Preparation, Purification, and NMR Spectra of Some Mono- and Dihemisuccinates of the Trichothecene Mycotoxin Nivalenol

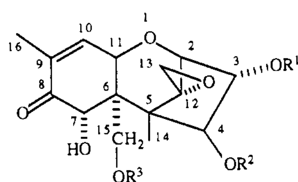
Denis R. Lauren,*,† Wendy A. Smith,† and Alistair L. Wilkins‡

Horticulture and Food Research Institute of New Zealand Ltd., Ruakura Research Centre, Hamilton, New Zealand, and Chemistry Department, University of Waikato, Hamilton, New Zealand

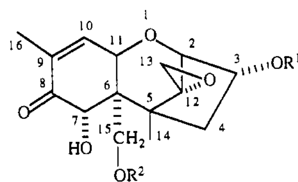
4,15-Diacetylnivalenol (DANIV) was produced in liquid culture and purified by fractionation over silica followed by preparative liquid chromatography. Hydrolysis gave nivalenol (NIV), a trichothecene mycotoxin found as a common contaminant in grain grown in the North Island of New Zealand. Reaction of NIV with succinic anhydride in the presence of *n*-butylboronic acid and (dimethylamino)pyridine produced a mixture of three mono- and two dihemisuccinates. These were separated by semipreparative HPLC. The major product was 3-hemisuccinyl-NIV (3-HS-NIV). Other products were 4-HS-NIV, 15-HS-NIV, 3,4-diHS-NIV, and 3,15-diHS-NIV. 3-HS-NIV was also produced by enzymatic hydrolysis of 3-HS-DANIV.

INTRODUCTION

Nivalenol (NIV) (**1a**) is one of two major group B trichothecene mycotoxins. These are characterized by the presence of a ketone group at the C-8 position of the 12,13-epoxytrichothecene skeleton. NIV and the related group B trichothecene deoxynivalenol (DON) (**2a**) occur



1a $R^1 = R^2 = R^3 = H$
1b $R^1 = R^3 = H, R^2 = Ac$
1c $R^1 = H, R^2 = R^3 = Ac$
1d $R^1 = R^3 = C(O)CH_2CH_2COOH, R^2 = H$
1e $R^1 = R^2 = C(O)CH_2CH_2COOH, R^3 = H$
1f $R^1 = C(O)CH_2CH_2COOH, R^2 = R^3 = H$
1g $R^2 = C(O)CH_2CH_2COOH, R^1 = R^3 = H$
1h $R^3 = C(O)CH_2CH_2COOH, R^1 = R^2 = H$
1i $R^1 = C(O)CH_2CH_2COOH, R^2 = R^3 = Ac$



2a $R^1 = R^2 = H$
2b $R^1 = Ac, R^2 = H$
2c $R^1 = H, R^2 = Ac$

worldwide as natural contaminants of cereal grains (Tanaka et al., 1988). Natural occurrence of the monoacetates fusarenone X (FX) (**1b**), 3-acetyl-DON (3ADON) (**2b**), and 15-acetyl-DON (15ADON) (**2c**) has also been reported [see, for example, Abbas et al. (1988), Luo et al. (1990), Ramakrishna et al. (1990), and Hietaniemi and Kumpulaineu (1991)].

In New Zealand, NIV and DON both occur commonly in grain, particularly maize, grown in the North Island. A small but significant proportion is present as ester forms (Lauren et al., 1991). NIV-related trichothecenes have been shown to be produced by New Zealand isolates of *Fusarium crookwellense*, *Fusarium graminearum*, and *Fusarium culmorum* (Lauren et al., 1992). Analysis of grain and grain-based human and animal foods for NIV and DON is necessary to protect consumers.

Common analytical methods for NIV and DON rely on analysis by HPLC or GC after extensive cleanup of sample extracts. Methods that convert all esterified trichothecenes to parent alcohols prior to analysis have been published (Rood et al., 1988; Kroll et al., 1988; Lauren and Agnew, 1991) and allow easier multitoxin screening. However, such instrumental methods are complex and sample throughput is slow. A simpler, more rapid method would allow quality assessment of crops and foods on a routine basis and allow for more rapid diagnosis of animal problems. Immunochemical methods would fulfill this requirement.

Published immunochemical methods for NIV rely on analysis of a product presumed to be the tetraacetate (Ikebuchi et al., 1990; Teshima et al., 1990; Wang and Chu, 1991). These assays were developed because of reported difficulties in raising antibodies against NIV in a nonacetylated form. Similar difficulties have been reported with DON, but antibodies to DON have been raised (Casale et al., 1988; Mills et al., 1990; Usleber et al., 1991), albeit producing assays with lower sensitivities than those reported for acetylated derivatives. Despite these problems, it is appropriate to endeavor to produce antibodies and sensitive assays to NIV (and DON) in the unacetylated form. If successful, the resulting assay system would be simpler and more rapid than methods involving acetylation. It is therefore important to test the immunological properties of toxin–protein conjugates made from as many alternative haptens as possible. Also, by correct orientation of coupling groups, either selective or generic antibodies may potentially be raised. Coupling of NIV through the C-4 position, for example, should produce antibodies with activity for NIV and DON, allowing screening for both toxins to be performed in a single assay. Reliable interpretation of any immunochemical studies will require production of the initial haptens in pure form.

