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DEVELOPMENT OF GENERAL ANTISERA FOR TRICHOTHECANES

Annual Report

C. E. Cook
M. C. Wani
C. C. Whisnant

July 1, 1986

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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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. This project is exploring the relationship between immunogen (hapten) structure and the selectivity of resulting antibodies to various of the trichothecenes. Immunogens are synthesized and used to immunize rabbits. The polyclonal sera are then examined for their selectivity for various trichothecene compounds before attempts are made to prepare monoclonal antibodies.

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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, anguidine was converted to its 8β alcohol analog by treatment with selenium dioxide. Further oxidation with pyridinium chlorochromate yielded the unsaturated 8-ketone. This ketone was readily converted to its oximino acetic acid analog and coupled to protein by a mixed anhydride procedure. The bovine serum albumin (BSA) conjugate yielded an incorporation of 25 trichothecene units per molecule of protein and the bovine thyroglobulin (BTg) conjugate yielded 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 have been analyzed in a competitive inhibition radioimmunoassay (CIRIA) to determine crossreactivities for T-2 toxin and related trichothecenes.

Two simple syntheses of T-2 toxin from readily available anguidine were developed. Use of 3-methyl-3-butenoic acid instead of isovaleric acid in this sequence followed by selective reduction with tritium gas has provided a means of preparing radiolabeled T-2 toxin with high specific activity.

After numerous unsuccessful attempts, the synthesis of the 4-keto analog of HT-2 toxin was achieved. All attempts to introduce the oximino acetic acid moiety at this position were unsuccessful. The synthesis of the 3-hemisuccinate of HT-2 toxin was accomplished in two simple steps, and it was coupled to BSA by the mixed anhydride procedure. An incorporation ratio of 19 moles of hapten per mole of BSA was obtained. This conjugate will be used to immunize rabbits.

see Keywords on page

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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 have started exploring the relationship between immunogen (hapten) structure and the selectivity of resulting antibodies to various of the trichothecenes. We are synthesizing immunogens, using them to immunize rabbits and examining the polyclonal sera for their selectivity for various trichothecene compounds before attempting to prepare monoclonal antibodies.

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 8Y 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 yielded an incorporation of 25 trichothecene units per molecule of protein and the bovine thyroglobulin (BTg) conjugate yielded 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 have been analyzed in a competitive inhibition radioimmunoassay (CIRIA) to determine crossreactivities for T-2 toxin and related trichothecenes.

During this report period we have 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 has provided a means of preparing radiolabeled T-2 toxin with high specific activity.

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. This conjugate will be used to immunize rabbits.

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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Charts, Figure, and Table

1.0 Background

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

The trichothecane molecules are too small to be immunogenic per se, but must first be linked to a large molecule (carrier) such as a protein by a covalent bond. The resulting conjugate will then stimulate antibodies capable of binding the trichothecane (the ligand). 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 (the hapten) 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 hapten which are not directly involved in the link to the carrier.

The affinity of an antibody for the 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 fo 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 only for a group of compounds rather than 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 trichothecane 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 trichothecanes, 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 have begun to examine the influence of hapten structure on antibody selectivity in the tricothecanes.

3.0 Trichothecane Analog Synthesis (Chart A)

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 in 45% yield, after silica gel chromatography, as a foam. 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.

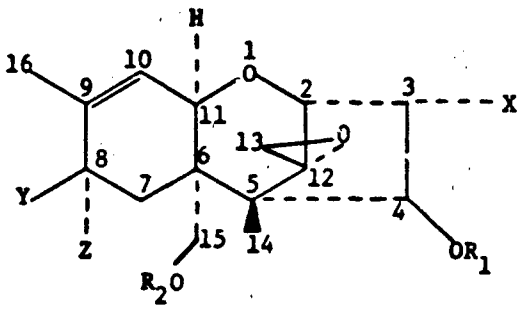
3.2 4β,15-Diacetoxy-8α-isovaleryloxyscirpen-3α-ol (T-2 toxin, A-12)

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.

CHART A

Structures of Tricothecanes



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

It was felt that the 3-OH position should be protected as a THP ether in order 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 diisobutyl-aluminium 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 (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 and GLC.

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.

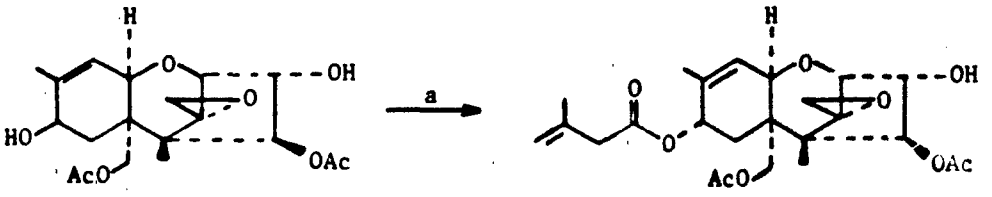
3.3 4β,15-Diacetoxy-8α-[(3,4-$^{3}H_2$)isovaleryloxy]scirpen-3α-ol(Tritiated T-2 toxin, B-3, Chart B)

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.

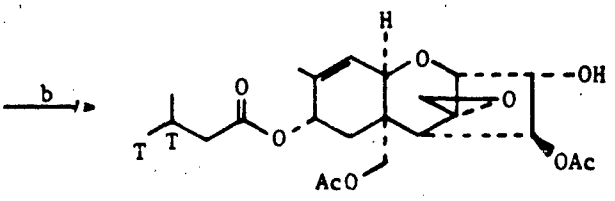
The tritiation step was initially studied using hydrogen gas instead of tritium gas, and subsequent analysis of the product by 250 MHz ^{1}H NMR. When 5% Pd/C was employed as the catalyst, the trisubstituted double bond at C-9 was also reduced along with the terminal double bond in the side chain. Fortunately, 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

CHART B

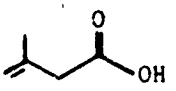
Synthesis of Tritiated T-2 Toxin



1 2



3

a) $(C_6H_5)_3P$, DEAD 

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

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 of TLC (silica gel, 2:1 ethyl acetate in hexanes) indicates the compound to be stable under these conditions for at least a month.

