the incubation itself. Fig. 2 also illustrates polyribosome profiles produced after 14 min incubation with the drug 3,15-didesacetyl-

calonecetrin at selected concentrations. Control with or without 1% dimethyl sulphoxide (●); scirpentriol or 3-desacetylcalonecetrin at 0.2 μ g/ml (Δ); 1 μ g/ml (■); 4 μ g/ml (○); 50–150 μ g/ml (▲). (c) Time-courses showing inhibition of protein synthesis by T-2 toxin, diacetoxyscirpenol and 15-acetoxyscirpendiol at selected concentrations. Control with or without 1% dimethyl sulphoxide (●); T-2 toxin, diacetoxyscirpenol or 15-acetoxyscirpendiol all at 0.2 μ g/ml (□); T-2 toxin (1–150 μ g/ml), diacetoxyscirpenol (1–20 μ g/ml) or 15-acetoxyscirpendiol (1–5 μ g/ml) (▲); diacetoxyscirpenol (150 μ g/ml) or 15-acetoxyscirpendiol (20 μ g/ml) (○); 15-acetoxyscirpendiol (150 μ g/ml) (■). (d) Time-courses showing inhibition of protein synthesis by calonecetrin, 15-desacetylcalonecetrin, 9,10-dihydrocalonecetrin and 3,15-diacetoxy-12-hydroxytrichothec-9-ene at selected concentrations. Control with or without 1% dimethyl sulphoxide (●); 3,15-diacetoxy-12-hydroxytrichothec-9-ene (20 μ g/ml) (Δ); 9,10-dihydrocalonecetrin (20 μ g/ml) (■); calonecetrin (20 μ g/ml) (○); 15-desacetylcalonecetrin (20 μ g/ml) (▼); calonecetrin (150 μ g/ml) (□); 15-desacetylcalonecetrin (150 μ g/ml) (▲).

Fig. 2. Effect of 3,15-didesacetylcalonecitrin on polyribosome profiles in reticulocyte lysates

Incubation mixtures were prepared as described in the Experimental section, and unless otherwise stated drugs were added after 2 min incubation and samples removed after a further 14 min incubation with the drug. Samples were analysed on sucrose gradients as described in the Experimental section. (a) Control with or without 1% dimethyl sulphoxide incubated for 0 min, 2 min or 16 min or in the presence of 3,15-didesacetylcalonecitrin at 20–150 µg/ml; 3,15-didesacetylcalonecitrin at (b) 12 µg/ml; (c) 4 µg/ml; (d) 1 µg/ml; (e) 0.2 µg/ml; (f) 3,15-didesacetylcalonecitrin at 1 µg/ml and T-2 toxin at 20 µg/ml. In all cases the position of the 80S ribosome peak is marked with a vertical arrow. The horizontal arrow under the abscissa represents the direction of sedimentation of polyribosomes. In this and subsequent Figures, the 80S ribosome peak is sometimes 'cut'. In each case the absorbance of the peak above the 'cut' is off the scale indicated on the ordinate, the peak being drawn arbitrarily.

calonecitrin. Drug concentrations in the range 20–150 µg/ml produced polyribosome profiles identical with the control, with a corresponding 100% inhibition of protein synthesis (Fig. 1a). However, further lowering of the concentration of 3,15-didesacetylcalonecitrin caused a partial disaggregation of polyribosomes, which approached a maximum of 30–35% 'run off' when the drug was present at a concentration of 1 µg/ml (Fig. 2d); at this same concentration protein synthesis was inhibited by approx. 75% (Fig. 1a). 3,15-Didesacetylcalonecitrin, added at a concentration of 0.2 µg/ml, inhibited protein synthesis only poorly and there was a corresponding decrease in the extent of 'run off'. The kinetics of inhibition of protein synthesis at those

drug concentrations studied below 4 µg/ml show an initial fall in the rate of protein synthesis, followed by a linear but lower rate of synthesis continuing for the remaining 15–20 min of the incubation period. This steady-state situation, coupled with the fact that 3,15-didesacetylcalonecitrin at no concentration induces more than 35% 'run off', indicates that this drug behaves in a manner qualitatively identical with that observed for trichodermin (Carter *et al.*, 1976).