As part of this aim, we have been studying selective derivatization reactions of NIV. Two innovative and

† Horticulture and Food Research Institute.
‡ University of Waikato.

0021-8561/94/1442-0828$04.50/0 © 1994 American Chemical Society

Table 1. ^{1}H NMR Chemical Shift Assignments (δ in CD_3OD unless Otherwise Stated) and Coupling Constants (Hz) (in Parentheses) of Nivalenol and Some Nivalenol Hemisuccinates

position	1a[a]	1f	1g	1h	1e	1d
2 (d)	3.61 (4.7)	3.89 (4.7)	3.67 (4.7)	3.60 (4.7)	3.95 (4.6)	3.92 (4.6)
3 (d,d)	4.13 (3.3, 4.7)	4.96 (3.3, 4.7)	4.27 (3.3, 4.7)	4.14 (3.4, 4.7)	5.12 (3.5, 4.6)	4.98 (3.5, 4.6)
4 (d)	4.41 (3.4)	4.81 (3.5)	5.64 (3.3)	4.39 (3.4)	5.98 (3.5)	4.65 (3.5)
7 (s)	4.80	4.79	4.79	4.89	4.78	4.87
10 (d,q)	6.57 (5.9, 1.5)	6.60 (5.9, 1.5)	6.59 (5.9, 1.5)	6.60 (6.0, 1.6)	6.62 (6.0, 1.6)	6.64 (6.0, 1.5)
11 (d)	4.59 (5.9)	4.57 (5.9)	4.71 (5.9)	4.63 (6.0)	4.64 (6.0)	4.53 (6.0)
13 (ABq)	2.96, 3.01 (4.4)	3.02 (s)	3.02, 3.05 (4.3)	2.98, 2.99 (4.3)	3.07, 3.10 (4.2)	3.02, 3.04 (4.3)
14 (s)	1.07	1.12	1.00	1.05	1.06	1.09
15 (ABq)	3.72, 3.78 (12.1)	3.68, 3.86 (12.0)	3.78, 3.93 (12.3)	4.22, 4.43 (12.3)	3.72, 3.93 (12.3)	4.23, 4.43 (12.3)
16 (br s)	1.83	1.83	1.84	1.83	1.84	1.86
CH_2CH_2 (m)		2.62–2.76	2.46–2.70	2.46–2.70	2.55–2.80	2.40–2.76

[a] In $CD_3OD/CDCl_3$ (1:1).

potentially selective techniques were earlier employed for DON, namely, the use of a cyclic boronate ester to protect the 7- and 15-hydroxyl groups and allow selective reaction of the 3-hydroxyl group (Casale et al., 1988) and the use of an acetyl esterase to selectively deacetylate the 15-hemiglutarate and 15-hemisuccinate of 3ADON (Mills et al., 1990). To compare the success of these, the other "selective" synthetic routes, quantities of possible products need to be prepared and purified for use as chemical standards in order to allow full characterization of reaction product mixtures.

This paper describes the production, separation, purification, and NMR spectral characterization of three mono- and two dihemisuccinates of NIV from the reaction of NIV with succinic anhydride. Procedures for the bulk production and purification of 4,15-diacetylnivalenol (DANIV) (**1c**) and NIV of starting materials are also presented.

MATERIALS AND METHODS

Materials. Standard materials of NIV and DANIV were available as described elsewhere (Lauren and Agnew, 1991). Bulk quantities were prepared as described below. Succinic anhydride was recrystallized from chloroform and stored in a desiccator prior to use. 4-(Dimethylamino)pyridine (DMAP) was purchased from Sigma Chemical Co. (St. Louis, MO). *n*-Butylboronic acid and Davisil silica (60 Å, 149–250 m) were purchased from Alltech Associates, Inc. (Deerfield, IL). Solvents were all of either AR or HPLC grade. Methyl-d_3 alcohol-*d* (CD_3OD) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetylesterase enzyme suspension was purchased from Sigma and stored at 4 °C.

Apparatus. High-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6A gradient system fitted with an autosampler, column oven, variable-wavelength UV detector and C-R3A data processor. Unless stated otherwise, analyses were done using a Zorbax C_8 (250 mm × 4.6 mm i.d.) column held at 35 °C with a mobile phase flow rate of 1 mL/min and detection of 245 nm. Gas chromatography with flame ionization detector (GC/FID) was performed on a Varian 3700 instrument using conditions as described by Lauren et al. (1992). NMR spectra, including two-dimensional COSY spectra, double quantum filtered COSY spectra, and ^{13}C–^1H correlated spectra, optimized for one-bond and for long-range couplings, were determined in CD_3OD, $CDCl_3$, or CD_3OD–$CDCl_3$ (1:1) at 300.13 MHz (^1H) and at 75.47 MHz (^{13}C) using a Bruker AC-300 spectrometer. Chemical shifts are reported relative to TMS. Low-resolution electron impact (EI) mass spectra (MS) were determined on a Kratos MS80RFA instrument. Sample introduction was by direct probe. Chemical ionization (CI; isobutane) MS were also determined for selected compounds.

