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DEVELOPMENT OF GENERAL ANTISERA FOR TRICHOTHECENES

Annual/Final Report

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Summary

Antibodies with narrow structural requirements for binding are useful in specific assays. Antibodies which bind a variety of structurally related compounds could be used in detection of classes of compounds. Therefore, we began exploring the relationship between immunogen (hapten) structure and the selectivity of resulting antibodies to various of the trichothecenes. We synthesized immunogens, used them to immunize rabbits and examined the polyclonal sera for their selectivity for various trichothecene compounds before preparing monoclonal antibodies. Keywords:)

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19. ABSTRACT - continued

The first position examined for linkage to protein was the 8-position of the trichothecene molecule. Following a published procedure, we converted anguidine (A-1) to the 8ß alcohol A-2 by treatment with selenium dioxide. The alcohol was further oxidized with pyridinium chlorochromate to the unsaturated ketone A-3. The ketone was readily converted to its oximino acetic acid analog A-4 and coupled to protein by a mixed anhydride procedure. The bovine serum albumin (BSA) conjugate (C8-T-2-BSA) had an incorporation of 25 trichothecene units per molecule of protein and the bovine thyroglobulin (BTg) conjugate (C8-T-2-BTg) had 60 units of trichothecene per molecule of protein. These two conjugates were used to immunize rabbits. Anti-T-2 toxin activity in the sera was demonstrated in an enzyme immunoassay (EIA). Selected sera were analyzed in a competitive inhibition radioimmunoassay (CIRIA) to determine crossreactivities for T-2 toxin and related trichothecenes. These antisera cross-reacted strongly with tricothecene compounds varying in structure at C-8 but not with variants at other positions (e.g., 0.6-0.8% with HT-2 toxin).

After numerous unsuccessful attempts, the synthesis of the 4-keto analog of HT-2 toxin (H-7) was achieved. Unfortunately, all attempts to introduce the oximino acetic acid moiety at this position were unsuccessful. The synthesis of the 4-hemisuccinate I-3 from HT-2 toxin (I-1) was accomplished in two simple steps and I-3 was coupled to BSA by the mixed anhydride procedure. An incorporation ratio of 19 moles of hapten per mole of BSA was obtained in this product (C4-T-2-BSA). A second conjugate with chick gamma globulin (C4-T-2-CGG) was also prepared. These two conjugates were used to immunize rabbits. Anti-T-2 toxin activity in the sera was demonstrated by EIA. Selected antisera were analyzed by CIRIA to determine cross-reactivities. These antisera cross-reacted strongly (34-71%) with HT-2 toxin, but not with variants at positions other than C-4 (e.g. 0.6-0.8% with 3'-hydroxy-T-2 toxin).

Mice were immunized with either C8-T-2-BSA or C4-T-2-BSA and splenic lymphocytes fused with myeloma cells. Use of C8-T-2-BSA led to unusually low yields of hybridomas and no stable cell lines producing antibodies to T-2 toxin were obtained. In contrast, use of immunogen C4-T-2-BSA resulted in a high fusion efficiency; seventy-six percent of seeded wells produced hybridoma colonies and 42 of the positive hybridoma colonies were selected for further study based on EIA results. Three hybridoma colonies were cloned once by limiting dilution and supernatant culture media from two of the resulting first generation clones (one producing IgG_1 and one producing IgG_3 antibodies) were tested for cross-reactions by CIRIA. One antibody was highly selective for T-2 toxin vs a wide variety of structures (cross-reacting only 0.9% with HT-2 toxin and <0.2% with 3'-hydroxy T-2 toxin) whereas the other had significant (13%) cross-reaction with HT-2 toxin.

As part of this project we developed two simple syntheses of T-2 toxin (A-12) from readily available anguidine (A-1). Use of 3-methyl-3-butenoic acid instead of isovaleric acid in this sequence followed by selective reduction with tritium gas provided a means of preparing radiolabeled T-2 toxin with high specific activity. With this ligand and the polyclonal antisera, T-2 toxin could be analyzed with a sensitivity of 32 pg. Plasma did not interfere at 100 uL per tube. The monoclonal antibodies permitted practical sensitivities of 29 pg/tube. These analytical limits could likely be lowered by variations in experimental techniques.

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Foreword

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

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1.0 Background

The object of this work was to prepare the necessary immunogens and use them to generate polyclonal and monoclonal antibodies to the class of trichothecene toxins. Class-specific antisera rather than compound-specific antisera were the goal. The purpose was to provide antisera which could be used to detect this class of compounds at low enough concentrations to permit protective action or to determine the safety of the environment after decontamination procedures are undertaken in a chemical warfare situation.

The trichothecene molecules are too small to be immunogenic per se, but must first be linked by a covalent bond to a large molecule (carrier) such as a protein. The resulting conjugate will then stimulate antibodies capable of binding the trichothecene. The ability of the antisera to discriminate among similar molecules (antibody selectivity) is very much influenced by the position and properties of the group which links the small molecule to the carrier. Most of the work in this area is based on the pioneering studies of Landsteiner (1962) who showed that in general an antibody is most selective for those portions of the small molecule which are not directly involved in the link to the carrier.

The affinity of an antibody for its ligand (antigen) is of considerable importance in developing an immunoassay procedure, since the sensitivity of the analytical method is dependent, among other things, upon the antibody affinity constant. Hydrophobic and hydrophilic interactions between antibody and ligand contribute to this affinity as do the presence of electrically charged groups and π electron interactions. The greater the resemblance between the hapten used for immunization and the molecule to be analyzed, the higher the affinity that can be expected for the antibody.

2.0 Rationale for the Proposed Work

In order to achieve high antibody selectivity (that is to obtain antibodies which can discriminate well among similar molecules), one normally positions the linkage between hapten and carrier on a portion of the molecule which remains invariant among the differing substances which may interact with the antibody. To obtain antibody selectivity for a group of related compounds rather than for a single member of that group, the principles discussed would dictate that the link from hapten to carrier should be in a region of a hapten molecule which varies within the series. Functional groups and structural features which are essentially invariant among the series should be left free so that they can contribute maximally to enhancement of antibody affinity.

Application of these precepts to the trichothecene series leads to the following conclusions:

(1) The 3-hydroxyl group which is a common feature of this series should be left free.

(2) The epoxide structure, which is common to all of the trichothecenes, should also be left untouched.

(3) It has been our experience that "leaving a hole" in the antibody binding site is less disruptive of binding than is trying to insert a group which is bulkier than that found in the hapten. Thus the bulkier group should be left in the molecule if there is a choice (for example leave the 4-acetoxy group of T-2 toxin in preference to a 4-hydroxy group of HT-2 toxin).

(4) A position in the molecule where variation occurs among the series might be converted to a structure slightly at variance with all of the compounds of interest. This would probably reduce overall affinity but may result in a more uniform binding across the series of compounds.

Using these concepts, we began to examine the influence of hapten structure on antibody selectivity in the trichothecenes. Three haptens were synthesized: the 8-oximinoacetic acid analog (A-4) of T-2 toxin, the 4-hemisuccinate analog (I-3), and the 15-hemisuccinate analog (J-11). The first two haptens were conjugated to two proteins each. Polyclonal antisera were produced by challenging rabbits with all four conjugates. Hybridoma colonies secreting antibodies to T-2 toxin were produced from mice immunized with a conjugate of I-3. Antibody selectivity was then examined.

3.0 Trichothecene Analog Synthesis

3.1 4β,15-Diacetoxy-3α-hydroxyscirpen-8-(O-carboxymethyl)oxime (A-4)

The hydroxylation of anguidine (A-1, see Chart A) proceeded smoothly as reported (Kaneko et al., 1982) by reaction with selenium dioxide in refluxing aqueous dioxane to give A-2 in 57% yield as crystals after silica gel chromatography. Oxidation was effected using pyridinium chlorochromate and sodium acetate in methylene chloride to give A-3 in 88% yield as a foam, after silica gel chromatography. The reported ketone (Kaneko et al., 1982) was characterized by TLC, GLC, and NMR and determined to be pure despite the failure to obtain crystals. Treatment of A-3 with carboxymethoxyamine hemihydrochloride in anhydrous pyridine for 40 h at 35°C gave the oxime acid A-4 as a foam in 45% yield, after silica gel chromatography. The physical properties (IR, NMR and MS) of A-4 were consistent with the assigned structure. The oxime acid A-4 was coupled with bovine serum albumin (BSA) and bovine thyroglobulin (BTg) (vide infra). Stability of the acid A-4 (e.g., epoxide cleavage, hydrolysis, etc.) to the reaction conditions required for conjugation was determined by reacting the mixed anhydride obtained from isobutyl chloroformate with ethyl amine to give A-5.

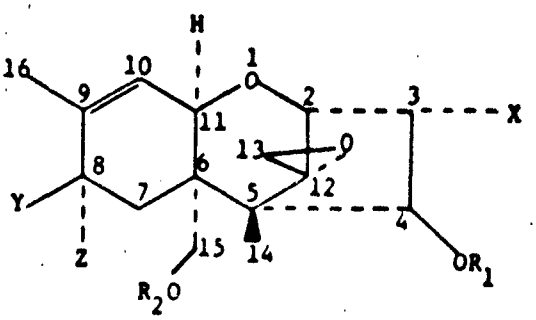
3.2 4β,15-Diacetoxy-8α-isovaleryloxyscirpen-3α-ol (T-2 toxin, A-12)

The synthesis of haptens based on link at C-4 required T-2 toxin (A-12) as the starting material. Although T-2 toxin is commercially available, it is rather expensive. We are happy to report that during this work we have developed two simple syntheses of T-2 toxin from anguidine.

The synthesis of T-2 toxin (A-12) from anguidine (A-1) requires the introduction of an α-OH function at C-8 and acylation with isovaleric acid. It was felt that the 3-OH position should be protected as a THP ether in order

CHART A

Structures of Tricothecenes



Compound	R_1	R_2	X	Y	Z
1	Ac	Ac	OH	H	H
2	Ac	Ac	OH	OH	H
3	Ac	Ac	OH	-O-	
4	Ac	Ac	OH	$=N-O-CH_2-COOH$	
5	Ac	Ac	OH	$=NOCH_2CONHEt$	
6	Ac	Ac	OTHP	H	H
7	Ac	Ac	OTHP	OH	H
8	Ac	Ac	OTHP	-O-	
9	Ac	Ac	OTHP	H	OH
10	Ac	Ac	OH	H	OH
11	Ac	Ac	OTHP	H	O-iVal
12	Ac	Ac	OH	H	O-iVal

to selectively functionalize the C-8 position. Thus, anguidine (A-1) was treated with dihydropyran and a catalytic amount of tosic acid in tetrahydrofuran to give A-6 as an oil. No attempt at purification was made at this point. The THP ether A-6 was hydroxylated as described above with selenium dioxide to give A-7 as an orange oil in 60% yield from A-1. This material was characterized by NMR and determined to be of 95% purity by TLC and GLC.

It has been reported (Kaneko et al., 1982) that the diisobutylaluminum hydride reduction of the 8-keto compound A-3 gives predominantly the 8α-OH isomer A-10. Thus, A-7 was oxidized as described above with pyridinium chlorochromate and sodium acetate in methylene chloride to give A-8 in 72% yield after silica gel chromatography. However, when the diisobutyl-aluminium hydride reduction was carried out with A-8, the major product was determined by GLC analysis to be A-7 instead of the desired A-9 in a ratio of 2:1. This was further confirmed by cleaving the THP ether with dilute hydrochloric acid in methanol to give a mixture of A-2 and A-10 in the same ratio as before. The isomers A-7 and A-9 were also found to be homogeneous by silica gel TLC in various solvent systems.

Therefore, an alternate approach for the inversion of stereochemistry at C-8 was explored. In this approach A-7 was treated with triphenylphosphine, isovaleric acid, and diethyl azodicarboxylate (cf. Bose et al., 1973) in tetrahydrofuran to give exclusively the 8α isovalerate ester A-11 in moderate yields (54% based on recovered A-7) after silica gel chromatography. The material was characterized by TLC, GLC, and NMR and determined to be of 95% purity. Its structure was further confirmed by the cleavage of the THP ether with pyridinium tosylate in 95% ethanol at reflux for 1 h to give the known T-2 toxin (A-12); m.p. 145-146°, reported (Bamburg et al., 1968) 150-151°. This product was found to be identical with an authentic sample of T-2 toxin by TLC, GLC and NMR.

After the completion of the above synthesis, it was discovered that the inversion of stereochemistry at C-8 can be accomplished by the procedure of Bose et al. (1973) without protection of the hydroxyl function at C-3. Thus, treatment of A-2 with triphenylphosphine, diethyl azodicarboxylate and isovaleric acid gave the T-2 toxin (A-12) in 40% yield.

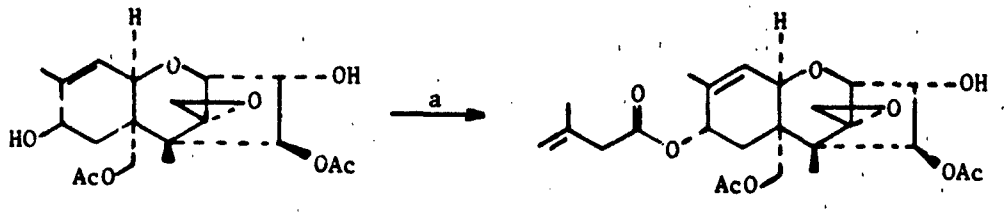
3.3 4β,15-Diacetoxy-3α-[(3,4-$^3H_2$)isovaleryloxy]scirpen-3α-ol(Tritiated T-2 toxin, B-3, Chart B)

It was possible to accomplish the synthesis of the high specific activity tritiated T-2 toxin (B-3) by employing 3-methyl-3-butenoic acid in the above sequence. Thus, B-1 (obtained by the SeO_2 oxidation of anguidine) was converted to the olefin B-2 (Chart B) in 40% yield using triphenylphosphine, diethylazodicarboxylate and 3-methyl-3-butenoic acid. The physical properties (IR, NMR and HRMS) of B-2 were consistent with the assigned structure.

The tritiation step was initially studied using hydrogen gas instead of tritium gas, and subsequent analysis of the product by 250 MHz ^1H NMR. When 5% Pd/C was employed as the catalyst, the trisubstituted double bond at C-9 was also reduced along with the terminal double bond in the side chain. Fortunately, when B-2 was stirred overnight with H_2 in the presence of tris-(triphenylphospine)rhodium (I) chloride in toluene, only T-2 toxin was isolated, completely free from either starting material or the over-reduced product. Thus, the tritiation was carried out on 20 mg of B-2 in the presence of 5 Ci of tritium gas and tris(triphenylphosphine)rhodium (I) chloride in toluene overnight. Subsequent purification by PTLC eluted with 2:1 ethyl acetate in hexanes yielded 13.2 mg of tritiated T-2 toxin (B-3) with a specific activity of 34.3 Ci/mmol. This material was diluted to one liter in toluene and stored in the refrigerator. Analysis by TLC (silica gel, 2:1

CHART B

Synthesis of Tritiated T-2 Toxin



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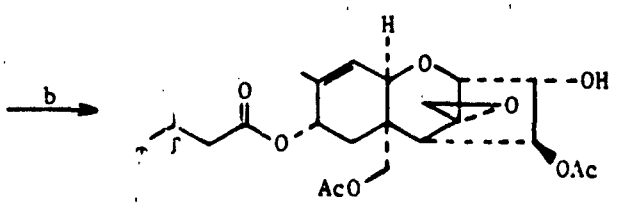
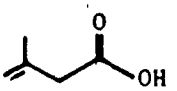


3

a) $(C_6H_5)_3P$, DEAD 

b) $^3H_2$, $[(C_6H_5)_3P]_3RhCl$

ethyl acetate in hexanes) indicates the compound to be stable under these conditions for at least a year.

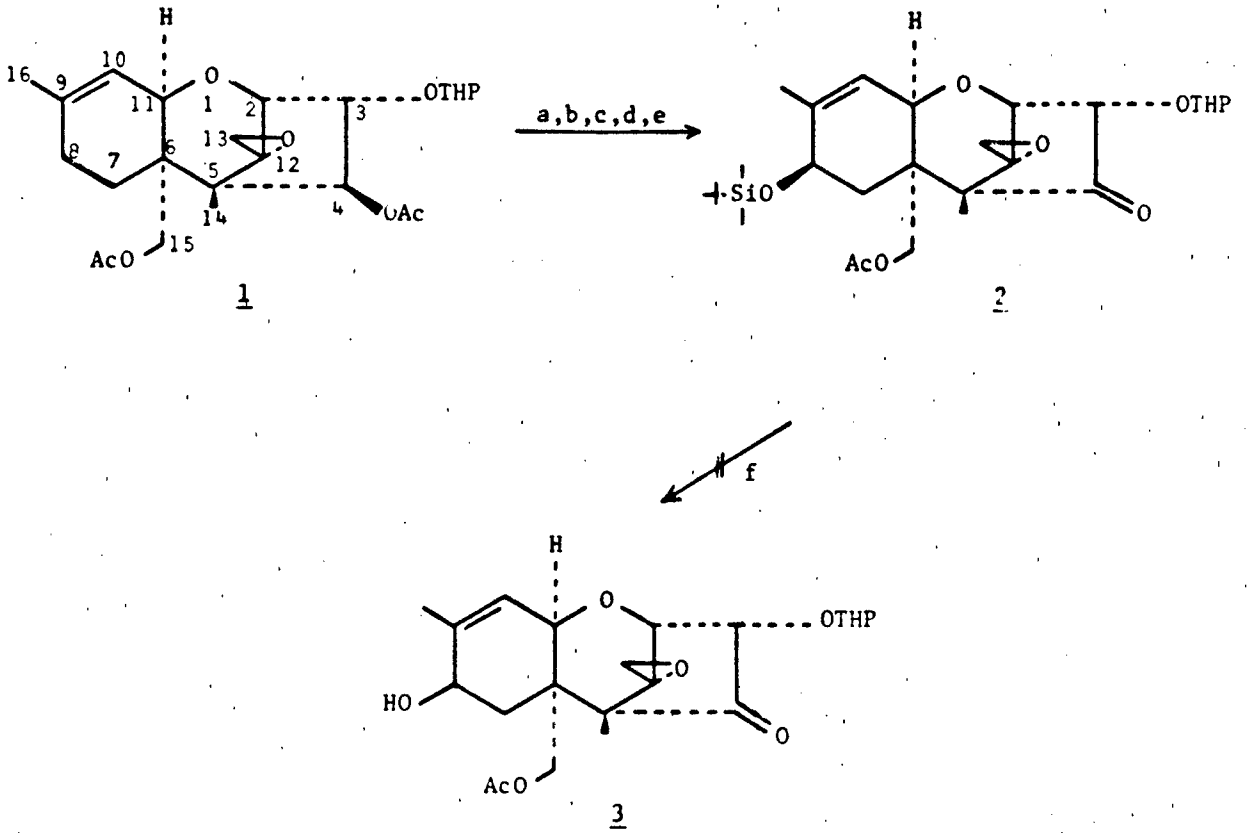
3.4 3α-Hydroxy-8α-isovaleryloxy-15-acetoxyscirpen-4-one (H-7)

After having developed a convenient two-step synthesis of T-2 toxin from anguidine, we had planned to employ the same substrate for the synthesis of haptens for linkage at C-4. It was hoped to effect the selective removal of the acetate funtions without affecting the isovalerate moiety. However, when T-2 toxin was treated with a catalytic amount of sodium methoxide in anhydrous methanol, the isovalerate functionality was also cleaved in addition to the acetates. Therefore, we explored alternate routes (Charts C-H) to the synthesis of haptens based on linkage at C-4. All the routes are based on selective protection-deprotection methodology. The first five approaches (Charts C-G) were unsuccessful. The sixth route yielded the desired 4-keto derivative of T-2 toxin.