We next turned our attention to the two drugs 3-desacetylcalonecitrin and scirpentriol, which are closely related chemically to 3,15-didesacetylcalonecitrin (Table 1). The effect of these two drugs on polyribosomes was studied, and, as shown for 3,15-didesacetylcalonecitrin, both drugs added at high

Fig. 3. Effects of scirpentriol or 3-desacetylcalonecetrin on polyribosome profiles in reticulocyte lysates. Incubation conditions and analysis of polyribosomes were as described under Fig. 2. Scirpentriol or 3-desacetylcalonecetrin at (a) 50–150 $\mu\text{g/ml}$; (b) 20 $\mu\text{g/ml}$; (c) 12 $\mu\text{g/ml}$; (d) 4 $\mu\text{g/ml}$; (e) 1 $\mu\text{g/ml}$; (f) 0.2 $\mu\text{g/ml}$.

concentrations (50–150 $\mu\text{g/ml}$) caused complete stabilization of polyribosomes (Fig. 3a), and progressive decrease in the concentration of both drugs induced 'run off'. However, in contrast with the maximum value of 35% obtained with 3,15-didesacetylcalonecetrin, both 3-desacetylcalonecetrin and scirpentriol at 1 $\mu\text{g/ml}$ produced much more extensive disaggregation of polyribosomes. The corresponding time-courses (Fig. 1b) show total inhibition of protein synthesis at the high drug concentrations, whereas addition of 1 μg of the drugs/ml caused a gradual decrease in the rate of protein synthesis, approaching total inhibition 5–6 min after drug addition. With the drug added at 4 $\mu\text{g/ml}$ an intermediate effect was apparent, showing a greatly decreased rate of protein synthesis; the incorporation approached, but never reached, the plateau value obtained in the presence of 1 μg of drug/ml. Further lowering of the concentration of the drugs to 0.2 $\mu\text{g/ml}$ induced less 'run off', and inhibition of protein synthesis was correspondingly less effective.

We next extended our studies by using seven more

trichothecene antibiotics, all these compounds again being chemically closely related to the three drugs described above and to each other. These compounds, 15-acetoxyscirpentiol, diacetoxyscirpenol, T-2 toxin, calonecetrin, 15-desacetylcalonecetrin, 9,10-dihydrocalonecetrin and 3,15-diacetoxy-12-hydroxytrichothec-9-ene, were again examined for their effects on protein synthesis and polyribosome profiles. Of these antibiotics, 15-acetoxyscirpentiol alone stabilized polyribosomes totally (Fig. 4a), but only at a concentration (150 $\mu\text{g/ml}$) that inhibited protein synthesis by 100% (Fig. 1c). This compound, at a concentration of 20 $\mu\text{g/ml}$, shared with diacetoxyscirpenol, added at the high concentration of 150 $\mu\text{g/ml}$, the property of causing only partial stabilization of polyribosomes (Figs. 4b and 4c), even though both these drugs, at the concentrations stated, are potent inhibitors of protein synthesis (Fig. 1c). In an attempt to induce further 'run off', experiments were carried out with lower concentrations of the two drugs, and the results are again illustrated in Fig. 4. Diacetoxyscirpenol at 20 $\mu\text{g/ml}$ caused extensive disaggregation

Fig. 4. Effects of T-2 toxin, diacetoxyscirpenol, 15-acetoxyscirpenol, calonectrin, 15-desacetylcalonectrin, 9,10-dihydrocalonectrin and 3,15-diacetoxyscirpenol on polyribosome profiles in reticulocyte lysates. Incubation conditions and analysis of polyribosomes were as described under Fig. 2. (a) 15-Acetoxyscirpenol (150 $\mu\text{g/ml}$); (b) diacetoxyscirpenol (150 $\mu\text{g/ml}$) or 15-desacetylcalonectrin (20 $\mu\text{g/ml}$); (c) 15-acetoxyscirpenol (20 $\mu\text{g/ml}$) or 15-desacetylcalonectrin (20 $\mu\text{g/ml}$); (d) calonectrin (20 $\mu\text{g/ml}$), T-2 toxin (0.2 $\mu\text{g/ml}$), diacetoxyscirpenol (0.2 $\mu\text{g/ml}$) or 15-acetoxyscirpenol (0.2 $\mu\text{g/ml}$); (e) calonectrin (150 $\mu\text{g/ml}$) or diacetoxyscirpenol (20 $\mu\text{g/ml}$); (f) 9,10-dihydrocalonectrin (20 $\mu\text{g/ml}$); (g) 3,15-diacetoxyscirpenol (20 $\mu\text{g/ml}$); (h) T-2 toxin (1–150 $\mu\text{g/ml}$), diacetoxyscirpenol (1–10 $\mu\text{g/ml}$) or 15-acetoxyscirpenol (1–5 $\mu\text{g/ml}$).