Production and Purification of 4,15-Diacetylnivalenol (DANIV) (1c). Batchwise 2.4-L preparations were made using a nivalenol-producing strain of *F. graminearum* grown in MYRO medium. The fungal strain was isolated from wheat grown in the Manawatu region of New Zealand and is stored at Ruakura Research Centre as isolate 188W19-4F4. Each batch of culture was extracted as described by Lauren et al. (1992), and typically afforded 700–900 mg of crude extract material. A total of 4.8 g from six batches was fractionated in a glass column (4-cm i.d.) dry packed with silica (200 g).

The crude extract was applied to the column in ethyl acetate–chloroform (10:90, 20 mL, plus five 20-mL rinses), and the column was fully wetted by application of a further ca. 160 mL of the same solvent. Thirteen fractions (S1–S13) were collected using applications of solvent mixtures as follows: ethyl acetate–chloroform (10:90, 2 × 400 mL), (20:80, 2 × 400 mL), (50:50, 2 × 400 mL), ethyl acetate (3 × 400 mL), methanol–chloroform (5:95, 400 mL, and 10:90, 400 mL), methanol–ethyl acetate (50:50, 400 mL), and methanol (400 mL). An aliquot of each fraction was analyzed by GC/FID.

The fraction containing DANIV (S6, 1.2 g) was further fractionated by preparative LC on a Lobar silica (Merck LiChroprep Si60, 40–63 μm) column (length 310 mm, 25-mm i.d.) at ambient temperature (ca. 22 °C). The fraction was dissolved in dichloromethane (5 mL), and portions of up to 800 μL were injected. The elution solvent was 2-propanol–dichloromethane (5:95) at 4 mL/min. The chromatogram was monitored using a UV detector set at 245 nm. The column void time under these conditions was about 20 min. DANIV was collected as a peak which eluted from 32 to 44 min. The column was then flushed out with 2-propanol–dichloromethane (25:75) for a further 40 min before it was reset to the elution solvent. The DANIV fractions from all injections were combined and evaporated to dryness. GC/FID analysis showed a single major peak at the correct retention time for DANIV.

The DANIV fraction from LC (0.7 g) was recrystallized from ethyl acetate–hexane (1:2, ca. 30 mL) to yield white crystals (300 mg), mp 134–136 °C [lit. mp 135–136 °C (Grove, 1970)], and, after the mother liquor was evaporated, a yellow oil (370 mg).

Hydrolysis of DANIV to NIV. An aliquot of the mother liquor oil (120 mg) was dissolved in methanol plus water (5 + 13, 18 mL) to give a cloudy solution. The solution was treated with 0.5 M aqueous sodium carbonate (2 mL) and stirred at ambient temperature (ca. 23 °C). Progress of the reaction was monitored by HPLC analysis of 5-μL aliquots taken at time intervals and diluted to 1 mL with methanol–water (5:95). The mobile phase was methanol–water (40:60), and the reaction products at different time intervals were compared with an external standard mixture of DANIV (retention time 12.0 min), FX (5.6 min), and NIV (3.5 min). After 3 h, the reaction was complete, and the reaction mixture was applied to a ChemElut 1020 extraction column (Analytichem International) and extracted with ethyl acetate (18 × 20 mL) to yield NIV (87 mg) as a single peak by HPLC. See Tables 1 and 2 for ^1H and ^{13}C NMR data.

Preparation of Hemisuccinates of NIV. In a screw-top tube with a Teflon-lined cap, NIV (35 mg, 0.112 mmol) was dissolved in dry pyridine (1.5 mL), treated with *n*-butylboronic acid (114 mg, 1.12 mmol), and thoroughly mixed. After standing overnight at ambient temperature, the mixture was further treated with succinic anhydride (18 mg, 0.18 mmol) and DMAP (44 mg, 0.36 mmol), thoroughly shaken to dissolve all reagents, and then allowed to stand at ambient temperature. Progress of the reaction was followed by HPLC using a mobile phase of methanol–1% aqueous acetic acid (36:64). Once approximately half the NIV (retention time 3.7 min) had reacted (ca. 4 h), the

Table 2. ^{13}C NMR Chemical Shift Assignments (δ in CD_3OD unless Otherwise Stated) of Nivalenol and Some Nivalenol Hemisuccinates

position	1a[a]	1f	1g	1e	1d
2	81.4[b]	79.4	78.0	79.7	79.3
3	81.6[b]	83.6	81.5	80.2	83.3
4	80.8	78.3	83.8	80.4	78.3
5	50.5	49.9	50.7	50.2	50.3
6	54.6	54.4	54.8	54.6	53.4
7	75.4	75.2	75.1	75.0	74.6
8	201.9	201.4	201.1	201.0	200.9
9	137.3	137.2	137.3	137.4	137.2
10	139.4	138.9	138.8	138.6	139.3
11	71.0	70.9	71.2	71.1	70.6
12	66.5	65.8	65.8	65.5	65.4
13	46.8	46.5	46.9	47.1	46.5
14	8.7	8.3	8.2	8.2	8.2
15	62.0	61.6	61.7	61.4	63.3
16	16.0	15.2	15.3	15.2	15.3
other		30.1	30.9	29.9	29.9
		30.2	30.9	30.0	30.0
		173.7	174.1	30.1	30.2
		176.2	174.7	30.2	30.2
				173.2	173.6
				173.7	173.7
				176.0	176.2
				176.1	176.2

[a] In $CD_3OD/CDCl_3$ (1:1). [b] Assignments are interchangeable.

reaction was stopped by the addition of water (200 μL). After a further 30 min, toluene (2 mL) was added and the mixture blown to dryness (N_2, 45 °C). The residue was dissolved in ethyl acetate (4 mL) and applied to a glass column (10-mm i.d.) dry packed with silica (4 g). Unreacted NIV was eluted with 2-propanol–chloroform (25:75), and then the column was washed with 2-propanol–chloroform (50:50) before the product was eluted with 2-propanol–chloroform–acetic acid (10:90:1). HPLC analysis of the product showed three main peaks (Figure 1), the first of which contained more than one component. Products from several such reactions were combined for separation and characterization.