The first approach, as shown in Chart C, involved the introduction of a β-hydroxy group at C-8 by the selenium dioxide oxidation of the known THP ether of anguidine (Kaneko, et al., 1982) as described above. The next step was to protect the 8β-hydroxy group as a t-butyldimethylsilyl ether. Attempted silylation of the 8β-hydroxy function with t-butyldimethylsilyl chloride in the presence of imidazole was unsuccessful. However, the silyl ether could be obtained in excellent yields when the corresponding triflate was employed in the reaction (cf. Corey et al., 1981). It was then planned to convert this intermediate to the 4-keto compound C-2 by a series of standard transformations. All of these reactions proceeded smoothly. However, when the cleavage of the silyl ether was attempted with tetrabutylammonium fluoride in anhydrous tetrahydrofuran to obtain C-3, extensive decomposition was observed.

Chart C

First Attempted Synthesis of 4-Keto Analog of T-2 Toxin



1 2 3

a) SeO_2

b) t-Butyldimethylsilyl triflate (Corey, et al., 1981)

c) OH^-

d) AcCl, Et_3N

e) Pyridinium chlorochromate

f) $Bu_4N^+F^-$

A modification of this approach is shown in Chart D. Instead of protecting the 8ß-hydroxy group as a silyl ether, it was hoped that the C-8 hydroxy group, being allylic, would be more reactive than the hindered C-4 hydroxy group, and therefore selective introduction of a formate ester at C-8 should be possible. This subsequently could be removed selectively under very mild conditions without affecting the primary acetate function at C-15. Unfortunately, the conversion of D-2 to D-3 using acetic-formic anhydride was not clean and therefore this approach was also abandoned.

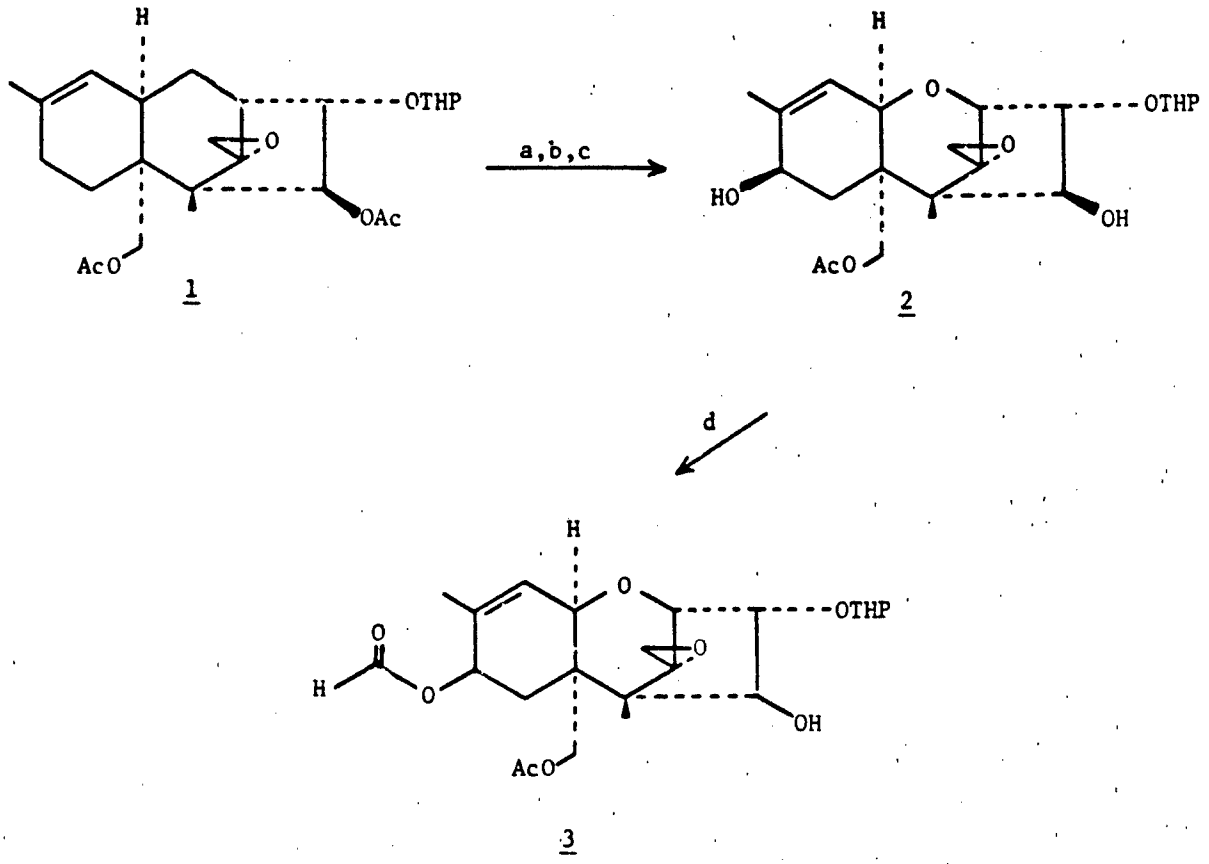
Another approach is shown in Chart E. The silyl ether at C-4 was prepared in good yields following a series of standard transformations. However, when hydroxylation with selenium dioxide was attempted, very poor yields were encountered. Apparently hydroxylation at C-8 is very sensitive to the functionality at C-4. This approach was therefore abandoned.

Another approach is shown in Chart F. Instead of using a protection-deprotection scheme as employed in the earlier approaches, the C-4 hydroxy functionality was smoothly oxidized to give the corresponding ketone, F-2, before the introduction of the C-8 ß-hydroxy group. However, when hydroxylation of F-2 with selenium dioxide was attempted, very poor yields along with extensive decomposition were encountered, probably due to the presence of ketone at C-4. Therefore, this approach was also abandoned.

Another approach involving the use of a silyl ether as a protecting group is shown in Chart G. The THP ether of anguidine (G-1) was converted to the protected intermediate G-2 by a series of standard transformations a-e. Thus, G-1 was subjected to selenium dioxide hydroxylation to give the 8ß-hydroxy compound. The stereochemistry at C-8 was inverted, as discussed above, using triphenylphosphine, diethyl azodicarboxylate (DEAD) and formic acid (cf. Bose, et al., 1973). This intermediate was then subjected to hydrolysis using

Chart D

Second Attempted Synthesis of 4-Keto Analog of T-2 Toxin



1 2 3

a) OH^-

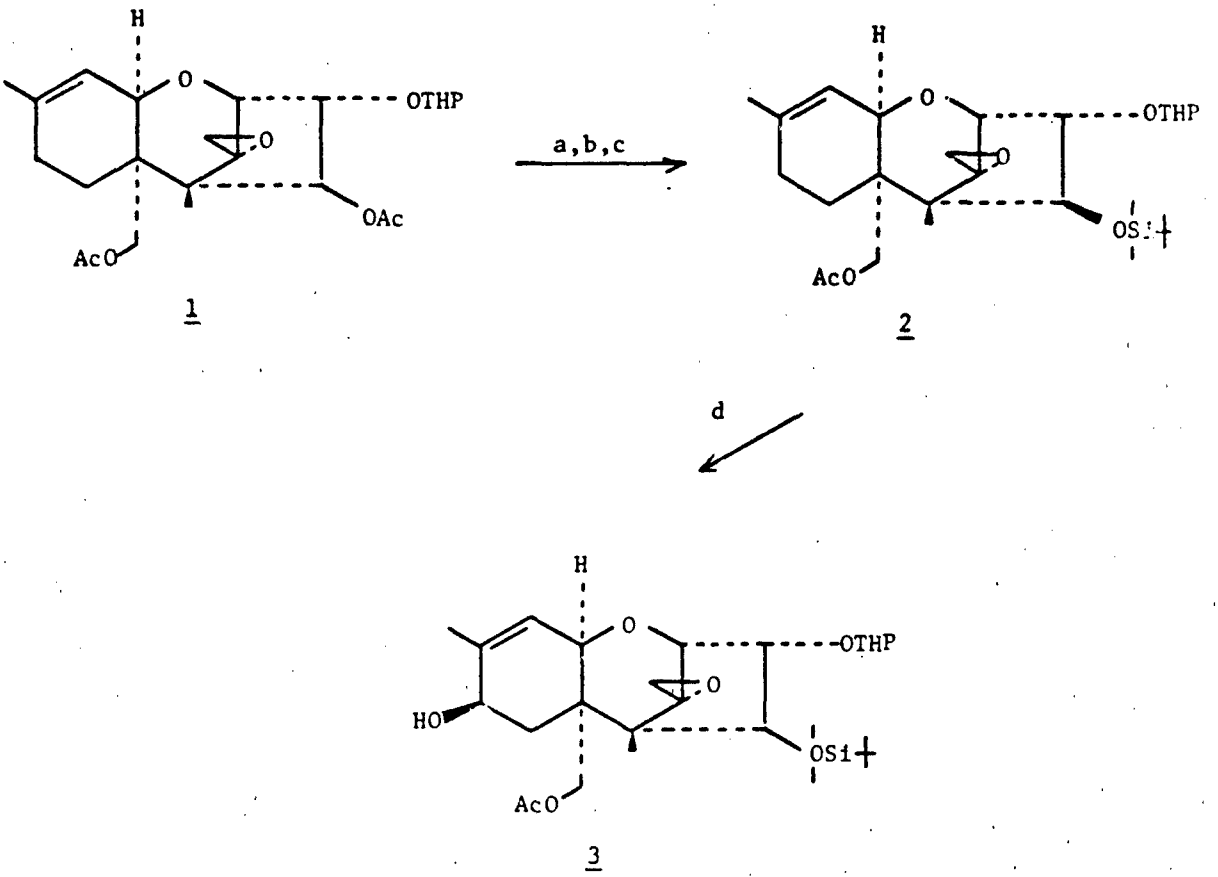
b) AcCl, Et_3N

c) SeO_2

d) $CH_3-\overset{O}{\overset{\|}{C}}-O-\overset{O}{\overset{\|}{C}}-H$, py

Chart E

Third Attempted Synthesis of 4-Keto Analog of T-2 Toxin



1 2 3

a) OH^-

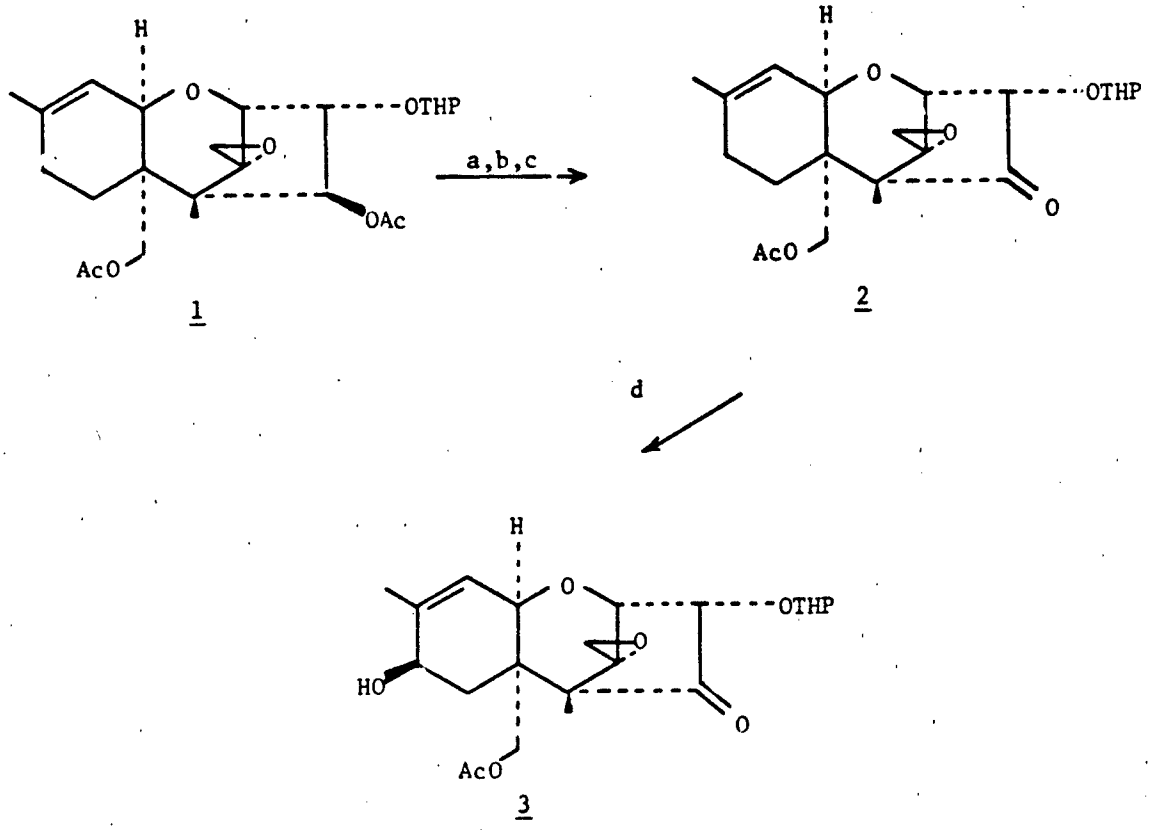
b) AcCl, Et_3N

c) t-Butyldimethylsilyl triflate

d) SeO_2

Chart F

Fourth Attempted Synthesis of 4-Keto Analog of T-2 Toxin



1 2 3

a) ^{-}OH

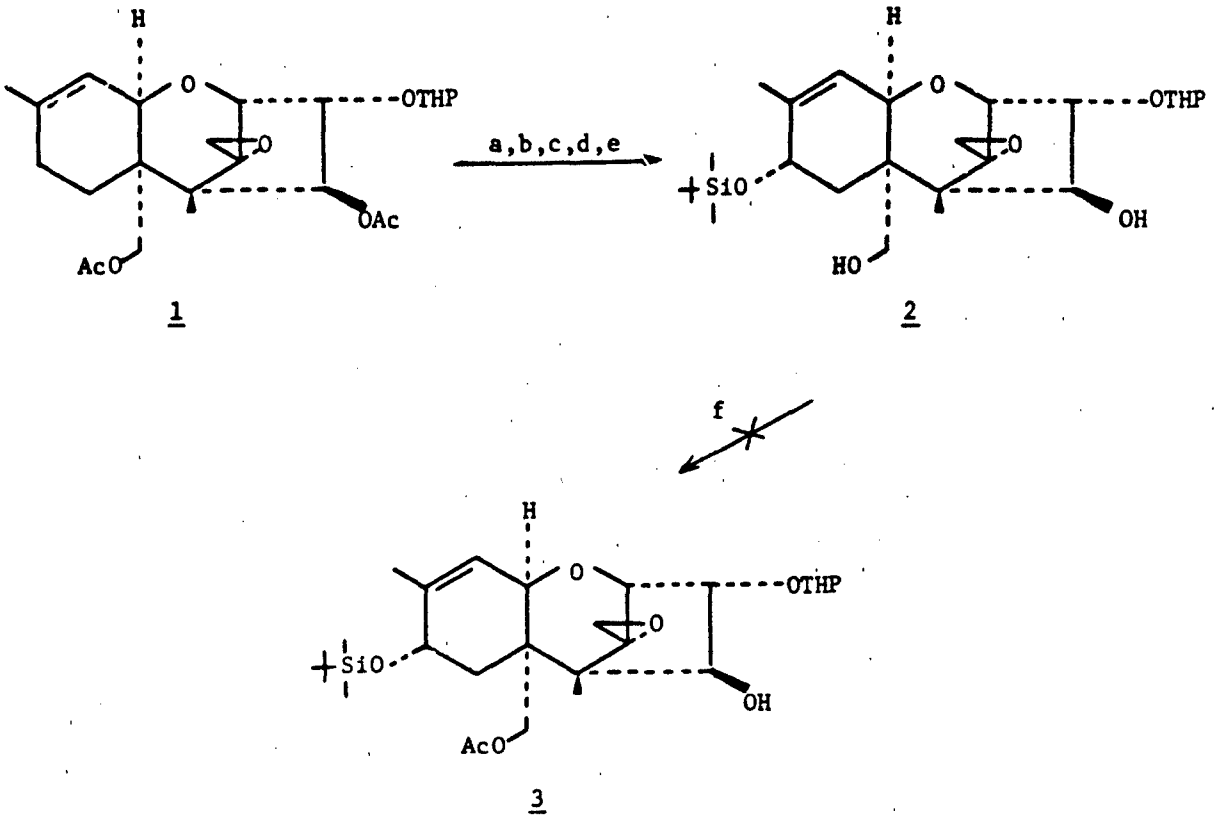
b) AcCl, Et_3N

c) Pyridinium chlorochromate

d) SeO_2

Chart G

Fifth Attempted Synthesis of 4-Keto Analog of T-2 Toxin



1 2 3

a) SeO_2

b) Formic acid, ϕ_3P, diethyl azodicarboxylate (DEAD)

c) $NaHCO_3$

d) t-Butyldimethylsilyl triflate

e) ^-OH

f) AcCl, Et_3N

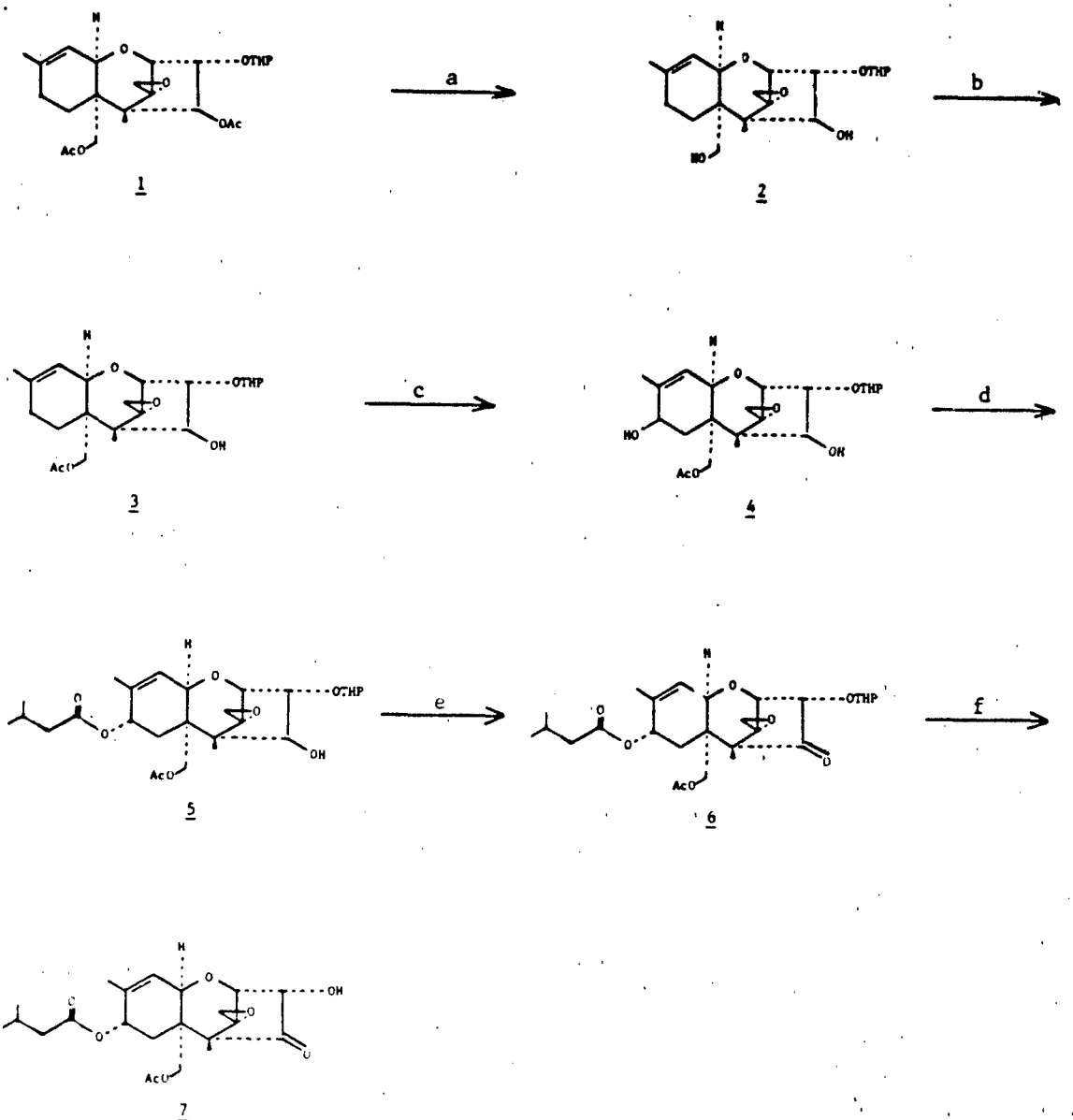
aqueous sodium bicarbonate in refluxing methanol to yield the naturally occurring trichothecene, solaniol (Ishii, et al., 1971). This compound was then protected as a t-butyldimethylsilyl ether before the alkaline hydrolysis of the diacetates to give G-2. However, we were unable to selectively re-acetylate the primary alcohol at C-15 in preference to the secondary alcohol at C-4. Upon examination of a model of G-2, it became apparent that, with the introduction of the silyl group at C-8, the C-15 alcohol is very hindered, and hence the lack of selectivity. Therefore, this approach was abandoned.

