INHIBITION OF PROTEIN SYNTHESIS IN *SACCHAROMYCES CEREVISIAE* BY THE 12,13-EPOXYTRICHOTHECENES TRICHODERMOL, DIACETOXYSCIRPENOL AND VERRUCARIN A

REVERSIBILITY OF THE EFFECTS

FRANCISCO HERNÁNDEZ* and MICHAEL CANNON

Department of Biochemistry, University of London King's College, Strand, London, WC2R 2LS, U.K.

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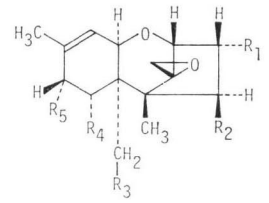
Inhibition of protein synthesis by trichodermol, diacetoxyscirpenol and verrucarin A in cells and spheroplasts of *Saccharomyces cerevisiae* was investigated. Inhibition was reversible for trichodermol and diacetoxyscirpenol, both drugs being removed from their target site(s) by washing, but was irreversible for verrucarin A. These results are interpreted in relation to variations in chemical structure between these trichothecenes.

The 12,13-epoxytrichothecenes (Fig. 1) specifically inhibit eukaryotic protein synthesis both *in vivo* and *in vitro*. The group includes more than 30 distinct compounds and their inhibitory actions have been reviewed[1]. They have a common binding site(s) on eukaryotic 80S ribosomes and inhibit peptidyl transferase activity. From *in vivo* and *in vitro* results involving polyribosomes the compounds have been divided into four general groups[2], the modes of action observed in each group often being critically dependent on the drug concentration selected[2,3]. Group I compounds stabilize polyribosome profiles at high drug concentrations by inhibiting elongation. At low drug concentrations, however, a limited disaggregation ("run off") of polyribosomes occurs, with the compounds subsequently affecting initiation of protein synthesis or becoming bound effectively only to those ribosomes carrying short nascent polypeptides[4]. Group II compounds produce effects ranging from total stabilization to extensive "run off", Group III compounds may induce total "run off" but never produce more than partial stabilization and Group IV compounds induce total polyribosome "run off" at all inhibitory concentrations tested. Inhibitory responses are controlled by chemical modification of the sub-

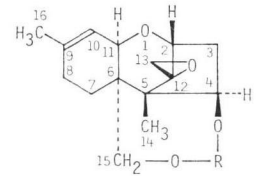
Fig. 1. Chemical structures of some trichothecene antibiotics.

a



	R_1	R_2	R_3	R_4	R_5
Trichodermol	H	OH	H	H	H
Trichodermin	H	OAc	H	H	H
Diacetoxyscirpenol	OH	OAc	OAc	H	H
T-2 Toxin	OH	OAc	OAc	H	$OOCCH_2$-$CHMe_2$

b



Verrucarin A

R = -C-CH-CH-CH_2-CH_2-O-C-CH=CH-CH=CH-C-
‖ | | ‖ ‖
O OH CH_3 O O

* Permanent address: Instituto de Bioquímica de Macromoléculas, Centro de Biología Molecular, C.S.I.C. y Universidad Autónoma de Madrid, Canto Blanco, Madrid 34, Spain

stituent groups R_1, R_2, R_3 and R_4 located on each trichothecene.

Observed inhibitory effects may relate to reversibility or irreversibility of the interaction of a particular trichothecene with its receptor site(s). Accordingly, we have used trichodermol, diacetoxyscirpenol and verrucarin A to inhibit protein synthesis in yeast cells and spheroplasts. These three inhibitors have been placed in Groups I, III and IV respectively[2], (M. CANNON, personal communication). We have determined if inhibition produced by each of the three drugs is easily reversed by washing and resuspension of cells or spheroplasts in drug-free medium. Data are discussed in relation to variations in chemical structure between these trichothecenes.

Materials and Methods

Cells of *Saccharomyces cerevisiae*, strains Y166 or A224A, were cultured at 30°C with shaking in synthetic medium[5] supplemented with 40 mg/liter of both histidine and tryptophan (for Y166) or with leucine (for A224A).

Cells, grown to a concentration of approximately 10^7 cells/ml, were converted into spheroplasts[6] with recovery in synthetic medium for 2.5 hours.