Separation of Hemisuccinate Reaction Mixture. The combined hemisuccinate mixture from several reactions (130 mg) was dissolved in methanol–1% aqueous acetic acid (5:95, 3 mL) and separated by semipreparative HPLC on the analytical Zorbax C_8 column. The mobile phase was methanol–1% aqueous acetic acid (32:68). The separation used repeat injections of 100 μL with fractions collected at 6–7.25 min (peak 1-1 of Figure 1), 11.1–12.3 min (peak 1-2), and 12.9–14.7 min (peak 1-3). All solutions were stored at −15 °C during any pauses in the separation procedure (e.g., overnight) to avoid any hydrolysis of products to NIV. The separated fractions each showed essentially one peak by HPLC analysis. The fractions were evaporated to dryness under vaccum (≤65 °C) using acetone to assist evaporation. The residues were treated with toluene and then blown to dryness (N_2, 45 °C) to remove traces of acetic acid.

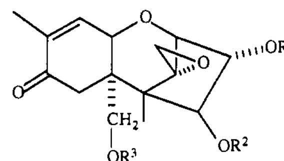
The fractions yielded, in elution order, a mixture of monohemisuccinylnivalenol (HS-NIV) isomers (40 mg), 3,15-dihemisuccinylnivalenol (3,15-diHS-NIV) (**1d**) (5 mg), and 3,4-dihemisuccinylnivalenol (3,4-diHS-NIV) (**1e**) (17 mg). See Tables 1 and 2 for ^{1}H and ^{13}C NMR data.

A subsample (200 μg) of 3,15-diHS-NIV was methylated (in 1 mL of methanol plus 5 μL of concentrated HCl, shaken and then left at ambient temperature for 3 h, then evaporated thoroughly under N_2) for MS determination: EI-MS, m/z 101 (33), 115 (100), 136 (29), 349 (11), 361 (4.2), 377 (8.5), 396 (6.5), 408 (3.5), 509 (2.8), 522 (0.4), 540 (0.5, M^+); CI-MS, m/z 101 (32), 115 (100), 133 (21), 277 (6.6), 349 (10), 367 (3.3), 391 (5.5), 409 (2.7), 523 (4.3), 541 (11, MH^+).

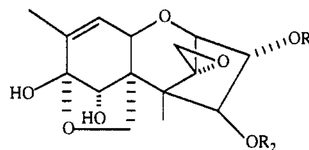
A subsample of 3,4-diHS-NIV was methylated as described above for MS determination: EI-MS, m/z 101 (38), 115 (100), 360 (1.1), 378 (3.1), 396 (3.2), 408 (0.55), 425 (0.29), 492 (0.68), 510 (3.5), 522 (0.14), 540 (0.07, M^+); CI-MS, m/z 101 (20), 115 (100), 133 (30), 247 (6.4), 361 (2.1), 379 (4.8), 427 (8.4), 509 (2.3), 541 (4.7, MH^+).

Separation of HS-NIV Isomeric Mixture. Analysis by HPLC using methanol–1% aqueous acetic acid (9:91) showed that the monohemisuccinate fraction was comprised of three major components (Figure 2). The mixture (40 mg) was dissolved in methanol–1% aqueous acetic acid (5:95, 2 mL) and separated on the analytical Zorbax C_8 column. The mobile phase was methanol–0.5% aqueous acetic acid (11:89). Using repeat injections of 100 μL, fractions were collected at 41.5–46.5 min (peak 2-1 of Figure 2), 46.5–50 min (mixture of peaks 2-1, 2-2, plus 2-3 at about 2:1:1), 50–56.5 min (mixture of peaks 2-2 plus 2-3 at about 1:2), and 57.5–63 min (a minor component that became detectable with large injections). All solutions were stored at −15 °C during pauses in the separation process. The four fractions collected were evaporated and traces of acetic acid removed as previously described.