The successful approach to the synthesis of a T-2 toxin analog with a 4-keto functionality is outlined in Chart H. This procedure is a modification of the previous scheme. The THP ether of anguidine, H-1, was subjected to basic hydrolysis to give H-2 which upon re-acetylation using acetyl chloride and triethylamine in methylene chloride yielded H-3. These two steps proceeded in essentially quantitative yields. The intermediate H-3 was then subjected to selenium dioxide hydroxylation to give the 4β, 8β diol H-4 in 52% yield. This compound was then successfully treated with diethyl azodicarboxylate, triphenylphosphine and isovaleric acid in anhydrous tetrahydrofuran to give exclusively the isovalerate ester H-5 in 43% yield. The ester H-5 was then subjected to pyridinium chlorochromate oxidation to give H-6 which upon treatment with a catalytic amount of pyridinium tosylate in refluxing 95% aqueous ethanol to give the 4-ketone H-7 in 75% yield.

A small amount (10 mg) of H-7 was treated with carboxymethoxyamine hemihydrochloride in anhydrous pyridine for 40 h at 35°C. An examination of the reaction mixture by TLC indicated that all of the starting material, H-7, was consumed; and a major product (50%), slightly more polar than H-7, was isolated. It appears that under these conditions, epimerization of the C-3 α-hydroxy group to the C-3 β-position is taking place. When this reaction was

Chart H

Successful Synthesis of 4-Keto Analog of T-2 Toxin



a) ^{-}OH

b) AcCl, Et_3N

c) SeO_2

d) Isovaleric acid, Φ_3P, DEAD

e) Pyridinium chlorochromate

f) Pyridine·TsOH, 95% EtOH

repeated while retaining the THP ether at C-3 (in the hope that this would prevent the observed side reactions) only unchanged starting material H-6 was isolated. Neither increasing the reaction temperature to 100°C nor adding a catalytic amount of N,N-dimethylaminopyridine produced the desired oxime acid. A final attempt to prepare the oxime under acidic conditions using carboxymethoxyamine hemihydrochloride in refluxing toluene under azeotropic removal of water resulted in extensive decomposition. It was concluded that the 4-ketone is too hindered to undergo a reaction to the oxime, and thus this preparation was abandoned.

3.5 15-Acetoxy 3α,4β-dihydroxy 8α-isovaleryloxyscirpen,4-hemisuccinate (I-3)

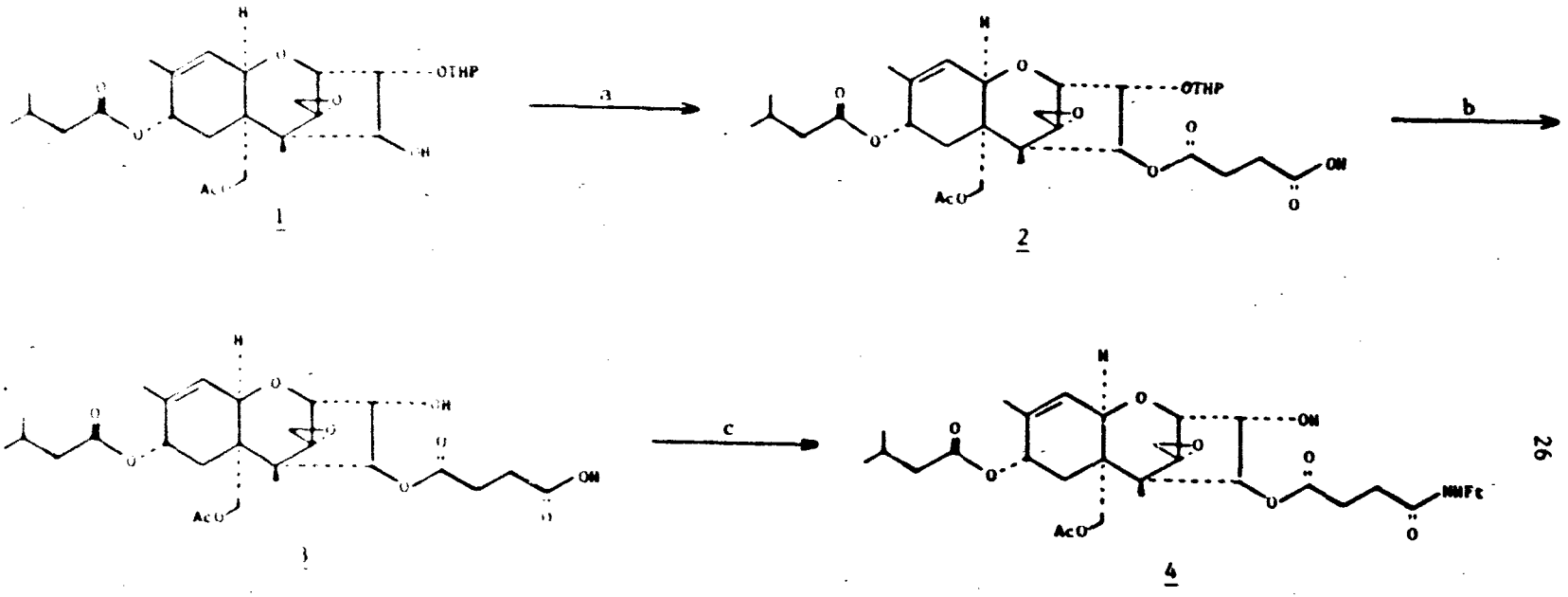
The synthesis of the hapten derived from HT-2 toxin is shown in Chart I. The reaction of the 3-THP ether of HT-2 toxin (I-1) with succinic anhydride in pyridine proceeded smoothly in 70% yield to give the hemisuccinate I-2. The THP ether was cleaved by pyridinium tosylate in refluxing EtOH to give the hemisuccinate derivative of HT-2 toxin (I-3) as a solid in 51% yield, after silica gel chromatography. The physical properties (IR, NMR, and MS) were consistent with the assigned structure. The hemisuccinate was coupled with bovine serum albumin (BSA, vide infra) and chicken gamma globulin (CGG, vida infra). Stability of the acid I-2 (e.g., epoxide cleavage, hydrolysis, etc.) to the reaction conditions required for conjugation was determined by preparing the N-ethyl amide I-4 under the same conditions.

3.6 4β Acetoxy 8α isovaleryloxyscirpene 3α,15 diol,15 hemisuccinate (J 11)

The synthesis of the 15-hemisuccinate hapten from anguidine is based on the selective protection-deprotection methodology (Chart J). Anguidine (J 1) was thus converted to TBDMS-ether J-2 by treatment with t-butyldimethylsilyl

Chart I

Synthesis of 4-Hemisuccinate of HT-2 Toxin



1 2 3 4

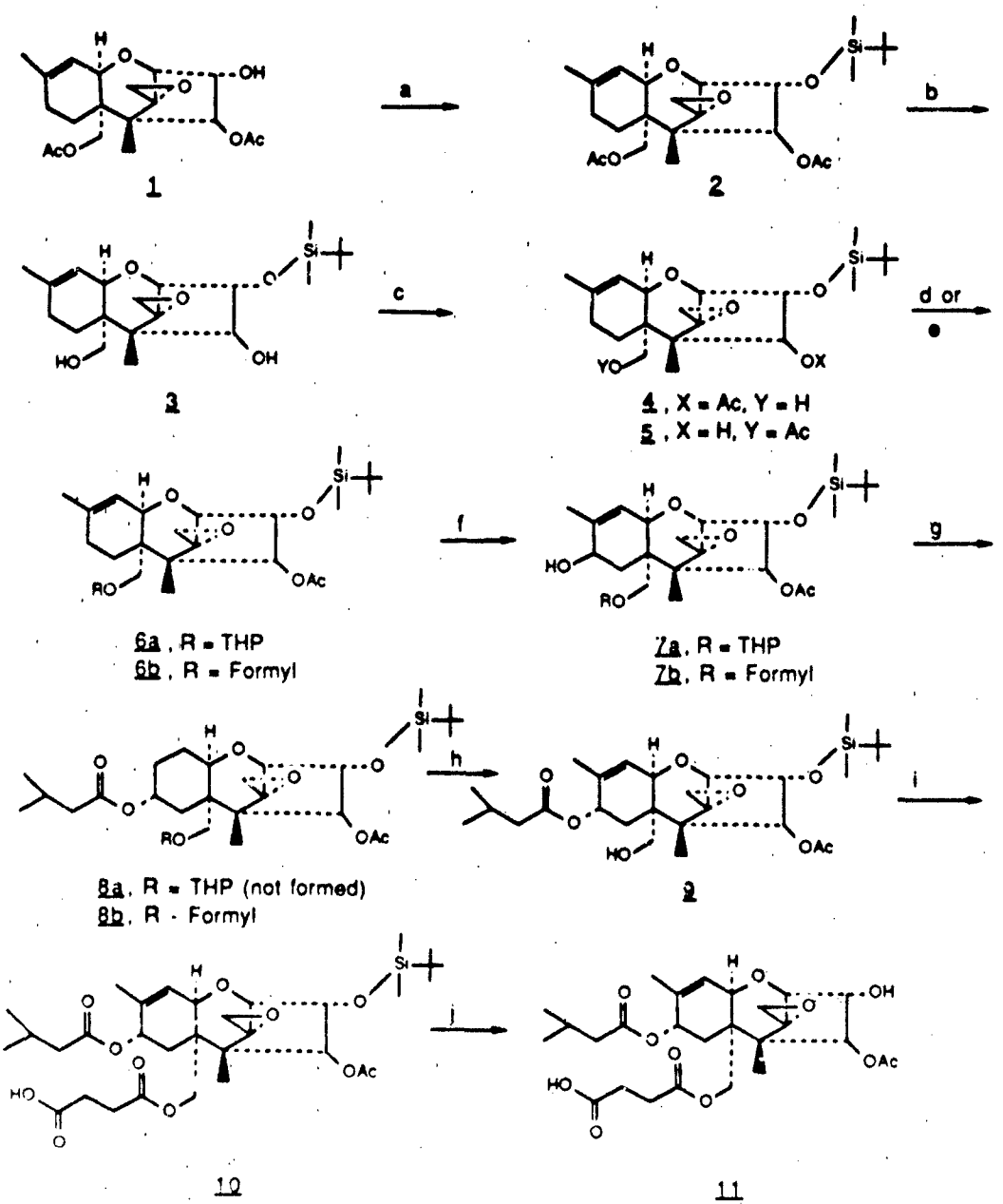
a) Succinic annydride, pyridine, DMAP

b) Pyridine · TsOH, 95% EtOH

c) Et_3N, t-BuOCOCl; $EtNH_2$

CHART J

Synthesis of 15-Hemisuccinate of T-2 Toxin



a) t-Butyldimethylsilyl trifluoromethanesulfate, lutidine
b) OH
c) Acetylimidazole
d) DHP, TsOH
e) Formylimidazole
f) SeO_2
g) $(C_6H_5)_3P$, DEAD, isovaleric acid
h) MeOH, Et_3N
i) Succinic anhydride, pyridine
j) $(nBu)_4NF$

(TBDMS) trifluoromethane sulfonate in the presence of lutidine. The silyl ether J-2 was then subjected to basic hydrolysis to give diol J-3. These two steps proceeded in essentially quantitative yields. The 4-hydroxy functionality was then selectively reacetylated in 23% yield following the procedure of Roush, et al. (1985) to give J-4. This reaction also resulted in the formation of the diacetate J-2, along with minor amounts of the positional isomer J-5. This mixture of compounds can be separated by elution from SiO_2 with 40% Et_2O in hexanes. It is possible to recycle the esters J-2 and J-5 by conversion to the diol J-3. Initially it was planned to protect the 15-OH as a THP ether, thus the THP ether J-6a was formed by treatment with dihydropyran in the presence of TsOH. This compound was then converted to J-7a in 7(% yield by reaction with SeO_2 in refluxing aqueous dioxane. Unfortunately, when J-7a was subjected to $(C_6H_5)_3P$, DEAD and isovaleric acid, none of J-8a was isolated. It appears that the THP ether is too bulky to permit inversion esterification reaction at the C-8 position. We therefore considered protecting the 15-OH group as a formate ester. It was anticipated that the formate ester should present no difficulties in the esterification-inversion reaction at C-8.

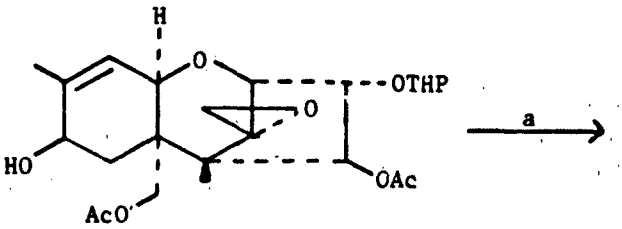
Accordingly, the formate ester J-6b was prepared in quantitative yield by reaction of J-4 with formylimidazole in pyridine. THis compound was converted to J-7b in 32% yield by reaction with SeO_2 in refluxing dioxane. Treatment of J-7b with triphenylphosphine, DEAD and isovaleric acid gave the isovaleryl ester J-8b which was selectively methanolyzed to give J-9 in 40% yield. Reaction with succinic anhydride followed by cleavage of the silyl ether at C-3 yielded the target hemisuccinate J-11. The physical properties (IR, NMR and MS) were consistent with the assigned structure.

3.7 4β,15-Diacetoxy-8α-(3-hydroxy-3-methylbutyryloxy)scirpene,3α-ol(K-3;3'-Hydroxy T-2 Toxin)

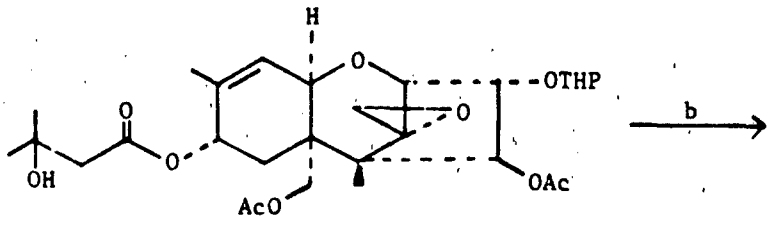
In order to explore the cross-reactivity of several varied trichothecenes in RIA, it was considered beneficial to test a metabolite of T-2 toxin, 3'-hydroxy T-2 toxin (Yoshizawa, et al., 1982). Since this compound is commercially unavailable, we utilized the synthetic route employed earlier for the synthesis of tritiated T-2 toxin (Chart K). Thus, K-1 (obtained by the SeO_2 oxidation of the THP ether of anguidine) was converted to K-2 by use of triphenylphosphine, diethyl azodicarboxylate and 3-methyl-3-hydroxybutanoic acid. The desired compound K-3 was then isolated after hydrolysis of the THP ether by pyridinium tosylate in refluxing 95% EtOH. The physical properties (IR, NMR and HRMS) of K-3 were consistent with the assigned structure.

Chart K

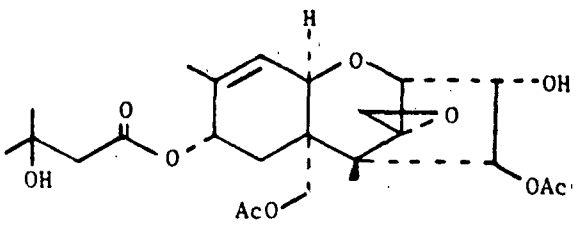
Synthesis of 3'-Hydroxy T-2 Toxin



1



2



3

a) $(C_6H_5)_3P$, DEAD, 3-methyl-3-hydroxybutanoic acid

b) pyridinium tosylate, 95% EtOH

4.0 Immunogen Synthesis

The oxime acid A-4 was used to synthesize immunogens L-1 and L-2 from bovine serum albumin (BSA) and bovine thyroglobulin (BTg) respectively. Conjugation of acid to protein was carried by the mixed anhydride procedure of Erlanger et al. (1957) as shown in Chart L.

Conjugates were purified by dialysis using Spectra/Por tubing with a molecular weight cutoff of 12-14,000. Ultraviolet analysis of the conjugate with BSA (L-1) indicates an incorporation ratio of 25 and with BTg (L-2) a ratio of 60, with no detectable unbound A-4 in either conjugate.

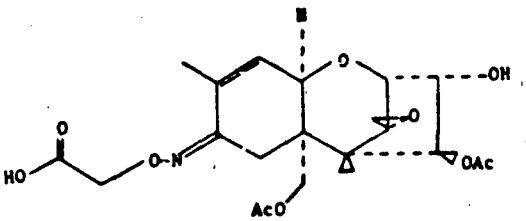
The hemisuccinate I-3 was used to synthesize the immunogen M-1 from bovine serum albumin (BSA). Conjugation of I-3 to protein was carried out by the same mixed anhydride procedure as shown in Chart M.

The conjugate was purified by dialysis using Spectra/Por tubing with a molecular weight cutoff of 12-14,000. An incorporation ratio of 19 moles of hapten per mole of BSA was obtained, as determined by the procedure of Habeeb (1966).

Using similar procedures, the hemisuccinate I-3 was conjugated to chicken gamma globulin (CGG) and purified by dialysis as above to give the conjugate M-2. Attempts to determine the molar ratio between the hapten and CGG by the Habeeb procedure resulted in inconsistent incorporation values. Hence, the ratio remains undetermined.