of polyribosomes (Fig. 4e), and the 'run off' was complete when the concentration of diacetoxyscirpenol was lowered further (1–10 µg/ml). Total 'run off' was also achieved by adding 15-acetoxyscirpentiol at 1–5 µg/ml, or T-2 toxin, observed by Cundliffe *et al.* (1974) to be an I-type inhibitor (Schindler, 1974), at any concentration between 1 and 150 µg/ml (Fig. 4h). The time-courses showing inhibition of protein synthesis by 15-acetoxyscirpentiol, diacetoxyscirpenol and T-2 toxin are illustrated in Fig. 1(c). The similarities between these time-courses and those illustrated in Fig. 1(b), for inhibition of protein synthesis by both 3-desacetylcalonecetrin and scirpentriol, are apparent. It should be remembered that the latter compounds also produce similar concentration-dependent effects on polyribosomes directly comparable with those produced particularly by 15-acetoxyscirpentiol. In another experiment with 15-acetoxyscirpentiol, diacetoxyscirpenol and T-2 toxin, the drug concentration was again decreased to the low value of 0.2 µg/ml. As in some of the experiments reported above, the extent of 'run off' was decreased (for typical effect see Fig. 4d) and the compounds inhibited protein synthesis less efficiently (Fig. 1c).

Figs. 4(d) and 4(e) show polyribosome profiles resulting from addition of calonecetrin at concentrations of 20 µg/ml and 150 µg/ml respectively, and indicate that a substantial, although not total, 'run off' had occurred, the effect being slightly more pronounced with the higher drug concentration. The corresponding time-course studies (Fig. 1d) indicate that calonecetrin present at 20 µg/ml produces kinetics very similar to those observed in Fig. 1(c) with very low concentrations (0.2 µg/ml) of T-2 toxin, diacetoxyscirpenol or 15-acetoxyscirpentiol, showing that calonecetrin is a poor inhibitor of protein synthesis at this concentration. Although slightly more inhibition was observed when the drug concentration was increased to 150 µg/ml, this was still far from complete, and indicates that even extremely high concentrations of calonecetrin fail to inhibit protein synthesis efficiently.

Figs. 4(b) and 4(c) show that the compound 15-desacetylcalonecetrin, closely related to calonecetrin, and added also at concentrations of 150 µg/ml and 20 µg/ml respectively, induces rather less 'run off', or more stabilization, than in the corresponding experiments with calonecetrin. Fig. 1(d) shows that 15-desacetylcalonecetrin, although similar to calonecetrin in having a low potency, shows different kinetics from this compound, which resemble those obtained with low concentrations of 3,15-didesacetylcalonecetrin.

In an attempt to gain more information about the modes of action of calonecetrin and 15-desacetylcalonecetrin, studies were carried out on these compounds in combination with T-2 toxin. T-2 toxin (20 µg/ml) was added 3 min after addition of either