The fractions yielded, in elution order, 3-hemisuccinylnivalenol (3-HS-NIV) (**1f**) (20 mg), a ca. 2:1:1 mixture (9 mg) of 3-HS-NIV, 15-hemisuccinylnivalenol (15-HS-NIV) (**1h**), and 4-hemisuccinylnivalenol (4-HS-NIV) (**1g**), a ca. 1:2 mixture (6 mg) of 15-HS-NIV and 4-HS-NIV, and 3-hemisuccinyl-7-deoxynivalenol (3-HS-7-DON) (**3c**) (2 mg): ^{1}H NMR ($CDCl_3$) δ 0.82 (s, H_3-14),



3a R1 = R2 = R3 = H
3b R1 = H, R2 = R3 = Ac
3c R1 = C(O)CH2CH2COOH, R2 = R3 = H



4a R1 = R2 = H
4b R1 = C(O)CH2CH2COOH, R2 = H
4c R1 = R2 = C(O)CH2CH2COOH

1.78 (br s, H_3-16), 2.37 and 2.81 (ABq, J = 16.2 Hz, H_2-7), 2.40–2.72 (m, 4 H, CH_2CH_2COOH), 2.83 and 3.00 (ABq, J = 4 Hz, H_2-13), 3.60 and 3.66 (ABq, J = 12.4 Hz, H_2-15), 3.83 (d, J = 4.8 Hz, H-2), 4.41 (d, J = 5.9 Hz, H-11), 4.75 (d, J = 3.2 Hz, H-4), 4.95 (d,d, J = 3.2, 4.8 Hz, H-3), 6.67 (d,q, J = 6.0, 1.5 Hz, H-10). The third fraction was used to obtain ^{1}H and ^{13}C NMR data for 4-HS-NIV and 15-HS-NIV. See Tables 1 and 2 for NMR data of all HS-NIV isomers.

A subsample (200 μg) of 3-HS-NIV was methylated as described above for MS determination: CI-MS, m/z 101 (15), 115 (100), 133 (15), 247 (15), 265 (9), 277 (7.6), 295 (5.3), 313 (9.9), 379 (15), 409 (2.9), 427 (9.7, MH^+).

A subsample of 3-HS-7-DON (200 mg) was methylated as described for MS determination: CI-MS, m/z 101 (4.9), 115 (100), 133 (11), 231 (32), 279 (9.1), 297 (5.6), 339 (4.2), 345 (1.7), 363 (31), 393 (6.5), 411 (19, MH^+).

Preparation of 3-Hemisuccinyl-4,15-diacetylnivalenol (3-HS-DANIV) (1i). DANIV (2 mg, 5 μmol) was dissolved in dry pyridine (0.5 mL) with succinic anhydride (2.5 mg, 25 μmol). DMAP (6.2 mg, 50 μmol) was added and the mixture swirled by vortex mixer and allowed to stand at ambient temperature. After 24 h, HPLC showed that the reaction was complete. Water (100 μL) was added to destroy excess reagent. After mixing, toluene (2 mL) was added and the solution evaporated to dryness (N_2, 45 °C). The residue was dissolved in ethyl acetate (3 mL) and added to a silica (4 g) column as described earlier. Any untreated DANIV was eluted with ethyl acetate, and then the column was washed with 2-propanol–chloroform (10:90) before the product was eluted with 2-propanol–chloroform–acetic acid (10:90:1). The product (2.6 mg, 90% purity by HPLC) was 3-HS-DANIV: ^{1}H NMR ($CDCl_3$) δ 1.00 (s, H_3-14), 1.91 (br s, H_3-16), 1.92 (s, OAc), 2.13 (s, OAc), 2.65–2.80 (m, 4 H, CH_2CH_2COOH), 3.09 (br s, H_2-

13), 4.03 (d, J = 4.7 Hz, H-2), 4.16 and 4.68 (ABq, J = 12.4 Hz, H_2-15), 4.56 (d, J = 5.8 Hz, H-11), 4.82 (s, H-7), 5.24 (d,d, J = 3.5, 4.7 Hz, H-3), 5.80 (d, J = 3.5 Hz, H-4), 6.63 (d,q, J = 5.8, 1.5 Hz, H-10).

Enzymatic Deacetylation of 3-HS-DANIV. 3-HS-DANIV (0.5 mg) from the above reaction was dissolved in acetonitrile (100 μL) and diluted with phosphate buffer (900 μL of a solution of 0.41 g of anhydrous sodium dihydrogen orthophosphate and 2.39 g of disodium hydrogen orthophosphate in 200 mL of water) containing orange peel acetylesterase (4.8 units). This was mixed in a 1.5-mL autosampler vial and allowed to react at ambient temperature. Progress of the reaction was monitored by HPLC (mobile phase 44:56 methanol–1% aqueous acetic acid) using external standards of NIV, DANIV, and a mixture of HS-NIV isomers. A more dilute solution of 3-HS-DANIV in buffer alone (no enzyme) was used for comparison as time zero.

The reaction proceeded slowly (10% in 24 h). After 3 days (35% reaction) and 9 days (80% reaction), a prominent peak at the retention time of HS-NIV isomers could be seen plus a prominent intermediate peak (presumably a 3-HS-monoacetyl nivalenol) and several other minor peaks, none at the retention time of DANIV. At 13 days (88% reaction), the product was approximately 50% HS-NIV. HPLC analysis of the mixture using a mobile phase of methanol–1% aqueous acetic acid (11:89) showed that the only HS-NIV isomer present was 3-HS-NIV. At 29 days no 3-HS-DANIV remained and the major product (estimated at 70%) was 3-HS-NIV. However, a significant proportion (estimated at 20%) of the reaction had proceeded to produce NIV, and this increased further with more time. The time zero comparative sample was analyzed at each date that the enzyme reaction was assessed. This solution also showed hydrolysis products but in a different pattern. DANIV was one of the hydrolysis products after day 3 but never a major one. Very little 3-HS-NIV was ever produced, and NIV was a major product by day 21. The main intermediate products were at different retention times than those for the enzymatic reaction.

