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TRICHOTHECENES - LITERATURE REVIEW

by

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June 1983

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This is a broad overview of the literature on the trichothecene subset of mycotoxins.
PREFACE

The work described in this report was authorized under Project 1L162706A553, CB Defense and General Investigations, Technical Area 3-L, Toxin Defense Technology. This work was started in February 1982 and completed in June 1982.

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In addition, it is appropriate to thank Mrs. Marguerite Brooks and Dr. Emory Sarver for their helpful suggestions and support and Mr. Harold Z. Sommer for his fruitful critique.
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CONTENTS

1 INTRODUCTION ........................................... 7

2 TRICHTHOCENES - DESCRIPTIVE OVERVIEW ................ 7

2.1 Definition ........................................... 7
2.2 History ............................................. 7
2.3 Structures ........................................... 11
2.4 Physical Properties ................................... 11
2.5 Occurrence .......................................... 17
2.6 Pathological Significance .............................. 18
2.7 Relative Toxicities and Toxin Lipophilicity ............ 18
2.8 Synergistic Effects ................................... 20

3 TRICHTHOCENES - TECHNOLOGICAL ASPECTS ............. 21

3.1 Procedures for Purification ............................ 21
3.1.1 Preparation of the Media ......................... 21
3.1.2 Isolation and Purification .......................... 22
3.1.3 Extraction ......................................... 22
3.1.4 Isolation and Purification - TLC ................ 22
3.2 Methods of Analysis .................................. 24
3.2.1 Spectrophotometric ................................ 24
3.2.2 Polarography ...................................... 24
3.2.3 TLC ............................................... 24
3.2.5 Gas-Liquid Chromatography/Flame Ionization Detector 25
3.2.6 Gas-Liquid Chromatography/Electron Capture Detector 25
3.2.7 Gas Chromatography/Mass Spectrometry ............. 26
3.2.8 Fluorescent Monitoring Methods .................... 26
3.2.9 Mass Spectrometry/Mass Spectrometry .............. 27
3.3 Chemical Reactions .................................... 27
3.4 Model Compounds of the Trichothecene “Active” Site 28
3.4.1 Chemical Reactions of Epoxide Systems ............ 28
3.5 Biochemistry ......................................... 36
3.5.1 Biochemical Detection ............................. 36
3.5.2 Biotransformations ................................ 37
3.5.3 Action on Deoxyribonucleic Acid ................... 37
3.5.4 Structure-Function Relationships Based on In Vitro Studies 38

LITERATURE CITED ....................................... 39

DISTRIBUTION LIST ....................................... 49

LIST OF TABLES ........................................... 49

Table

1 Summary of Mycotoxin-Induced Toxicoses ................ 8
2 Examples of Mycotoxins Other Than the Trichothecenes 10
3 Physical Properties of Selected Trichothecenes ........ 16
4 Trichothecene-Producing Fungi .......................... 17
5 Trichothecene Contamination Levels Found in Agricultural Products 17
Table

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Trichothece-Fungus and Their Diagnosis on Laboratory Animals</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>Toxicities of Trichotheccenes</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>Intraperitoneal Toxicities</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>Detection Limits of Trichotheccenes</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>Potential Reagent-Reaction Conditions for the Trichotheccenes</td>
<td>36</td>
</tr>
<tr>
<td>11</td>
<td>Trichotheccenes and Their Stage of Protein Synthesis Inhibition</td>
<td>38</td>
</tr>
</tbody>
</table>

LIST OF FIGURES

Figure

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group A Trichotheccenes (T-2 Type)</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Group B Trichotheccenes (Nivalenol Type)</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Group C - Macrocyclic Trichotheccenes</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Group D - The Trichotheccene Crotocin</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Isobologram Showing Synergistic Effects of Mycotoxins</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>Distinction Between Trichotheccenes Based on Solubility</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Chemical Reactions of the Trichotheccenes</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>Structural Analogues of the Region Imparting Toxicity in the Trichotheccene Compounds</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>Anti-Coplanar Mode of Attack of a Nucleophile with an Epoxide System</td>
<td>35</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

During the past century, documented outbreaks of massive intoxication and death, resulting from mycotoxin-infected foodstuffs all over the world, have been recorded in the literature. The data, summarized in Table 1, show that the mycotoxin-producing fungi are pathogens found in various plants and in the fruit that they bear. These mycotoxins (from the Greek word "mykes" meaning "fungus" and the Latin word "toxicum" meaning "poison") are a threat to the well-being of humans and animals.

Whenever outbreaks of poison-producing bacteria, viruses, or fungi occur or when unusually large amounts of the poisons themselves are found in nature, whether of natural or artificial origin, a careful and complete investigation is warranted. In the last decade, the possibility of artificially induced mycotoxin threats to humans in various parts of the world has come to the attention of the general public.

The mycotoxins chosen for concentrated review in this report are the trichothecenes because the majority of the work performed to date (with the possible exception of the aflatoxins) has been associated with the trichothecenes in the agricultural, chemical, biological, and toxicological fields. However, a sampling of other naturally occurring mycotoxin compounds and a brief review of each are presented in Table 2. Due to not only the toxic properties but also the carcinogenic or therapeutic potential of the naturally occurring compounds discussed in this report, extensive scientific work has been done over the past two decades. The scientific community continues to address the problem in order to control the occurrence of the toxins in human and animal food sources, including the possibility of long-term exposure to foodstuffs.

This literature review attempts to supply a concise information source as an aid to investigators faced with problems of trichothecene detection, analysis, and decontamination.

2. TRICHOTHECENES - DESCRIPTIVE OVERVIEW

2.1 Definition.

The trichothecenes encompass a broad group of naturally occurring compounds, a subset of the mycotoxins, and they are produced by the fungal genera Fusarium, Trichoderma, Myrothecium, Stachybotrys, Cephalosporium, and Verticimonosporium.

2.2 History.

The verrucarin group, initially named glutinosin, were the first trichothecenes to be isolated (1946), whereas, it was more than a decade later (1960) before isolation of the next trichothecene, diacetoxyscirpenol. In 1967, the scirpene compounds nivalenol and fusarenon-X were isolated from Fusarium nivale by Morooka and Tatsuno. In the late 1960's, a toxic compound was isolated from F. tricinctum-infected corn. It was analyzed as T-2 toxin and it induced edema and intradermal hemorrhage on rat skin.

* Personal communication, C. J. Mirocha, December 1981
<table>
<thead>
<tr>
<th>Year</th>
<th>Toxicosis</th>
<th>Location</th>
<th>Species</th>
<th>Symptoms&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Food</th>
<th>Fungus</th>
<th>Mycotoxin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1890</td>
<td>Taumelgetreide</td>
<td>Ussuri district of Siberia, USSR</td>
<td>Man. farm animals</td>
<td>Headache, vertigo, nausea, vomiting</td>
<td>Millet, barley</td>
<td>Gibberella saubinettii, Cladosporium herbarum, Fusarium roseum</td>
<td>Trichothecces</td>
<td>1</td>
</tr>
<tr>
<td>1923</td>
<td></td>
<td>European Russia</td>
<td>Humans</td>
<td>Weakness, vertigo, headache, vomiting</td>
<td>Bread made from moldy rye</td>
<td>F. roseum</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1931-1940</td>
<td>Stachybotryotoxicosis</td>
<td>Ukraine, Hungary</td>
<td>Cattle&lt;sup&gt;c&lt;/sup&gt;, calves, humans, horses, swine, poultry</td>
<td>Shock, stomatitis, hemorrhage, leukopenia, nervous disorders, abortions, death from respiratory failure</td>
<td>Straw. oats, beans, chaff, hay</td>
<td>Stachybotrys alternans</td>
<td>Satratoxins, roridins</td>
<td>3-6</td>
</tr>
<tr>
<td>1940-1946</td>
<td>Stachybotryotoxicosis</td>
<td>Central Europe</td>
<td>Hens</td>
<td>Shock, dermal neurosis, hemorrhage, respiratory failure</td>
<td>Feed</td>
<td>S. alternans</td>
<td>Stachybotryotoxin</td>
<td>7</td>
</tr>
<tr>
<td>1942-1947</td>
<td>Alimentary toxic aleukia (ATA)</td>
<td>Orenburg, USSR</td>
<td>10% Human population</td>
<td>Vomition, skin inflammation, diarrhea, leukopenia, necrotic angina, bone marrow exhaustion</td>
<td>Overwintered millet, wheat, barley</td>
<td>F. sporotrichioides, F. poae, C. epiphyllum</td>
<td>T-2 toxin</td>
<td>8, 9</td>
</tr>
</tbody>
</table>
### Table 1. Summary of Mycotoxin-Induced Toxicoses (Cont'd)