Protein synthesis in both cells and spheroplasts was measured at 30°C by determining uptake of ^{3}H-labelled phenylalanine (specific activity 522 Ci/mole: The Radiochemical Centre, Amersham, Bucks., U. K.). At times indicated in the Figures, samples (0.5 ml) were mixed with ice-cold 10% trichloroacetic acid (3 ml) and precipitates containing radioactive protein held at 90°C for 10 minutes. Tube contents were cooled and precipitates collected on Whatman GF/C glass fiber filters. Filters were washed 3 times with 3 ml portions of ice-cold 5% trichloroacetic acid, dried and finally counted in a toluene based scintillation fluid at 14% efficiency. Various drugs were added to incubation mixtures, as indicated in the Figures where drug concentrations are expressed as final concentrations in reaction mixtures. For Figs. 2~6 inclusive, 100% incorporation levels represent c.p.m. of ^{3}H-labelled phenylalanine incorporated 0.5 ml of control incubation mixtures.

Trichothecenes were provided by JULIAN DAVIES (Department of Biochemistry, University of Wisconsin–Madison, U.S.A.). They were dissolved in 50% (v/v) dimethylsulfoxide (DMSO) with the concentration of the latter in incubation mixtures never exceeding 1% (v/v). DMSO did not contribute to any of the effects observed.

Results

Protein Synthesis in Yeast Cells

Trichodermol, diacetoxyscirpenol and verrucarin A all inhibit phenylalanine incorporation into protein in cells of *S. cerevisiae* strains Y166 and A224A (Fig. 2). For trichodermol and diacetoxyscirpenol linear rates of inhibition were observed at all drug levels tested but at high concentration (100 μg/ml) trichodermol is a more effective inhibitor in Y166 than is diacetoxyscirpenol (120 μg/ml), relative inhibitory values being 80% and 59% respectively. In Y166 5 μg/ml of verrucarin A inhibited phenylalanine incorporation very strongly with concentrations above 10 μg/ml producing inhibitory levels approaching 100%. Verrucarin A (30 μg/ml) completely inhibited phenylalanine incorporation in Y166 with the amount of trichloroacetic acid-precipitable material present at the time of drug addition declining with time, possibly as a result of proteolytic activity within these highly inhibited cells. There was no obvious evidence of cell lysis as checked microscopically. Verrucarin A pinpointed interesting differences between the two yeast strains studied since phenylalanine incorporation in A224A but not in Y166 was totally inhibited at all drug concentrations selected (0~15 μg/ml). This variation presumably reflects a permeability difference(s) between the two strains associated with the cell wall or membrane.

Fig. 2. Inhibition of protein synthesis in yeast cells.

Protein synthesis was assayed (see Materials and Methods) with drugs added after 40 minutes incubation.

a) Strain Y166: Control without drug (●). Trichodermol at 10 μg/ml (□), 30 μg/ml (▲), 100 μg/ml (○). (100%=2,975 cpm).

b) Strain Y166: Control (●). Diacetoxyscirpenol at 40 μg/ml (□), 80 μg/ml (▲), 120 μg/ml (○). (100%=6,700 cpm).

c) Strain Y166: Control (●). Verrucarin A at 5 μg/ml (□), 10 μg/ml (▲), 30 μg/ml (○). (100%=6,100 cpm).

d) Strain A224A: Control (●). Verrucarin A at 5 μg/ml (□), 10 μg/ml (▲), 15 μg/ml (○), (100%=15,050 cpm).

Fig. 3. Inhibition of protein synthesis in yeast spheroplasts.

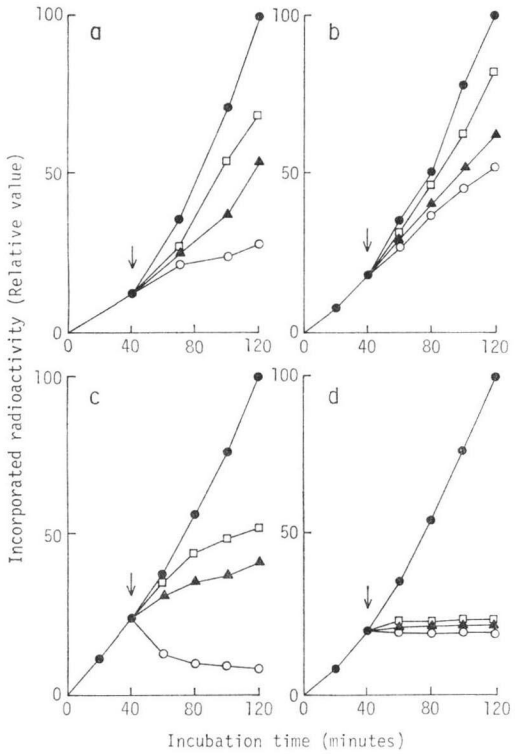
Protein synthesis was assayed (see Materials and Methods) with drugs added after 40 minutes incubation.