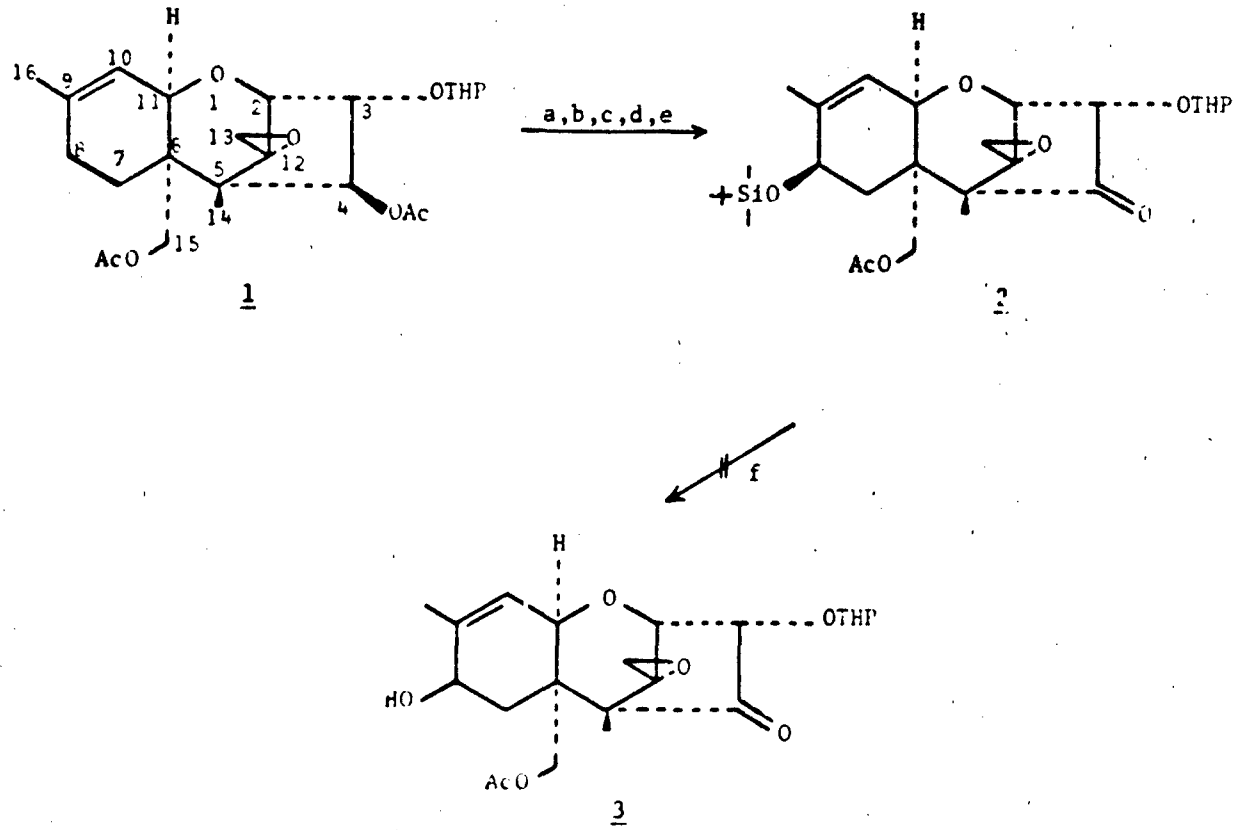
3.4 3α-Hydroxy-8α-isovaleryloxy-15-acetoxyscirpen-4-one (H-7)

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 before (Table A). 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

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) $Bu_4N^+F^-$

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.

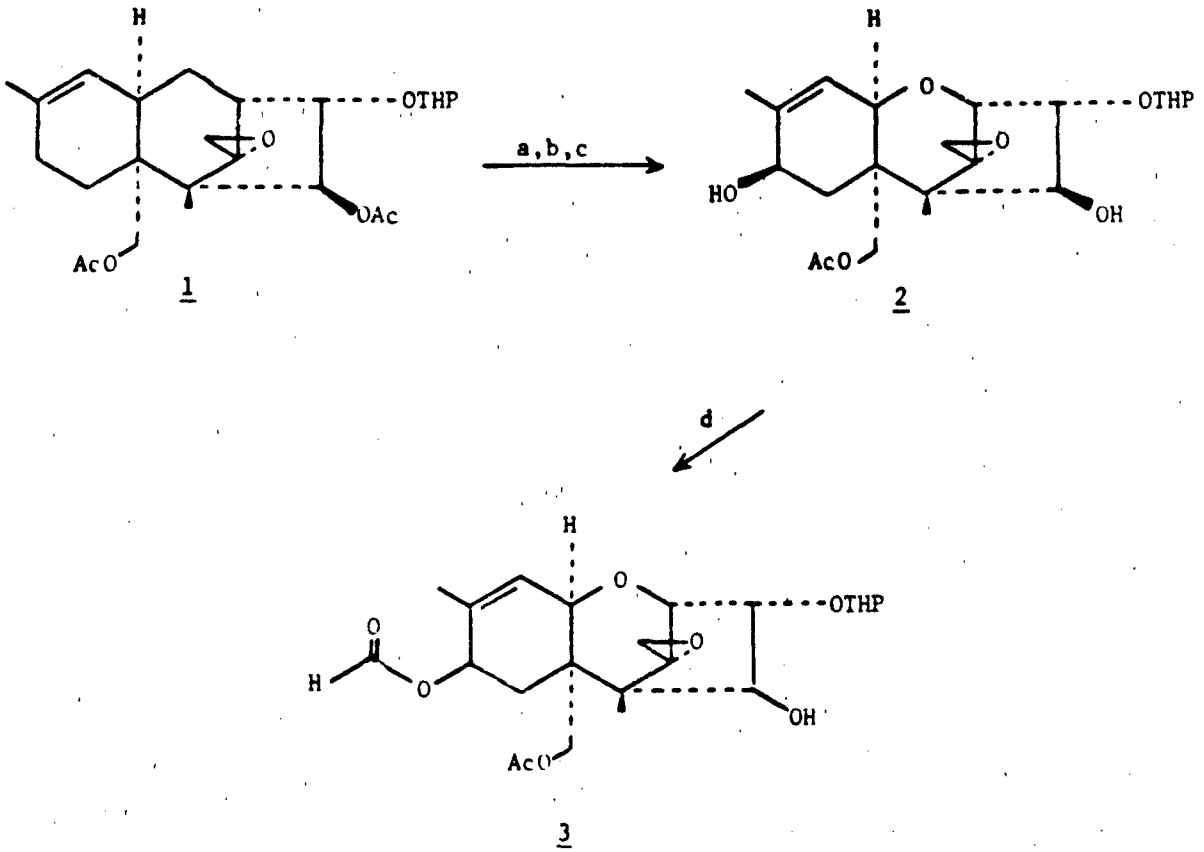
A modification of this approach is shown in Chart D. Instead of protecting the 8ß-hydroxy group as a silyl ether, it was hoped that the C-8 hydroxy group, being allylic, would be more reactive than the hindered C-4 hydroxy group, and therefore selective introduction of a formate ester at C-8 should be 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.