<table>
<thead>
<tr>
<th>Year</th>
<th>Toxicosis</th>
<th>Location</th>
<th>Species</th>
<th>Symptoms&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Food</th>
<th>Fungus</th>
<th>Mycotoxin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1958-1959</td>
<td></td>
<td>Ukraine</td>
<td>Horses, cattle</td>
<td>Same as stachybotryotoxicosis</td>
<td>Straw, hay,</td>
<td>S. alternans</td>
<td>Stachybotryotoxins A and B, Satratoxin</td>
<td>10</td>
</tr>
<tr>
<td>1960-1975</td>
<td>Bean hull poisoning</td>
<td>Hokkaido, Japan</td>
<td>Horses</td>
<td>Convulsion, cyclic movement, disturbed respiration, decrease in heart rate</td>
<td>Bean hulls</td>
<td>F. solani</td>
<td>Neosolanil, T-2 toxin</td>
<td>11,12</td>
</tr>
<tr>
<td>1971</td>
<td>Moldy corn toxicosis</td>
<td>Wisconsin</td>
<td>Cattle</td>
<td>Hemorrhage</td>
<td>Corn</td>
<td>F. tricinctum</td>
<td>T-2 toxin</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Dendrodochium toxicosis</td>
<td>Russia</td>
<td>Horse, sheep, pigs</td>
<td>Nervous disorder, internal hemorrhage</td>
<td>Feedstuffs</td>
<td>Dendroechium toxicum</td>
<td>Macroyclic roridins and verrucarins</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Akakabihyo (red-mold) poisoning</td>
<td>Japan</td>
<td>Humans, animals</td>
<td>Vomiting, diarrhea, refusal of feed, hemorrhage of various bodily organs</td>
<td>Wheat, barley,</td>
<td>F. nivale</td>
<td>Nivalenol fusarenon-X</td>
<td>15-17</td>
</tr>
</tbody>
</table>

<sup>a</sup> Additionally, necrotization of the skin is a common symptom of most trichothecene toxicoses in animals. Most toxins also induce feed refusal in animals, but this could be due to inflammation of the oral cavity and gastrointestinal tract.<sup>9</sup>

<sup>b</sup> For a more complete account of the various toxicoses, the reader is referred to the review by Draper.<sup>187</sup>

<sup>c</sup> For cattle, it was found that stachybotryotoxicosis is enhanced with an enrichment of the feed with either carbohydrates or acid silage. On the other hand, their alkaline saliva inhibited the stachybotrys toxin as opposed to that of horses.<sup>188,189</sup>

<sup>d</sup> For swine, it was shown<sup>190,191</sup> that the addition of iron into the red blood cells is inhibited by the toxin.
<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Structure</th>
<th>Occurrence</th>
<th>Fungus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B</td>
<td><img src="image1" alt="Structure" /></td>
<td>Edible nuts (especially peanuts)</td>
<td>Aspergillus flavus Link A. parasiticus Speare</td>
<td>22</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td><img src="image2" alt="Structure" /></td>
<td>Coffee beans</td>
<td>A. ochraceus</td>
<td>22</td>
</tr>
<tr>
<td>Patulin</td>
<td><img src="image3" alt="Structure" /></td>
<td>Grains</td>
<td>Penicillium palitans P. viridicatum</td>
<td>22</td>
</tr>
<tr>
<td>Penicilllic acid</td>
<td><img src="image4" alt="Structure" /></td>
<td>Hay, pork, poultry</td>
<td>Aspergillus and Penicillium species</td>
<td>22</td>
</tr>
<tr>
<td>Rubratoxin</td>
<td><img src="image5" alt="Structure" /></td>
<td>Grains</td>
<td>P. purpureum</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corn, fermented sausage</td>
<td>P. rubrum A. flavus</td>
<td>22</td>
</tr>
</tbody>
</table>
2.3 Structures.

The over 47 naturally occurring trichothecenes as a group have a number of elements in common. The most obvious is the trichothecan ring system. In fungi, the trichothecenes are derived from a cyclization of the precursor molecule farnesyl pyrophosphate, involving a 1,2-dimethyl double migration.

They belong to a family of sesquiterpenoids (i.e., one and one-half terpene units in the A-ring of the trichothecenes) (figure 1) with ester and alcohol functions residing on the periphery of the toxin molecules. They also have a C-9,10 double bond and an epoxy group at C-12,13; hence, they are usually described as 12,13 epoxytrichothecenes.

Various modes of trichothecene classification are employed in the literature. Figures 1 through 4 present the toxins and their chemical-structural relationships.

The trichothecenes are arbitrarily classified into the four groups: A, B, C, and D. The group A trichothecenes (figure 1, A and B) have a hydroxyl or acetoxy function at R1, R3, R4, and R5. Figure 1, B, depicts the stereochemical structure of a typical group A trichothecene, diacetoxyscirpenol, with the α-face being above the molecular plane and the β-face being below. Note that the six-membered B ring has a chair conformation and the five-membered C ring adopts an envelope form with carbon-12 as the flap. Also, the R2 acetyl carbonyl oxygen hydrogen-bonds (dashed line in figure 1, B) with the α-hydrogen of carbon-3 forming a six-membered ring. The group B trichothecenes (figure 2) contain a carbonyl at R5 (C-8) in addition to the group A functions at R1, R2, R3, and R4. The macrocyclic trichothecenes (figure 3) comprise group C, attaching their broad-based, carbon-oxygen ring system to the R2 and R3 positions of the basic trichothecene structure. Figure 3 portrays examples of the more than 30 known macrocyclic members.

Crotocin (figure 4), a nonmacro cyclic, diepoxy trichothecene is in a class by itself, group D. Groups A, B, and D comprise the simple ester and alcoholic trichothecenes; whereas, group C encompasses the macrocyclic compounds.

Trichothecene structure and absolute configuration determinations were first resolved for trichothe cole, trichodermol, and verrucarol. The primary analytical techniques used were X-ray diffraction of the p-bromobenzoate of trichodermol and nuclear magnetic resonance (NMR) spectroscopy. By inference, the three-dimensional structures of the other simple trichothecenes were assumed. The determination of three-dimensional structures of the macrocyclic molecules commenced with verrucarin A, followed by baccharin and then verrucarin B and roridin D.

2.4 Physical Properties.

The trichothecenes in general are colorless, crystalline, optically active, and stable in the solid state. Presented in table 3 are some physical properties of selected trichothecenes. The trichothecenes listed can be stored at room temperature for years or heated at 100°C for up to 1 hour with no loss of activity. A concise compendium of trichothecenes and other mycotoxin physical constants and spectral data can be found in the handbook by Cole and Cox.
A. T-2 Type

![Chemical structure of T-2 Type]

<table>
<thead>
<tr>
<th>Trichothecene</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic Trichothecene</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Trichodermol (roridin C)</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Trichodermin</td>
<td>H</td>
<td>OAc</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Verrucarol</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Scirpentriol</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Monoacetoxysscirpenol (MAS)</td>
<td>OH</td>
<td>OH</td>
<td>OAc</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Diacetoxysscirpenol (anguidine) (DAS)</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>7-Hydroxy DAS</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Calonectrin</td>
<td>OAc</td>
<td>H</td>
<td>OAc</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>15-Diacetylcalonectrin</td>
<td>OAc</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Dihydroxy trichothecene</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>T-2 tetraol</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Neosolaniol (solaniol)</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Monoacetylneosolaniol</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>OAc</td>
</tr>
<tr>
<td>7,8-Dihydroxy DAS</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>OH</td>
<td>OH</td>
<td>OAc</td>
<td>H</td>
<td>b</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>b</td>
</tr>
<tr>
<td>Acetyl T-2 toxin</td>
<td>OAc</td>
<td>OAc</td>
<td>OAc</td>
<td>H</td>
<td>b</td>
</tr>
</tbody>
</table>

*OAc, acetate

B. The Stereochemical Structure of Diacetoxysscirpenol

![Chemical structure of Diacetoxysscirpenol]