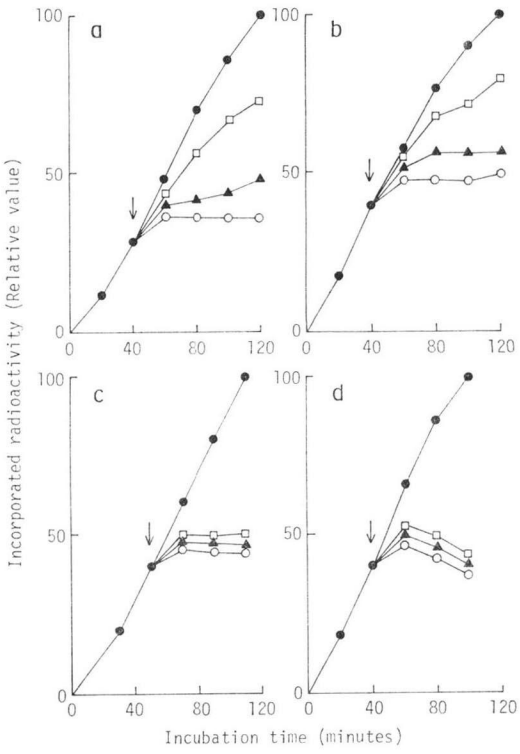
a) Y166 Spheroplasts: Control without drug (●). Trichodermol at 10 μg/ml (□), 30 μg/ml (▲), 100 μg/ml (○). (100%=9,500 cpm).

b) Y166 Spheroplasts: Control (●). Diacetoxyscirpenol at 40 μg/ml (□), 80 μg/ml (▲), 120 μg/ml (○). (100%=12,150 cpm).

c) Y166 Spheroplasts: Control (●). Verrucarin A at 5 μg/ml (□), 10 μg/ml (▲), 20 μg/ml (○). (100%=13,150 cpm).

d) A224A Spheroplasts: Control (●). Verrucarin A at 2 μg/ml (□), 4.5 μg/ml (▲), 9 μg/ml (○). (100%=9,050 cpm).





Cells from both strains were, however, similarly inhibited by trichodermol and diacetoxyscirpenol (results not shown).

Protein Synthesis in Yeast Spheroplasts

The above inhibitory studies were then repeated using spheroplasts (Fig. 3) and the data obtained were essentially similar to those shown in Fig. 2. In general, however, spheroplasts were more sensitive to inhibition than were cells, particularly for diacetoxyscirpenol with 120 μg/ml inhibiting protein synthesis by 85%. The differences shown previously between the two yeast strains for verrucarin A inhibition (Fig. 2c & d) were also abolished using spheroplasts. Thus spheroplasts from both strains were

Fig. 4. Reversible and irreversible inhibition of protein synthesis in yeast cells by trichodermol, diacetoxyscirpenol and verrucarin A.

Y166 cells were incubated with trichodermol (100 μg/ml) for 20 minutes (a) diacetoxyscirpenol (160 μg/ml) for 80 minutes (b) or verrucarin A (15 μg/ml) for 60 minutes (c). A224A cells were incubated with verrucarin A (5 μg/ml) for 30 minutes (d). Cells were pelleted, washed twice and resuspended in medium. Protein synthesis was assayed (see Materials and Methods) with drugs added after 40 minutes incubation.

a) Strain Y166: Control cells (●). Cells plus trichodermol (100 μg/ml) (▲). (100%=5,100 cpm).

b) Strain Y166: Control cells (●). Cells plus diacetoxyscirpenol (160 μg/ml) (▲). (100%=4,375 cpm).

c) Strain Y166: Control cells (●). Cells plus verrucarin A (15 μg/ml) (▲). Cells not incubated with verrucarin A but washed (○). (100%=4,475 cpm).

d) Strain A224A: Control cells (●). Cells plus verrucarin A (5 μg/ml) (▲). Cells not incubated with verrucarin A but washed (○). (100%=5,200 cpm).

Incorporations in cells for Figs a) and b), not previously incubated with drug but washed are not shown but were almost identical to those for cells incubated with drug followed by washing.