RESULTS AND DISCUSSION

Production of Toxins. The trichothecene mycotoxin DANIV was produced in liquid culture medium by *F. graminearum*. A simple silica fractionation of the crude product followed by preparative HPLC gave essentially pure DANIV (yield 14%) from which crystalline material was obtained. DANIV is a useful starting material for selective reactions of the NIV skeleton. Although rice culture medium can often produce a greater per kilogram yield of toxin, other coextractives commonly present from solid media make cleanup more difficult.

Mild basic hydrolysis of DANIV produced NIV in high yield. Isolation was achieved by using a solid-phase extraction column. NIV was difficult to recover off the column, and 18 volumes of ethyl acetate was needed to obtain a good recovery.

Preparation and Separation of Hemisuccinates. NIV was treated with succinic anhydride in the presence of n-butylboronic acid. This reagent was employed by Casale et al. (1988) to allow selective reaction of succinic anhydride with the 3-hydroxyl group of DON. The reagent was used for in-situ protection of the 7- and 15-hydroxyl groups of DON, presumably as the 7,15-boronate. Casale et al. (1988) reported that without protection they obtained at least three products, believed to be isomers of mono-hemisuccinyl-DON plus dihemisuccinyl-DON. In theory, n-butylboronic acid should have protected the 7- and 15-hydroxyl groups of NIV and restricted reaction to the 3- and 4-hydroxyl groups. In practice, some reaction of the 15-hydroxyl occurred also, although the ratio was small when compared with unprotected NIV reactions (authors' unpublished information). This suggests that n-butylboronic acid is not a completely efficient protecting group for NIV. Conformational and intramolecular effects, such as the relative ease of hemiketal formation between the

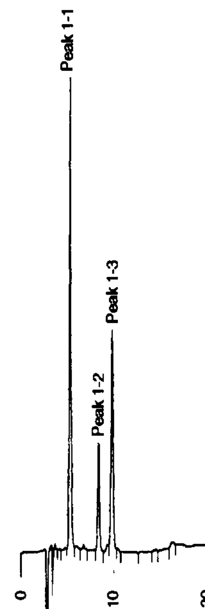


Figure 1. HPLC analysis of product mixture from the reaction of NIV with succinic anhydride in the presence of n-butylboronic acid. Chromatographic conditions are as in the text. Peak identification: 1-1, mixture of HS-NIV isomers; 1-2, 3,15-diHS-NIV; 1-3, 3,4-diHS-NIV.

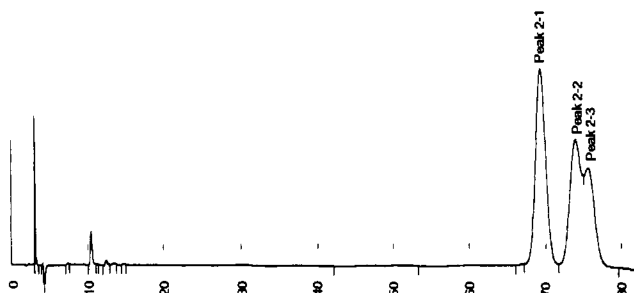


Figure 2. HPLC separation of the three isomers of HS-NIV. Chromatographic conditions are as in the text. Peak identification: 2-1, 3-HS-NIV; 2-2, 15-HS-NIV; 2-3, 4-HS-NIV.

15-hydroxyl group and C8 (Jarvis et al., 1990), may explain the difference between NIV and DON reactivity with n-butylboronate.

The mixture of three mono- and two dihemisuccinates of NIV was readily separated into a mixture of monohemisuccinate isomers (peak 1-1 of Figure 1) and two dihemisuccinates (peaks 1-2 and 1-3) by semipreparative HPLC. Further separation of the monohemisuccinate mixture was more difficult (Figure 2), and only the major product, 3-HS-NIV, and a fourth minor compound, 3-HS-7-DON, were obtained as pure compounds. The isolation of 3-HS-7-DON as a fourth component from the mixture of monohemisuccinyl-NIV isomers can be explained by contamination of the DANIV used as starting material with 4,15-diacetyl-7-deoxynivalenol (4,15-DA-7-DON) (**3b**). 4,15-DA-7-DON has been observed previously as a minor component in extracts of liquid cultures of NIV-chemotypes of *F. graminearum* (Lauren et al., 1992). Derivatives of 7-deoxynivalenol (7-DON) (**3a**), could be expected to have chromatographic properties similar to those of equivalent NIV derivatives.

All of the hemisuccinates of NIV were prone to slow, but noticeable, hydrolysis in the HPLC analysis solutions of methanol–water (5:95) when left at room temperature for several days. DANIV behaved in a similar way. This behavior needs to be noted as a risk factor in the use of these products as immunogenic protein conjugates. It is

a possible contributing factor to the difficulties reported by previous workers to raise antibodies against NIV.