Figure 1. Group A Trichothecenes (T-2 Type)
<table>
<thead>
<tr>
<th>Trichothecene</th>
<th>R(_1)</th>
<th>R(_2)</th>
<th>R(_3)</th>
<th>R(_4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nivalenol</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>MonoacetylNivalenol (fusarenon-X)</td>
<td>OH</td>
<td>OAc</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>DiacetylNivalenol (DAN)</td>
<td>OH</td>
<td>OAc</td>
<td>OAc</td>
<td>OH</td>
</tr>
<tr>
<td>Deoxynivalenol (DON) (vomitoxin)</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Monoacetyl DON</td>
<td>OAc</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Diacetyl DON</td>
<td>OAc</td>
<td>H</td>
<td>OAc</td>
<td>OH</td>
</tr>
<tr>
<td>Trichothecin</td>
<td>H</td>
<td>*</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Trichotheclone</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
</tbody>
</table>

\(\text{O} \quad \text{O} - \text{C} - \text{CHCH}_3, \text{isocrotonate.}\)

Figure 2. Group B Trichothecenes (Nivalenol Type)
Figure 3. Group C – Macro cyclic Trichothecenes
Figure 4. Group D – The Tricothecene Crotocin
Table 3. Physical Properties of Selected Trichothecenes

<table>
<thead>
<tr>
<th>Trichothecone</th>
<th>State or shape</th>
<th>Recrystallizing medium</th>
<th>Melting point</th>
<th>[d]$<em>{25}^{a}$ D$</em>{solvent}$</th>
<th>ε$<em>{solvent}^{b}$ λ$</em>{max}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>Needles</td>
<td>Ethylacetate and petroleum ether</td>
<td>151-153</td>
<td>+6.35 EtOH</td>
<td>4,500 EtOH 218</td>
<td>42,43</td>
</tr>
<tr>
<td>DON triacetate</td>
<td>Colorless needles</td>
<td>Ethylacetate and petroleum ether</td>
<td>155-157</td>
<td></td>
<td></td>
<td>42,43</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>Crystals</td>
<td>Methanol</td>
<td>222-223</td>
<td>+21.5 EtOH</td>
<td>7,500 MeOH 218</td>
<td>44,45</td>
</tr>
<tr>
<td>DON monoacetate</td>
<td></td>
<td>Ether and n-pentane</td>
<td>185.5-186</td>
<td>+430 MeOH</td>
<td>5,900 EtOH 219</td>
<td>42,43,46</td>
</tr>
<tr>
<td>Diacetyl DON</td>
<td></td>
<td>Ethanol</td>
<td>119-120</td>
<td></td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>White needles</td>
<td>Benzene</td>
<td>151-152</td>
<td>+15 EtOH</td>
<td></td>
<td>31,47,48</td>
</tr>
<tr>
<td>Satratoxin G</td>
<td></td>
<td></td>
<td>167-170</td>
<td></td>
<td>6,500 MeOH 256</td>
<td>49</td>
</tr>
<tr>
<td>Satratoxin H</td>
<td></td>
<td></td>
<td>162-166</td>
<td></td>
<td>10,400 MeOH 220</td>
<td>49</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>Yellow oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>Fusarenon-X</td>
<td>Hexagonal</td>
<td>CH$_2$Cl$_2$ and n-pentane</td>
<td>91-92</td>
<td>+58 MeOH</td>
<td>6,500 MeOH 220</td>
<td>52,53</td>
</tr>
<tr>
<td>DAN</td>
<td>Crystals</td>
<td>Acetone and hexane</td>
<td>135-136</td>
<td>+64.3 EtOH</td>
<td>6,200 MeOH 220</td>
<td>54,55</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>Crystals</td>
<td>Ethylacetate and n-hexane</td>
<td>171-172</td>
<td></td>
<td></td>
<td>56</td>
</tr>
<tr>
<td>DAS</td>
<td>Crystals</td>
<td>Ether</td>
<td>162-164</td>
<td></td>
<td></td>
<td>57,58</td>
</tr>
<tr>
<td>MAS</td>
<td>Crystals</td>
<td>Isooctane and ethyl acetate</td>
<td>172-173</td>
<td></td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>Baccharin</td>
<td>Crystals</td>
<td>CH$_2$Cl$_2$ and MeOH</td>
<td>200-230</td>
<td>+41.5 CHCl$_3$</td>
<td>18,700 EtOH 259</td>
<td>40</td>
</tr>
</tbody>
</table>

$^a$ The specific optical rotation at 25°C using the sodium D line

$^b$ The molar extinction coefficient of the compound as measured at the listed wavelength in the given solvent

$^c$ . . . absorption, <230 mm
2.5 Occurrence.

The production of these toxins by fungi is somewhat diverse. Each of the species produces a number of toxins as their natural metabolites as listed in table 4. \(^3\), \(^34\), \(^61\)-\(^64\) The fungi themselves are found all over the world in agricultural products and plants. Table 5 lists the results of studies of toxin concentrations in different agricultural samples. \(^13\), \(^42\), \(^65\), \(^66\)

<table>
<thead>
<tr>
<th>Trichothecene and their sources</th>
<th>A</th>
<th>B</th>
<th>Group</th>
<th>A and B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarium tricinctum</td>
<td>T-2 toxin</td>
<td>Nivalenol</td>
<td>DAS</td>
<td>DAN</td>
<td>Roridins</td>
</tr>
<tr>
<td>F. roseum</td>
<td>HT-2 toxin(^b)</td>
<td>Fusarenon-X</td>
<td></td>
<td>7-Hydroxy DAS</td>
<td>Satratoxins</td>
</tr>
<tr>
<td>F. sporotrichoides</td>
<td>Fusarone-2</td>
<td>Neosolaniol</td>
<td></td>
<td>7,8-Hydroxy DAS</td>
<td>Verrucarins</td>
</tr>
<tr>
<td>F. poae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. solani</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) References 31, 34, 61-64

\(^b\) A metabolite

Table 5. Trichothecene Contamination Levels Found in Agricultural Products

<table>
<thead>
<tr>
<th>Trichothecene</th>
<th>Sample</th>
<th>Place</th>
<th>Yield</th>
<th>Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-2 toxin</td>
<td>Corn</td>
<td>Wisconsin</td>
<td>2 mg/kg</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>DON</td>
<td>Corn</td>
<td>Ohio</td>
<td>3 mg/kg</td>
<td></td>
<td>65</td>
</tr>
<tr>
<td>DON</td>
<td>Corn</td>
<td>Illinois</td>
<td>8 mg/kg</td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>DON</td>
<td>Corn</td>
<td>Indiana</td>
<td>8 ppm</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>DON</td>
<td>Barley</td>
<td>Kagawa, Japan</td>
<td>4 ppm</td>
<td></td>
<td>42</td>
</tr>
</tbody>
</table>

It is interesting to note that the triepoxy trichothecene baccharin, isolated from *Baccharis megapotamica* Spreng (Asteraceae), was the first and only trichothecene to have been isolated in higher plants without the fungus responsible for its production.\(^40\) The yield of the macrocyclic trichothecene was 0.02% (w/w) from the dried plant which is high enough to kill tomatoes, peppers, and artichokes.\(^67\)
2.6 Pathological Significance.

Mycotoxin infestation occurs in basic foodstuffs, e.g., oats, hay, rye, and corn (table 1). These foodstuffs, in turn, are ingested by farm animals and humans and if toxin elimination methods (chemical or physical) are not undertaken, dire consequences can result. Various symptoms range from headache, nausea, and vomiting to death. Table 6 summarizes a few of the experiments that were undertaken in order to assess the effects of different trichothecenes and their fungal sources on laboratory animals. Their acute pathology resembles that caused by radiation (radiomimetic agents) or of alkylating agents such as nitrogen mustard. No obvious symptoms relating to carcinogenic effects could be seen. Further corroborating evidence of a noncarcinogenic effect was shown by using T-2 toxin in long-term, low-dose feedings in rainbow trout. However, Nagao has found fusarenon-X to be mutagenic to bacteria.