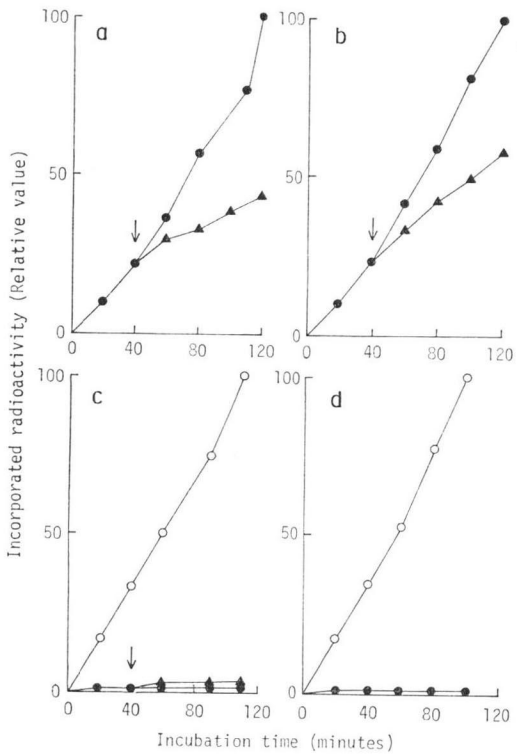


Fig. 5. Reversible and irreversible inhibition of protein synthesis in yeast spheroplasts by trichodermol, diacetoxyscirpenol and verrucarin A.

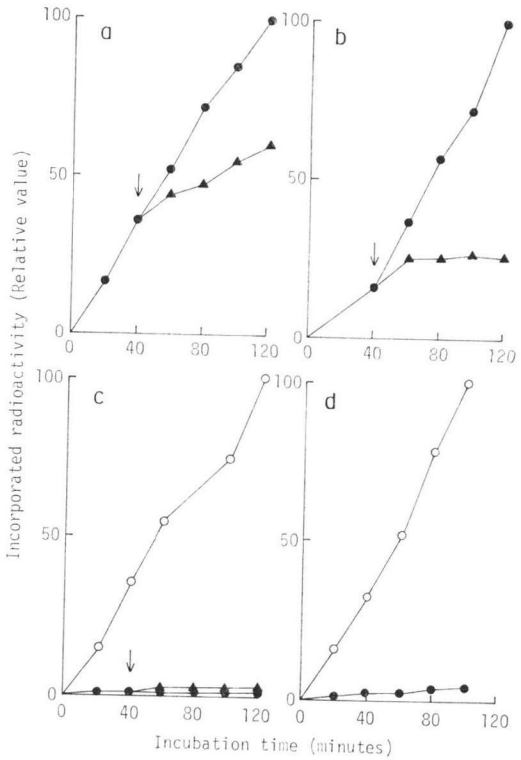
Y166 spheroplasts (a~c) were treated with drugs as described under Fig. 4 for cells. A224A spheroplasts were incubated with verrucarin A (2 μg/ml) for 30 minutes (d). Spheroplasts were pelleted and treated as described for cells under Fig. 4.

a) Conditions as for Fig. 4a. (100%=9,700 cpm).

b) Conditions as for Fig. 4b. (100%=2,300 cpm)

c) Conditions as for Fig. 4c but using verrucarin A (5 μg/ml). (100%=7,000 cpm).

d) Conditions as for Fig. 4d but using verrucarin A (2 μg/ml). (100%=4,600 cpm). Other controls as for Fig. 4.



strongly inhibited by low verrucarin A concentrations (Fig. 3c & d) suggesting more precisely that the differences observed between cells of Y166 and A224A are associated with cell wall permeability.

Reversible and Irreversible Inhibition of Protein Synthesis

Reversibility of inhibitory effects induced by the drugs *in vivo* was next studied. Cells or spheroplasts were incubated with a selected high concentration of each drug, washed twice and finally

