STRUCTURE-FUNCTION RELATIONSHIP IN THE 12,13-EPOXYTRICHOTHECENES
Novel Inhibitors of Protein Synthesis

Cha-Mer Wei and Calvin S. McLaughlin
Department of Molecular Biology
University of California, Irvine; Irvine, California 92664

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An important group of mycotoxins, the 12,13-epoxytrichothecenes (Fig. 1), are among the most active inhibitors of protein synthesis in eucaryotic cells. Some of these compounds inhibit a step required for the initiation of protein synthesis, while other compounds inhibit a step required for the termination or elongation of protein synthesis. Structure-function studies on the various derivatives indicate which areas of the molecule are important in the inhibition of initiation and termination or elongation.

INTRODUCTION

The 12,13-epoxytrichothecenes (Fig. 1) form an important group of mycotoxins produced by several species of fungi involved in grain and fruit spoilage. Several of them are among the most cytotoxic agents known and have been implicated in a number of significant mycotoxicoses that afflict man and domestic animals (1,2,3). A comprehensive review of these compounds as toxic agents is available (4). Our studies have indicated that one of the 12,13-epoxytrichothecenes, trichodermin, is a potent specific inhibitor of protein synthesis and inhibits the chain termination step of protein synthesis by inhibiting the peptidyl transferase activity of the ribosome required for chain termination (5,6,7). However, studies by other workers have indicated that some of the 12,13-epoxytrichothecenes inhibit a reaction required for the initiation of protein synthesis (8,9). Since it is unusual to have a class of closely related compounds acting on two different phases of protein synthesis, we have surveyed 16 of the 12,13-epoxytrichothecenes to determine if they inhibit the initiation or the elongation-termination steps of protein synthesis.

MATERIALS AND METHODS

Labeling conditions and cell fractionation-Rabbit reticulocytes were prepared as described (10). The rate of protein synthesis in intact reticulocytes and the polyribosome profiles in these cells was determined as described (11).

In vitro protein synthesis-Reticulocyte ribosomes and S100 were prepared according to Schreier and Staehelin (12). The reaction mixture for poly U directed polyphenylalanine synthesis contains in a total volume of 0.25 ml, 0.03 mg poly U, 2.3 μg of pyruvate kinase, 0.75 μCi of ^{14}C-phenylalanine, 50 mCi/mmole, 0.15 mg of crude reticulocyte ribosomes, 0.1 ml of dialysed reticulocyte S100 and the following concentration of reactants: 0.05 M Tris-HCl (pH 7.6), 0.05 M KCl, 7 mM $MgCl_2$, 0.9 mM ATP, 0.03 mM GTP, 0.2 mM amino acids minus phenylalanine, 5.44 mM 2-mercaptoethanol and 4.94 mM phosphenolpyruvate. The reaction mixture was incubated at 30°C for 30 minutes and the tricholoroacetic acid precipitable radioactivity was determined. Assays for peptidyl transferase and chain termination were according to the methods developed by Caskey and co-workers (13,14).

RESULTS

Trichodermol is one of the simplest of the 12,13-epoxytrichothecenes. The basic ring structure is shown in Fig. 1 (R1 = H, R2 = OH, R3 = H). A comparison of a number of derivatives of trichodermol in table I, outlines the structural requirements for activity as inhibitors of protein synthesis. Reduction of the double bonds between C-9 and C-10 or the introduction of an epoxy ring between C-7 and C-8 results in a substantial reduction in activity. The substitution of a carbonyl group at C-8 causes a moderate loss of activity. Modification of the R2 group causes profound changes in activity. Conversion of the hydroxyl group to a carbonyl group in trichodermone results in complete loss of activity. The configuration of the hydroxyl group itself is important. Thus, 4-epitrichodermol is virtually without activity. Polyribosomes are stable during inhibition with all of the active inhibitors in Table I. This indicates that these inhibitors act on either the elongation or termination steps in protein synthesis. An initiation inhibitor would cause rapid conversion of polyribosomes to monoribosomes (15).

Esterification of the R2 hydroxyl results in a substantial increase of the biological activity. Thus, trichodermin is a more effective inhibitor of protein synthesis *in vivo* and *in vitro* than trichodermol (Table II). Trichothecin with a large ester group is an even better inhibitor. Reduction of the 9,10 double bond

Table I. Derivatives of Trichodermol (R1 = H, R2 = OH, R3 = H) as Inhibitors of Protein Synthesis.

	% Activity Remaining								
	Termination assay		Peptidyl transferase assay		Polyphenylalanine synthesis assay		in vivo protein synthesis		% Polysomes at* 90% inhibition
	20μg/ml	100μg/ml	5μg/ml	20μg/ml	5μg/ml	20μg/ml	1μg/ml	10μg/ml	
Control	100	100	100	100	100	100	100	100	--
Trichodermol (R2 = OH)	92	76	73	40	73	49	40	13	>90
9,10-Dihydrotrichodermol (R2 = OH)	100	100	97	87	95	88	100	52	--
4-Epitrichodermol (R2 = OH)	100	100	98	93	93	88	100	100	--
Trichodermone (R2 = O)	100	100	100	99	100	100	100	83	--
Crotocol (C-7,C-8 = β-epoxy)	100	100	98	94	99	95	79	49	>90
Trichothecolone (C-8 = O, C-7 = H)	96	86	88	68	84	63	74	23	90

*The effect of each inhibitor on the polysome profile of the cell was tested at the concentration required to give 90% inhibition of in vivo protein synthesis. The polysomes that remain after 15 minutes of incubation in the presence of an inhibitor are compared with those in a control culture.