Table 6. The Effects of Trichothecenes/Fungi on Laboratory Animals

<table>
<thead>
<tr>
<th>Animal</th>
<th>Trichothecene</th>
<th>Fungus</th>
<th>Mode of application</th>
<th>Diagnosis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats, mice</td>
<td>F. nivale</td>
<td>Oral – feed</td>
<td>Atrophy of thymus, spleen, bone marrow, and testicles; bronchopneumonia</td>
<td>68,69</td>
<td></td>
</tr>
<tr>
<td>Rats, mice</td>
<td>Fusarenon-X</td>
<td>Oral</td>
<td>Same as above</td>
<td>68,69</td>
<td></td>
</tr>
<tr>
<td>Rats</td>
<td>T-2 toxin</td>
<td>Oral – feed</td>
<td>Lesions and extreme papillary growth inside the gastrointestinal tract</td>
<td>12,70</td>
<td></td>
</tr>
</tbody>
</table>

2.7 Relative Toxicities and Toxin Lipophilicity.

Determination of the cause of the toxicoses such as those presented in table 1 was accomplished by the isolation and identification of the toxin(s) by reproducing the illness in different experimental animals and arriving at toxic doses of the trichothecene poisons.

Bamburg and Strong and Ueno et al. performed skin irritability experiments on the shaved backs of guinea pigs, mice, and rabbits with the results that the group A toxins were ten times as effective as the group B toxins. Antiprotozoal activity (Tetrahymena pyriformis GL) studies were undertaken in which it was found that the group A toxins are collectively 50 times as toxic as the group B toxins, T-2 and DAS being ten times as effective as HT-2 and neosolaniol; whereas, the latter two are approximately 20 times as effective as the group B compounds. In the cytotoxicity experiments utilizing the inhibition of protein synthesis assay in rabbit reticulocytes (vide infra), the verrucarins and roridins (group C) are three times as potent as the group A toxins; whereas the latter is approximately 10 times as toxic as the group B compounds (table 7).
Table 7. Toxicities of Trichothecenes

<table>
<thead>
<tr>
<th>Assay</th>
<th>Group</th>
<th>Number of lipophilic R-functions</th>
<th>Trichothecenes</th>
<th>MED and ID50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Guinea pigs</td>
</tr>
<tr>
<td>Skin necrotization</td>
<td>A</td>
<td>3,2</td>
<td>T-2, HT-2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAS, neosolaniol</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1,2</td>
<td>Fusarenon-X, DAN</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nivalenol</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MED&lt;sup&gt;a&lt;/sup&gt; (µg/spot)</td>
<td></td>
</tr>
<tr>
<td>Protozoal</td>
<td>A</td>
<td>3,2</td>
<td>T-2, DAS</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HT-2, neosolaniol</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>2</td>
<td>DAN</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fusarenon-X, DON</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nivalenol</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DON monoacetate</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ID50&lt;sup&gt;b&lt;/sup&gt; (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>C</td>
<td>0,0</td>
<td>Verrucarin A, roridin A</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>3,2,2</td>
<td>T-2, HT-2, DAS</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Neosolaniol</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>2,1,1</td>
<td>DAN, fusarenon-X, trichothecin</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nivalenol, DON</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Trichothecolone</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ID50&lt;sup&gt;c&lt;/sup&gt; (µg/ml)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Minimum effective dose  
<sup>b</sup> Dose for 50% inhibition of protozoal multiplication  
<sup>c</sup> Dose for 50% inhibition in the “whole cell” assay system

The above results lead one to conclude that in organisms, since group A is more toxic than group B, greater activity is achieved when the R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub> functions are esterified. Within group A, the reduced toxicity of neosolaniol, in all three experimental cases, can be ascribed to the lack of the methylbutyryl group which T-2 toxin possesses. Increased toxicity is realized by acetyllating the side groups of nivalenol to fusarenon-X and DAN.

Hence, increasing the lipophilicity upon esterification results in a concomitant increase in toxic activity. Work done in cell cultures<sup>78</sup> and chick embryos<sup>79</sup> reinforces this belief. This trend is observed in groups A and B, both collectively and separately (table 7).

Inspection of the results of intraperitoneal (ip) studies presented in table 8 indicates that the acute toxicity of the macrocyclic compounds is greater than that observed in the A- and B-group trichothecenes, and slightly higher oral toxicity exists over an ip administration with most of the trichothecenes. However, two further noteworthy points which are in conflict with the data in table 7 are: (1) The hydroxyl group at carbon-4 (R<sub>2</sub>) of the trichothecene nivalenol imparts greater or similar toxicity in comparison to its esterified forms and (2) within each of the two toxin series, less lipophilicity generally imparts greater toxicity.
### Table 8. Intraperitoneal Toxicities

<table>
<thead>
<tr>
<th>Group</th>
<th>Trichotheccene</th>
<th>Acetate location</th>
<th>LD50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mg/kg</td>
</tr>
<tr>
<td>C</td>
<td>Verrucarins, roridins</td>
<td>-</td>
<td>0.50</td>
</tr>
<tr>
<td>A</td>
<td>T-2 toxin</td>
<td>-</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>HT-2 toxin</td>
<td>-</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>Neosolaniol</td>
<td>-</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>DAS</td>
<td>-</td>
<td>23.0</td>
</tr>
<tr>
<td>Bc</td>
<td>DON</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>3-Acetyl DON</td>
<td>R₁</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>3,15-Acetyl DON</td>
<td>R₁,R₃</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>Nivalenol</td>
<td>-</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>4-Acetyl nivalenol</td>
<td>R₂</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>(fusarenon-X)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4,15-DAN</td>
<td>R₂,R₃</td>
<td>9.6</td>
</tr>
</tbody>
</table>

*a Dose for 50% of the mice to succumb.

*b Dose for 50% of the 1-day-old broiler chicks to succumb (group A) and dose for 50% of the mice to succumb (group B). Chi, M. S., Robison, T. S., Mirocha, C. J., and Reddy, K. R. Appl. Env. Microbiol. 35, 636 (1978).


#### 2.8 Synergistic Effects.

Lindenfelser et al.\(^70\) performed experiments showing the synergistic effects of mixtures of different mycotoxins. Toxins were introduced into female mice (ip). A graph of similar parameters (isobologram) was constructed in order to depict the response of T-2 toxin when combined with aflatoxin B (figure 5). The dashed line shows what LD50 values would be expected, assuming an additive response. The solid curve represents the experimentally observed synergistic effect that occurs with the two mycotoxins. The isobologram shows that one toxin greatly enhances the other. A similar effect is also observed with the mycotoxins ochratoxin and penicillic acid.\(^80\)

Wogan et al.\(^81\) studied the combined effects of rubratoxin B and aflatoxin B\(_1\). One group of rats was fed three times a week with rubratoxin B. Another group was fed aflatoxin B\(_1\). A third group was fed the two mycotoxins simultaneously. No fatalities were observed when the mycotoxins were administered independently. A high mortality rate resulted from simultaneous feeding of the two mycotoxins. An additive as opposed to a synergistic effect was noticed by Ohtsubo et al.\(^82\) when mice were fed fusarenon-X and penicillic acid simultaneously.
3. TRICHOTHECENES - TECHNOLOGICAL ASPECTS

3.1 Procedures for Purification.

3.1.1 Preparation of the Media.

A problem common to the toxin-producing fungi exists in the difference in the storage conditions of the foodstuffs that they infect. Depending on the environmental and nutrient conditions, varying amounts of toxin can be produced in addition to the generation of different types of toxins. For example, in the laboratory, *F. nivale* produces fusarenon-X on a peptone liquid medium while on a moistened rice medium, greater amounts of nivalenol are produced.\(^3\) *F. tricinctum* produces DAS and T-2 at 8°C; whereas, at 25°C, a preponderance of HT-2 is produced.\(^3\) This is observed also in field conditions where the fungus can easily produce different toxins depending on the locale. Also, by changing conditions such as substrate, humidity, temperature, light intensity, and duration of culture, the same toxic fungus strain can produce different compounds in different yields.\(^4\)

A complete account of an investigation is reported in the literature by Joffe.\(^4\) It includes obtaining *Fusarium sporotrichioides* 921 (directly involved in the fatal ATA cases), growing the monoconidial fungal cultures, and isolating and purifying T-2 toxin, which was responsible for the ATA deaths. Particular care was observed concerning the temperature and length of time associated in growing quantities of the fungal species on potato-dextrose agar.
Another experiment, which was designed to investigate the potential of trichothecene production on 27 different kinds of commercially available foods, consisted of flow charts from fungal growth, under varied conditions, to thin-layer-chromatography (TLC) purification. From the analyzed foods, only pepper seed, cinnamon, and roasted coffee beans inhibited fungal growth and, therefore, toxin production. The implication is that the foods might contain toxin-inhibitory compounds.