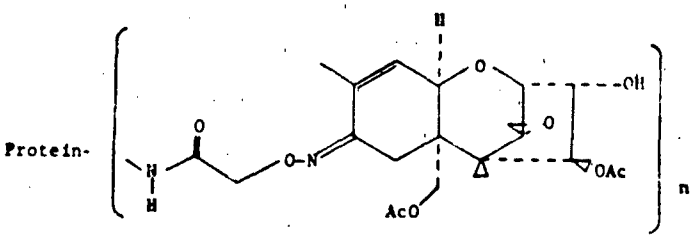
Chart L

Synthesis of 8-Linked Immunogen



A-4

a,b,c



1 Protein = BSA

2 Protein = BTg

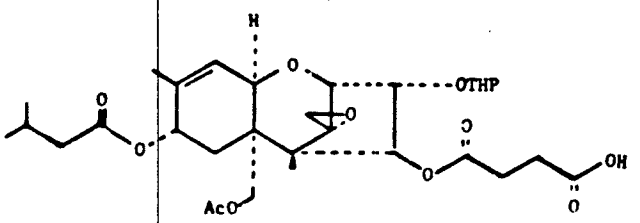
a) Et_3N

b) i-BuOCOCl

c) Protein in 0.1 M $NaHCO_3$

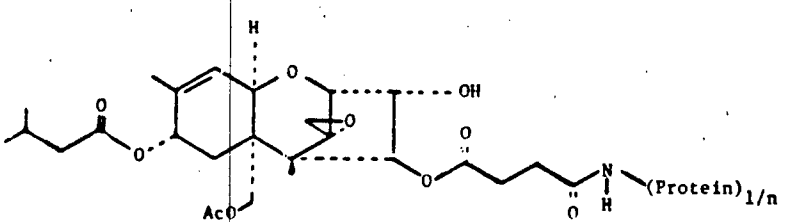
Chart M

Synthesis of 4-Linked Immunogen



I-3

a,b,c



1, Protein = BSA
2, Protein = CGG

a) Et_3N

b) i-BuOCOCl

c) Protein in 0.1 M $NaHCO_3$

5.0 Polyclonal Antibodies to T-2 Toxin

5.1 Production of Polyclonal Antisera in Rabbits Using T-2 Toxin Coupled to Bovine Serum Albumin (BSA) and Bovine Thyroglobulin (BTg) at the C-8 Position

We have completed long-term immunization protocols for producing polyclonal antisera in rabbits by use of the C8-T-2-BSA and C8-T-2-BTg immunogens. We have collected 8 antisera from each of 7 rabbits and 6 antisera from one rabbit (animal #532 died on 12/31/85 from causes unrelated to the immunization protocol). These antisera, except for small reference samples, have all been shipped to USARMDC. The titers (defined as the antiserum dilution giving 50% of maximal binding) for these 62 antisera as measured in an enzyme immunoassay (EIA) are shown in Tables I and II. Patterns of antibody response differ among individual rabbits. Of the 4 rabbits immunized with C8-T-2-BSA, 2 animals (#532, #547) produced high-titered sera early in and throughout the immunization protocol, one (#533) produced consistently high-titered sera only after several booster injections and one (#531) had serum titers which fluctuated during the immunization protocol. Two of the rabbits immunized with C8-T-2-BTg (#545, #546) failed to produce high-titered sera, while the other two (#543, #544) had fluctuating titers.

We have further analyzed four of these sera (531-4, 532-4, 544-4, 545-4) by competitive inhibition radioimmunoassay (CIRIA) to determine average affinity constants for T-2 toxin and cross-reactivities with related trichothecenes (see Table XIII for structures). Results are shown in Tables III and IV. Curve fitting for determination of concentrations of T-2 toxin and related trichothecenes giving 50% inhibition of binding (IC_{50}) was done by use of the 4-parameter logistic equation of Rodbard (Rodbard and Hutt, 1974); affinity constants were calculated by the method of Mueller (1983).

TABLE I. Titers[a] of Antisera from Rabbits Immunized with C8-T-2 BSA as Measured in Enzyme Immunoassay (EIA)

	Animal #			
Bleed	531	532	533	547
1	13,165	4,594	5,256	14,247
2	5,188	18,997	5,939	18,159
3	11,285	23,240	9,591	16,308
4	16,943	16,762	5,911	26,267
5	5,715	20,670	22,134	18,086
6	8,741	27,533	17,879	16,961
7	6,632	---[b]	20,991	18,146
8	18,396	---[b]	28,810	17,593

[a] Titers are expressed as the reciprocal of the antiserum dilution giving 50% of maximal binding in the EIA.

[b] Animal #532 died on 12/31/85.

TABLE II. Titers[a] of Antisera from Rabbits Immunized with C8-T-2 BTg as Measured in Enzyme Immunoassay (EIA)

	Animal #			
Bleed	543	544	545	546
1	3,344	13,408	4,003	6,426
2	5,510	3,738	6,888	4,278
3	7,383	14,198	2,761	916
4	9,657	11,714	4,711	4,227
5	28,345	2,087	6,965	4,975
6	11,388	11,829	2,497	2,047
7	1,545	25,793	3,109	4,970
8	3,284	5,605	3,728	4,047

[a] Titers are expressed as the reciprocal of the antiserum dilution giving 50% of maximal binding in the EIA.

TABLE III. Average Affinity Constants for Four Antisera from Rabbits Immunized with C8-Coupled T-2 Toxin Immunogens

Serum	Ka,av (M^{-1})
531-4[a]	6.1×10^9 (1.4×10^9; n = 7)[c]
532-4[a]	6.0×10^9 (1.6×10^9; n = 7)
544-4[b]	6.6×10^9 (5.0×10^9; n = 7)
545-4[b]	6.0×10^9 (2.4×10^9; n = 7)

[a] Rabbits immunized with C8-T-2 BSA.

[b] Rabbits immunized with C8-T-2 BTg.

[c] Standard deviation and number of replicate experiments.

TABLE IV. Cross-Reactivities of Four Antisera from Rabbits Immunized with C8-Coupled T-2 Toxin Immunogens

Hapten	Serum			
	531-4[a]	532-4[a]	544-4[b]	545-4[b]
T-2 Toxin	100%[c]	100%	100%	100%
3'-Hydroxy T-2 Toxin	102% (6)[d]	100% (10)	140% (4)	95% (0)
3',4'-Dehydro T-2 Toxin	106% (10)	101% (16)	75% (33)	104% (4)
Anguidine	42% (8)	58% (13)	62% (21)	89% (11)
Neosolaniol	14% (3)	26% (8)	44% (13)	26% (6)
HT-2 Toxin	0.6% (0.2)	0.6% (0.2)	0.6% (0.3)	0.8% (0.1)
Fusarenon-X	<0.2%	<0.2%	0.5% (0.2)	0.2% (0.03)
T-2 Triol	<0.2%	<0.2%	<0.2%	<0.2%
T-2 Tetraol	<0.2%	<0.2%	<0.2%	<0.2%
Verrucarin A	<0.2%	<0.2%	<0.2%	<0.2%
Vomitoxin	<0.2%	<0.2%	<0.2%	<0.2%
Scirpentriol	<0.2%	<0.2%	<0.2%	<0.2%
Nivalenol	<0.2%	<0.2%	<0.2%	<0.2%

[a] Rabbits immunized with C8-T-2 BSA.

[b] Rabbits immunized with C8-T-2 BTg.

[c] Cross-reactivity = (IC_{50}, T-2 Toxin ÷ IC_{50}, Hapten) x 100%.

[d] Standard deviation; number of replicate experiments = 2-4.

The sensitivity of our RIA using these sera for detection of T-2 toxin is 34 pg per tube at 10% displacement and 260 pg per tube at 50% displacement. The presence of up to 100 uL per tube of human plasma, mouse plasma or mouse serum has no adverse effect on binding.

5.2 Production of Polyclonal Antisera in Rabbits Using T-2 Toxin Coupled to Bovine Serum Albumin (BSA) and Chicken Gamma Globulin (CGG) at the C-4 Position

We have terminated our protocol for immunization of rabbits with the C4-hemisuccinate conjugates of T-2 toxin with BSA and CGG. We have collected 5 bleeds each from 8 rabbits. The fourth bleed from each rabbit has been analyzed in a radioimmunoassay (RIA); approximate serum dilutions giving 50% of maximum binding are given below. These are initial dilutions, of which 0.05 mL is included in a total incubation volume of 0.56 mL.

Rabbit-Bleed #	Dilution
680-4	1:1500
683-4	1:500
684-4	1:2500
685-4	1:1000
686-4	1:2500
688-4	1:2500
689-4	1:4000
690-4	1:2000

We have analyzed four of these antisera (684-4, 685-4, 689-4, 690-4) in CIRIA to determine binding affinities for T-2 toxin and cross-reactivities with related trichothecenes (Table XIII). Results are shown in Tables V and VI. The sensitivity of our RIA using these sera for detection of T-2 toxin is 30 pg per tube at 10% displacement and 260 pg per tube at 50% displacement.

Table V. Average Affinity Constants for Four Antisera from Rabbits Immunized with C4-Coupled T-2 Toxin Immunogens

Serum	Ka,av (M^{-1})
684-4[a]	12×10^9 (4.9×10^9; n = 4)[c]
685-4[a]	9.2×10^9 (7.8×10^9; n = 4)
689-4[b]	14×10^9 (8.5×10^9; n = 4)
690-4[b]	7.0×10^9 (3.0×10^9; n = 4)

[a] Rabbits immunized with C4-T-2 BSA.

[b] Rabbits immunized with C4 T-2 CGG.

[c] Standard deviation and number of replicate experiments.

Table VI. Cross-Reactivities of Four Antisera from Rabbits Immunized with C4-Coupled T-2 Toxin Immunogens

Hapten	Serum			
	684-4[a]	685-4[a]	689-4[b]	690-4[b]
T-2 Toxin	100%[c]	100%	100%	100%
HT-2 Toxin	42% (0.6)[d]	34% (7)	48% (5)	71% (2)
3',4'-Dehydro T-2 Toxin	25% (5)	25%	18% (2)	20% (5)
3'-Hydroxy T-2 Toxin[e]	0.7% (0.1)	0.8% (0.3)	0.6% (0.5)	0.6% (0.1)
T-2 Triol	0.4% (0.05)	0.3% (0.1)	0.7% (0.1)	2.4% (1.2)
T-2 Tetraol	<0.2%	<0.2%	<0.2%	<0.2%
Anguidine	<0.2%	<0.2%	<0.2%	<0.2%
Neosolaniol	<0.2%	<0.2%	<0.2%	<0.2%
Verrucarin A	<0.2%	<0.2%	<0.2%	<0.2%
Vomitoxin	<0.2%	<0.2%	<0.2%	<0.2%
Scirpentriol	<0.2%	<0.2%	<0.2%	<0.2%
Nivalenol	<0.2%	<0.2%	<0.2%	<0.2%
Fusarenon-X	<0.2%	<0.2%	<0.2%	<0.2%

[a] Rabbits immunized with C4-T-2 BSA.

[b] Rabbits immunized with C4-T-2 CGG.

[c] Cross-reactivity = (IC_{50}, T-2 Toxin + IC_{50}, Hapten) x 100%.

[d] Standard deviation; number of replicate experiments = 1-4.

[e] Data analyzed by logit-log method.

6.0 Monoclonal Antibodies to T-2 Toxin

6.1 Production of Hybridomas/Monoclonal Antibodies from Mice Immunized with C8-T-2-BSA

We have immunized 15 BALB/c mice with T-2 toxin coupled via the C-8 position to bovine serum albumin (C8-T-2-BSA) according to the schedule given in Table VII. Anti-(T-2 toxin) antibodies in preimmune sera and in bleeds #1-4 from these mice were measured in an enzyme immunoassay (EIA) as the difference between binding to T-2 toxin-conjugated bovine thyroglobulin (BTg) and to unmodified BTg. As shown in Figure 1, all 15 mice produced antibodies to T-2 toxin. Mice #454, 458, and 460 have been sacrificed and their spleen cells fused with a murine myeloma in attempts to produce antibody-secreting hybridomas as discussed below.

We have fused spleen cells from mice #454 and #458 (which had been immunized and then boosted 7 days prior to fusion with 30 ug of the C8-T-2-BSA conjugate in incomplete Freund's adjuvant) with the murine myeloma cell line Sp2/O-Ag14UJ. No viable hybridomas were derived from this fusion. On August 1, 1986, we performed a fusion using a different subline of Sp2/O-Ag14 (Ag14DU) and spleen cells from mouse #460 which had been immunized with the C8-T-2 BSA conjugate and boosted with 60 ug immunogen in incomplete Freund's adjuvant 7 days previously. A few viable hybridomas were produced in this fusion--79 out of 1,820 wells contained dividing cells. Supernatants from 3 of these wells were initially positive for T-2 antibody activity in EIA but were negative when retested 2 weeks later. Cloned cell lines derived from these three wells were also negative for anti-(T-2) activity. The surprising difficulty in obtaining antibody-secreting hybridomas from these mice has yet to be explained. The fusion results described in Section 6.2 are much more typical of those we normally observe.

TABLE VII. Schedule for Immunization of BALB/c Mice with T-2 Toxin BSA Conjugate (May-July, 1986)

5/19/86	(M)	Preimmune bleed and ear tag
5/27/86	(T)	Primary immunization* of mice 453-468
6/10/86	(T)	Bleed 1 of mice 453-468
6/24/86	(T)	Boost+ mice 453-468
7/01/86	(T)	Bleed 2 of mice 453-468
7/08/86	(T)	Boost+ mice 453-468
7/15/86	(T)	Bleed 3 of mice 453-468
7/22/86	(T)	Boost+ mice 453-468
7/29/86	(T)	Bleed 4 of mice 453-468

* 50 ug immunogen and 50 uL Bordetella pertussis in CFA, injected IP.

+ 25 ug immunogen in IFA, injected IP.

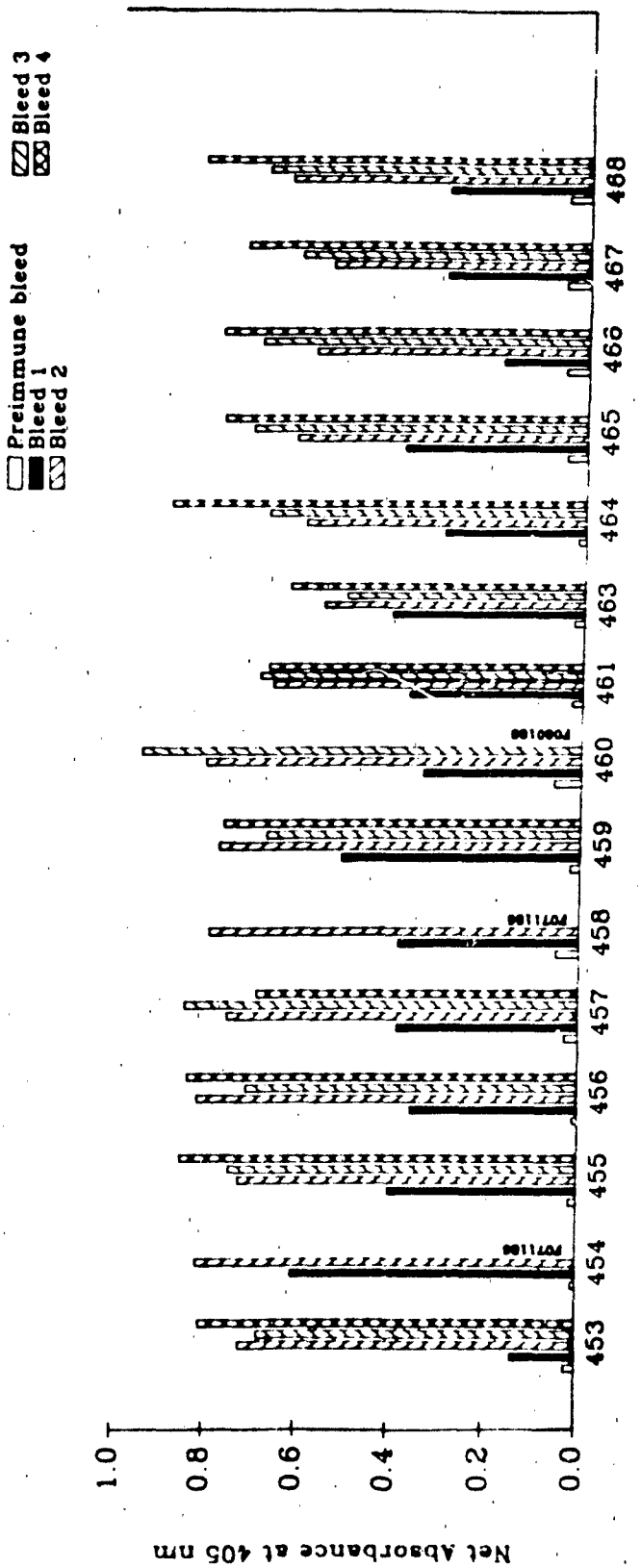


Figure 1. Anti-T-2 Toxin Activity as Measured by EIA in Sera from Mice Immunized with C8-T-2-BSA. Net Absorbance is Difference in Antibody Binding to C8-T-2-BTg and BTg. All Samples Diluted 1:1000.

6.2 Production of Hybridomas/Monoclonal Antibodies from Mice Immunized with C4-T-2-BSA

We have been more successful in deriving hybridomas using the C4-T-2 BSA conjugate as immunogen. We have immunized eight BALB/c mice with the C-4 hemisuccinate T-2 toxin BSA conjugate according to the schedule given in Table VIII. Antibody activities in the preimmune bleed and in bleeds 1-4 from these mice have been measured in an enzyme immunoassay (EIA) as the difference between binding to the C-4 hemisuccinate T-2 toxin CGG conjugate and to unmodified CGG. The EIA results shown for 1:10,000 serum dilutions in Figure 2 indicate high levels of antibody activity in these murine antisera. Spleen cells from mouse #470 were used for hybridoma formation as discussed below.

On September 2, 1986, spleen cells from mouse #470 were fused with the Sp2/0-Ag14DU subline. From this fusion 912 out of 1,200 wells contained dividing hybridomas. Supernatants from 250 of these wells reacted strongly with C4-T-2-CGG; 42 of these supernatants exhibited much higher binding to C4-T-2-CGG than to underivatized CGG and therefore were potential sources of anti-(T-2 toxin) monoclonal antibodies. Twelve of these hybridomas have been screened for reactivity with unconjugated T-2 toxin in a competitive inhibition EIA, and two (F090286.11F7 and F090286.16G4) have been selected for more extensive analysis. Results of the competitive inhibition EIA for 16G4 and 11F7 are shown in Figures 3 and 4, where binding activity is plotted as a function of dilution of supernatant both in the absence and in the presence of free T-2 toxin at 100 ng/well. Sub-isotyping for murine immunoglobulin (Ig) heavy chains indicated that multiple populations of Ig-producing cells are present in these cell lines. The supernatant from 16G4 was positive for μ, γ_1, and γ_{2b}, while the supernatant from 11F7 was positive for μ, γ_{2b}, and γ_3.

Table VIII. Schedule for Immunization of BALB/c Mice with T-2 Hemisuccinate BSA Conjugate (July-September, 1986)

7/14/86	(M)	Preimmune bleed and ear tag
7/22/86	(T)	Primary immunization* of mice
8/05/86	(T)	Bleed 1 of mice
8/19/86	(T)	Boost+ mice
8/26/86	(T)	Bleed 2 of mice
9/02/86	(T)	Boost+ mice
9/09/86	(T)	Bleed 3 of mice
9/16/86	(T)	Boost+ mice
9/23/86	(T)	Bleed 4 of mice

* 50 ug immunogen at. 50 uL Bordetella pertussis in CFA, injected IP.

+ 25 ug immunogen in IFA, injected IP.