Chart D

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



a) OH^-

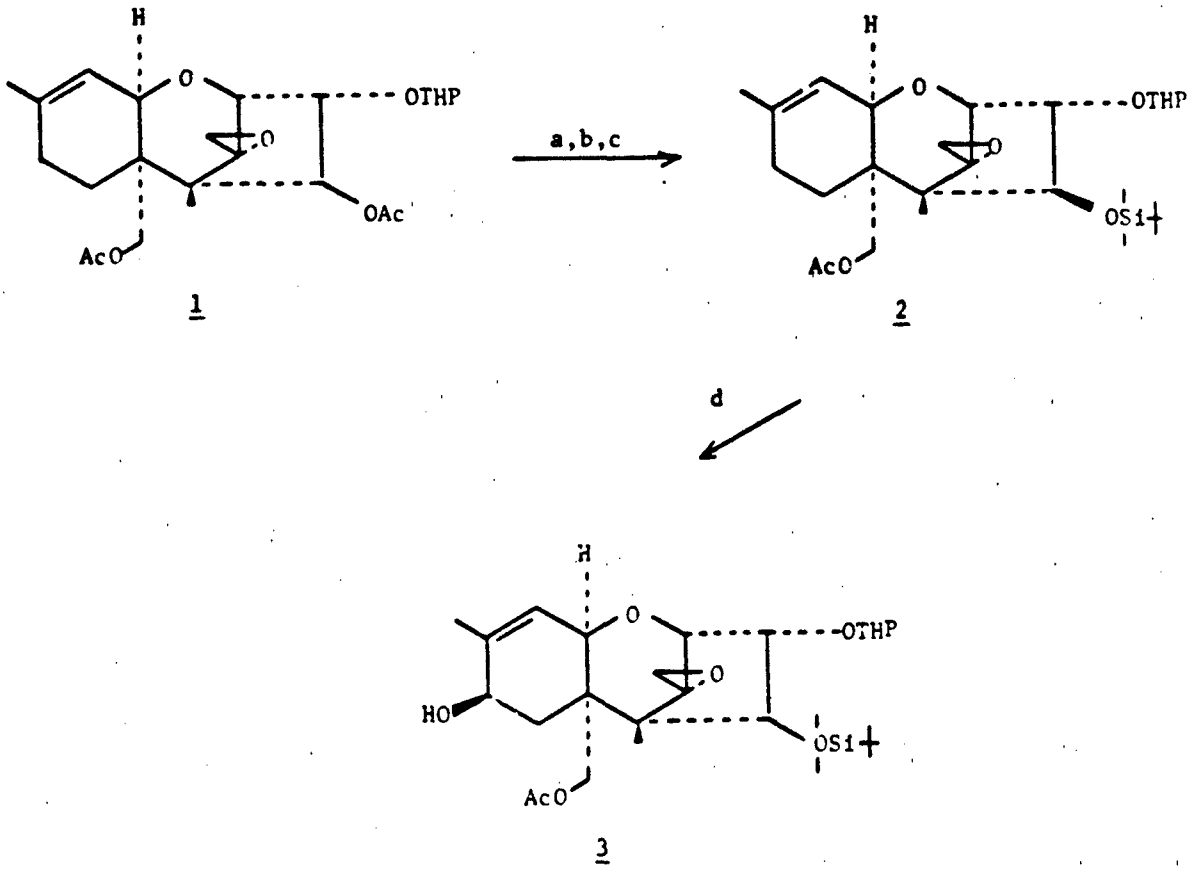
b) AcCl, Et_3N

c) SeO_2

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

Chart I

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



1 2 3

a) OH^-

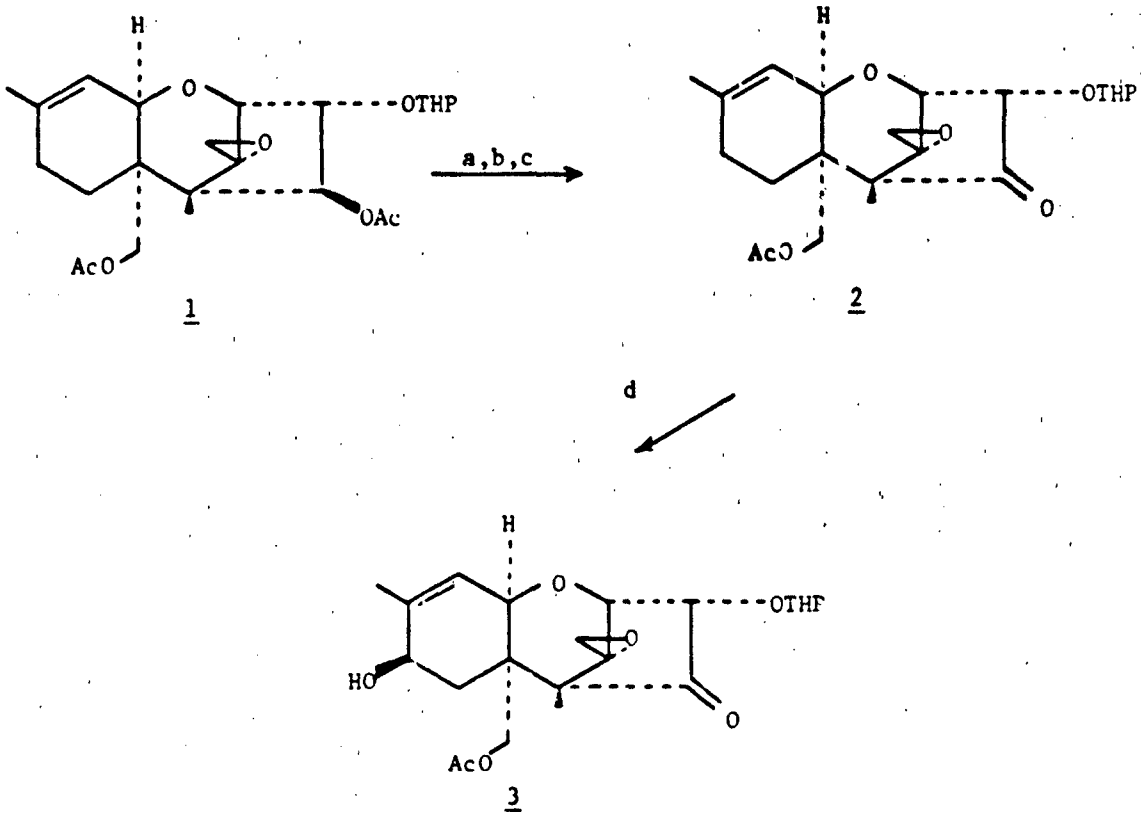
b) AcCl, Et_3N

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