3.1.2 Isolation and Purification.

The trichothecenes can be classified as belonging to either group I or group II depending on their solubility in various solvents. Group I (figure 6, A) toxins are very soluble and, hence, efficiently extractable from mixtures in aprotic solvents such as chloroform, methylene chloride, ethyl acetate, diethyl ether, and acetone. Group II toxins (figure 6, B), due to their greater abundance of polar side groups, are soluble in such solvents as ethanol, methanol, water, aqueous methanol, and aqueous acetonitrile.

It was found that the solvent system of choice for the extraction of a mixture of group I and group II compounds is ethyl acetate and acetonitrile. The drawback to this procedure is the extensive purification needed to remove the ethyl acetate from the group I fraction.

3.1.3 Extraction.

Separation techniques of the trichothecenes from their extracts consist of different liquid/liquid or solid/liquid partitions. Some liquid/liquid systems in use are a 1:1 (v/v) solution of 50% MeOH/ethyl acetate-CHCl₃ and a solution of aqueous MeOH/petroleum ether (60°-70°C). Both systems are currently in use in the extraction of T-2 toxin. A mixture of H₂SO₄ (0.8 N)/ethyl acetate is in use for DAS and a 1:1 (v/v) solution of acetonitrile/petroleum ether (60°-70°C) can be conveniently used in the separation of MAS, DAS, or T-2 toxin. Other frequently used extraction solvents and partition systems for trichothecene compounds can be found in a previous report.

Subsequent isolation of the toxins from the solvents should be approached with caution if chemical methods are to be used. For example, a weak base (5% NaHCO₃) is the choice over a strong base (NaOH) in the extraction of vomitoxin from a methanol/acetone system because of the α, β-ene reactivity of the toxin.

The solid/liquid system has been used with success in the purification of trichothecene crude extract mixtures. Some of the systems used were silica gel with chloroform/methanol (97:3) for fusarenon-X, ferric gel with aqueous acetonitrile for T-2 toxin and DAS, and charcoal with methanol for a majority of the trichothecenes.

3.1.4 Isolation and Purification – TLC.

Extensive analysis of toxin mixtures by TLC has been done yielding qualitative and quantitative information on the types and concentrations of the toxins present in the sample. However, the main drawback of this technique is the interference of other substances (lipophilic substituents) in a complex mixture. Szathmary et al. and Mirocha and Pathre circumvented this problem by multiple TLC analyses. Virtually no movement of the trichothecenes is observed on a silica gel developed in petroleum ether-diethyl ether-glacial acetic acid (70:30:2) while various lipids and pigments...
A. Representative Group I Trichothecenes – Soluble in Aprotic Solvents

\[
\begin{align*}
\text{T-2 toxin} & : & \text{OA}c & & \text{OA}c & & \text{Isovalerate} \\
\text{HT-2 toxin} & : & \text{OH} & & \text{OA}c & & \text{Isovalerate} \\
\text{Neosolaniol} & : & \text{OA}c & & \text{OA}c & & \text{OH} \\
\text{DAS} & : & \text{OA}c & & \text{OA}c & & \text{H} \\
\text{MAS} & : & \text{OH} & & \text{OA}c & & \text{H} \\
\text{Trichothecin} & : & \text{H} & & \text{H} & & \text{H}
\end{align*}
\]

B. Representative Group II Trichothecenes – Soluble in Protic Solvents

\[
\begin{align*}
\text{T-2 tetraol} & : & \text{OH} & & \text{OH} & & \text{H} & & \text{OH} \\
\text{Scirpentriol} & : & \text{OH} & & \text{OH} & & \text{H} & & \text{H} \\
\text{DON} & : & \text{H} & & \text{OH} & & \text{OH} & & =\text{O} \\
\text{Fusarenon-X} & : & \text{OA}c & & \text{OH} & & \text{OH} & & =\text{O} \\
\text{Nivalenol} & : & \text{OH} & & \text{OH} & & \text{OH} & & =\text{O}
\end{align*}
\]

Figure 6. Distinction Between Trichothecenes Based on Solubility
are cleanly and effortlessly removed. Using a different solvent system, a second chromatogram can be produced. The sensitivity of this technique is limited to approximately 0.1 \, \mu g \text{ and } 1.0 \, \mu g \text{ for the group I and group II trichothecenes, respectively.}

Other procedures, including mass fungal growth on solid and in liquid media and isolation and purification of the toxins involved, are described by Mirocha et al.\textsuperscript{,*} Bamburg and Strong.\textsuperscript{51} Kamimura et al.,\textsuperscript{97} Ishii et al.,\textsuperscript{98} Stahr et al.,\textsuperscript{99} Scott,\textsuperscript{100} and Josefsson and Moller.\textsuperscript{101}

3.2 Methods of Analysis.

Spectroscopic methods of trichothecene detection are not available chiefly due to the lack of chromophores on the molecule. Hence, fluorometric, spectrophotometric, and colorimetric analyses cannot be done.

3.2.1 Spectrophotometric.

A spectrophotometric assay for the trichothecenes was devised by reacting them with chromotropic acid.\textsuperscript{102,103} The absorbance of the resulting solution was measured at 583 nm. Fusarenon-X and T-2 toxin could be determined in the 1- to 20-\mu g range in a 20-\mu l sample.

3.2.2 Polarography.

Detection of trichothecin was accomplished using polarography at the 0.2-\mu g/ml level.\textsuperscript{103} However, to circumvent spectral limitations, one routinely turns to several chromatographic determinations.

3.2.3 TLC.

The simplest quantitative analysis is by using TLC. The resulting spots are visualized by exposing the plate either to iodine vapor or concentrated sulfuric acid spray, heating to approximately 105°C, and noting the blue fluorescence under longwave UV light (\approx 356 nm). By spraying the plates with either a concentrated sulfuric acid or p-anisaldehyde solution, the trichothecenes exhibit characteristic colors. The group B toxins, because of their \alpha,\beta-enone system, produce a nonfluorescent brown spot.\textsuperscript{64} Naoi et al.\textsuperscript{104} has found that by spraying a TLC plate with 50% AlCl\textsubscript{3} and heating, fusarenon-X reacts with ZrO(NO\textsubscript{3})\textsubscript{2} in the presence of ethylene diamine to produce an adduct in which 25 ppb can be detected by fluorescence.

Recently three new and more sensitive TLC techniques have been developed.

1. The first procedure utilizes the fact that the pyridine nitrogen of the compound 4-(p-nitrobenzyl) pyridine (NBP or DB-3) attacks epoxide moieties and conjugates with the C-12 portion of the trichothecene epoxide.\textsuperscript{105} The resulting blue spot observed on the plate is then analyzed by spectrophotodensitometry. Linear standard curves of group A and B trichothecenes are attained at levels of 0.05 to 10 \, \mu g/spot. Positive results are realized with this procedure as opposed to negative results from the application of reagents such as Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}, MgCl\textsubscript{2}, HCl, and picric acid. Its specificity is defined by negative NBP results with trichothecene-like compounds lacking the epoxide ring.

* Personal communication, C. J. Mirocha, December 1981
2. The second procedure, a sensitive and specific fluorometric one, is based on alkylation using the reagent nicotinamide.\(^{106,107}\) Briefly, the epoxide-containing compound is added to a solution of nicotinamide, a ketone (e.g., acetophenone) and alcoholic KOH. The fluorescent species is subsequently generated with the addition of formic acid. It is approximately 100 times as sensitive as the NBP method in that 0.1 to 2.0 ng of epoxy compounds can be detected. Another desirable property of this technique is that it is performed under ambient conditions versus the requisite one-half hour heating at 150\(^\circ\)C for the NBP analysis.

3. The third technique, continuous multiple-development high-performance TLC (HPTLC),\(^{108}\) provides an extremely sensitive and yet rapid screening method without prior sample derivatization. Thirteen UV-absorbing, short wavelength VIS fluorescing mycotoxin compounds (not including the trichothecenes) were successfully separated by Lee et al.\(^{109}\) Detection limits were in the nanogram and picogram range for UV-VIS and fluorescence detection methods, respectively. For nonfluorescing species (e.g., the trichothecenes) various sprays (\textit{vide supra}) can be used in certain plate development stages to induce sample fluorescence and therefore detection.