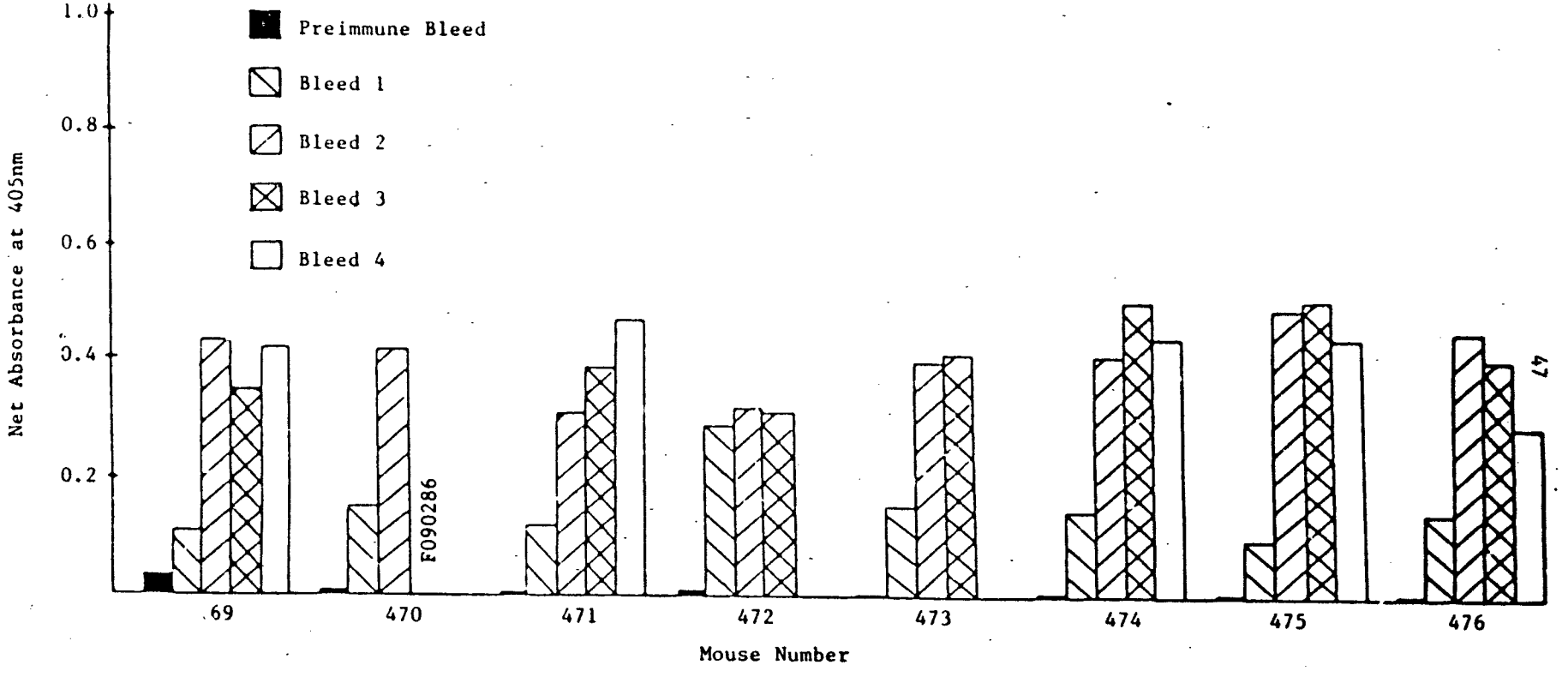


Figure 2: Sera from mice immunized with C4-T-2-BSA as analyzed in EIA. Net absorbance is difference in binding to C4-T-2-CGG and CGG. Sera diluted 1:10,000.

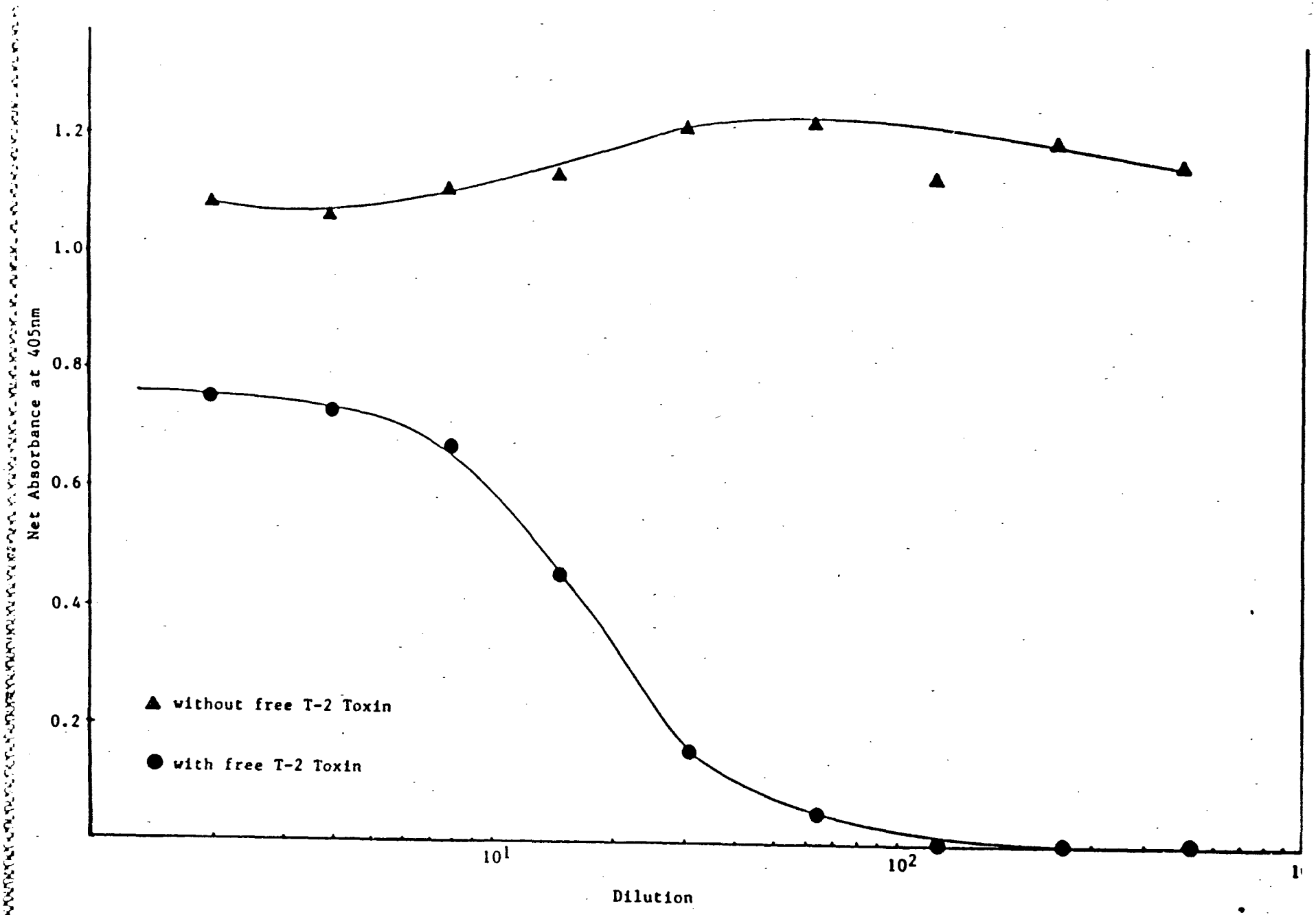


Figure 3: CIEIA for supernatant from F090286.16G4

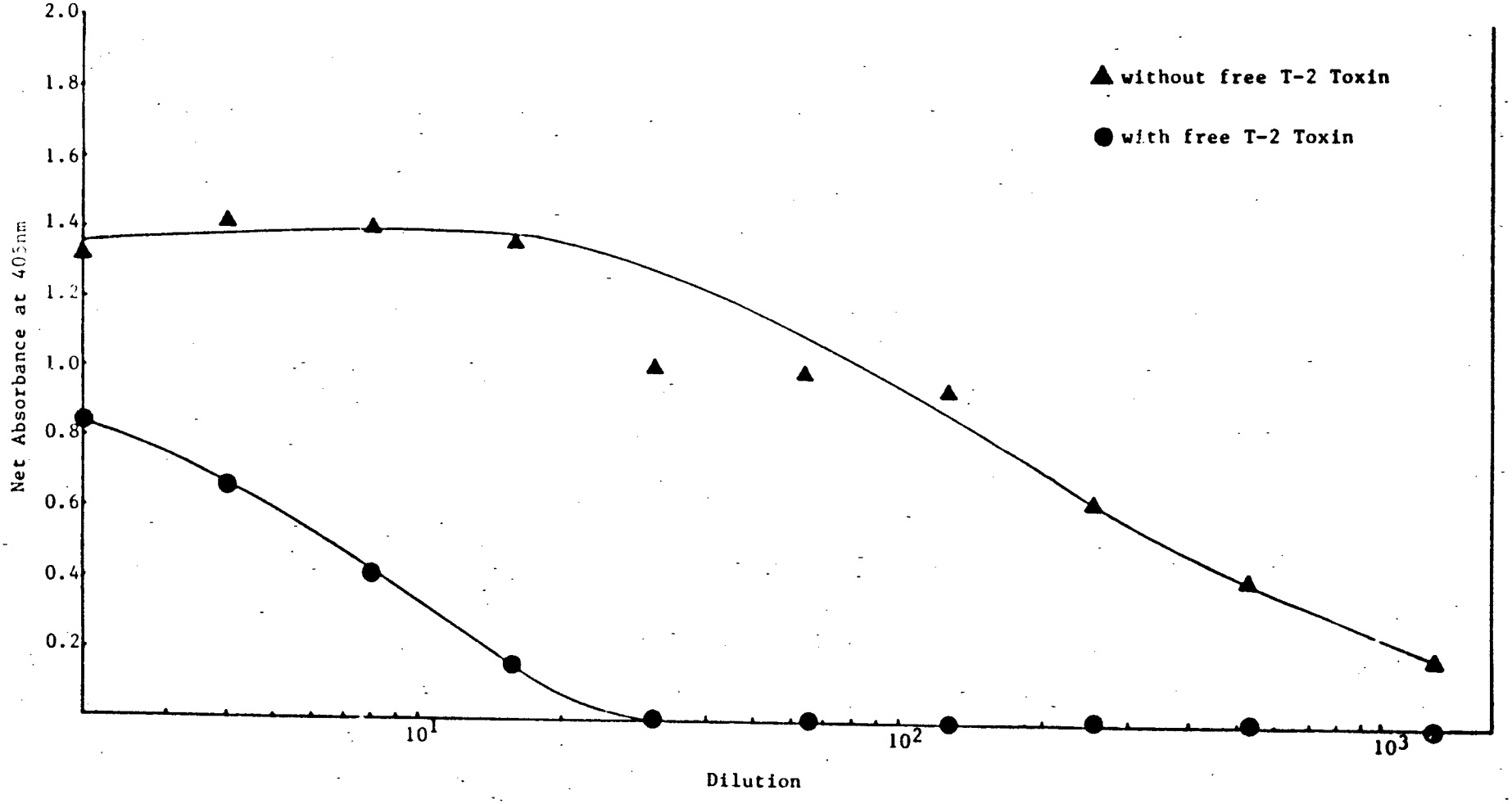


Figure 4: CIEIA for supernatant F090286.11F7

We have cloned the hybridoma lines F090286.11F7 and F090286.16G4 by limiting dilution. Twenty-nine first generation clones (FGC) have been obtained from the 11F7 parent line and 25 from the 16G4 parent line. We have analyzed culture supernatants from these FGC for T-2 toxin binding activity and for immunoglobulin isotypes. Results are shown in Tables IX and X.

We have analyzed culture supernatants from the first generation clones 11F7.E6B and 16G4.F2C by CIRIA to determine binding affinities for T-2 toxin (Table XI) and cross-reactivities (Table XII) with related trichothecenes (Table XIII). Frozen cells from these two hybridoma lines have been supplied to USARMDC. The sensitivity of our RIA for detection of T-2 toxin using these culture supernatants is as follows: for 11F7.E6B, 33 pg per tube at 10% displacement and 260 pg per tube at 50% displacement; for 16G4.F2C, 25 pg per tube at 10% displacement and 220 pg per tube at 50% displacement.

Table IX. T-2 Toxin Binding Activity and Isotype Analysis of First Generation Clones from F090286.11F7

			Isotype/Subisotype Analysis					
FGC	EIA	CIEIA	μ	γ_1	γ_{2a}	γ_{2b}	γ_3	α
B3B	+	+	+	-	-	+	+	-
B4B	+	+	+	-	-	+	+	-
C5B	+	+	+	-	-	+	+	-
D2B	+	+	+	-	-	+	+	-
D3B	-	-	NT[a]	NT	NT	NT	NT	NT
D5B	+	+	+	-	-	+	+	-
D6B	+	±	+	-	-	+	+	-
E7B	+	±	NT	NT	NT	NT	NT	NT
E6B	+	+	-	-	-	-	+	-
E5B	+	+	+	-	-	+	+	-
E4B	+	+	-	-	-	+	-	-
E3B	+	+	+	-	-	+	+	-
F4B	+	+	+	-	-	+	-	-
F6B	+	+	+	-	-	+	+	-
F7B	+	+	+	-	-	+	±	-
F8B	+	+	+	-	-	+	+	-
G7B	+	+	+	-	-	+	+	-
G2B	+	+	+	-	-	+	+	-
B4C	-	-	NT	NT	NT	NT	NT	NT
C7C	+	+	+	-	-	+	+	-
C3C	+	-	+	-	-	+	+	-
D2C	+	+	+	-	-	+	+	-
D5C	+	+	+	-	-	+	+	-
E5C	+	+	+	-	-	+	+	-
E4C	+	+	+	-	-	+	+	-
E2C	+	±	NT	NT	NT	NT	NT	NT
F5C	+	+	+	-	-	+	+	-
F11C	+	+	+	-	-	+	+	-
G3C	+	-	NT	NT	NT	NT	NT	NT

[a] NT = not tested

Table X. T-2 Toxin Binding Activity and Isotype Analysis of First Generation Clones from F090286.16G4

			Isotype/Subisotype Analysis					
FGC	EIA	CIEIA	μ	γ_1	γ_{2a}	γ_{2b}	γ_3	α
C8B	+	+	+	+	-	+	-	-
D7B	+	+	-	+	-	+	-	-
F8B	+	+	-	+	-	-	-	-
F7B	+	+	-	+	-	-	-	-
B5B	+	+	-	+	-	-	-	-
B9B	+	+	+	+	-	+	-	-
B10	+	+	-	+	-	-	-	-
C11B	+	+	+	+	-	+	-	-
C1CB	+	+	-	+	-	-	-	-
C7B	+	+	-	+	-	-	-	-
C5B	+	+	+	+	-	+	-	-
D6B	+	+	-	+	-	+	-	-
D9B	+	+	-	+	-	+	-	-
G10B	+	+	-	+	-	+	-	-
B9C	+	+	-	+	-	+	-	-
C7C	+	+	-	+	-	+	-	-
C8C	+	+	-	+	-	-	-	-
D10C	+	+	+	+	-	+	-	-
E3C	+	+	+	+	-	+	-	-
F8C	+	+	-	+	-	-	-	-
G6C	+	+	-	+	-	+	-	-
D7C	+	+	-	+	-	+	-	-
F2C	+	+	-	+	-	-	-	-
G11C	+	+	+	+	-	-	-	-
G5C	+	+	+	+	-	-	-	-

Table XI. Affinity Constants for T-2 Toxin of Antibodies Secreted by First Generation Clones of Hybridoma Cell Lines

FGC	Ka (M^{-1})
F090286.11F7.E6B	4.7×10^9 (0.3×10^9; n = 2)[a]
F090286.16G4.F2C	7.6×10^9 (1.1×10^9; n = 2)

[a] Standard deviation and number of replicate experiments.

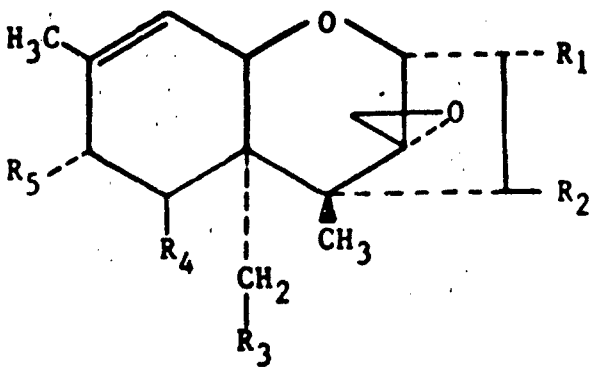
Table XII. Cross-Reactivities of Antibodies Secreted by First Generation Clones of Hybridoma Cell Lines

	FGC	
Hapten	F090286.11F7.E6B	F090286.16G4.F2C
T-2 Toxin	100%	100%
HT-2 Toxin	13%[a] (0.3)[b]	0.9% (0.2)
3'-Hydroxy T-2 Toxin	0.8% (0.0)	<0.2%
3',4'-Dehydro T-2 Toxin	27% (4)	71% (6)
T-2 Triol	<0.2%	<0.2%
T-2 Tetraol	<0.2%	<0.2%
Anguidine	<0.2%	<0.2%
Neosolaniol	<0.2%	<0.2%
Verrucarin A	<0.2%	<0.2%
Vomitoxin	<0.2%	<0.2%
Scirpentriol	<0.2%	<0.2%
Nivalenol	<0.2%	<0.2%
Fusarenon-X	<0.2%	<0.2%

[a] Cross-reactivity = (IC_{50}, T-2 Toxin + IC_{50}, Hapten) x 100%.

[b] Standard deviation; number of replicate experiments = 2.

Table XIII. Structures of Trichothecenes Used in CIRIA



	R_1	R_2	R_3	R_4	R_5
T-2 Toxin	OH	OAc	OAc	H	X
HT-2 Toxin	OH	OH	OAc	H	X
T-2 Triol	OH	OH	OH	H	X
T-2 Tetraol	OH	OH	OH	H	OH
3'-Hydroxy T-2 Toxin	OH	OAc	OAc	H	Y
3',4'-Dehydro T-2 Toxin	OH	OAc	OAc	H	Z
Neosolaniol	OH	OAc	OAc	H	OH
Scirpentriol	OH	OH	OH	H	H
Anguidine (Diacetoxyscirpenol)	OH	OAc	OAc	H	H
Nivalenol	OH	OH	OH	OH	-O-
Vomitoxin (Deoxynivalenol)	OH	H	OH	OH	-O-
Fusarenon-X	OH	OAc	OH	OH	-O-
Verrucarin A	H	-XX-		H	H

X = $OOCCH_2CH(CH_3)_2$

Y = $OOCCH_2C(OH)(CH_3)_2$

Z = $OOCCH_2C(CH_2)CH_3$

XX = $OOCCH(OH)C(CH_3)CH_2CH_2OCH(OH)CHCH_2CHCH_2COO$

7.0 Conclusions and Remarks

1. As part of the synthetic effort of this contract, we developed a new and simple route to T-2 toxin and its analogs from the readily available anguidine. A synthesis of high specific activity tritium-labeled T-2 toxin was developed based on this approach.

2. We modified different positions on the T-2 toxin molecule (4-,8- and 15-) to produce T-2 analogs suitable for conjugation to protein. Two of these were conjugated to proteins for use as immunogens.

3. The T-2 toxin-protein conjugates used as immunogens in this study elicit good antibody responses in rabbits and mice with no apparent toxicity to immunized animals. Levels of antibody activity (titers) vary in individual animals and with the time course of immunization.

4. We have developed a radioimmunoassay (RIA) using ^{3}H-T-2 toxin as the tracer and have analyzed eight selected polyclonal antisera for detection of T-2 toxin and related trichothecenes. The average affinity constants of these antisera are from 6 x 10^{9} M^{-1} to 14 x 10^{9} M^{-1}, and the practical sensitivity of the RIA for T-2 toxin is approximately 32 pg per tube at 10% displacement and 260 pg per tube at 50% displacement.

5. The patterns of cross-reactivity with related trichothecenes observed for these 8 antisera are determined by the position of linkage of T-2 toxin to carrier protein in the immunogen. Antisera generated by immunization with C8-T-2-protein conjugates cross-react significantly with 3'-hydroxy T-2 toxin, anguidine and neosolaniol. These antisera have very low cross-reactivity with HT-2 toxin. In contrast, antisera generated by immunization with C4-T-2-protein conjugates cross-react significantly with HT-2 toxin, have very low cross-reactivity with 3'-hydroxy T-2 toxin and exhibit negligible cross-reactivity with anguidine and neosolaniol

6. We have produced hybridomas secreting anti-T-2 toxin antibodies. These hybridomas are derived from a mouse immunized with C4-T-2-BSA. Three of these hybridoma cell lines have been cloned by limiting dilution, and the antibodies produced by two first generation clones have been analyzed in RIA for detection of T-2 toxin and related trichothecenes. One clone produces an IgG_3 antibody which has an affinity constant for T-2 toxin of 5×10^9 M^{-1} and detects T-2 toxin at a sensitivity of 32 pg per tube at 10% displacement and 260 pg per tube at 50% displacement. This antibody cross-reacts with HT-2 toxin. A second antibody (of the IgG_1 subclass) has an affinity constant of 8×10^9 M^{-1} and detects T-2 toxin at a sensitivity of 25 pg per tube at 10% displacement and 220 pg per tube at 50% displacement. This antibody is highly selective for T-2 toxin and does not cross-react significantly with any other trichothecenes used in this study. Most of the monoclonal anti-T-2 toxin antibodies derived from hybridoma cell lines have not been characterized with respect to binding affinity and cross-reactivity. These antibodies are a potentially valuable resource for the development of antibody-based detection devices.