calonecetrin (150 µg/ml) or 15-desacetylcalonecetrin (150 µg/ml) to see if total 'run off' could be induced with such drug combinations, an effect always observed with T-2 toxin added alone (Fig. 4h). The combination of calonecetrin and T-2 toxin produced total polyribosome degradation over the 14 min incubation period. In contrast, however, the combination of 15-desacetylcalonecetrin and T-2 toxin caused very little additional degradation over that observed with 15-desacetylcalonecetrin (150 µg/ml) alone, and a similar result was obtained by using a combination of 3,15-didesacetylcalonecetrin (1 µg/ml) and T-2 toxin (Figs. 1a and 2f). We can in fact show that the addition of a well-characterized elongation inhibitor, such as anisomycin, together with any concentration of a trichothecene that on its own induces polyribosome disaggregation, causes stabilization of polyribosomes, indicating that such disaggregation is a result of ribosomal 'run off', a process that can be stopped by inhibiting elongation (results not shown). Apparently, therefore, both 15-desacetylcalonecetrin and 3,15-didesacetylcalonecetrin at concentrations causing partial (50–90%) inhibition of protein synthesis produce incomplete 'run off' of polyribosomes because they also affect elongation at these concentrations. Trichothecene concentrations above 150 µg/ml may produce results that are artifacts, since such high concentrations, in addition to the corresponding increase in the amounts of dimethyl sulphoxide in reaction mixtures, could exert non-specific effects on protein synthesis. Accordingly, further increasing of the dose of calonecetrin or 15-desacetylcalonecetrin was not attempted, but it is clear that, over the concentration range tested, 100% inhibition of protein synthesis could not be achieved with either of these compounds.

Finally, we carried out experiments with the compounds 9,10-dihydrocalonecetrin and 3,15-diacetoxy-12-hydroxytrichothec-9-ene (Fig. 1d; Figs. 4f and 4g). 9,10-Dihydrocalonecetrin (20 µg/ml) is somewhat less inhibitory than calonecetrin at the same concentration, whereas 3,15-diacetoxy-12-hydroxytrichothec-9-ene (20 µg/ml) is virtually without activity as an inhibitor of protein synthesis. These results are consistent with only a very slight 'run off' observed in the corresponding profiles.

Discussion

Eight of the trichothecenes tested can be placed into one of four distinct groups, the mode of action observed in each group being critically dependent on the drug concentration. Group (i) compounds show effects ranging from total stabilization of polyribosomes (E-type responses) to an induction of 35% 'run off'. Group (ii) compounds produce effects ranging from total stabilization to extensive 'run off'. Group (iii) compounds may induce total 'run off' (I-type responses) but never produce more than partial

stabilization of polyribosomes. Group (iv) compounds induce total 'run off' of polyribosomes at all inhibitory concentrations tested.

Typical E-type responses (i.e. instantaneous and essentially complete inhibition of protein synthesis) are observed with high concentrations of 3,15-didesacetylcalonecetrin (20–150 µg/ml), 3-desacetylcalonecetrin (50–150 µg/ml), scirpentriol (50–150 µg/ml) and 15-acetoxyscirpentiol (150 µg/ml), polyribosome profiles being totally stabilized. Typical I-type responses (i.e. a gradual onset of inhibition of protein synthesis, becoming complete 5–6 min after drug addition) are observed with T-2 toxin (1–150 µg/ml), diacetoxyscirpenol (1–10 µg/ml) and 15-acetoxyscirpentiol (1–5 µg/ml), inhibition being accompanied by total 'run off' of polyribosomes. Some of these compounds added at certain concentrations show intermediate effects, which we consider represent an I-type response accompanied by a secondary partial inhibition of elongation, producing kinetics shown in Fig. 1(b) for scirpentriol (4 µg/ml) or 3-desacetylcalonecetrin (4 µg/ml) and in Fig. 1(c) for diacetoxyscirpenol (150 µg/ml) or 15-acetoxyscirpentiol (20 µg/ml). 'Run off' takes longer and incorporation of radioactivity approaches, but never exceeds, the plateau value obtained when lower drug concentrations produce total 'run off' without affecting elongation. Profiles corresponding to these mixed responses show partial stabilization of polyribosomes (i.e. Fig. 4b) even after 14 min incubation with suitable drug concentrations. Polyribosome profiles alone are not, however, sufficient to distinguish between such mixed responses, with 90% inhibition of protein synthesis, and 'leaky' I-type responses, where protein synthesis is inhibited poorly. Thus T-2 toxin (0.2 µg/ml) inhibits protein synthesis by approx. 30% (Fig. 1c) but fails to induce complete 'run off' (Fig. 4d). Data from both time-courses and profile studies must, therefore, be carefully compared.