3.2.4 NMR and IR.

NMR and IR absorption are routinely used in the detection and identification stages of analysis.

3.2.5 Gas-Liquid Chromatography/Flame Ionization Detector.

Gas-liquid chromatography (GLC) is routinely utilized in the identification of a mixture of trichothecenes. In an analysis, the derivatization of the hydroxyl functions is vital so as to impart sample volatility so that separation and hence identification of the toxins in the mixture may be accomplished. Sample analyses are routinely performed using a flame ionization detector (FID). A few examples of derivatization and GLC analyses follow. Various agents were reacted with a mixture of N-trimethylsilylimidazole (SIM) and trimethylchlorosilane (TMCS) in pyridine which resulted in the complete silylation of the group II toxins. Satisfactory retention times resulted in chromatographic peaks of good resolution. Incomplete silylation of group II toxins occurred with the use of bis(trimethylsilyl)acetamide (BSA) or hexamethyldisilazane.\(^{110}\) Mirocha et al.\(^{90}\) found that a 3:2:3 mixture of BSA, TMCS, and SIM ensured complete silylation of DON in the absence of solvent. Ohta et al.\(^{111}\) accomplished the separation of a mixture of seven group I and four group II toxins by the judicious choice of proportionate amounts of silylating agents and application of the derivatized toxins onto a column packed with 1.5% OV-17. The silylation reagent, composed of a 5:1:5 mixture of BSA, TMCS, and SIM (compare with the data of Mirocha et al.\(^{90}\)) completely derivatized the toxins in 5 minutes at room temperature.

The group I toxins themselves can be analyzed by derivatizing them with N-methylbis(trifluoroacetamide), (CF\(_3\)CO)\(_2\)NCH\(_3\),\(^{91}\) provided that interfering compounds from the original extract are satisfactorily removed.

3.2.6 Gas-Liquid Chromatography/Electron Capture Detector.

Another GLC method utilizes the electron capture detector (ECD). A number of research groups\(^{112-115}\) have used the GLC/ECD technique in the detection of the trichothecenes. By using either the SIM/TMCS or heptafluorobutyric acid derivatizing agents, detection limits of approximately 5 pg and 400 pg of a number of the group II and group I toxins, respectively, have been obtained.
However, by derivatizing the sample with more efficient electrophoric silyl functions and using the ECD mode, greater sensitivity is observed than with a sample derivatization using an alkylsilyl function and detected by flame ionization. Various research groups using this technique have reported picogram to femtogram limits of sample detection. Summaries of these research efforts follow.

Poole et al.\textsuperscript{116-121} have prepared flophemesyl (pentafluorophenyldimethylsilyl) derivatizing reagents which react with a handful of functionalities in compounds. Although the reagent does not react with epoxide and ether moieties, susceptible groups are alcohols, phenols, carboxylic acids, and amines, which make this reagent a fairly specific label. Greater specificity can be given to individual functionalities by using certain flophemesyl and pentafluorophenyl derivatives.

The pentafluorophenyl moiety exhibits electrophoric properties which make this a convenient probe in the GLC/ECD technique. The sensitivity of the electron capture detector to flophemesyl-derivatized compounds is on the order of picogram to femtograms. Together with hydrolyzing the trichothecene epoxide group to a diol moiety and the inherent hydroxyl groups in the toxins, the flophemesyl-GLC/ECD combination appears to have a potential application in trichothecene detection.

Greater specificity can be attained by using either the substituted benzeneboronic acids (BB)\textsuperscript{116,122-124} or ethylphosphonothioic dichloride (EPTD)\textsuperscript{125} as derivatizing agents. They both react with bifunctional compounds wherein both functions are either OH, NH\textsubscript{2}, or COOH separated by up to two methylene units. Picogram detection is realized with BB derivatization; and, by using a phosphorus detector with the EPTD reagent instead of a thermal electron detector, femtogram quantities can be attained.

Using GLC/ECD, Corkill et al.\textsuperscript{126} have presented experimental evidence of approximately \(10^{-17}\) gm (90 attograms) detection with a signal-to-noise ratio of 2 of a new compound that has the potential to act as a derivatizing reagent. The compound, N,N-dipentafluorobenzoylpentafluoroaniline (DPPA) is essentially a modified version of N-methyl-bis(trifluoroacetamide) with flophemesyl features.

### 3.2.7 Gas Chromatography/Mass Spectrometry

Even though GLC/FID is a sensitive tool, it was found by Mirocha et al.\textsuperscript{127} that three samples of feed analyzed in different laboratories resulted in negative T-2 toxin diagnosis using gas chromatography/mass spectrometry (GC/MS) when a GLC technique gave false positive findings of the toxins. This usually occurs at the limit of detection level and is compounded by the problem of a moderate amount of interfering lipid substances that co-chromatograph with the toxin from the sample. Because a normal GC/MS analysis can circumvent this problem, it provides more reliable information with greater sensitivity. The selected ion monitoring spectrometry\textsuperscript{90} (SIMS) GC/MS technique produces an even greater resolution of the trichothecene derivatives because it can be used in detecting single or multiple ion signals. Table 9 gives a sampling of the resolution one can obtain using the previously stated methods of trichothecene isolation and identification.

### 3.2.8 Fluorescent Monitoring Methods

Various chromatographic techniques coupled with sample-derivatized, fluorescence monitoring methods have been reported in the literature. One technique involves the usage of high
Table 9. Detection Limits of Trichothecenes

<table>
<thead>
<tr>
<th>Trichothecene</th>
<th>GC/MS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GLC&lt;sup&gt;a&lt;/sup&gt;/FID, μg/μl injection</th>
<th>TLC&lt;sup&gt;b&lt;/sup&gt;, μg/spot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIMS mode, μg/μl injection</td>
<td>Normal scanning, μg/μl injection</td>
<td></td>
</tr>
<tr>
<td>DON</td>
<td>0.007</td>
<td>0.02</td>
<td>0.025</td>
</tr>
<tr>
<td>MAS</td>
<td>0.015</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>DAS</td>
<td>0.009</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Neosolaniol</td>
<td>0.02</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>0.02</td>
<td>0.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data presented from TMS ether derivatives of the toxins

<sup>b</sup> H<sub>2</sub>SO<sub>4</sub> spray

Performance liquid chromatography (HPLC)<sup>128-130</sup> or TLC<sup>131</sup> with video fluorimetry (VF). Video fluorimetry employs a novel irradiation geometry and image detector to simultaneously collect excitation and emission spectra of fluorescent compounds. Along with the retention time, the excitation and emission matrix can subsequently be treated to resolve known and unknown spectrally overlapping, fluorescent compounds for further analyses. Coupling the HPLC-separated, nicotinamide-derivatized sample with VF, a potentially sensitive and specific technique for epoxy-containing toxins exists.

A second technique utilizes TLC. The intensities of the fluorescent spots can be monitored with a silicon intensified target vidicon camera by irradiating the plate with UV radiation or with a laser for even greater sensitivity. Picogram quantities of the sample can be detected. In a second TLC format, an emission spectrum from each position along the elution axis of the plate is obtained and this results in spectral fingerprinting and R<sub>f</sub> information of the entire spotted sample.

3.2.9 Mass Spectrometry/Mass Spectrometry.

Both GC/MS techniques (<i>vide supra</i>) are being expanded to multiple-stage, MS analyses or mass spectrometry/mass spectrometry (MS/MS).<sup>132</sup> The strength of this technique lies in the fact that a greatly increased signal-to-noise ratio, despite overall loss of signal strength, is achieved from one MS stage to the next by a reduction in chemical noise. Most applications of this technique involve chemical ionization, mass selection of a parent ion, collisionally induced fragmentation and subsequent recording of a daughter ion MS/MS spectrum. Very low detection limits have resulted for various compounds. For example, 2,3,7,8-tetrachlorodibenzodioxin, despite the presence of an excess of the interferential compound polychlorinated biphenyl, has been detected at less than 50 pg.