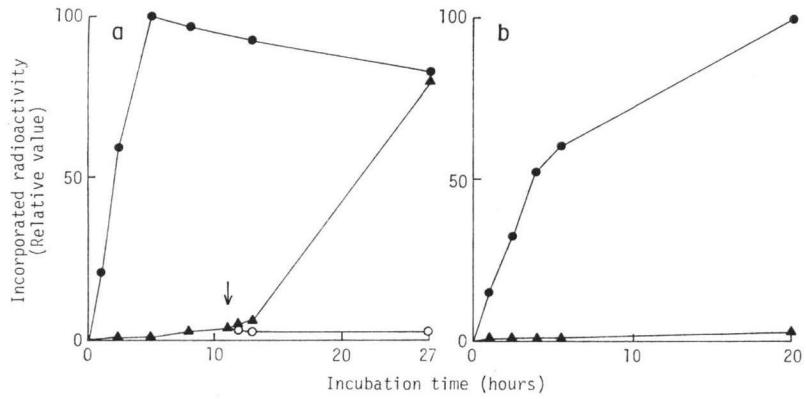
resuspended in fresh medium. Protein synthesis was then assayed. Drug concentrations and incubation times are indicated in the Figs. Inhibition of protein synthesis by both trichodermol and diacetoxyscirpenol is fully removed by washing and resuspension of cells or spheroplasts in fresh medium (Figs. 4 & 5). Recovered cells and spheroplasts are, however, fully susceptible to inhibition if the relevant drug is added back to the cultures. However, inhibition by verrucarin A in both cells and spheroplasts of A224A and Y166 is irreversible. Thus after a two hour incubation with drug and subsequent washing to remove excess verrucarin A no phenylalanine was incorporated (Figs. 4 & 5). Finally, cells and spheroplasts of A224A were inhibited with verrucarin A, washed free from excess drug and phenylalanine incorporation followed over 24 hours (Fig. 6). Control cells incorporate all added phenylalanine after approximately 6 hours. Cells previously treated with verrucarin A remain inhibited relative to controls for approximately 6 hours but then commence phenylalanine uptake with a high incorporation level reached by 23-hour incubation. In contrast, spheroplasts remained highly inhibited relative to controls over the total incubation period. Recovery of cells from verrucarin A inhibition does not result from the presence in inhibited cultures of a small proportion of cells resistant to the drug since addition of verrucarin A to recovering cells restores inhibition (Fig. 6a). Drug release from its receptor site(s) also seems unlikely since spheroplasts remain strongly inhibited over the long incubation period. Possibly initial cultures were less than 100% inhibited and with removal of excess verrucarin A any few viable cells could multiply. There is, in fact, a very noticeable increase in the optical density of 'inhibited' cultures between 10 and 23 hours incubation (M. CANNON, unpublished observations) but not in spheroplasts where cell division does not take place. Nevertheless spheroplasts, although remaining highly inhibited relative to controls, do incorporate a very small amount of radioactivity linearly over 20 hours incubation, taken to represent background incorporation by a very small proportion of unaffected spheroplasts. A similar very small, but steady incorporation is seen with 'inhibited' cells between 0 and 11 hours incubation.

Fig. 6. Reversible and irreversible inhibition of protein synthesis in yeast cells and spheroplasts by verrucarin A.

A224A cells or spheroplasts were incubated with verrucarin A at 10 μg/ml (a) or 5 μg/ml (b) respectively for 30 minutes. Cells or spheroplasts were pelleted, washed, resuspended and assayed for protein synthesis all as described under Fig. 4. Verrucarin A (10 μg/ml) was added to a sample of cells at 11-hour of incubation.

a) Reversible inhibition by verrucarin A in cells. Control cells (▲). Cells plus verrucarin A (10 μg/ml) (○). Cells not incubated with verrucarin A but washed (●). (100%=84,401 cpm).

b) Irreversible inhibition by verrucarin A in spheroplasts. Control spheroplasts (▲). Spheroplasts not incubated with verrucarin A but washed (●). (100%=108,489 cpm).



Discussion

In HeLa cells, verrucarin A rapidly induced total "run off" of polyribosomes and inhibited leucine incorporation into protein[7]. A further Group IV compound, T-2 toxin, induced only slow "run off" in yeast spheroplasts, an effect presumably related to cell membrane permeability, although in reticulocyte cell-free systems, protein synthesis can be inhibited by approximately 60% using T-2 toxin at concentrations as low as 0.2 μg/ml[2]. Inhibition of cell-free protein synthesis was also studied[2] using diacetoxyscirpenol (Group III). This compound was a more effective inhibitor than T-2 toxin, particularly at high drug concentrations. Group IV compounds inhibit recently-initiated ribosomes very strongly but do not prevent elongation of nascent polypeptide chains that have reached a certain minimal chain length. A residual amount of protein synthesis can thus take place before inhibition becomes complete. Diacetoxyscirpenol also inhibits recently-initiated ribosomes but can, at high concentrations, efficiently block elongation. Our results for diacetoxyscirpenol (Figs. 2 & 3) are thus surprising since a more pronounced inhibitory effect is to be expected. Less is known about the potency of trichodermol as an inhibitor of protein synthesis *in vitro*. However, other Group I compounds produce, at high drug concentrations, instantaneous and essentially complete inhibition of elongation. Since trichodermol inhibits the peptidyl transferase center on ribosomes and chemically is closely related to trichodermin (Group I) its potency should resemble that of diacetoxyscirpenol[2] a prediction supported by our *in vivo* data (Fig. 3a & b).