3,15-Didesacetylcalonecetrin and 15-desacetylcalonecetrin behaved differently from all the other drugs tested here, and the former inhibited protein synthesis about 100 times more potently than the latter. Total 'run off' was never observed at any concentration tested and time-courses with 3,15-didesacetylcalonecetrin (0.2–1 µg/ml) and 15-desacetylcalonecetrin (20–150 µg/ml) did not reach a plateau, but took up a 'steady state' of decreased protein synthesis. Incomplete 'run off' of polyribosomes, combined with 'steady-state' kinetics, could result from either a 'leaky' I-type response alone or a combination of 'leaky' I/'leaky' E-type responses (Carter *et al.*, 1976). Accordingly, either 3,15-didesacetylcalonecetrin (1 µg/ml) or 15-desacetylcalonecetrin (150 µg/ml) were added in combination with T-2 toxin (20 µg/ml). At this concentration T-2 toxin normally induces total 'run off' without affecting elongation, and the incomplete 'run off' observed with the drug combinations

must have resulted from a partial inhibition of elongation by the two calonecetrin derivatives rather than to 'leaky' I-type action. In mode of action these compounds therefore resemble trichodermin (Carter *et al.*, 1976), trichothecin (C. J. Carter, unpublished work) and pactamycin (Carter *et al.*, 1976), although the latter drug is chemically unrelated to the trichothecenes and binds to a different ribosomal receptor site (Macdonald & Goldberg, 1970; Schindler *et al.*, 1974; Barbacid & Vazquez, 1974). These drugs apparently exert an early preferential inhibition on initiation, producing partial 'run off', followed by a secondary effect on elongation, both effects being incomplete and occurring at drug concentrations producing 50–90% inhibition of protein synthesis. Thus trichodermin and 3,15-didesacetylcalonecetrin (and trichothecin, not studied here) can be placed in group (i), being compounds stabilizing polyribosomes at most drug concentrations and never causing more than 30–35% 'run off'. 15-Desacetylcalonecetrin is also placed in this group, as concentrations up to 150 µg/ml produce kinetics and profiles most closely resembling those for other group (i) members. In terms of potency, trichodermin > 3,15-didesacetylcalonecetrin ≥ 15-desacetylcalonecetrin.

Scirpentriol, 3-desacetylcalonecetrin and 15-acetoxyscirpentiol are placed in group (ii), as they appear to be truly intermediate compounds. High drug concentrations totally stabilize polyribosomes, low concentrations induce extensive 'run off' and intermediate concentrations produce mixed effects. In terms of potency scirpentriol ≈ 3-desacetylcalonecetrin > 15-acetoxyscirpentiol, the last compound being a less effective stabilizer in the concentration range 20–50 µg/ml although inducing more 'run off' in the concentration range 1–10 µg/ml than the other two.

Diacetoxyscirpenol is placed in group (iii), since at very high concentrations (up to 150 µg/ml) it partially stabilizes polyribosomes but induces total 'run off' at concentrations below 20 µg/ml. Higher concentrations of diacetoxyscirpenol (>150 µg/ml) might, however, stabilize polyribosomes totally, implicating diacetoxyscirpenol as a group (ii) compound. We did not use such excessively high concentrations for the reasons described in the Results section.

Calonecetrin, like 15-desacetylcalonecetrin, is far less potent than most of the antibiotics tested here, and a concentration of 150 µg/ml produces a 'leaky' I-type response. T-2 toxin in combination with this drug concentration produces total 'run off', suggesting that calonecetrin has no effect on elongation. Possibly higher calonecetrin concentrations could partially inhibit elongation, as for diacetoxyscirpenol (150 µg/ml), or give solely an I-type response, as for T-2 toxin. Since drug concentrations above 150 µg/ml were not investigated, it is not possible to assign with total certainty either group (iii)- or group (iv)-type activity

to calonectrin, so for structure-function analysis it is placed between diacetoxyscirpenol and T-2 toxin. The latter compound shows true group (iv)-type activity, producing total 'run off' at all inhibitory concentrations tested.