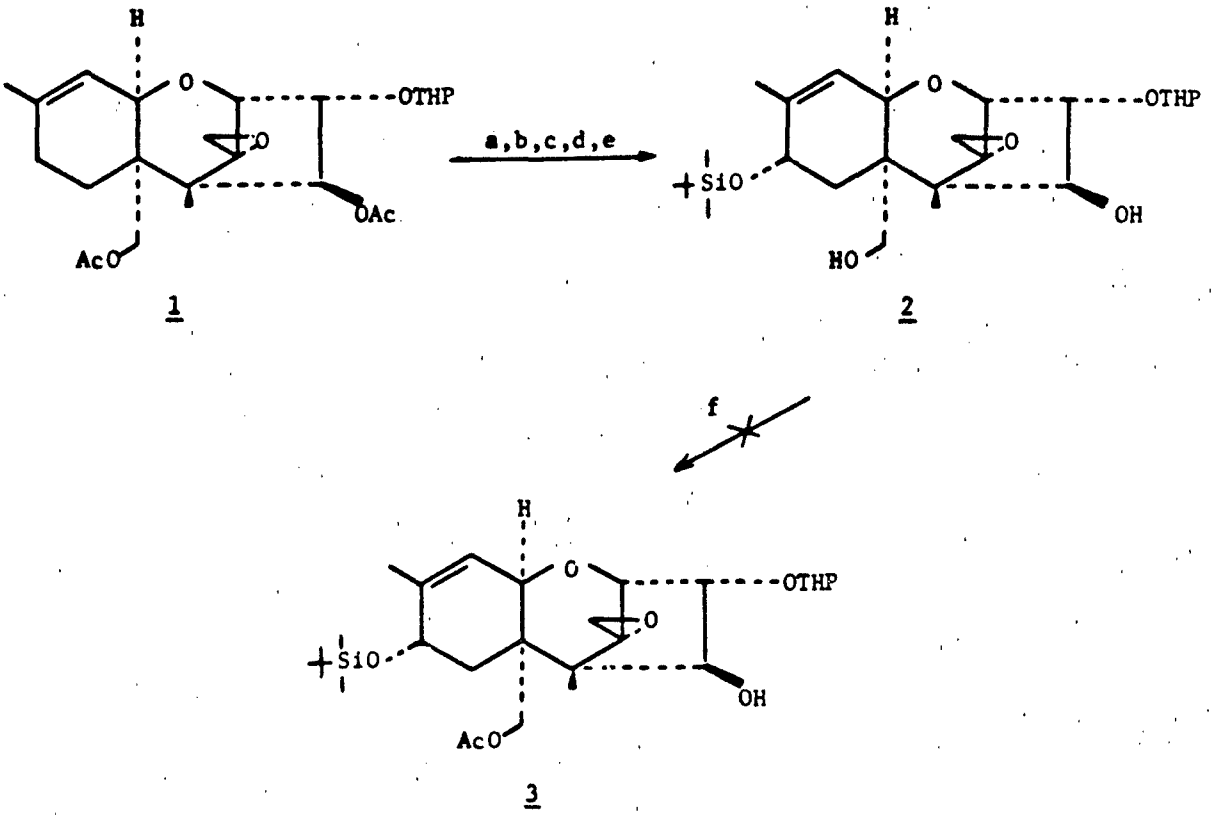
d) SeO_2

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-c. Thus, G-1 was subjected to selenium dioxide hydroxylation to give the 8β-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 aqueous sodium bicarbonate in refluxing methanol to yield the naturally occurring trichothecane, 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