Trichodermol, diacetoxyscirpenol and verrucarin A are particularly effective inhibitors of protein synthesis in yeast spheroplasts with diacetoxyscirpenol marginally the least and verrucarin A certainly the most potent of these three drugs. Inhibition by trichodermol and diacetoxyscirpenol is readily reversed whereas that by verrucarin A is irreversible. Similar experiments were carried out with HeLa cells and rabbit reticulocyte lysates[8] using diacetoxyscirpenol, trichodermin and T-2 toxin. Protein synthesis in HeLa cells was strongly and irreversibly inhibited by both T-2 toxin and diacetoxyscirpenol whereas inhibition by trichodermin was weak and reversible. These results[8] for diacetoxyscirpenol are clearly at variance with ours and others[9] have claimed that inhibition by diacetoxyscirpenol *in vivo* in both reticulocytes and tumour cells can be markedly reversed by washing. Other data from LIAO *et al.*[8] also contradict those reported by other groups and claim that T-2 toxin at high concentration inhibits elongation. Others[2,7,10] have excluded this possibility.

The published literature concerning trichothecenes is typified by similar contradictions since inhibition can vary depending upon the type and source of experimental system employed and whether *in vivo* or *in vitro* conditions are selected. Thus diacetoxyscirpenol is claimed to be at least 20 times more active in whole cells than in cell-free systems whereas other trichothecenes (including fusarenon-X) show no detectable difference in potency[9]. Furthermore, T-2 toxin can strongly inhibit several systems *in vivo* with a potency similar to that of diacetoxyscirpenol although verrucarin A is shown relatively to be a poor inhibitor[9]. The variability observed may in part result from certain trichothecenes exerting *in vivo* some influence on cell membranes and affecting uptake of certain precursors[9]. Selected trichothecenes may have a particularly high affinity for biomembrane structures.

Trichodermol and diacetoxyscirpenol are chemically very similar (Fig. 1) but there are structural differences at R_1, R_2 and R_3. Although esterification of a R_2 hydroxyl group, as in trichodermol to trichodermin, increases inhibitory activity this may only happen when R_1=H (as in trichodermol being less active than trichodermin). For diacetoxyscirpenol, however, where R_1=OH the critical group may be R_3[2]. Thus diacetoxyscirpenol with R_3=O–CO–CH_3 has Group III activity whereas in the Group I compound trichodermol R_3=H. The presence of R_3=O–CO–CH_3 in diacetoxyscirpenol allows total "run off" at low drug concentrations since this trichothecene binds very weakly to ribosomes carrying nascent polypeptide chains. At high drug concentrations, however, diacetoxyscirpenol binds to ribosomes more efficiently and partially stabilizes polyribosomes.

Such chemical differences apparently critically control drug activity *in vitro* but structural considerations are more difficult to apply to inhibition *in vivo*. Indeed, although LIAO *et al.*[8] have implied that R_3=O–CO–CH_3 in diacetoxyscirpenol causes irreversible inhibition of protein chain initiation our data contradict this hypothesis. Nevertheless, verrucarin A (*cf.* T-2 toxin) irreversibly inhibits protein syn-

thesis in our experiments. This Group IV compound induces total "run off" *in vivo*[7] but it is impossible at this stage to explain precisely why in chemical terms inhibition of protein synthesis in spheroplasts is also irreversible. Irreversibility may well be an inaccurate term in describing inhibition by trichothecenes. Trichodermin binds with dissociation constants of 2.10 μM and 0.72 μM respectively to yeast polyribosomes and "run off" ribosomes[11]. Thus even for this Group I compound its affinity for ribosomes is considerably enhanced when these carry no bound nascent polypeptide chains. Trichodermol, trichodermin and verrucarin A also have a common receptor site(s) which we assume is shared by diacetoxyscirpenol. The affinity of a given trichothecene for this site(s) must vary and the chemical structure of verrucarin A presumably affords a particularly favourable dissociation constant for ribosome binding such that an apparent binding irreversibility can be demonstrated under selected conditions. Furthermore, the dissociation constant for a given trichothecene may vary for 80S ribosomes depending upon their source accounting at least in part for some of the apparent discrepancies reported in the literature and considered here.

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