8.0 Experimental Section

8.1 Chemistry

Melting points were obtained on a Kofler Hot Stage and are uncorrected. The infrared spectra were determined in CH_2Cl_2 on a Perkin-Elmer 267 Infrared Spectrometer. Proton NMR spectra were obtained in $CDCl_3$ with a Varian EM-360 at 60 MHz or with a Bruker WM-250 spectrometer at 250 MHz. Chemical shifts are expressed in ppm with tetramethylsilane as an internal standard. Mass spectra were determined with an AEI MS902 spectrometer at an ionizing voltage of 70 eV.

All reactions were run under dry nitrogen. Tetrahydrofuran (THF) was freshly distilled from lithium aluminum hydride prior to use. Column chromatography was performed on slurry packed silica gel (Kieselgel 60, 70-230 mesh) columns or E. M. Merck Lobar columns.

4β,15-Diacetoxyscirpene-3α,8β-diol (A-2). This compound was prepared by the literature method (Kaneko et al., 1982): yield 57%; mp 116-120°C (lit. mp 114-116°C).

4β,15-Diacetoxy-3α-hydroxyscirpen-8-one (A-3). This compound was prepared by the literature method (Kaneko et al., 1982): yield 88%; foam (lit. foam).

4β,15-Diacetoxy-3α-hydroxyscirpen-8-(O-carboxymethyl)oxime (A-4). To a solution of A-3 (105 mg, 0.28 mmol) in anhydrous pyridine (2 mL) was quickly added carboxymethoxyamine hemihydrochloride. The reaction vessel was stoppered and heated in an oil bath at 35°C for 40 h. The solvent was then removed in vacuo and the residue dissolved in CH_2Cl_2 (10 mL) and washed with cold (0°C) 0.5 N HCl (5 mL). The organic layer was dried (Na_2SO_4) and the solvent removed in vacuo. The residue was eluted from a silica gel (6 g) column using toluene-dioxane-acetic acid (64:35:0.1) to give 57 mg (45% yield)

of A-4 as a foam. Although it was homogeneous by TLC [silica gel; toluene-dioxane-acetic acid (9:5:0.5)], all attempts to crystallize A-4 were unsuccessful; IR 3600, 1735, 1710, 1590 cm^{-1}; UV (MeOH) 243 nm (ε, 13,750); NMR δ 0.83 (s, 3, C-14), 1.78 (s, 3, C-16), 1.95 (s, 3, OAc), 2.07 (s, 3, OAc), 2.77 (d, 1, J = 4Hz, C-13), 3.00 (d, 1, J = 4Hz, C-13), 3.63 (d, 1, J = 5Hz, C-2), 3.98 (s, 2, C-15), 4.17 (m, 1, C-3), 4.50 (d, 1, J = 6Hz, C-11), 4.58 (s, 2, O-CH_2-COOH), 5.17 (m, 1, C-4), 5.87 (d, 1, J = 6Hz, C-10).

N-Ethyl Amide of 4β,15-Diacetoxy-3α-hydroxyscirpen-8-(O-carboxymethyl)-oxime (A-5). To a cold (15°C) solution of A-4 (22 mg, 0.06 mmol) in anhydrous dioxane (2 mL) was added Et_3N (14 μL, 0.1 mmol). After stirring for 3 min, isobutylchloroformate (14 μL, 0.1 mmol) was added, and the reaction was allowed to proceed for an additional 20 min, after which time this reaction mixture was added to cold (0°C) stirred solution of 70% $EtNH_2$ in H_2O. The reaction mixture was stirred at 0°C for 1 h and at room temperature for 1 h. The reaction mixture was then diluted with a saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2 (3x). The organic phase was dried (Na_2SO_4) and the solvent removed in vacuo. Analysis by TLC (silica gel, EtOAc) and HPLC (Partisil 10, RCM; 85:15 EtOAc-hexane, 265 nm) indicated that only one product was formed; IR 3550, 3440, 1735, 1710, 1670 cm^{-1}; NMR δ 0.89 (s, 3, C-14), 1.20 (t, 3, J = 5Hz, N-CH_2-CH_3), 1.88 (s, 3, C-16), 2.02 (s, 3, OAc), 2.16 (s, 3, OAc), 2.88 (d, 1, J = 4Hz, C-13), 3.07 (d, 1, J = 4Hz, C-13), 3.39 (m, 2, N-CH_2-CH_3), 4.03 and 4.20 (ABq, 2, J = 12Hz, C-15), 4.25 (m, 1, C-3), 4.44 (d, 1, J = 5Hz, C-11), 4.55 (s, 2, O-CH_2-CONHEt), 5.19 (d, 1, J = 3Hz, C-4), 6.03 (d, 1, J = 5Hz, C-10). Anal. ($C_{23}H_{32}N_2O_9$): Found m/z 480.211. Required m/z 480.211.

4β-15-Diacetoxy-3α-O-(2-tetrahydropyranyl)scirpene (A-6). This compound was prepared according to a literature procedure (Kaneko et al., 1982).

4β-15-Diacetoxy-3α-O-(2-tetrahydropyranyl)scirpen-8β-ol (A-7). Solution of A-6 (630 mg, 1.4 mmol) and SeO_2 (171 mg, 1.5 mmol) in dioxane (34 mL) containing water (1.4 mL) was refluxed for 22 h. The solvents were removed in vacuo and the residue dissolved in CH_2Cl_2 (3 mL) and filtered through Celite. This material was then eluted from a Merck Lobar silica gel column (size B) using a gradient of 10% EtOAc in CH_2Cl_2 to 50% EtOAc in CH_2Cl_2 to yield A-7 (393 mg, 60%) as a foam; IR 3600, 1735 cm^{-1}; NMR δ 0.72 (s, 3, C-14), 1.78 (s, 3, C-16), 2.02 (s, 3, OAc), 2.05 (s, 3, OAc), 2.73 (d, 1, J = 4Hz, C-13), 3.00 (d, 1, J = 4Hz, C-13), 5.47 (m, 2, C-4, C-10).

4β-15-Diacetoxy-3α-O-(2-tetrahydropyranyl)-8α-isovaleryloxyscirpene (A-11). To a solution of A-7 (120 mg, 0.27 mmol), $(C_6H_5)_3P$ (141 mg, 0.54 mmol) and isovaleric acid (55 mg, 0.54 mmol) in anhydrous THF (2 mL) was slowly (1.5 h) added a solution of diethyl azodicarboxylate (95 mg, 0.54 mmol) in anhydrous THF (2 mL). After stirring at room temperature for an additional hour, the reaction mixture was diluted with CH_2Cl_2 (15 mL) and shaken with a saturated solution of $NaHCO_3$ (5 mL). Removal of the dried (Na_2SO_4) solvent in vacuo gave crude A-11 which was purified by elution from a Merck Lobar silica gel column (size A) using 50% EtOAc in hexanes to yield 71 mg (49%) of pure A-11 as a foam; IR 1735 cm^{-1}; NMR δ 0.72 (s, 3, C-14), 0.95 (m, 7, $(CH_3)_2$-CH-CH_2-CO-), 1.74 (s, 3, C-16), 2.06 (s, 3, OAc), 2.09 (s, 3, OAc), 2.78 (d, 1, J = 4Hz, C-13), 3.03 (d, 1, J = 4Hz, C-13), 5.29 (d, 1, J = 4Hz, C-8), 5.80 (m, 2, C-4, C-10).

4β,15-Diacetoxy-8α-isovaleryloxyscirpen-3α-ol (T-2 toxin, A-12). A solution of A-11 (20 mg, 0.04 mmol) and pyridinium tosylate (3 mg, 0.02 mmol) in 95% EtOH (1 mL) was heated to reflux for 1 h. After removal of the solvent in vacuo, the residue was eluted from silica gel (1 g) with a gradient of 25%

EtOAc in CH_2Cl_2 to EtOAc to yield 12 mg (67%) of T-2 toxin (A-12), mp 145-146°C (lit. mp 150-151°C, Bamburg et al., 1968); IR 3600, 1735 cm^{-1}; NMR δ 0.81 (s, 3, C-14), 0.96 (m, 7, $(CH_3)_2CH$-CO), 1.75 (s, 3, C-16), 2.03 (s, 3, OAc), 2.15 (s, 3, OAc), 2.80 (d, 1, J = 4Hz, C-13), 3.06 (d, 1, J = 4Hz, C-13), 3.80 (d, 1, J = 4Hz, C-2), 4.05 and 4.30 (ABq, 2, J = 12Hz, C-15), 4.17 (m 1, C-3), 4.35 (d, 1, J = 6Hz, C-11), 5.30 (m, 2, C-4, C-8), 5.81 (d, 1, J = 6Hz, C-10).

4β,15-Diacetoxy-8α-O-(3-methyl-3-buteroyl)scirpen-4β-ol (B-2). To a solution of 520 mg (1.4 mmol) of B-1 ((Kaneko et al., 1982), $(C_6H_5)_3P$ (734 mg, 2.08 mmol) and 280 mg of 3-methyl-3-butenoic acid (Smith et al., 1981) in anhydrous THF (25 mL) was slowly (1.5 h) added a solution of diethyl azodicarboxylate (409 mg, 2.8 mmol) in anhydrous THF (5 mL). After stirring at room temperature for an additional 3 h, the reaction mixture was diluted with CH_2Cl_2 (150 mL) and shaken with a saturated solution of $NaHCO_3$ (50 mL). Removal of the dried (Na_2SO_4) solvent in vacuo gave crude B-2 which was purified by elution from silica gel (30 g) using a gradient of 50% Et_2O in hexanes to Et_2O to yield 255 mg (40%) of pure B-2 as a foam: IR 3500, br 1730 cm^{-1}; NMR δ 0.81 (s, 3, C-14), 1.75 (s, 3, C-16), 1.82 (s, 3, CH_3-$\dot{C}$=CH_2), 2.04 (s, 3, OAc), 2.15 (s, 3, OAc), 2.80 (d, 1, J = 4Hz, C-13), 2.99 (s, 2, C(0)-CH_2-$\dot{C}$=CH_2), 3.06 (d, 1, J = 4Hz, C-13), 3.69 (d, 1, J = 5Hz, C-2), 4.87 (s, 1, CH_2=$\dot{C}$-CH_2), 4.93 (s, 1, CH_2=$\dot{C}$-CH_2), 5.30 (m, 2, C-4 and C-8), 5.81 (d, 1, J = 6Hz, C-10). Required for $C_{24}H_{32}O_9$: m/z 464.2046. Found: m/z 464.2049.

4β,15-Diacetoxy-8α-[3,4-$^3H_2$-isovaleryloxy]scirpen-3α-ol (Tritiated T-2 Toxin, B-3). A solution of B-2 (20 mg, 0.04 mmol) and tris(triphenylphosphine)rhodium(I) chloride (8 mg) in toluene (0.75 mL) was exposed to 5 Ci of tritium gas at room temperature for 24 h. The solution was transferred to a vial with MeOH and the solvents removed with a stream of nitrogen. The

residue was chromatographed on a 20 x 20 x 0.1 cm silica gel plate (E. M. Merck) with 2:1 EtOAc in hexanes.

The band corresponding to T-2 toxin was eluted with about 10 mL of chloroform/ethanol (1/1). The resulting solution was then diluted to 50 mL with ethanol and then to 1 liter with toluene.

An aliquot (100 mL) was withdrawn and the solvent concentrated *in vacuo* to 1.0 mL. This aliquot was then transferred to a flask containing 1.0 mg of anguidine, to be used as an internal standard for GLC analysis. This mixture was then analyzed on an OV-17 GLC column at a programmed temperature of 200°C to 250°C. The integral of the known concentration (1 mg/mL) of anguidine was 626 and the integral of the unknown concentration of T-2 toxin was 825 for a ratio of 1:1.32. Thus, the 10% aliquot of the total sample contains 1.32 mg of T-2 toxin, and therefore, the total recovered tritiated T-2 toxin was 13.2 mg. An aliquot was counted using a Minaxi Tri-Carb® 4000 series scintillation counter to give an activity of 73.9 mCi per mg, or 34.3 Ci per mmol.

3α-O-(Tetrahydropyranyl)scirpene-4β,15-diol (H-2). To a cold (0°C) solution of 1.26 g (2.8 mmol) of 3α-O-tetrahydropyranylscirpene-4β,15-diol diacetate (*H-1*) (Kaneko *et al*., 1982) in THF (25 mL) and MeOH (15 mL) was added 0.3 N NaOH (40 mL). The reaction flask was stoppered and allowed to stand at 5°C for 18 h. The reaction mixture was then diluted with CH_2Cl_2 (200 mL) and shaken with H_2O (2 x 100 mL) and brine (50 mL). Removal of the dried (Na_2SO_4) solvent *in vacuo* yielded 956 mg of *H-2* (93%) as an oil (Roush *et al*., 1985).

3α-O-tetrahydropyranyl-15-acetoxyscirpen-4β-ol (H-3). To a cold (0°C) solution of *H-2* (0.96 g, 2.6 mmol) in Et_3N (1 mL) and CH_2Cl_2 (40 mL) was added AcCl (0.35 mL). After being stirred at 0°C for 2 h, the reaction mixture was diluted with CH_2Cl_2 (200 mL) and shaken with aqueous $NaHCO_3$ (2 x 50 mL), H_2O (50 mL) and brine (50 mL). Removal of the dried (Na_2SO_4) solvent *in vacuo*

yielded 1.08 g (96%) of H-3 as a foam; IR 3600, 1735 cm^{-1}; NMR δ 0.81 (s, 3, C-14), 1.74 (s, 3, C-16), 2.00 (s, 3, OAc), 2.72 (d, 1, J = 4Hz, C-13), 2.97 (d, 1, J = 4Hz, C-13), 5.42 (m, 1, C-10). Molecular ion was not observed; required for M^+-THP ($C_{17}H_{23}O_6$); m/z 323.149. Found: m/z 323.149.

3α-O-Tetrahydropyranyl-15-acetoxyscirpen-4β-8β-diol (H-4). A solution of 1.08 g (2.4 mmol) of H-3 and 333 mg (3.0 mmol) of freshly sublimed SeO_2 in dioxane (64 mL) and H_2O (2.8 mL) was refluxed for 17 h. After removal of the solvent in vacuo, the residue was eluted from silica gel (15 g) using a gradient of 10% EtOAc in $CHCl_3$ to 50% EtOAc in $CHCl_3$ to yield 635 mg (68%) of H-4 as a foam; IR 3600, 1735 cm^{-1}; NMR δ 0.85 (s, 3, C-14), 1.83 (s, 3, C-16), 2.00 (s, 3, OAc), 2.72 (d, 1, J = 4Hz, C-13), 2.97 (d, 1, J = 4Hz, C-13), 5.42 (m, 1, C-10). Required for $C_{22}H_{32}O_8$; m/z 424.2097. Found: m/z 424.2094.

3α-O-Tetrahydropyranyl-8α-isovaleryloxy-15-acetoxyscirpen-4β-ol (H-5). To a stirred solution of H-4 (600 mg, 1.4 mmol), $(C_6H_5)_3P$ (750 mg, 2.9 mmol) and isovaleric acid (290 mg, 2.9 mmol) in THF (13 mL) was slowly (2 h) added a solution of diethyl azodicarboxylate (DEAD) (417 mg, 2.9 mmol) in THF (2 mL). After stirring at room temperature for 3 h, the solvent was removed in vacuo and the residue taken up in CH_2Cl_2 (200 mL). The excess isovaleric acid was removed by shaking with aqueous $NaHCO_3$ (2 x 50 mL) and the organic layer dried over Na_2SO_4. The crude product obtained after removal of the solvent in vacuo was purified by elution from silica gel (10 g) with Et_2O in hexane (2:1) to give 223 mg (35%) of pure H-5 as a foam; IR 3600, 1735, 1725 cm^{-1}; NMR δ 0.83 (s, 3, C-14), 0.98 [d, 6, J = 5Hz, $(CH_3)_2CH-CH_2$], 1.72 (s, 3, C-16), 2.00 (s, 3, OAc), 2.02 [d, 2, J = 4Hz, $(CH_3)_2-CH-CH_2-C=O$], 2.72 (d, 1, J = 4Hz, C-13), 2.97 (d, 1, J = 4Hz, C-13), 5.18 (d, 1, J = 6Hz, C-8), 5.58 (d, 1, J = 6Hz, C-10). Molecular ion was not observed; required for M+1 ($C_{27}H_{41}O_9$); m/z 509.2748. Found: m/z 509.2748.

3α-O-Tetrahydropyranyl-8α-isovaleryloxy-15-acetoxyscirpen-4-one (H-6). To a stirred mixture of H-5 (22 mg, 0.044 mmol) and anhydrous NaOAc (4 mg, 0.048 mmol) in CH_2Cl_2 (1 mL) was added 22 mg (0.1 mmol) of pyridinium chlorochromate. After stirring at room temperature for 17 h, the solvent was removed in vacuo. The crude ketone H-6 was purified by elution from silica gel (1 g) using CH_2Cl_2/EtOAc/hexane (1:1:1) to yield 14 mg (64%) of a pure H-6 as a foam; IR 3580, 1740, 1730 cm^{-1}; NMR δ 0.90 [m, 9, C-14, $(CH_3)_2$-CH-], 1.72 (s, 3, C-16), 1.96 (s, 3, OAc), 2.00 [d, 2, J = 4Hz, $(CH_3)_2$-CH-CH_2-C=O], 2.87 (d, 1, J = 4Hz, C-13), 3.12 (d, 1, J = 4Hz, C-13), 5.20 (m, 1, C-8), 5.55 (m, 1, C-10).

3α-Hydroxy-8α-isovaleryloxy-15-acetoxyscirpen-4-one (H-7). A solution of H-6 (153 mg, 0.3 mmol) and pyridinium tosylate (8 mg, 0.04 mmol) in 95% EtOH (8 mL) was refluxed for 3 h. Removal of the solvent gave the crude product which was purified by elution from silica gel (10 g) with EtOAc in hexane (2:3) to yield 110 mg (88%) of pure H-7; IR 3600, 1740, 1730 cm^{-1}; NMR δ 0.89 [m, 9, C-14, $(CH_3)_2$-CH-CH_2], 1.67 (s, 3, C-16), 1.95 (s, 3, OAc), 2.00 (d, 2, J = 4Hz, $(CH_3)_2$-CH-CH_2-C=O), 2.87 (d, 1, J = 4Hz, C-13), 3.12 (d, 1, J = 4Hz, C-13), 3.90 (s, 2, C-15), 4.20 (d, 1, J = 4Hz, C-11), 5.20 (m, 1, C-8), 5.55 (m, 1, C-10). Required for $C_{22}H_{30}O_8$: m/z 422.1940. Found: m/z 422.1945.