NMR Spectral Analysis. An analysis of the ^1H and ^{13}C NMR spectral data determined for the hemisuccinates readily defined the sites at which the succinyl groups had been introduced. For example, succinylation of the 15-hydroxyl group results in the H-15 protons experiencing a downfield shift to resonate in the vicinity of 4.22–4.43 ppm in **1d** and **1h**, compared to 3.68–3.93 ppm in **1a**, **1e**, **1f**, and **1g**. H-7 is also mildly sensitive to changes in substitution on C15; typically, succinylation of the 15-hydroxyl group results in a downfield shift of 0.1 ppm (see Table 1). Succinylation of the 4-hydroxyl group results in H-4 experiencing a marked downfield shift to resonate at 5.64 ppm in 4-HS-NIV (**1g**) compared to 4.41 ppm in NIV (**1a**), while succinylation of the 3-hydroxyl group results in H-3 experiencing a lesser downfield shift to resonate at 4.96 ppm in 3-HS-NIV (**1f**) compared to 4.13 ppm in NIV (**1a**). In the 3,4-dihemisuccinate (**1e**), H-3 and H-4 occur at 5.12 and 5.98 ppm, respectively. The two-dimensional COSY and double quantum filtered COSY NMR spectra of the hemisuccinates were in all cases fully consistent with assignments given in Table 1.

The ^{13}C NMR assignments presented in Table 2 for **1e** and **1f** were also substantiated in two-dimensional ^{13}C–^1H correlated NMR experiments optimized for ^1J and for long-range (^2J and ^3J) couplings. For example, in 3-HS-NIV (**1f**) the H-14 protons (1.12 ppm) exhibited correlations at the resonance frequencies of C4, C5, C6, and C12 (78.3, 49.9, 54.4, and 65.8 ppm, respectively).

An intriguing aspect of the ^1H NMR spectra of 3-HS-NIV (**1f**) and 3,4-diHSNIV (**1e**) when determined in CD_3OD (but not when determined in 1:1 CD_3OD–$CDCl_3$) was the appearance of signals attributable to a ca. 10% contribution from the 8,15-hemiketal analogues, **4b** and **4c**, respectively, of the parent 8-keto-15-hydroxy compounds.

Jarvis et al. (1990) have observed that while published ^1H NMR spectral data for NIV are entirely consistent with structure **1a**, crystallization from methanol–water affords the corresponding hemiketal form (**4a**). In our hands, the ^1H NMR spectrum of NIV, when determined in CD_3OD, gave broad H-15 (3.78 ppm), H-4 (4.47 ppm), H-11 (4.62 ppm), H-7 (4.80 ppm), and H-10 (6.56 ppm) signals, while in 1:1 CD_3OD–$CDCl_3$ it gave a complete series of sharp, well-defined signals consistent with structure **1a** (see Table 1). Cooling the CD_3OD solution to 240 K resulted only in further line broadening and not in the detection of signals attributable to discrete populations of conventional and hemiketal forms of NIV. Similar observations were made with ^{13}C NMR spectra of NIV, where 1:1 CD_3OD–$CDCl_3$ was again required to obtain sharp line resonances for all signals (see Table 2).

We interpret these observations to indicate that in CD_3OD the conventional and hemiketal forms of NIV are interconverting at a moderate rate, hence broad line signals are observed, while in 1:1 CD_3OD–$CDCl_3$ interconversion is appreciably faster, hence sharp, well-defined, time-averaged signals are observed. The introduction of hemisuccinate groups appears to reduce the rate of interconversion in CD_3OD; hence, signals attributable to both the conventional (ca. 90%) and hemiketal (ca. 10%) forms are observed.

Enzymatic Deacetylation of 3-HS-DANIV. Identification of the predominant monohemisuccinate as 3-HS-NIV was also verified by producing 3-HS-DANIV (by succinylation of DANIV) and subjecting this to enzymatic deacetylation. HPLC assessment of the reaction products showed that the only monohemisuccinate produced was that assigned as 3-HS-NIV by NMR analysis. The reaction pathway to 3-HS-NIV was very slow and produced a mixture of products, including components presumed to be 3-HS-monoacetyl-NIV, and eventually led to the fully hydrolyzed product, NIV. When 3-HS-DANIV was kept at ambient temperature in buffer without enzyme, it slowly hydrolyzed to a mixture of products with 3-HS-NIV as a very minor component. In the absence of enzyme the hemisuccinate group is more readily hydrolyzed, and the fully hydrolyzed product, NIV, was produced more rapidly.

Enzymatic deacetylation has been used by Mills et al. (1990) in the production of DON hemisuccinate and hemiglutarate from the 3ADON derivatives with 80% efficiency. Although we obtained relatively complex mixtures from a single deacetylation experiment with 3-HS-DANIV, the results suggest that further studies with alternative acetylesterase enzymes or buffer conditions are warranted. Selective hydrolysis could be a useful tool in the production of alternative NIV haptens from starting products such as DANIV and FX.

Conclusion. DANIV may be produced in liquid culture in good yield and purified by a combination of silica fractionation, preparative LC, and recrystallization. Hydrolysis of DANIV in mild base produces NIV, which may be separated by solid-phase extraction techniques. The reaction of NIV with succinic anhydride produces a complex mixture, even in the presence of *n*-butylboronic acid. However, this mixture may be separated by preparative HPLC, and the method described is capable of producing useful quantities of 3-HS-NIV. Similar methodology should also be suitable for the production of other 3-hydroxyl derivatives such as the hemiglutarate which may be required in immunochemical experiments. Selective reaction schemes will need to be developed for the production of the 4- and 15-hydroxyl derivatives, since they are extremely difficult to separate, even by analytical HPLC.