3.3 Chemical Reactions.

A number of chemical reactions have been reported in the literature using the trichothecenes including oxidation, reduction, catalytic hydrogenation, and acid and base reactions. Using the numbered ring systems as shown in figure 1, various chemical reactions are listed in figure 7, A through D, by reaction type, reagent used, and a representative group A or B trichothecene.<sup>31,133-154</sup>
The oxidation reaction utilizing perbenzoic acid [figure 7, A (10)] is interesting in that the α-epimer (epoxide ring facing away from the viewer) is more stable than the β-epimer (epoxide ring racing toward the viewer). The latter undergoes intramolecular nucleophilic substitution to form another six-membered ring. Various other ring closure reactions are presented, destroying the 12,13-epoxy function in the process, thereby eliminating toxicity. Also the apotrichotheceine ring system (devoid of toxicity) is such that the epoxide is destroyed [(figures 7, B (1), 7C) without generating another ring, which is contrary in the case of ring closure reactions in figure 7 D (3, 4).

The reactions portrayed in figure 7 A (5, 6) would afford the α-stereocchemical substitution of a hydroxyl group since that particular face of the molecule is less sterically hindered. Reaction 7 B (4) (figure 7, B) is particularly interesting in that it is a very specific, stereocchemically oriented reaction in which the \( R_1 \) mesityl and \( R_2 \) mesityloxy groups are removed with subsequent ketone formation at \( R_1 \).

3.4 Model Compounds of the Trichothecene “Active” Site.

A study of the chemistry of the trichothecan ring system compounds would not be complete without an understanding of what makes the molecule “tick.” There is evidence portraying the R groups (sections “Relative Toxicities and Toxin Lipophilicity” and “Structure-Function Relationships Based on In Vitro Studies”) as contributing to trichothecene toxicity. The 9,10-olefinic portion of the toxin molecule also influences toxicity since reduction by hydrogenation leads to a reduction in activity.\(^{78,155,156}\) However, a total loss of activity is realized when the epoxide function is destroyed.\(^{157-160}\)

As a further means of addressing this subject, model compounds (1,5-dioxaspiro[2,5] octanes) containing the oxiran function were produced and evaluated as trichothecene mimics of anticancer (cytostatic) activity. Figure 8 portrays eight compounds modeling the active site of the family of trichothecenes.

Two studies were undertaken in the assessment of activity, the first being the Ehrlich ascites carcinoma activity screen\(^{161}\) in mice. As shown in figure 8, only structures 2 and 3 imparted significant activity; whereas, compounds 6 and 8 were slightly active. The rest displayed no activity. Compound 2, the most active model, was 25 times less active than T-2 toxin. These results suggest that sterically hindered epoxides presumably have high activity along with the specific epoxide stereochemistry as illustrated between 3 and 4.

The four most active spirooctanes were then screened for lymphocytic leukemia P388 activity. They all displayed slight activity with compound 2 registering at a maximum\(^{162,163}\) (see also reference 161).

3.4.1 Chemical Reactions of Epoxide Systems.

The purpose for describing the various reactions of the epoxide ring (oxiran ring) system is because it is possible to chemically target the ring functionality, which is important because it occupies a central role in toxicity. This ease of defining the oxiran ring functionality is in contrast to the various R groups and the olefinic bond which, as a whole, topically exist in a more diverse arrangement about the trichothecene molecule. Modifying either one or several of the groups reduces toxicity as opposed to eliminating it with the destruction of the epoxide ring.
Figure 7. Chemical Reactions of the Trichothecenes

A. Oxidation Reactions

1. \( \text{T-2 TETRAOL} \xrightarrow{\text{MnO}_2^a} \text{AN ENONE} \)

2. \( \text{VERRUCAROL} \xrightarrow{\text{CrO}_3^b \text{aq.}} \text{KETOALDEHYDE} \)

3. \( \text{ANGUIDINE ACETATE} \xrightarrow{\text{CrO}_3^c \text{pyr}} \)

4. \( \text{DIACETYLAPOVERRUCAROL} \xrightarrow{\text{mild oxidant}} \)

Reference: 133, 134, 135
5. ENONE OF T-2 → ENOLATE
   \[ \text{MoO}_3\text{PH or } \text{O}_2 \]
   \[ \alpha\text{-HYDROXYENONE} \]

6. ENONE OF T-2 → Silyl ENOL ETHER
   \[ \alpha\text{-HYDROXYENONE} \]

7. ANGUIDINE
   1. NBS, H\(_2\)O, \(h\nu\)
   2. Na\(_2\)CO\(_3\)/H\(_2\)O

8. ACETYL T-2 TOXIN
   \[ \text{SeO}_2^+ \text{t-BuOH} \]
Selective oxidation of the allylic alcohol occurs.

Other hydroxyl groups present must be protected by the acetyl groups.

Allylic methylene oxidation

The acetyl moieties act as protecting groups if the original trichothecene had a hydroxyl group.

Li:diisopropylamide or Li:hexamethyldisilazide

Trimethylsilyltrifluoromethanesulfonate

OsO$_4$/N-methylmorpholine N-oxide

The R$_5$ ester is more susceptible to oxidation by SeO$_2$ than the other positions containing an ester group.
B. Reduction Reactions

1. VERRUCAROL $\xrightarrow{\text{LiAIH}_4}$

2. $\xrightarrow{\text{Li}/\text{NH}_3(\text{liq})}$ ROH $\xrightarrow{\text{NaBH}_4}$

3. $\xrightarrow{\text{Ca}/\text{NH}_3(\text{liq})}$ or $\text{CrCl}_2/\text{acetone}$

4. $\xrightarrow{\text{mesitylCl}}$ pyr

C. Lewis Acid and Base Reactions

1. KETOALDEHYDE

\[ \text{KETOALDEHYDE} \xrightarrow{\text{Na}_2\text{CO}_3} \text{Reference} \]

2. KETOALDEHYDE

\[ \text{KETOALDEHYDE} \xrightarrow{\text{K}_2\text{CO}_3 \text{aq.}} \text{Reference} \]

3. KETOALDEHYDE

\[ \text{KETOALDEHYDE} \xrightarrow{\text{K}_2\text{CO}_3 \text{MeOH}} \text{Reference} \]

4. T-2 TOXIN

\[ \text{T-2 TOXIN} \xrightarrow{1. \text{NaOH/EtoH} \atop 2. \text{H}^\ominus} \text{T-2 TETRAOL} \]

5. VERRUCAROL

\[ \text{VERRUCAROL} \xrightarrow{\text{a}} \text{APOVERRUCAROL} \]

*H\textsubscript{2}O, r.t., overnight and either 0.5N H\textsubscript{2}SO\textsubscript{4} or 50% CF\textsubscript{3}COOH
D. Other Reactions

1. **ACETYLATION**
   
   ![Acetylation Reaction]
   
   **APOVERRUCAROL**
   
   Reference

2. **CATALYTIC HYDROGENATION**
   
   ![Hydrogenation Reaction]
   
   **VERRUCAROL**
   
   Reference

3. **HYDRATION, RING CLOSURE**
   
   ![Hydration Reaction]
   
   **VERRUCAROL**
   
   Reference

4. **RING CLOSURE**
   
   ![Ring Closure Reaction]
   
   **APOVERRUCAROL**
   
   Reference
To the best of the authors' knowledge, no systematic study of the chemical reactions of the oxirane ring of the toxins has been reported, including such parameters as kinetics and temperature and solvent effects with varying agents. The literature describes trichothecene-epoxide reactions dealing primarily with simple acids (e.g., HCl, H$_2$SO$_4$, HBr, and CF$_3$COOH), bases (e.g., KOH, NaOH, Na$_2$CO$_3$, and NH$_4$OH), (reference 164) and oxidation and reduction reactions. In particular, it has been observed that, using either concentrated HCl/EtOH and concentrated HBr/aq. or H$_2$CrO$_3$/acetone, trichothecin and verrucarol, respectively, entered into reactions rapidly (within minutes) at room temperature.

Greater understanding of some of the reactions concerning their mechanistic behavior could be of significance. Under basic or neutral conditions, the mode of attack of a nucleophile follows an anti-coplanar arrangement of the reagent, oxygen, and carbon-$\alpha$ and carbon-$\beta$ atoms in the epoxy ring (figure 9). An S$_\text{N}2$ reaction takes place in that the nucleophile approaches the least substituted carbon (C-$\beta$). Under acidic conditions, protonation of the ring oxygen occurs and the nucleophile attacks the $\alpha$-carbon in order to relieve its electron debt, following an S$_\text{N}1$-type reaction.