Table II. Comparison of the Ester Derivatives of Trichodermol as Inhibitors of Protein Synthesis.

	% Activity Remaining								
	Termination assay		Peptidyl transferase assay		Polyphenylalanine synthesis assay		in vivo protein synthesis		% Polysomes at 90% inhibition
	20μg/ml	100μg/ml	5μg/ml	20μg/ml	5μg/ml	20μg/ml	1μg/ml	10μg/ml	
Control	100	100	100	100	100	100	100	100	--
Trichodermol (R2 = OH)	92	76	73	40	73	49	40	13	>90
Trichodermin (R2 = OAc)	45	32	12	7	41	28	50	7	>90
9,10-Dihydrotrichodermin (R2 = OAc)	100	100	98	90	90	88	100	65	--
Crotocin (R2 = OOCCH = $CHCH_3$)	70	42	34	22	44	32	11	2	>90
Trichothecin (C-8 = O, R2 = OOCCH= $CHCH_3$)	24	14	8	3	25	15	6	2	>90

in trichodermin results in a substantial loss of activity, indicating again that this feature of the molecule is required for activity. However, introduction of a epoxide group at C-8 does not result in a large drop in activity in between

crotocin and trichothecene, which suggests that the nature of the ester component can influence the effect of modification at C-8. All of the compounds in Table II are inhibitors of the elongation or termination phase of protein synthesis. The compounds all have the same pattern of inhibition of the assays as trichodermin, which suggests that all the simple ester derivatives of trichodermol may be inhibitors of termination (16).

Table III compares the effect of substituents in the R1, R2, and R3 position. Comparison of trichodermol with verrucarol inidicates that introduction of a hydroxyl group on C-3 (R1 position) in scirpentriol results in a substantial increase in in vitro and in vivo activity and causes a profound change in the mechanism of action of the antibiotic. Scirpentriol and several of its acetylated derivatives inhibit the initiation of protein synthesis. Although verrucarol itself is not an inhibitor of the initiation step in protein synthesis, a number of its derivatives are. All of the verrucarins we have tested (verrucarins A, E, J, and H) are inhibitors of the initiation step in protein synthesis.

Calonectrin and 15-desacetylcalonectrin (Table III) are potent inhibitors of protein synthesis, even though they lack a hydroxyl in the R2 position. However, these compounds are very poor inhibitors of peptidyl transferase in vitro. This suggests that the presence of a hydroxyl group or an ester group on C-4 is a requirement for the inhibition of peptidyl transferase. Interestingly, these two compounds differ markedly in their ability to inhibit initiation compared to elongation and termination. Calonectrin inhibits initiation while 15-desacetylcalonectrin is an inhibitor of the elongation-termination steps. Presumably, this difference is due to the acetylation of the R3 hydroxyl group.

There appears to be two sites on the molecule which are important in determining the potency of a compound as an inhibitor of initiation. Our data enables us to refine a previous suggestion that oxygen containing substituents on C-15 are inhibitors of initiation (8). A simple hydroxyl group on C-15 is not sufficient for an inhibition of initiation as shown by verrucarol. However, the larger and more bulky diester groups between C15 and C-4 appear to be inhibitors of initiation. The

Table III: Comparison of Derivatives of the R1, R2 and R3 Groups of the 12,13-Epoxytrichothecenes as Inhibitors of Protein Synthesis

	% Activity Remaining								
	Termination assay		Peptidyl transferase assay		Polyphenylalanine synthesis assay		*in vivo* protein synthesis		% Polysomes at 90% inhibition
Control	20μg/ml 100	100μg/ml 100	5μg/ml 100	20μg/ml 100	5μg/ml 100	20μg/ml 100	1μg/ml 100	10μg/ml 100	--
Trichodermol (R1 = H, R2 = OH, R3 = H)	92	76	73	40	73	45	40	13	>90
Verrucarol (R1 = H, R2 = OH, R3 = OH)	95	85	91	67	96	94	68	63	>90
Scirpentriol (R1 = OH, R2 = OH, R3 = OH)	49	46	9	5	33	27	11	2	<10
15-Acetoxyscirpendiol (R1 = OH, R2 = OH, R3 = OAc)	33	33	19	11	22	19	9	1	<10
Diacetoxyscirpenol (R1 = OH, R2 = OAc, R3 = OAc)	36	36	26	15	38	32	4	0	<10
Verrucarin A (R1 = H, R2, R3 = Dicarboxylic acid ester: O ‖ -CCHOHCHCH3CH2CH2- O O ‖ ‖ OCCH=CHCH=CHC-)	76	51	42	30	9	8	23	0	<10
15-Desacetylcalonectrin (R1 = OAc, R2 = H, R3 = OH	99	95	99	97	93	78	71	29	100
Calonectrin (R1 = OAc, R2 = H, R3 = OAc)	100	98	93	83	64	54	48	21	<10

other important site on the molecule concerned with initiation is represented by substituents on C-3. This site lies close to the R3 group and on the same side of the molecule. Substitution of an hydroxyl group in the R1 position can lead to the inhibition of initiation.

