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MYCOTOXINS PRODUCED BY *FUSARIUM NIVALE* (FRIES) CESATI ISOLATED FROM TALL FESCUE (*FESTUCA ARUNDINACEA* SCHREB.)

SYNTHESIS OF 4-ACETAMIDO-4-HYDROXY-2-BUTENOIC ACID-γ-LACTONE

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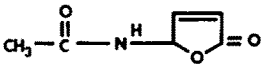
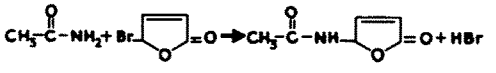
Abstract—D,L-4-Acetamido-4-hydroxy-2-butenoic acid-γ-lactone has been synthesized in 21% yield by reaction of 4-bromo-4-hydroxy-2-butenoic acid-γ-lactone and acetamide in chloroform to confirm the structure of a new mycotoxin.

A NEW mycotoxin has recently been isolated from *Fusarium nivale* culture medium[1] and D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone (I) proposed as its structure. This report describes the synthesis of I, its degradation products, confirmation of structure by spectral techniques and identity with the natural product isolated from *F. nivale*.



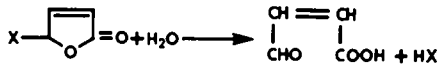
RESULTS AND DISCUSSION

D,L-4-Acetamido-4-hydroxy-2-butenoic acid-γ-lactone has been obtained as a minor reaction product by refluxing 4-bromo-4-hydroxy-2-butenoic acid-γ-lactone with acetamide in chloroform under anhydrous conditions according to equation II. It has been isolated from the complex reaction mixture in 21% yield through a series of solvent partitions, extractions, and fractional crystallizations.



The successful synthesis of D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone reported here resulted from rapid, easy detection of the reaction product even when present below 1% by means of analytical TLC. After methods and preliminary conditions were found that yielded minute quantities of D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone, the results of changes in conditions were studied by quantitative TLC to obtain maximum yield. The yield was increased from below 1% in the first synthesis to nearly 21% as reported here.

4-Substituted-2-butenoic acid-γ-lactones are reported to yield *cis*-formyl acrylic acid upon hydrolysis according to equation III.[2] It was found, however, that mixtures of *cis*- and *trans*-isomers of formyl acrylic acid were formed upon hydrolysis of the



synthesized D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone as well as of 4-bromo-4-hydroxy-2-butenoic acid-γ-lactone when used as a model compound. The *cis*-isomer was obtained in pure form by column chromatography on aluminum oxide, and characterized as its phenylhydrazone. Pure *trans*-formyl acrylic acid phenylhydrazone was isolated from a mixture of *trans*- and *cis*-phenylhydrazone by preparative TLC. Identical isomers were obtained from natural and synthetic D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone which gave no m.p. depression and identical IR spectra.

Confirmation of the structure of the synthesized compound was also given by four spectral techniques (MS, IR, NMR, UV). Interpretations of the UV and IR spectra were identical to those presented earlier[1] for the natural substance. The mass spectrum is shown in Fig. 1, and accurate mass determination was obtained for

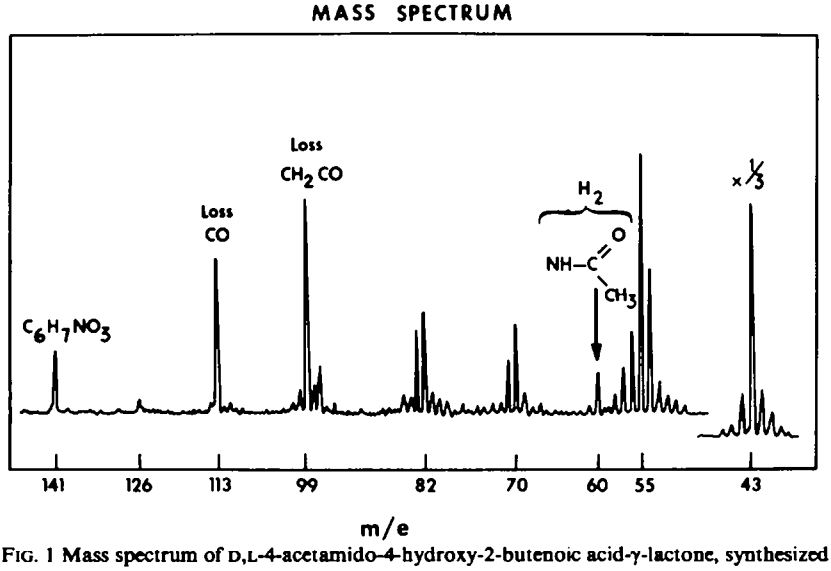


FIG. 1 Mass spectrum of D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone, synthesized and natrual.