Hemisuccinate of 3α-O-Tetrahydropyranyl-8α-isovaleryloxy-15-acetoxyscirpen-4β-ol (I-2). A solution of 83 mg (0.16 mmol) of 3α-O-tetrahydropyranyl-8α-isovaleryloxy-15-acetoxyscirpen-4β-ol (I-1) and 400 mg (4 mmol) of sublimed succinic anhydride and a catalytic amount (2 mg) of N,N-dimethylamino pyridine in 4 mL of pyridine (distilled from BaO) was heated at 100°C for 1 h. The reaction mixture was then cooled to 40°C and the solvent removed in vacuo. The residue was then triturated with Et_2O and filtered to remove the precipitated succinic anhydride. The crude hemisuccinate was purified by elution

from silica gel (10 g) using a gradient of 34% EtOAc in $CHCl_3$ to 50% EtOAc in $CHCl_3$ to yield 68 mg (70%) of pure I-2 as a foam; IR 3500, 1735 cm^{-1}; NMR δ 0.70 (s, 3, C-14), 0.95 [m, 7, $(CH_3)_2CH-CH_2$], 1.80 (s, 3, C-16), 2.00 (s, 3, OAc), 2.02 [s, 2, $(CH_3)_2-CH-CH_2-C=O$], 2.67 [s, 4, $C(O)-CH_2-CH_2-C(O)$], 2.79 (d, 1, J = 4 Hz, C-13), 3.06 (d, 1, J = 4 Hz, C-13), 5.30 (d, 1, J = 6 Hz, C-8), 5.80 (m, 2, C-4 and C-10). Molecular ion was not observed; required for [$M^+-C_5H_9O$ (isovaleroyl)-$C_4H_5O_3$(succinoyl)] $C_{22}H_{30}O_8$: m/z 422.1940. Found: m/z 422.1945.

15-Acetoxy-3α,4β-dihydroxy-8α-isovaleryloxyscirpene, 4 hemisuccinate (I-3). A solution of I-2 (150 mg, 0.25 mmol) and pyridinium tosylate (10 mg, 0.05 mmol) in 95% EtOH (10 mL) was refluxed for 4 h. The solvent was removed in vacuo, and the residue redissolved in CH_2Cl_2. The pyridinium tosylate was removed by shaking with H_2O, and the organic layer was dried (Na_2SO_4). Removal of the solvent in vacuo gave 94 mg of slightly impure I-3, which was purified by elution from silica gel (10 g) using 10% EtOH in EtOAc to yield 53 mg (40%) of the hemisuccinate I-3 as a foam; IR 3500, br 1730 cm^{-1}; NMR δ 0.78 (s, 3, C-14), 0.95 [m, 7, $(CH_3)_2CH-CH_2$], 1.75 (s, 3, C-16), 2.03 (s, 3, OAc), 2.14 [s, 2, $(CH_3)_2-CH-CH_2-C=O$], 2.68 [s, 4, $C(O)-C-H_2-CH_2-C(O)$], 2.79 (d, 1, J = 4 Hz, C-13), 3.05 (d, 1, J = 4 Hz, C-13), 3.69 (d, 1, J = 5 Hz, C-2), 5.29 (d, 1, J = 6 Hz, C-8), 5.43 (d, 1, J = 3 Hz, C-4), 5.80 (d, 1, J = 6 Hz, C-10). Molecular ion was not observed; required for [$M^+-C_5H_9O$ (isovaleroyl)] $C_{21}H_{28}O_{10}$: m/z 440.1682. Found: m/z 440.1685.

N-Ethyl Amide of 15-Acetoxy-3α,4β-dihydroxy-8α-isovaleryloxyscirpene, 4 hemisuccinate (I-4). To a cold solution of I-3 (15 mg, 0.029 mmol) in anhydrous dioxane (2 mL) was added Et_3N (5 μL, 0.036 mmol). After stirring for 3 min, isobutyl chloroformate (5 μL, 0.036 mmol) was added, and the reaction was allowed to proceed for an additional 20 min after which time the reaction

was added to a cold (0°) solution of $EtNH_2$ in 0.1 M $NaHCO_3$. The reaction was stirred at 0° for 1 h and at room temperature for 1 h. The reaction mixture was then diluted with a saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2 (3X). The organic phase was dried (Na_2SO_4) and the solvent removed in vacuo. Analysis by TLC (silica gel, EtOAc) indicted that only one product was formed; IR 3440, 1730, 1670 cm^{-1}; NMR δ 0.70 (s, 1, C-14), 0.95 [m, 7 $(CH_3)_2$-CH-CH_2], 1.74 (s, 3, C-16), 2.05 (s, 3, OAc), 2.40 [m, 2, C(O)-CH_2-CH_2-N], 2.70 [m, 2, C(O)-CH_2-CH_2-N], 2.79 (d, 1, J = 4 Hz, C-13), 3.02 (d, 1, J = 4 Hz, C-13), 5.28 (d, 1, J = 6 Hz, C-8), 5.69 (m, 1, C-4), 5.74 (d, 1, J = 6 Hz, C-10). Required for $C_{33}H_{49}NO_{11}$; m/z 635.3305. Found: m/z 635.3300.

4β,15-Diacetoxy-3α-O-(t-butyldimethylsily)scirpene (J-2). To a cold (0°C) solution of J-1 (600 mg, 1.7 mmol) and lutidine (0.55 mL, 4.9 mmol) in anhydrous CH_2Cl_2 (6 mL) was added t-butyldimethylsilyl trifluoromethanesulfonate (0.65 mL, 2.8 mmol). After stirring for 5 min, the reaction mixture was diluted with CH_2Cl_2 (50 mL) and shaken with a saturated solution of $NaHCO_3$. Removal of the dried (Na_2SO_4) solvent in vacuo yielded 803 mg (98%) of J-2 as a foam; IR 1735 cm^{-1}; NMR δ 0.71 (s, 3, C-14), 0.87 (s, 9, t-butyl Si), 1.74 (s, 3, C-16), 1.95 (s, 3, OAc), 2.00 (s, 3, OAc), 2.73 (d, 1, J = 4 Hz, C-13), 2.91 (d, 1, J = 4 Hz, C-13), 5.37 (d, 1, J = 6 Hz, C-10), 5.48 (d, 1, J = 3 Hz, C-4). Molecular ion was not observed; required for M^+-C_4H_9 ($C_{21}H_{31}O_7Si$); m/z 423.1838. Found: m/z 423.1835.

3α-O-(t-Butyldimethylsilyl)scirpene-4β,15-diol (J-3). To a cold (0°C) solution of J-2 (1.44 g, 3.0 mmol) in MeOH (16 mL) and THF (24 mL) was added 0.3 N NaOH (40 mL). The reaction flask was stoppered and allowed to stand at 5°C for 18 h and at room temperature for 4 h. The reaction mixture was then diluted with CH_2Cl_2 (200 mL) and shaken with H_2O (2 x 100 mL) and brine. Removal of the dried (Na_2SO_4) solvent in vacuo yielded 1.13 g (93%) of J-3 as

an oil; IR 3600 cm^{-1}; NMR δ 0.73 (s, 3, C-14), 0.88 (s, 9, t-butyl Si), 1.68 (s, 3, C-16), 2.69 (d, 1, J = 4 Hz, C-13), 2.89 (d, 1, J = 4 Hz, C-13), 5.35 (d, 1, J = 6 Hz, C-10). Molecular ion was not observed; required for $M^+-C_4H_9-H_2O$ ($C_{17}H_{25}O_4Si$); m/z 321.1522. Found: m/z 321.1528.

4β-Acetoxy-3α-O-(t-butyldimethylsilyl)scirpen-15-ol (J-5). To a solution of J-3 (240 mg, 0.6 mmol) in anhydrous toluene (4 mL) was added 1,5-diazabicyclo(5,4,0)undecene (0.04 ml, 0.3 mmol), followed by the addition of acetylimidazole (200 mg, 1.8 mmol). After stirring at room temperature for 5 h, the reaction mixture was diluted with Et_2O (20 mL) and shaken with H_2O (2 x 20 mL). Removal of the dried (Na_2SO_4) solvent in vacuo yielded 241 mg of crude residue, which was purified by elution from silica gel (5 g) using 30% Et_2O in hexanes to give 60 mg (23%) of J-5 as a foam; IR 3600, 1735 cm^{-1}, NMR δ 0.75 (s, 3, C-14), 0.91 (s, 9, t-butyl Si), 1.72 (s, 3, C-16), 2.01 (s, 3, OAc), 2.75 (d, 1, J = 4 Hz, C-13), 3.01 (1, d, J = 4 Hz, C-13), 5.54 (d, 1, J = 6 Hz, C-10), 5.75 (d, 1, J = 3 Hz, C-4). Molecular ion was not observed; required for $M^+-C_4H_9$ ($C_{19}H_{29}O_6Si$); m/z 381.1733. Found: m/z 381.1730.

4β-Acetoxy-15-formyloxy-3α-O-(t-butyldimethylsilyl)scirpene (J-6b). To a solution of J-5 (700 mg, 1.6 mmol) in anhydrous pyridine (10 mL) was added formylimidazole (540 mg, 6 mmol). After stirring at room temperature for 3 h, the solvent was removed in vacuo. The resulting residue was dissolved in $CHCl_3$ (200 mL) and washed with H_2O (2 x 100 mL). Removal of the solvent in vacuo yielded 710 mg (94%) of pure J-6b as a foam; IR 1735 cm^{-1}; NMR δ 0.72 (s, 3, C-14), 0.91 (s, 9, t-butyl Si), 1.72 (s, 3, C-16), 2.10 (s, 3, OAc), 2.76 (d, 1, J =4 Hz, C-13), 3.01(d, 1, J = 4 Hz, C-13), 4.16 (d, 1, J = 12 Hz, C-15), 4.44 (d, 1, J = 12 Hz, C-15), 5.45 (m, 2, C-4 and C-10). Molecular ion was not observed; required for $M^+-C_4H_9$ ($C_{20}H_{29}O_7S$); m/z 409.1682. Found: m/z 409.1685.

4β-Acetoxy-15-formyloxy-3α-O-(t-butyldimethylsilyl)scirpen-8β-ol (J-7b). A solution of J-6b (710 mg, 1.5 mmol) and SeO_2 (209 mg, 1.9 mmol) in dioxane (44 mL) containing H_2O (2 mL) was refluxed for 18 h. The solvents were removed in vacuo, and the residue eluted from silica gel (20 g) using 50% EtOAc in hexanes to yield 282 mg (36%) of J-7b as a foam; IR 3600, 1735 cm^{-1}, NMR δ 0.72 (s, 3, C-14), 0.91 (s, 9, t-butyl Si), 1.83 (s, 3, C-16), 2.11 (s, 3, OAc), 2.80 (d, 1, J = 4 Hz, C-13), 3.03 (d, 1, J = 4 Hz, C-13), 4.09 (d, 1, J = 12 Hz, C-15), 4.49 (d, 1, J = 12 Hz, C-15), 5.38 (d, 1, J = 3 Hz, C-4), 5.53 (d, 1, J = 6 Hz, C-10). Molecular ion was not observed; required for M^+-C_4H_9 ($C_{20}H_{29}O_8Si$): m/z 425.1631. Found: m/z 425.1628.

4β-Acetoxy-8α-isovaleryloxy-3α-O-(t-butyldimethylsilyl)scirpen-15-ol (J-9). To a solution of J-7b (282 mg, 0.58 mmol), $(C_6H_5)_3P$ (283 mg, 1.08 mmol) and isovaleric acid (110 mg, 1.08 mmol) in anhydrous THF (2 mL) was slowly (1.5 h) added a solution of diethyl azodicarboxylate (162 mg, 1.10 mmol) in anhydrous THF (1 mL). After stirring at room temperature for an additional 3 h, the solvent was removed in vacuo, and the residue purified by elution from silica gel (5 g) using 33% Et_2O in hexanes to yield 150 mg of J-8b, containing a small amount (≈20%) of $(C_6H_5)_3PO$. This material was dissolved in anhydrous MeOH (8 mL) containing Et_3N (0.2 mL) and refluxed for 2 h. The solvent was then removed in vacuo, and the residue eluted from silica gel (5 g) using 33% Et_2O in hexanes to yield 125 mg (40%) of J-9 as a foam; IR 3600, 1735 cm^{-1}; NMR δ 0.65 (s, 3, C-14), 0.85 (s, 9, t-butyl Si), 0.87 [m, 7, $(CH_3)_2$-CH CH_2], 1.67 (s, 3, C-16), 2.12 (s, 3, OAc), 2.71 (d, 1, J = 4 Hz, C-13), 2.93 (d, 1, J = 4 Hz, C-13), 3.56 (d, 1, J = 12 Hz, C-15), 3.91 (d, 1, J = 12 Hz, C-15), 5.28 (d, 1, J = 6 Hz, C-8), 5.70 (m, 2, C-4 and C-10). Molecular ion was not observed; required for M^+ C_4H_9 ($C_{24}H_{37}O_8Si$): m/z 481.2257. Found: m/z 481.2249.

Hemisuccinate of 4β-Acetoxy-8α-isovaleryloxy-3α-O-(t-butyldimethylsilyl)-scirpen-15-ol (J-10). A solution of J-9 (150 mg, 0.28 mmol) and sublimed succinic anhydride (300 mg, 3.0 mmol) in anhydrous pyridine (2 mL) containing a catalytic amount of N,N-dimethylamino pyridine (5 mg) was refluxed for 2 h. The solvent was then removed in vacuo and the residue purified by elution from silica gel (5 g) using 66% Et_2O in hexanes to yield 130 mg (72%) of J-10 as a foam; IR 3540, 3100, 1735 cm^{-1}; NMR δ 0.69 (s, 3, C-14), 0.85 (s, 9, t-butyl Si), 0.89 [m, 7, $(CH_3)_2$-CH-CH_2], 1.74 (s, 3, C-16), 2.09 (s, 3, OAc), 2.12 [s, 2, $(CH_3)_2$-CH-CH_2-CO], 2.68 (m, 4, CO-CH_2-CH_2-CO), 2.77 (d, 1, J = 4 Hz, C-13), 3.01 (d, 1, J = 4 Hz, C-13), 4.17 (d, 1, J = 12 Hz, C-15), 4.25 (d, 1, J = 12 Hz, C-15), 5.30 (d, 1, J = 6 Hz, C-8), 5.73 (d, 1, J = 6 Hz, C-10), 5.77 (d, 1, J = 3 Hz, C-4). Molecular ion was not observed; required for M^+-C_4H_9 ($C_{28}H_{41}O_{11}Si$); m/z 581.2417. Found: m/z 581.2398.

4β-Acetoxy-8α-isovaleryloxyscirpene-3α,15-diol,15 hemisuccinate (J-11). A solution of J-10 (125 mg, 0.20 mmol) and $(nBu)_4NF$ (150 mg, 0.60 mmol) in THF (10 mL) was stirred at room temperature for 1 h. The solvent was then removed in vacuo, and the residue eluted from silica gel (5 g) using 10% EtOH in EtOAc to yield 88 mg (84%) of J-11 as a foam: IR 3550, 3100, 1735 cm^{-1}; NMR δ 0.69 (s, 3, C-14), 0.94 [m, 7, $(CH_3)_2$-CH-CH_2-], 1.75 (s, 3, C-16), 2.10 (s, 3, OAc), 2.66 (m, 4, CO-CH_2-CH_2-CO), 2.79 (d, 1, J = 4 Hz, C-13), 3.06 (d, 1, J = 4 Hz, C-13), 4.09 (d, 1, J = 12 Hz, C-15), 4.31 (d, 1, J = 12 Hz, C-15), 5.30 (d, 1, J = 6 Hz, C-8), 5.44 (d, 1, J = 3 Hz, C-4), 5.81 (d, 1, J = 6 Hz, C-10). Molecular ion was not observed; required for M^+-C_5H_9O ($C_{21}H_{28}O_{10}$); m/z 440.1682. Found: m/z 440.1660.

4β,15 Diacetoxy 3α O (2 tetrahydropyranyl)scirpene 8β ol (K-1). A solution of 4β,15 diacetoxy-3α-O-(2-tetrahydropyranyl)scirpene (Kaneko et al.,

1982) (630 mg, 1.4 mmol) and SeO_2 (171 mg, 1.5 mmol) in dioxane (34 mL) containing water (1.4 mL) was refluxed for 22 h. The solvents were removed in vacuo and the residue dissolved in CH_2Cl_2 (3 mL) and filtered through Celite. This material was then eluted from a Merck Lobar silica gel column (size B) using a gradient of 10% EtOAc in CH_2Cl_2 to 50% EtOAc in CH_2Cl_2 to yield K-1 (393 mg, 60%) as a foam: IR 3600, 1735 cm^{-1}; NMR δ 0.72 (s, 3, C-14), 1.78 (s, 3, C-16), 2.02 (s, 3, OAc), 2.05 (s, 3, OAc), 2.73 (d, 1, J = 4 Hz, C-13), 3.00 (d, 1, J = 4 Hz, C-13), 5.47 (m, 2, C-4, C-10). Required for $C_{24}H_{34}O_9$: m/z 466.2203. Found: m/z 466.2201.

3'-Hydroxy T-2 Toxin (K-3). To a solution of K-1 (80 mg, 0.17 mmol) $(C_6H_5)_3P$ (80 mg, 0.34 mmol) and 3-hydroxy-3-methylbutanoic acid (Yoshizawa et al., 1982) (40 mg, 0.34 mmol) in anhydrous THF (2 mL) was slowly (1 h) added a solution of diethyl azodicarboxylate (50 mg, 0.34 mmol) in THF (1 mL). After stirring at room temperature for an additional 3 h, the reaction mixture was diluted with CH_2Cl_2 (20 mL) and shaken with a saturated solution of $NaHCO_3$ (5 mL). After removal of the dried (Na_2SO_4) solvent in vacuo, the residue was passed through a clean-up column (silica gel, 2 g) using Et_2O to remove unchanged starting material and $(C_6H_5)_3PO$. The partially purified material K-2 was then refluxed with pyridinium tosylate (20 mg) in 95% EtOH (5 mL) for 1 h. Removal of the solvent in vacuo gave the crude product which was purified by elution from silica gel (2 g) with Et_2O to give 17 mg (21%) of 3'-hydroxy T-2 toxin (K-3), whose spectral properties (IR and NMR) were identical to those reported (Yoshizawa et al., 1982).

Preparation of Bovine Serum Albumin (BSA) Conjugate (L-1). To a cold (15°C) solution of A-4 (22 mg, 0.05 mmol) in anhydrous dioxane (2 mL) was added Et_3N (14 uL, 0.1 mmol). After stirring for 3 min, isobutyl chloroformate (14 uL, 0.1 mmol) was added, and the reaction was allowed to proceed

for an additional 20 min, after which time the reaction mixture was added to a stirred, cold (0°C) solution of BSA (66 mg, 0.001 mmol) in aqueous $NaHCO_3$ (0.1 M, 3 mL). Stirring was continued for 3 h at 0°C and for 1 h at room temperature. The reaction mixture was diluted with distilled water (20 mL) and dialyzed against distilled water (2 L, 12 changes). The clear solution was lyophilized, yielding 60 mg of highly electrostatic material. Analysis by UV using the following formula (cf. Cook et al., 1980) indicates a hapten incorporation of 25 units:

$$\text{Molar Ratio} = \frac{A_{conjugate} \times M.W._{BSA} - \epsilon_{BSA} \times C_{conjugate}}{\epsilon_{hapten} \times C_{conjugate} - A_{conjugate} \times MW_{hapten\ residue}}$$

A	=	absorbance	= 1.55
C	=	concentration (mg/mL)	= 0.52
MW_{BSA}			= 66,000
$MW_{hapten\ residue}$	=	453-17	= 436
ϵ_{BSA} (at 260 nm)			= 25,750
ϵ_{hapten} (at 260 nm)			= 8,250

Extraction with $CHCl_3$ showed that the amount of unbound hapten was insignificant.

Preparation of Bovine Thyroglobulin (BTg) Conjugate (L-2). To a cold (15°C) solution of A-4 (14 mg, 0.03 mmol) in anhydrous dioxane (2 mL) was added Et_3N (9 µL, 0.06 mmol). After stirring for 5 min, isobutyl chloroformate (9 µL, 0.06 mmol) was added, and the reaction was allowed to proceed for an additional 25 min, after which time the reaction mixture was added to a stirred, cold (0°C) solution of BTg (100 mg, 0.00015 mmol) in aqueous $NaHCO_3$

(0.1 M, 3 mL). Stirring was continued for 3 h at 0° and for 2 h at room temperature. The reaction mixture was diluted with distilled water (20 mL) and dialyzed against distilled water (2 L, 12 changes). The clear solution was lyophilized, yielding 90 mg of highly electrostatic material. Analysis by UV using the formula in the experiment described for the BSA conjugate indicated a hapten incorporation of 60 units. Extraction with $CHCl_3$ showed that the amount of unbound hapten was insignificant.