DISCUSSION

Not all of the 12,13-epoxytrichothecenes that are active inhibitors of protein synthesis strongly inhibit the peptidyl transferase activity of the ribosomes. Certainly, based on the data in Tables I, II, III, the *in vitro* assays for protein synthesis; the termination assay, the peptidyl transferase assay, and the poly-

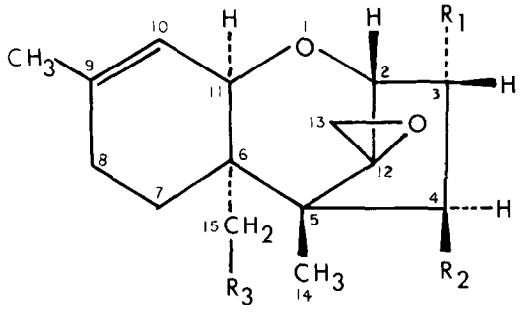

Fig. 1 General Structure of 12,13-Epoxytrichothecenes

phenylalanine synthesis assay; do not reliably distinguish between inhibitors of initiation and termination or elongation. As shown the ability of a 12,13-epoxytrichothecene to inhibit initiation depends on the presence of certain structural features in the molecule. However, the puzzle remains that these compounds are responsible for two different types of action--inhibition of initiation and inhibition of termination or elongation. Yet they have similar structures, a single binding site on the ribosome (unpublished observation) and inhibit peptidyl transferase. This difference in action cannot be explained by a concentration effect since a wide variation in the concentration of the initiation inhibitors did not affect their ability to inhibit initiation. At least two possible models can explain this difference in action. The first model proposes that these compounds all interact at the same site on the ribosome and that their ability to inhibit the peptidyl transferase reaction is in part fortuitous. They block either initiation or termination, depending on how the various substituent groups interfere with eihter the binding or the reactions of the appropriate initiation or termination factors. The alternative interpretation suggests that the compounds interfere specifically with termination or initiation because they are able to bind to the ribosome only at that particular point in the ribosome-polysome cycle and interfere with peptidyl transferase or one of the other factors required in the process. The 12,13-epoxytrichothecenes may represent a group of specific inhibitors for the different steps in the ribosome cycle. We are at present conducting experiments to distinguish between these two possibilities.

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REFERENCES

1. Joffe, A.A., in "Microbial Toxins", Vol. VII, Ed. S. Kadis, A. Ciegler, and S.J. Ajl, Academic Press, New York, 1971, pp. 139-190.
2. Yates, S.G., in "Microbial Toxins", Vol. VII, Ed. S. Kadis, A. Ciegler, and S.J. Ajl, Academic Press, New York, 1971, pp. 191-206.
3. Ueno, Y., Ueno, I., Tatsuno, T., Ohokubo, K. and Tsunoda, H., Experientia, 25, 1062 (1969).
4. Ueno, Y., Hosoya, M., Morita, Y., Ueno, I. and Tatsuno, T., J. Biochem., 64, 479 (1968).
5. Stafford, M.E. and McLaughlin, C.S., J. Cell. Physiol., 82, 121 (1973).
6. Wei, C. and McLaughlin, C.S., Federation Proceedings, 32, 494 (1973).
7. Hansen, B.S. and Vaughan, M.H., Federation Proceedings, 32, 494 (1973).
8. Cundliffe, E., Cannon, M., Davies, J., Proc. Natl. Acad. Sci., USA, in press.
9. Ohtsubo, K., Kadem, P., and Mittermayer, C., Biochim. Biophys. Acta 287, 520 (1972).
10. Lamfrom, H., McLaughlin, C.S. and Sarabhai, A., J. Mol. Biol., 22, 353 (1966).
11. Lamfrom, J. and Knopf, P.M., J. Mol. Biol., 11, 589 (1965).
12. Schreier, M.H. and Staehelin, T., J. Molec. Biol., 73, 329 (1973).
13. Beaudet, A.L. and Caskey, C.T., Proc. Natl. Acad. Sci. USA, 68, 619 (1971).
14. Caskey, C.T., Beaudet, A.L., Scolnick, E.M. and Rosman, M., Proc. Natl. Acad. Sci. USA, 68, 3163 (1971).
15. Hartwell, L.H., McLaughlin, C.S., J. of Bacteriol., 96, 1664 (1968).
16. Wei, C., Hansen, B.S., Vaughan, M.H., McLaughlin, C.S., Proc. Natl. Acad. Sci. USA, 71, (1974) in press.