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) $NaHCO_3$

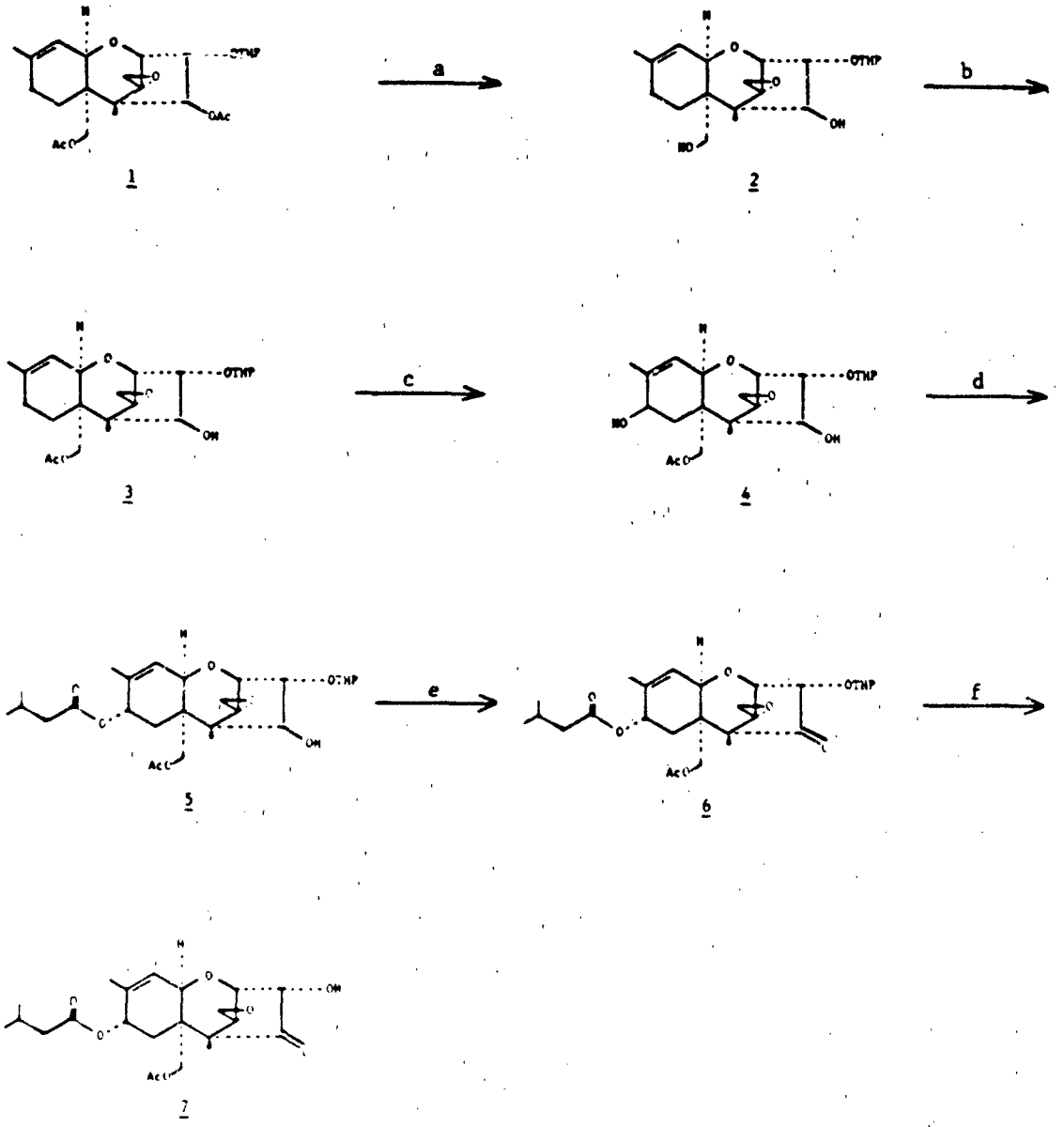
d) t-Butyldimethylsilyl triflate

e) ^-OH

f) AcCl, Et_3N

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

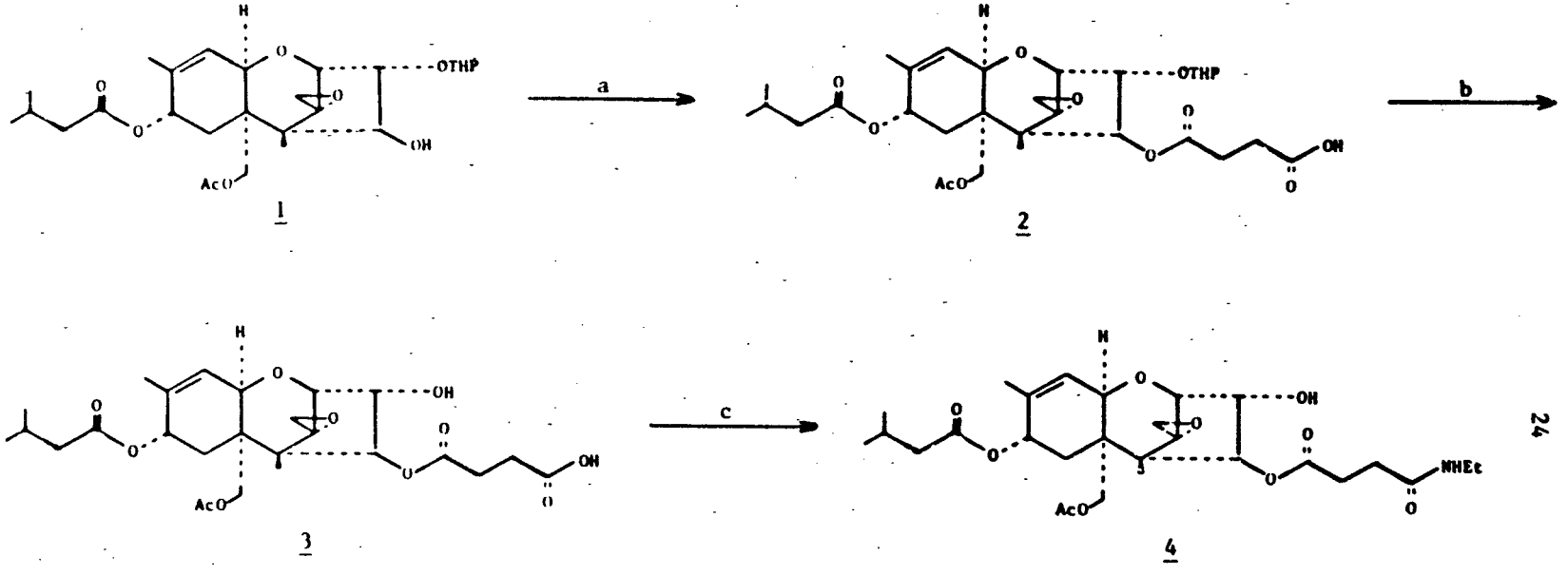
treatment with a catalytic amount of pyridinium tosylate in refluxing 95% aqueous ethanol to give the 4-ketone H-7 in 75% yield.

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 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

Chart I
Synthesis of 4-Hemisuccinate of HT-2 Toxin



a) Succinic anhydride, pyridine, DMAP

b) Pyridine · TsOH, 95% EtOH

c) Et_3N, i-BuOCOCl; $EtNH_2$

bovine serum albumin (BSA, vide 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.

4.0 Immunogen Synthesis

The oxime acid A-4 was used to synthesize immunogens J-1 and J-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 J.

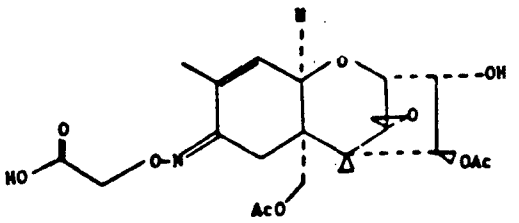