Table 1 indicates the general structure of a 12,13-epoxytrichothecene molecule, together with eight of the compounds under investigation arranged according to the group (i)–(iv) classification as outlined above. 9,10-Dihydrocalonectrin (reduced double bond between C-9 and C-10) and 3,15-diacetoxy-12-hydroxytrichothec-9-ene (reduced 12,13-epoxy group) were not included in Table 1, as only one concentration (20 µg/ml) of each of these compounds was tested. They were included in these experiments to allow direct comparison with calonectrin (from which they are both derived), to show what effect either (a) reduction of the double bond between C-9 and C-10 or (b) opening of the 12,13-epoxy group has on the activity of the molecule. Wei & McLaughlin (1974) have suggested that the double bond between C-9 and C-10 contributes to antibiotic activity, although they used different comparisons (i.e. trichodermin and 9,10-dihydrotrichodermin; trichodermol and 9,10-dihydrotrichodermol). Our results agree with their observations in that reduction of the double bond between C-9 and C-10, as in 9,10-dihydrocalonectrin, causes substantial loss of activity compared with that shown by calonectrin at the same concentration. Our data also indicate that an intact 12,13-epoxy group is equally important for antibiotic action, as the compound 3,15-diacetoxy-12-hydroxytrichothec-9-ene caused negligible inhibition of protein synthesis.

Our results allow us to examine how chemical modifications of the substituent groups R_1 , R_2 , R_3

and R_5 (Table 1) influence the observed inhibitory activity for a given compound. Wei & McLaughlin (1974) suggested that an oxygen-containing substituent at R_2 is essential for potent inhibition of the peptidyltransferase activity of eukaryotic ribosomes. Thus trichodermol ($R_2 = \text{OH}$) is a fairly active inhibitor of peptidyltransferase, and inhibitory activity is increased by esterification of this R_2 hydroxyl group as in trichodermin ($R_2 = \text{O}-\text{CO}-\text{CH}_3$) and trichothecin ($R_2 = \text{O}-\text{CO}-\text{CH}=\text{CH}-\text{CH}_3$). Even the configuration of the oxygen-containing substituent at R_2 is crucial for activity, as 4-epitrichodermol is virtually inactive. Since 3,15-didesacetylcalonectrin and 15-desacetylcalonectrin affect polyribosome profiles and time-courses of protein synthesis in a manner characteristic of trichodermin, all three compounds probably share a similar mode of action, even though the two former compounds are both deficient in an oxygen-containing substituent at R_2 . However, whereas trichodermin is a specific inhibitor of ribosomal activities involving peptidyltransferase (Tate & Caskey, 1973), this mode of action has not been proved for 3,15-didesacetylcalonectrin, although clearly this drug is a potent inhibitor of either elongation or termination of nascent peptides, as indicated by its ability to stabilize polyribosome profiles. Interestingly, other workers (Cundliffe & Davies, 1977) have suggested that the nature of the substituent at R_2 determines whether a compound inhibits elongation or termination, and they claim that the presence of either an H or an OH group at R_2 results in inhibition of termination (T-type response). Our results show no evidence of a T-type response with either scirpentriol or 15-acetoxyscirpendiol ($R_2 = \text{OH}$) or with any of the other compounds

Table 1. Chemical structures of trichothecene antibiotics

Group	R_1	R_2	R_3	R_4	R_5
(i) Trichodermin	H	OAc	H	H	H
3,15-Didesacetylcalonectrin	OH	H	OH	H	H
15-Desacetylcalonectrin	OAc	H	OH	H	H
(ii) Scirpentriol	OH	OH	OH	H	H
3-Desacetylcalonectrin	OH	H	OAc	H	H
15-Acetoxyscirpendiol	OH	OH	OAc	H	H
(iii) Diacetoxyscirpenol	OH	OAc	OAc	H	H
Calonectrin	OAc	H	OAc	H	H
(iv) T-2 toxin	OH	OAc	OAc	H	$\text{O}-\text{CO}-\text{CH}_2-\text{CH}(\text{CH}_3)_2$

($R_2 = H$), all tested over the concentration range 0.2–150 $\mu\text{g/ml}$, although we accept that our system may not distinguish between E-type and T-type responses in the way described by Cundliffe & Davies (1977).