Figure 8. Structural Analogues of the Region Imparting Toxicity in the Trichothecene Compounds

Figure 9. Anti-Coplanar Mode of Attack of a Nucleophile with an Epoxide System (See text for details)
Among the myriad of epoxy-compound reactions surveyed, several appear to be of potential use in characterization, identification, and decontamination of the trichothecene toxins. These are summarized in table 10.165-171 Even though the reagents vary somewhat, the reaction conditions are similar. Solvent systems range from polar to nonpolar, yet ambient temperature prevails and reaction completion times are short. Consequently, it is obvious that the reagents listed in table 10 should be screened for trichothecene-epoxide destruction.

<table>
<thead>
<tr>
<th>Epoxide compound</th>
<th>Reagent</th>
<th>Solvent</th>
<th>Reaction time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HgSO₄</td>
<td>H₂SO₄</td>
<td>Immediate</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>LiNEt₂</td>
<td>Ether-hexane</td>
<td>Immediate</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>BF₃·OEt₂</td>
<td>Benzene</td>
<td>1 Minute</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>BF₃·OEt₂</td>
<td>Benzene</td>
<td>20 Minutes</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>HClO₄</td>
<td>1:9 Dioxane-H₂O</td>
<td>–</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>LiBEt₃H</td>
<td>THF</td>
<td>5 Minutes</td>
<td>170,171</td>
</tr>
</tbody>
</table>

* All reactions were performed at ambient temperature (20°-25°C).

3.5 Biochemistry.

3.5.1 Biochemical Detection.

It was shown in an experiment that glutathione S-epoxide transferase catalyzes the conjugation reaction between a trichothecene and an excess of the thiol enzyme gonadotropic stimulating hormone (GSH).172 The amount of toxin bound was measured indirectly by determining the remaining concentration of GSH by reacting it with 5,5-dithiobis(2-nitrobenzoic acid) and then measuring the rate of formation of the colored ion of 5-mercapto(2-nitrobenzoic acid). Submicrogram quantities could be detected by this biological technique.103 In a similar vein, the trichothecene epoxide group also reacts with the thiol side group of the enzyme alcohol dehydrogenase173 in the absence of substrate.

A reliable assay technique9 was designed in which concentrations of 5 to 60 µg/ml of T-2 toxin could be determined by applying solutions of unknown concentration of T-2 toxin and
standard solutions of the toxin onto the shaved backs of test and control rats and noting the relative intensities of the reactions. Another method used is the rabbit reticulocyte assay. This technique involves the inhibition by the trichothecenes of $^{14}$C-leucine uptake by the cells. Toxin concentrations as low as 30 ppb have been detected.\textsuperscript{76,174}

The main drawback of these methods of trichothecene detection is that, although sensitive, they lack selectivity and specificity. However, this disadvantage has been overcome in the use of the enzyme-linked immunosorbent assay technique.\textsuperscript{175,176} In a relatively simple procedure, the toxin of interest is conjugated to either bovine serum albumin or horseradish peroxidase. The antibody sera are applied onto polystyrene microtissue culture plates simultaneously with conjugated standards on other plates. Minimum detection levels of this technique are of the order of 2.5 pg/assay. This extremely sensitive and specific assay has a disadvantage in that the preparation of specific antibodies to a particular toxin usually occurs over a period of several years.

3.5.2 Biotransformations.

The fate of administered toxins in animals was studied previously with radioactively labeled compounds. $^3$H-labeled fusarenon-X was seen to rapidly spread to the intestines, liver, and other organs of the body. Within one day, 25\% of the radioactivity was eliminated in the urine and upon analysis yielded nivalenol.\textsuperscript{177-179} The same treatment with $^3$H-labeled T-2 toxin produced HT-2 toxin with no amount of detectable T-2 toxin. Upon subsequent analysis, both radioactive compounds were found to be deacetylated at the R\textsubscript{2} function in the liver by microsomal esterase.\textsuperscript{178}

Nakano et al.\textsuperscript{180} conducted experiments consisting of iv injections into mice of the basic trichothecene compound ($R_1 = R_2 = R_3 = R_4 = R_5 = H$) which was $^{14}$C-labeled on the epoxide ring. The observed high radioactivity in the liver and kidney decreased rapidly, although it persisted in the bladder and intestines. Per os (oral) administration to the mother led to its appearance in the 7-day suckling mice stomachs, thus showing secretion of the toxin in the milk. Also, in rats, no radioactive $^{14}$CO\textsubscript{2} was detected in the expired breath when given either orally or intravenously, indicating no cleavage of the epoxide ring. Another study\textsuperscript{181} showed that labeled T-2 toxin injected into pregnant rats produced radioactivity in the thymus of the fetus and persisted for 1 week.

3.5.3 Action on Deoxyribonucleic Acid (DNA).

The effect of T-2 toxin action on DNA was studied by using Parodi's alkaline elution technique coupled with a microfluorimetric DNA determination.\textsuperscript{103,182,183} Briefly, the experiment relies on the fact that the greater the incidence of toxin-induced DNA cleavage, the greater the number of smaller DNA fragments are produced which subsequently elute through cellulose filters. The toxin was utilized in vivo and in vitro and its effects were studied on liver, spleen, and thymus tissues from rats. In both cases, no damage was observed on the hepatic DNA. However, with concentrations as low as 5 ng/ml of culture and 2-hour exposure times, the lymphoid organs were extensively damaged in that many breaks in the cellular DNA occurred. In vivo, as opposed to in vitro, reversibility occurred which indicated that DNA repair took place. Because other chemical compounds\textsuperscript{103} such as methyl nitrosourea and methyl methanesulfonate require concentrations of approximately 0.1 mM to induce DNA breaks, T-2 toxin is indeed a very potent substance.
3.5.4 Structure-Function Relationships Based on In Vitro Studies.

The trichothecenes are the most potent protein synthesis inhibitors in eucaryotic cells. They inhibit either the initiation or termination step of protein synthesis on the cellular level.

Table 11 summarizes at what stage different trichothecenes exert their inhibitory effect.

Table 11. Trichothecenes and Their Stage of Protein Synthesis Inhibition

<table>
<thead>
<tr>
<th>Initiation</th>
<th>Elongation or termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarenon-X</td>
<td>Verrucarol</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>Crotocol</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>Crotocin</td>
</tr>
<tr>
<td>Scirpentriol</td>
<td>Trichotheclin</td>
</tr>
<tr>
<td>MAS</td>
<td>Trichotheclone</td>
</tr>
<tr>
<td>DAS</td>
<td>Trichoderm</td>
</tr>
<tr>
<td>Verrucarin A,E,J,H</td>
<td>Trichodermol</td>
</tr>
</tbody>
</table>

The mode of action the trichothecenes undertake at the protein synthesis level can be explained by two biochemical models, one being favored over the other.\textsuperscript{34,43} A synopsis of the preferred model shows that the toxin binds throughout the protein synthesis cycle with equal affinities, only interfering with the crucial enzyme peptidyl transferase when the ribosome unit assumes a certain three-dimensional structure. Two of the substituent side groups of the toxin would determine the site of activity on the ribosomal unit.\textsuperscript{184,185}

Analyzing these facts,\textsuperscript{43,186} structural correlations can be assumed. $R_2$ substitution ($R_1 = R_3 = H$) is detrimental (inhibits peptidyl transferase activity) at the elongation or termination stage. $R_1$ and $R_3$, residing on the opposite side of the $R_2$ function in the toxin molecule (cf. figure 1), inhibit initiation when derivatized (OH, OAc). The toxin verrucarol ($R_1 = H, R_2 = R_3 = OH$) inhibits elongation while inhibiting initiation of protein synthesis when esterified.

The vital structure-function relationships of this model are: (1) $R_2$ substitution inhibits elongation and termination and (2) $R_1$ and $R_3$ substitution leads to initiation inhibition. Since the epoxide ring, which is crucial for toxicity, is also found on the same side of the molecule as the $R_1$, $R_2$, and $R_3$ functions, the toxin's ability to impart deleterious effects appears to be centered on the right half of the molecule (figure 1).
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123. Singhawangcha, S., Poole, C. F., and Zlatkis, A. Ibid. 183, 433 (1980).


134. Ibid, 133


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