Conjugates were purified by dialysis using Spectra/Por tubing with a molecular weight cutoff of 12-14,000. Ultraviolet analysis of the conjugate with BSA (14) indicates an incorporation ratio of 20 and with BTg (15) a ratio of 60, with no detectable unbound 4 in either conjugate.

The hemisuccinate I-3 was used to synthesize the immunogen K-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 K.

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).

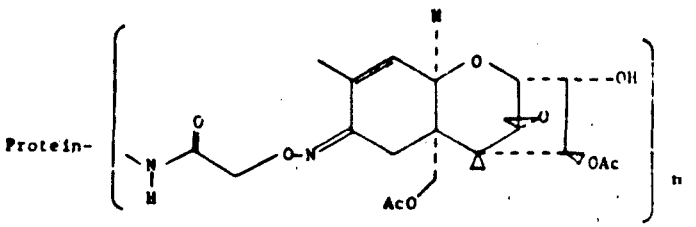
Chart J

Synthesis of 8-Linked Immunogen



A-4

a,b,c



1 Protein = BSA

2 Protein = BTg

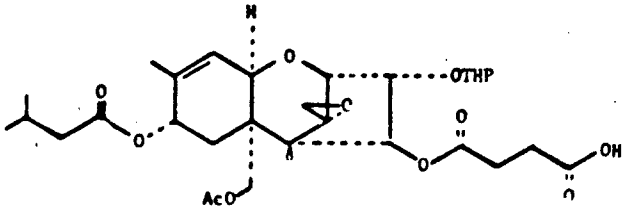
a) Et_3N

b) i-BuOCOCl

c) Protein in 0.1 M $NaHCO_3$

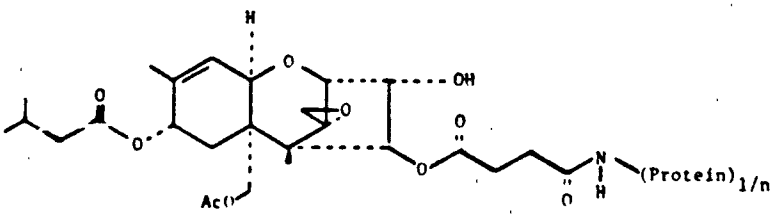
Chart K

Synthesis of 4-Linked Immunogen



I-3

a,b,c



1

Protein = BSA

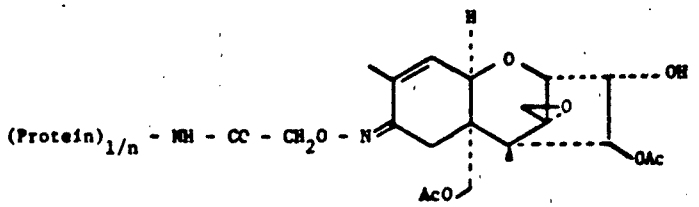
a) Et_3N

b) i-BuOCOCl

c) Protein in 0.1 M $NaHCO_3$

5.0 Preparation and Initial Characterization of Antibodies to T-2 Toxin

The immunogens shown below were each used to immunize four rabbits.