Wei & McLaughlin, (1974) have also shown that 15-desacetylcalonecetrin and calonecetrin are relatively poor inhibitors of protein synthesis *in vitro*, although they observed that both compounds inhibited protein synthesis far more efficiently *in vivo*. Comparison of 15-desacetylcalonecetrin and calonecetrin ($R_1 = O-CO-CH_3$) with 3,15-didesacetylcalonecetrin and 3-desacetylcalonecetrin ($R_1 = OH$) respectively reveals the latter pair of compounds to be approx. 100 times more potent than the former pair. Thus, when $R_2 = H$ (as in all four compounds), to be a potent inhibitor the molecule requires $R_1 = OH$, and esterification of this group (as in both calonecetrin and 15-desacetylcalonecetrin) leads to a considerable decrease in potency. Exactly the opposite happens after the esterification of an OH group at R_2 when $R_1 = H$ (as with trichodermol to trichodermin).

The only difference in structure between diacetoxyscirpenol and 15-acetoxyscirpentiol is at R_2 , where the groups are $O-CO-CH_3$ and OH respectively. From the behaviour of trichodermin and trichodermol we might predict that diacetoxyscirpenol would give a stronger E-type response than 15-acetoxyscirpentiol at high drug concentrations, whereas in fact the reverse is true. Thus an $O-CO-CH_3$ group at R_2 may only induce a more potent E-type response than an OH group in the same position when $R_1 = H$. When $R_1 = OH$, the presence of the $O-CO-CH_3$ substituent at R_2 appears to reverse the effect. As R_1 and R_2 are fairly closely situated on the same side of the molecule (as shown by molecular models), it is not surprising that the modification of one can influence the activity of the other.

If we compare any pair of compounds, such as calonecetrin and 15-desacetylcalonecetrin, 3-desacetylcalonecetrin and 3,15-didesacetylcalonecetrin or 15-acetoxyscirpentiol and scirpentiol, where $R_3 = O-CO-CH_3$ and OH respectively, the compound possessing $R_3 = O-CO-CH_3$ always exhibits a stronger I-type response at a given concentration than the corresponding compound where $R_3 = OH$. Also, all the compounds possessing $R_3 = O-CO-CH_3$ can at some concentration cause total or at least extensive 'run off', which is not always true when $R_3 = OH$ (e.g. 3,15-didesacetylcalonecetrin). Thus esterification of the hydroxyl at R_3 appears to induce an increased I-type response, in agreement with the work of Cundliffe *et al.* (1974), where it was suggested that the presence of an oxygen-containing substituent at R_3 is necessary to produce such an effect.

Another interesting comparison may be made between diacetoxyscirpenol and T-2 toxin, where the only difference in structure is at R_5 . Diacetoxys-

cirpenol ($R_5 = H$) induces an I-type response at low concentrations with a secondary effect on elongation at higher concentrations, whereas T-2 toxin [$R_5 = O-CO-CH_2-CH(CH_3)_2$] gives solely an I-type response even at very high drug concentrations. T-2 toxin can be excluded from its ribosomal binding site by nascent peptides greater than a certain unspecified chain length (Cannon *et al.*, 1976a), and thereby appears to inhibit 'initiation' in that it can only bind effectively to recently initiated ribosomes. This could be explained in terms of steric interactions between the longer ribosome-bound nascent peptides and the bulky oxygen-containing substituent at R_5 , and to a lesser extent with the $O-CO-CH_3$ group at R_3 (a prominent position in the molecule, as shown with space-filling models). Steric effects of the latter group could explain why diacetoxyscirpenol and any other compound with $R_3 = O-CO-CH_3$ induce an I-type response at low concentrations. At high concentrations such steric effects may be overcome and a secondary inhibition of elongation is observed. Clearly, combination of R_5 and R_3 steric effects, in T-2 toxin, prevent an E-type response being observed even at very high concentrations.

Our results clearly indicate that different modes of inhibition can often be observed for the same compound over a wide concentration range, and the relative amount of each type of inhibition at a given concentration is closely dependent on the chemical structures of certain substituent groups, in particular R_1 , R_2 , R_3 and R_5 . As all the 12, 13-epoxytrichothecenes have very similar structures, and studies suggest that they all have a common binding site (Cannon *et al.*, 1976b), we still consider that all these drugs inhibit the same ribosomal function, namely peptidyltransferase activity, although their ability to achieve this will depend on their ability to gain access to their binding site. This accessibility may be governed by a number of separate but interacting factors, such as the size and nature of ribosome-bound nascent peptides, substituent groups on the trichothecene molecule and the functional state of a given ribosome.

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