ACKNOWLEDGMENT

We thank P. T. Holland and D. E. McNaughton for mass spectra collection and for assistance with interpretation. We thank C. O. Miles for assistance with the enzymatic reaction.

LITERATURE CITED

Abbas, H. K.; Mirocha, C. J.; Meronuck, R. A.; Pokorny, J. D.; Gould, S. L.; Kommedahl, T. Mycotoxins and *Fusarium* spp. associated with infected ears of corn in Minnesota. *Appl. Environ. Microbiol.* **1988**, *54*, 1930–1933.

Casale, W. L.; Pestka, J. J.; Hart, L. P. Enzyme-linked immunosorbent assay employing monoclonal antibody specific for deoxynivalenol (vomitoxin) and several analogues. *J. Agric. Food Chem.* **1988**, *36*, 663–668.

Grove, J. F. Phytotoxic compounds produced by *Fusarium equiseti*. Part V. Transformation products of 4β,15-diacetoxy-3α,7α-dihydroxy-12,13-epoxytrichothec-9-en-8-one and the structures of nivalenol and fusarenone. *J. Chem. Soc. C*, **1970**, 375–378.

Hietaniemi, V.; Kumpulainen, J. Contents of *Fusarium* toxins in Finnish and imported grains and feeds. *Food Addit. Contam.* **1991**, *8*, 171–182.

Ikebuchi, H.; Teshima, R.; Hirai, K.; Sato, M.; Ichinoe, M.; Terao, T. Production and characterization of monoclonal antibodies to nivalenol tetraacetate and their application to enzyme-linked immunoassay of nivalenol. *Biol. Hoppe-Seyler* **1990**, *371*, 31–36.

Jarvis, B. B.; Mazzocchi, D. B.; Ammon, H. L.; Mazzola, E. P.; Flippen-Anderson, J. L.; Gilardi, R. D.; George, C. F. Con-

formational effects in trichothecenes: Structures of 15-hydroxy C4 and C8 ketones. *J. Org. Chem.* **1990**, *55*, 3660–3662.

Kroll, J.; Giersch, Ch.; Guth, S. Screening method for detection of trichothecene types A and B in cereal and cereal products. *Nahrung* **1988**, *32*, 75–77.

Lauren, D. R.; Agnew, M. P. Multitoxin screening method for *Fusarium* mycotoxins in grains. *J. Agric. Food Chem.* **1991**, *39*, 502–507.

Lauren, D. R.; Agnew, M. P.; Smith, W. A.; Sayer, S. T. A survey of the natural occurrence of *Fusarium* mycotoxins in cereals grown in New Zealand in 1986–1989. *Food Addit. Contam.* **1991**, *8*, 599–605.

Lauren, D. R.; Sayer, S. T.; di Menna, M. E. Trichothecene production by Fusarium species isolated from grain and pasture throughout New Zealand. *Mycopathologia* **1992**, *120*, 167–176.

Luo, Y.; Yoshizawa, T.; Katayama, T. Comparative study on the natural occurrence of *Fusarium* mycotoxins (trichothecenes and zearalenone) in corn and wheat from high- and low-risk areas for human esophageal cancer in China. *Appl. Environ. Microbiol.* **1990**, *56*, 3723–3726.

Mills, E. N. C.; Alcock, S. M.; Lee, H. A.; Morgan, M. R. A. An enzyme-linked immunosorbent assay for deoxynivalenol in wheat, utilizing novel hapten derivatization procedures. *Food Agric. Immunol.* **1990**, *2*, 109–118.

Ramakrishna, Y.; Bhat, R. V.; Vasanthi, S. Natural occurrence of mycotoxins in staple foods in India. *J. Agric. Food Chem.* **1990**, *38*, 1857–1859.

Rood, H. D., Jr.; Buck, W. B.; Swanson, S. P. Gas chromatographic screening method for T-2 toxin, diacetoxyscirpenol, deoxynivalenol, and related trichothecenes in feeds. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 493–498.

Tanaka, T.; Hasegawa, A.; Yamamoto, S.; Lee, U.-S.; Sugiura, Y.;. Ueno, Y. Worldwide contamination of cereals by the *Fusarium* mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. Survey of 19 countries. *J. Agric. Food Chem.* **1988**, *36*, 979–983.

Teshima, R.; Hirai, K.; Sato, M.; Ikebuchi, H.; Ichinoe, M.; Terao, T. Radioimmunoassay of nivalenol in barley. *Appl. Environ. Microbiol.* **1990**, *56*, 764–768.

Usleber, E.; Märtlbauer, E.; Dietrich, R.; Terplan, G. Direct enzyme-linked immunosorbent assays for the detection of the 8-ketotrichothecene mycotoxins deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol in buffer solutions. *J. Agric. Food Chem.* **1991**, *39*, 2091–2095.

Wang, C.-R.; Chu, F. A. Production and characterization of antibodies against nivalenol tetraacetate. *Appl. Environ. Microbiol.* **1991**, *57*, 1026–1030.

Received for review July 27, 1993. Revised manuscript received December 13, 1993. Accepted December 16, 1993.[⊗]

⊗ Abstract published in *Advance ACS Abstracts*, February 1, 1994.