8-T-2-BSA - Protein = bovine serum albumin
8-T-2-BTg - Protein - bovine thyroglobulin

A solid phase enzyme immunoassay (EIA) was used for initial characterization of antisera. Test sera were added to microtiter plate wells coated with T-2-protein conjugate, and bound antibody was detected by subsequent addition of peroxidase-coupled second antibody followed by substrate. Sera from rabbits immunized with 8-T-2-BSA were tested on wells coated with 8-T-2-BTg and conversely. Details of the immunization protocol and of the EIA are given in the experimental section. Activity of test sera with unconjugated protein and activity of normal rabbit serum with 8-T-2-protein conjugates were measured as controls for specific binding.

We have analyzed the sera obtained by bleeds 1 and 4 of all 8 rabbits immunized with 8-T-2-protein conjugates by measuring antibody activity as a function of serum dilution in the EIA. Typical titration curves are shown in Figure 1. The titer of each antiserum, defined as the reciprocal of the dilution giving 50% of maximum antibody binding, was derived by Probit analysis of experimental data. Results are shown in Table 1.

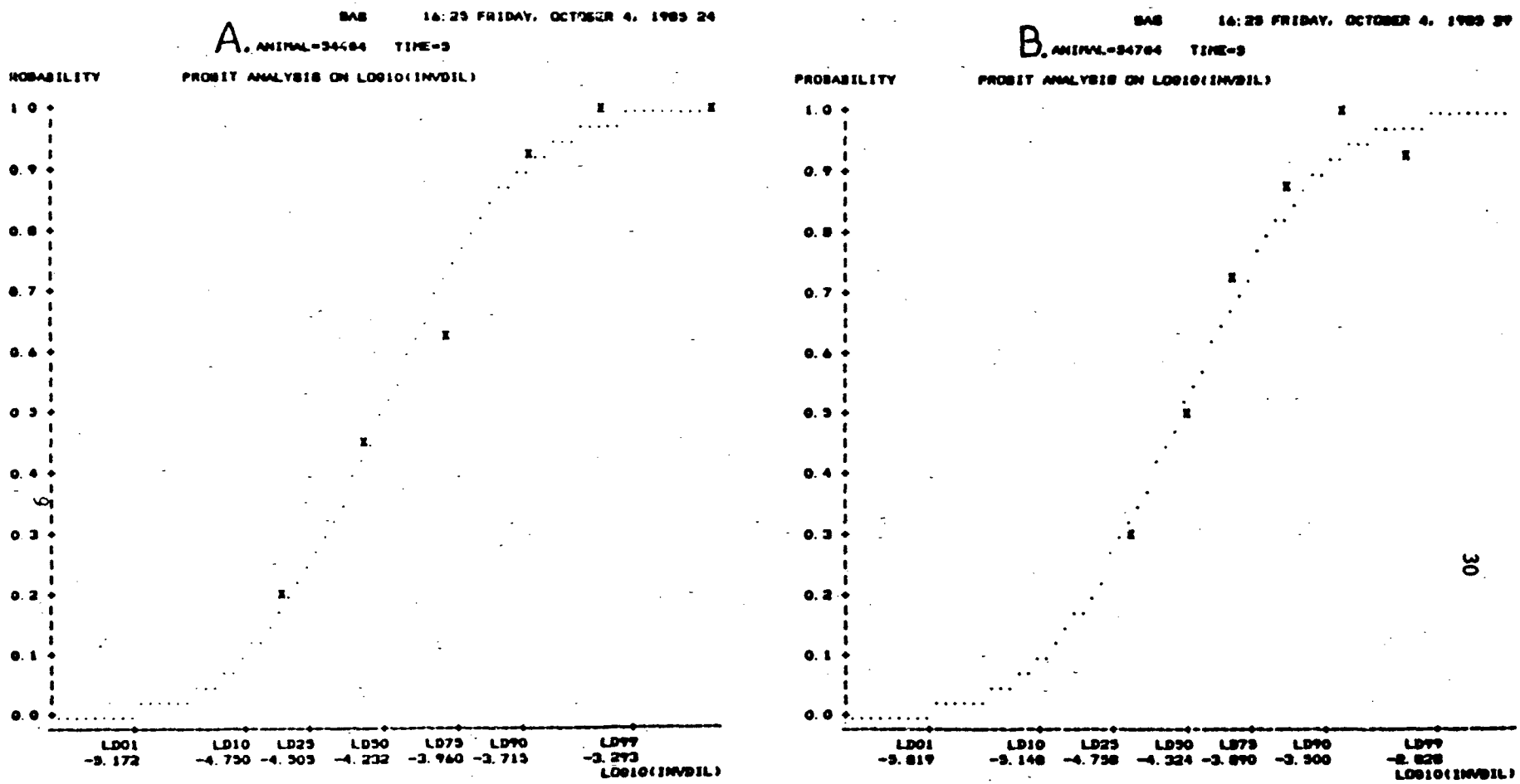


Figure 1. Titration curves for antisera 544.4 (a) and 547.4 (b): $(A/A_{max})_{t=5}$, as a function of log (dilution); for 544.4 A_{max} = 0.885-0.146 = 0.739, for 547.4, A_{max} = 0.835-0.249 = 0.586.

Table 1. Titers of Rabbit Antisera

A. Rabbits Immunized with 8-T-2-BSA

Animal	Titer[a] Bleed 1	Bleed 4
531	6,394	9,277
532	13,111	19,223
533	5,680	11,846
547	7,080	21,083

B. Rabbits Immunized with 8-T-2-BTg

Animal	Titer[a] Bleed 1	Bleed 4
543	10,688	29,824
544	7,642	17,079
545	5,890	6,376
546	4,719	3,934

[a] Titers are defined as the reciprocal of the dilution giving 50% of maximum antibody binding and are derived by probit analysis of the data.