Preparation of Bovine Serum Albumin (BSA) Conjugate M-1. To a cold (15°C) solution of I-3 (15 mg, 0.029 mmol) in anhydrous dioxane (2 mL) was added Et_3N (5 µL, 0.036 mmol). After stirring for 3 min, isobutyl chloroformate (5 µL, 0.036 mmol) was added, and the reaction was allowed to proceed for an additional 20 min, after which time the reaction mixture was added to a stirred cold (0°C) solution of BSA (46 mg, 0.0007 mmol)in aqueous $NaHCO_3$ (0.1 M, 3 mL). Stirring was continued for 3 h at 0°C and for 1 h at room temperature. The reaction mixture was diluted with distilled H_2O (20 mL) and dialyzed against distilled H_2O (2 L, 12 changes). The clear solution was lyophilized, yielding 42 mg of highly electrostatic material.

The molar ratio between hapten and BSA was 19:1 as determined by the free amino groups present in BSA before and after the conjugation (Habeeb, 1966). Thus, 1.0 mg of conjugate and 1.0 mg of BSA were each dissolved in 2 mL of 2% $NaHCO_3$. To these solutions was added 0.1% trinitrobenzenesulfonic acid (TNBS) (1 mL), and the solutions heated at 60°C for 4 h; then 1 mL of 10% sodium dodecyl sulfate was added to solubilize the protein and prevent its precipitation on addition of 0.5 mL of 1 N HCl. The absorbance of the solution was read at 335 nm against a blank treated as above. The reaction of BSA gave an absorbance of 2.70, and the conjugate gave an absorbance of 1.84. Thus, 68% of the amino groups of the protein conjugate reacted with TNBS, therefore 32%

of the amino groups have reacted with the hapten. Since there are 61 amino groups available (Habeeb, 1966) for reaction, an incorporation of 19 units is obtained (0.32 x 61 = 19). Although this calculation ignores MW differences between BSA and the conjugate, it gives reasonable accuracy.

Preparation of Chicken Gamma Globulin (CGG) Conjugate M-2. To a cold (15°C) solution of I-3 (5 mg, 0.010 mmol) in anhydrous dioxane (0.3 mL) was added Et_3N (1.6 µL, 0.012 mmol) in dioxane (0.2 mL). After stirring for 5 min, isobutyl chloroformate (1.5 µL, 0.012 mmol) was added, and the reaction was allowed to proceed for an additional 20 min, after which time the reaction mixture was added to a stirred cold (0°C) solution of CGG (80 mg, 0.0005 mmol) in aqueous $NaHCO_3$ (0.1 M, 3 mL). Stirring was continued for 3 h at 0°C and for 1 h at room temperature. The reaction mixture was diluted with distilled H_2O (25 mL) and dialyzed against distilled H_2O (2 L, 9 changes). The clear solution was lyophilized, yielding 74 mg of M-2 as highly electrostatic material.

Attempts to determine the molar ratio between hapten and CGG by the Habeeb procedure resulted in inconsistent incorporation values, and thus the ratio remains undetermined.

8.2 Immunology

8.2.1 Production and Characterization of Polyclonal Antibodies

8.2.1.1 Production of Polyclonal Antisera in Rabbits Using T-2 Toxin Coupled to Proteins via the C-8 Position

Female NZW rabbits were purchased from Hazleton Research Products, Denver, PA, and maintained on rabbit chow or certified high fiber rabbit chow and water ad libitum.

Four rabbits (3 kg weight) were immunized with C8-T-2-BSA and four with C8-T-2 BTg as follows: On days 1, 3, 6, and 15 each animal was injected intramuscularly in the thigh with 0.5 mL of an emulsion consisting of equal volumes of T-2 protein conjugate at 0.8 mg/mL in physiological saline and complete Freund's adjuvant (Miles Laboratories, Inc., Naperville, IL). On days 29 and 43 and thereafter at 4 week intervals through day 140 each animal received an intramuscular injection of 0.5 mL of emulsion containing equal volumes of T-2 protein conjugate (0.4 mg/mL in physiological saline) and incomplete Freund's adjuvant (Miles Laboratories). After day 140, animals were rested for 3 months and then boosted on days 233, 261 and 293. Beginning on day 41 and subsequently 11 days after each injection of immunogen in incomplete Freund's adjuvant, animals were bled from a peripheral ear vein. Sera were harvested and stored at -20°. During this 304 day protocol, 8 sera were collected from each of seven immunized rabbits. These animals appeared normal and healthy throughout the immunization procedure. Rabbit #532 died on day 245 of the immunization schedule. Necropsy revealed masses of hair in the stomach of this animal and no indication of toxicity due to T-2 toxin. Six sera were collected from this rabbit.

8.2.1.2 Production of Polyclonal Antisera in Rabbits Using T-2 Toxin Coupled to Proteins via the C-4 Position

Female NZW rabbits were purchased and maintained as above (Section 8.2.1.1) except that their diet consisted of certified high fiber rabbit chow supplemented with fresh carrot tops. Four rabbits (3 kg weight) were immunized with C4-T-2-BSA and four with C4-T-2-CGG as follows: On days 1, 3, 6 and 16 each animal was injected intramuscularly in the thigh with 0.5 mL of an emulsion consisting of equal volumes of T-2 protein conjugate at 0.8 mg/mL in physiological saline and complete Freund's adjuvant. On days 30 and 44 and thereafter at 4 week intervals through day 131 each animal received an intramuscular injection of 0.5 mL of emulsion containing equal volumes of T-2 protein conjugate (0.4 mg/mL in physiological saline) and incomplete Freund's adjuvant. Beginning on day 41 and subsequently 11 days after each injection of immunogen in incomplete Freund's adjuvant, animals were bled from a peripheral ear vein. Sera were harvested and stored at -20°C. During this 142 day protocol, 5 sera were collected from each immunized rabbit. The animals appeared normal and healthy throughout the immunization procedure except that rabbit #688 developed bilateral glaucoma two months into the immunization protocol. Rabbits #688 and 689 exhibited transient swelling of the popliteal lymph nodes on day 103.

8.2.1.3 Enzyme Immunoassay (EIA) and Competitive Inhibition Enzyme Immunoassay (CIEIA)

Anti-T-2 toxin activity in the above sera was measured in solid phase EIA. Test sera were added to microtiter plate wells coated with T-2 protein conjugate; bound antibody was detected by subsequent addition of peroxidase-coupled second antibody and substrate. Sera from rabbits immunized with T-2 BSA were tested on wells coated with T-2 BTg or T-2 CGG and conversely.

Activity of test sera with unconjugated protein and activity of normal rabbit serum with T-2 protein conjugates were measured as controls for nonspecific binding. Details of the EIA are given below.

Wells of 96-well microtiter plates (Dynatech Immulon 2 plates with flat bottom wells, Fisher Scientific) were pretreated for 30 min at room temperature with 100 µL per well of 0.1 M Na_2CO_3, pH=9. Plates were washed twice with deionized/distilled water and suctioned dry. To each well was added 50 µL of T-2 protein conjugate or unconjugated protein at 0.02 mg/mL in phosphate buffered saline (PBS), pH 7.2. Plates were incubated at 37° overnight or until wells were dry. To prevent further adsorption of protein, wells were "blocked" by incubation with 300 µL per well of 0.7% BSA/PBS (Kirkegaard and Perry Labs., Inc., Gaithersburg, MD) for 30 min at room temperature. Excess moisture was removed and 50 µL of dilutions of test sera or normal rabbit serum in PBS were added per well. Plates were incubated for 2 h at room temperature and washed once with 0.02 M imidazole buffered saline containing 0.02% Tween 20 (Kirkegaard and Perry Labs). Peroxidase-coupled second antibody (affinity purified goat antibodies to rabbit immunoglobulin G., heavy and light chain specific, horseradish peroxidase conjugated, Calbiochem Biochemicals, San Diego, CA) diluted 1:3000 in 1% BSA/PBS (Kirekegaard and Perry Labs) was added at 50 µL per well and incubated for 1 h at room temperature. Plates were washed five times with imidazole wash solution (see above), and 50 µL per well of ABTS substrate (Kirkegaard and Perry Labs) were added. Absorbance at 405 nm was read using a Multiskan Microplate Reader (Flow Laboratories, Inc., McLean, VA) after 2.5, 5, 15, and 30 min at room temperature.

Competitive inhibition enzyme immunoassay (CIEIA) was performed as described above except that test samples were diluted 1:2 and incubated in coated wells in the absence or presence of free T-2 toxin at 50 ng or 100 ng per well.

8.2.1.4 Competitive Inhibition Radioimmunoassay (CIRIA)

The reactivities of selected rabbit antisera with T-2 toxin and related trichothecenes were measured in a solution CIRIA using [3H]-T-2 toxin prepared as described in Section 3.3. Antiserum dilutions which bound ~40% of added radioligand were used in these assays. After incubation with antibody in the presence of varying concentrations of unlabeled inhibitors, bound and free radioligand were separated by charcoal adsorption. Details of the CIRIA are given below.

To 12 x 75 mm borosilicate glass tubes were added 0.40 mL buffer (phosphate buffered saline, pH 7.2, containing 0.1% w/v BSA), 0.010 mL radioligand,* 0.100 mL inhibitor solution and 0.050 mL antiserum dilution. For each assay binding was measured in the absence of added inhibitor, and non-specific binding was measured in the absence of serum and in the presence of normal rabbit serum. Tubes were capped, vortexed and incubated overnight at 4°C. Charcoal suspension (0.50 mL per tube @ 15.8 mg/mL in buffer) was added and tubes were capped, vortexed, incubated for 20 min at 4°C and then centrifuged for 10 min at 800 x g. One-half mL of supernatant was removed from each tube and thoroughly mixed with 10 mL of scintillation fluid (Scinti Verse II, Fisher Scientific Company). Radioactivity was measured in a Packard Tri-Carb 460 CD liquid scintillation counter.

*For use in the RIA, an aliquot of radioligand solution in toluene was removed, evaporated to dryness using N_2 gas, and redissolved in a small volume of 95% ethanol. The radioligand/ethanol solution was diluted in buffer (phosphate buffered saline, pH 7.2, containing 0.1% w/v bovine serum albumin) so that 0.010 mL of radioligand solution contained approximately 20,000 cpm of radioactivity.

8.2.2 Production and Characterization of Monoclonal Antibodies

8.2.2.1 Immunization of Mice with T-2 Toxin Coupled to Bovine Serum Albumin via the C-8 Position

Fifteen 6-8 week old female BALB/cByJ mice (The Jackson Laboratories, Bar Harbor, ME) were immunized with C8-T-2-BSA. For the primary immunization (day 1) each animal was injected intraperitoneally (ip) with 0.3 mL of an emulsion containing 50 µg of C8-T-2-BSA, 50 µL of Bacto-Bordetella Pertussis (Difco Laboratories, Detroit, MI), 100 µL of physiological saline and 150 µL of complete Freund's adjuvant. On days 29, 43 and 57, each mouse received an ip booster injection consisting of 25 µg C-8-T-2-BSA in 150 µL physiological saline emulsified in 150 µL of incomplete Freund's adjuvant. Animals were anesthetized with Metofane® and bled from the retro-orbital sinus fourteen days after the primary immunization and seven days after each booster injection. Sera were harvested and stored at -20°. All mice appeared normal and healthy during the immunization procedure.

Anti-T-2 toxin activity in the sera of mice immunized with C8-T-2-BSA was measured in solid phase EIA as the difference in binding to C8-T-2-BTg and BTg. Bound antibody was detected by the addition of peroxidase-coupled second antibody and substrate. The EIA was performed as described in Section 8.2.1.3 except that peroxidase-labeled, affinity-purified goat antibody to mouse IgG and IgM (H+L) (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) diluted 1:101 in 1% BSA/PBS was used as the second antibody.

8.2.2.2 Immunization of Mice with T-2 Toxin Coupled to Bovine Serum Albumin via the C-4 Position

Eight 6-8 week old female BALB/cByJ mice (The Jackson Laboratories, Bar Harbor, ME) were immunized with C4-T-2-BSA and sera were collected as described in Section 8.2.2.1. The mice seemed to suffer no adverse effects

from their exposure to the T-2 protein conjugate. Animal #473 died during retro-orbital bleeding from an apparent overdose of anesthetic, and animal #472 died after bleeding from an iatrogenic cerebral hemorrhage.

Anti-T-2 toxin activity in the sera of mice immunized with C4-T-2-BSA was measured in solid phase EIA as the difference in binding to C4-T-2-CGG and CGG. The assay is described in Sections 8.2.2.1 and 8.2.1.3.

8.2.2.3 Fusion of Spleen Cells from Mice Immunized Using T-2 Protein Conjugates with Sp2/0-Ag14 Cells

All cell culture experiments were carried out aseptically using conventional tissue culture techniques. The basic cell culture medium was high glucose Dulbecco's modified Eagle's medium (DMEM), pH 7.2-7.4, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and L-glutamine (2 x 10^{-3} M), sodium pyruvate (1 x 10^{-3} M) and gentamycin (0.05 mg/mL). Prior to fusion Sp2/0-Ag 14 cells were maintained in log phase growth at viability >95%. After fusion, cells were cultured in medium as above containing HMGT (hypoxanthine, 1 x 10^{-4} M; methotrexate 4 x 10^{-6} M; glycine, 3 x 10^{-6} M; thymidine, 1.6 x 10^{-5} M; and sodium bicarbonate, 1.3 x 10^{-5} M) unless otherwise indicated. Spleen cells from immunized mice were harvested aseptically in serum-free medium. Red blood cells were not removed. Just before fusion, spleen cells and Sp2/0-Ag 14 cells were mixed in serum-free medium at a ratio of two splenic lymphocytes to one Sp2/0-Ag 14 cell.

Cell fusion was achieved by application of a 540 kV pulse for 30 usec using a High Voltage Cell Processor (D.E.P. Systems, Inc., Troy, MI); in one experiment fusion was also accomplished by addition of 50% (w/v) polyethylene glycol (PEG).

All cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 7% CO_2.

For cryopreservation cells were suspended in DMEM containing 15% (v/v) FBS, 10% (v/v) dimethyl sulfoxide, L-glutamine and sodium pyruvate. Vials were frozen at -20°C, transferred to -80°C and stored at -135°C.

The three fusions performed for this project are described in more detail in the following paragraphs.

F071186. Spleen cells from mice #454 and #458 were fused with Sp2/0-Ag 14UJM, a nonsecretor BALB/c hybridoma line. These mice had been immunized (day 1) and boosted (day 29) with C8-T-2-BSA as described in Section 8.2.2.1. They were boosted ip with 30 ug of immunogen in incomplete Freund's adjuvant on day 38 and sacrificed on day 46 (day of fusion). Two fusion techniques were used: (a) electrofusion by a 30 usec pulse of 540 kV and (b) fusion by the addition of polyethylene glycol (PEG). After fusion, cells were gently suspended in medium without HMGT and plated both in 96-well plates at 1×10^4 Sp2 cells per well in the presence of 1×10^4 peritoneal exudate cells (PEC) per well and in 6-well plates at 4×10^5 cells/mL without PEC. Two days later HMGT was added to one half the concentrations indicated above. Seven days after fusion the HMGT concentration was increased to full strength. When 96-well plates were examined microscopically, very few hybridoma colonies were observed, and these died within a few days. From the 6-well plates, 41/48 wells had hybridoma colonies, but none of the supernatants from these wells had anti-T-2 toxin activity as measured in EIA. In conclusion, no hybridomas secreting anti-T-2 toxin antibodies were derived from this fusion.

F080186. Spleen cells from mouse #460 were fused with Sp2/0-Ag14 DU, an Sp2 subline which secretes γ_{2b} heavy chains. This mouse had been immunized on day 1 and boosted on days 29 and 43 as described in Section 8.2.2.1, boosted ip on day 60 with 60 ug of immunogen in incomplete Freund's adjuvant and

sacrificed for the fusion on day 67. Cell fusion was achieved by electrofusion at 540 kV for 30 usec. Cells were suspended in fusion medium containing HMGT and (a) plated at 1 x 10^4 Sp2 cells per well in 96-well plates containing 1 x 10^4 PEC per well and (b) plated at 4 x 10^5 cells/mL in 6-well plates without PEC. Plates were examined for growth seventeen days later, at which time 59 out of 1800 wells in the 96-well plates and 21 out of 21 wells in 6-well plates contained hybridoma colonies. When screened for anti-T-2 toxin activity in EIA 2/59 and 7/21 wells from 96-well and 6-well plates, respectively, were positive. Cell lines derived from these wells by subculturing or cloning by limiting dilution were unstable with respect to anti-T-2 toxin activity, and this activity, when tested, was not inhibitable by excess free T-2 toxin in competitive inhibition EIA. In conclusion, no stable hybridomas with anti-T-2 toxin activity were derived from this fusion.

F090286. In this fusion spleen cells from mouse #470 immunized with C4-T-2-BSA were fused with the Sp2/0-Ag14 DU hybridoma. This mouse received primary and booster injections of immunogen (as described in Section 8.2.2.2) on days 1 and 29, respectively, and an ip injection of 30 ug of immunogen in incomplete Freund's adjuvant on day 37 prior to being sacrificed for fusion on day 43. Cell fusion was accomplished by the electrofusion method as described above. After fusion cells were gently suspended in DMEM containing 10% (v/v) FBS and HMGT and plated in 96-well plates at 1 x 10^4 Sp2 cells per well in the presence of 1 x 10^4 PEC per well. When plates were examined microscopically 13 days after fusion 912/1200 wells contained hybridoma colonies. When supernatants were screened in EIA 15 days after fusion 250/1200 supernatants were positive for binding to C4-T-2 CGG. Of these, 176 supernatants were rescreened on C4-T-2 CGG and CGG, and 42 cell lines were selected for further analysis. Several of these lines have been cloned by limiting dilution; two

such hybridomas, F090286.11F7 and F090286.16G4, have been studied most extensively.

The hybridoma line F090286.11F7 was cloned by limiting dilution on 10-23-86. Cells were plated out in 96-well plates at concentrations of 1 cell/well ("B" plate) and 0.5 cell/well ("C" plate) in the presence of 5 x 10^4 PEC/well. Cloning plates were examined under the microscope 14 days later. Colonies were observed in 18 out of 60 wells on the B plate and in 11 out of 60 wells on the C plate. Supernatants harvested 18 days after cloning from the 18 "B" colonies and the 11 "C" colonies were tested in CIEIA; isotype/subisotype analysis was performed by EIA using a Mouse Hybridoma Subisotyping Kit (Calbiochem Immunochemicals, Behring Diagnostics, LaJolla, CA) according to manufacturer's directions. The first generation clone (FGC) F090286.11F7.E6B was selected for determination of binding affinity for T-2 toxin and cross-reactivities with related trichothecenes. These experiments were performed by CIRIA as described in Section 8.2.1.4 for analysis of polyclonal antibodies.

Hybridoma F090286.16G4 was cloned by limiting dilution on 10-21-86 at concentrations of 1 and 0.5 cell/well as described in the previous paragraph. When cloning plates were examined 15 days later, colonies were present in 14/60 wells of the B plate and in 11/60 wells of the C plate. Supernatants from all 25 wells were tested in CIEIA 20-22 days post-fusion, and isotype/subisotype analysis was performed on these same supernatants. The FGC F090286.16G4.F2C was further analyzed in CIRIA (Section 8.2.1.4) to determine the binding affinity for T-2 toxin and the cross-reactivities with related trichothecenes.

9.0 Literature Cited

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