T-2 toxin coupled to BSA and BTg *via* the C-8 position and administered to rabbits according to the described protocol elicited a strong immune response (Table 1) without apparent adverse effects on the immunized animals. This is in contrast to previous reports citing the difficulty of eliciting a vigorous immune response by injection of (T-2-C3 hemisuccinate) BSA (Hunter et al., 1985; Pontelo et al., 1983; Peters et al., 1982; Chu et al., 1979). The weak immune responses were attributed to possible toxic effects of (free) T-2 toxin, in particular on cells of the hemopoietic system undergoing the differentiation and proliferation necessary to produce an immune response (Hunter et al., 1985).

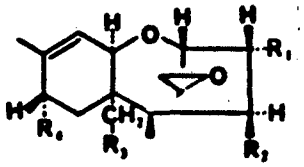
6.0 Reactivity of Polyclonal Rabbit Antisera with T-2 Toxin and Related Trichothecenes

Using a competitive inhibition radioimmunoassay (CIRIA), we have analyzed one antiserum from each of four rabbits for reactivity with T-2 toxin, HT-2 toxin, anguidine, neosolaniol, T-2 triol, T-2 tetraol, verrucarin A, and vomitoxin (Chart 4). See experimental section for details. Two of the antisera (531.4, 532.4) were from rabbits immunized with the 8-T-2-BSA conjugate and two (544.4, 545.4) were from rabbits immunized with the 8-T-2-BTg conjugate. Similar results were obtained with all four sera. Approximate percent cross-reactivities at 50% radioligand displacement were

T-2 toxin	100%
HT-2 toxin	0.6%
anguidine	60%
neosolaniol	38%
T-2 triol	<0.4%
T-2 tetraol	<0.4%
verrucarin A	<0.4%
vomitoxin	<0.4%

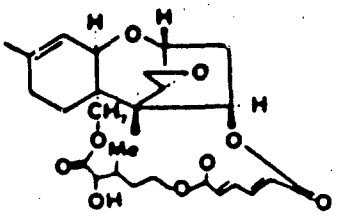
Chart L

Trichothecanes Tested for Antibody Crossreactivity

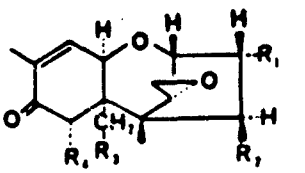


TRIVIAL NAME	R_1	R_2	R_3	R_4
T-2 Toxin	OH	OAc	OAc	X
HT-2 Toxin	OH	OH	OAc	X
Anguidine	OH	OAc	OAc	H
Neosolaniol	OH	OAc	OAc	OH
T-2 Triol	OH	OH	OH	X
T-2 Tetraol	OH	OH	OH	OH

X = $OOCCH_2CH(CH_3)_2$



VERRUCARIN A



TRIVIAL NAME	R_1	R_2	R_3	R_4
Vomitoxin	OH	H	OH	OH

7.0 Recommendations and Plans

1. To complete the long-term immunization protocols for rabbits immunized with 8-T-2-BSA and 8-T-2-BTg, which entails two additional immunizations (days 261 and 293) and bleedings (days 272 and 304). We will then have 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). All these antisera will be characterized in the EIA, and binding affinities and cross-reactivities of selected antisera will be determined in the RIA.

2. To produce and characterize trichothecane-reactive murine monoclonal antibodies using T-2 toxin coupled to BSA at the C-8 position.

3. To produce and characterize polyclonal antisera in rabbits immunized with the hemisuccinate derivative of HT-2 toxin coupled at the C-4 position to chick gamma globulin (CGG) and to BSA.

4. To begin production of murine monoclonal antibodies to the hemisuccinate derivative of anguidine coupled at the C-4 position to CGG and to BSA.

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, c, 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), 3.88 (s, 2, C-15), 4.17 (m, 1, C-3), 4.50 (d, 1, J = 6Hz, C-11), 4.58 (s, 2, O-$\underline{CH_2}$-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 0.39 (s, 3, C-14), 1.20 (t, 3, J = 5Hz, N-CH_2-$\underline{CH_3}$), 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-$\underline{CH_2}$-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-$\underline{CH_2}$-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)scirpene-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-butenoyl)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-$\overset{'}{C}$=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(O)-CH_2-$\overset{'}{C}$=CH_2), 3.06 (d, 1, J = 4Hz, C-13), 3.69 (d, 1, J = 5Hz, C-2), 4.87 (s, 1, CH_2=$\overset{'}{C}$-CH_2), 4.93 (s, 1, CH_2=$\overset{'}{C}$-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-$^{3}H_2$-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 intergal of the known concentration (1 mg/mL) of anguidine was 626 and the intergal 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-Tetrahydropyranylscirpene-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, 2, C-10). Required for $C_{22}H_{32}O_8$; m/z 424.2097. Found: m/z 424.2094.

3α-O-Tetrahydropyranyl-8α-isovaleryl-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 $CHCl_2$ (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 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 (T-1) (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 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 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.

Preparation of Bovine Serum Albumin (BSA) Conjugate (J-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 µL, 0.1 mmol). After stirring for 3 min, isobutyl chloroformate (14 µL, 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:

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

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 (J-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 K-1. To a cold (15°C) solution of C-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). 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).

8.2 Immunology

Production of Antisera. Four female rabbits (3 kg weight) were immunized with T-2 BSA and four with 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 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). 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°. The animals appeared normal and healthy throughout the immunization procedure.

Enzyme Immunoassay (EIA). 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 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 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 (Kirkegaard 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 Radioimmunoassay (CIRIA). The reactivities of rabbit antisera 531.4, 532.4, 544.4, and 545.4 with T-2 toxin and related trichothecenes were measured in a solution CIRIA using [3H]-T-2 toxin prepared as described above. 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 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). Samples were counted 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.

9.0 Literature Cited

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