several of the ion peaks using a CEC 21-110B double focusing mass spectrometer (Table 1). The peak at mass 141 was established as the expected molecular ion, $C_6H_7NO_3$. Loss of CO gives the peak at mass 113. Loss of CH_2CO occurs, by hydrogen rearrangement from the acetyl, to give the mass 99 peak. An interesting rearrangement ion occurs at mass 60. This ion was measured to be C_2H_6NO. It is due to a rearrangement reaction analogous to the well-known ester rearrangement which transfers two hydrogens to the acid moiety and thus is indicative of the MeCONH group.[3]

The NMR spectrum of natural 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone presented earlier[1] was determined in trideuteroacetonitrile and did not clearly show the amide proton. However, the spectrum of the compound (natural and synthetic) in DMSO-d_6 (Fig. 2), shows a low field doublet at $\delta = 8{\cdot}95$ which is typical, both

TABLE 1. ACCURATE MASS DETERMINATION OF SELECTED PEAKS IN THE MASS SPECTRUM OF D,L-4-ACETAMIDO-4-HYDROXY-2-BUTENOIC ACID-γ-LACTONE

Measured mass	Calculated mass	Proposed formula
141·0422[a]	141·0426	$C_6H_7NO_3$
113·0482[b]	113·0477	$C_5H_7NO_2$
99·0326[a]	99·0320	$C_4H_5NO_2$
60·0460[a]	60·0449	C_2H_6NO
43·0181[b]	43·0184	C_2H_3O

[a] Photoplate measurement.
[b] Peak matching method.

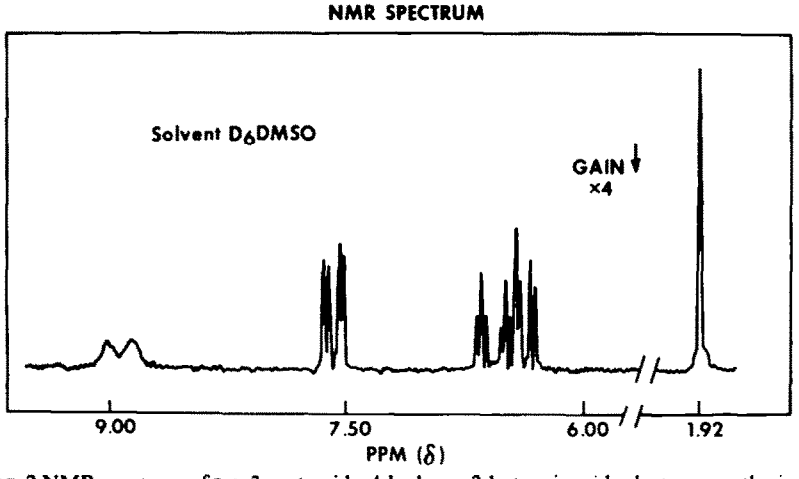


FIG. 2 NMR spectrum of D,L-3-acetamido-4-hydroxy-2-butenoic acid-γ-lactone, synthesized and natural.

in position and shape, of an amide proton. Other features of the spectrum are interpreted here in greater detail than space permitted in the preliminary communication. The spectrum in DMSO-d_6 showed from low to high field: a broad, incompletely resolved doublet at $\delta = 8{\cdot}95$ ($J = 9{\cdot}2$ Hz), a doublet of doublets at $\delta = 7{\cdot}59$ ($J =$ 5·5, 1·6 Hz), a doublet of triplets at $\delta = 6{\cdot}57$ ($J = 9{\cdot}4$, 1·7 Hz), a doublet of doublets at $\delta = 6{\cdot}38$ ($J = 5{\cdot}6$, 1·8 Hz), and a singlet at $\delta = 1{\cdot}92$. The area ratios were respectively 1:1:1:1:3 for a total of seven protons. The singlet at $\delta = 1{\cdot}92$ obviously arises from the methyl protons of the acetamido group. The 5·5 Hz coarse splitting of the double doublets at $\delta = 7{\cdot}59$ and $\delta = 6{\cdot}38$ shows that the protons giving rise to these lines must be located on adjacent carbons. The position of these bands are typical of protons on a C=C double bond conjugated to a CO group. An excellent model for this system is provided by 2-butenoic acid-γ-lactone (Varian Spectra Catalog No. 51), which shows olefinic resonances at $\delta = 6{\cdot}15$ ($J = 5{\cdot}9$, 2·2) and $\delta = 7{\cdot}63$ ($J = 5{\cdot}9$, 1·8). The almost identical triplet splittings of these resonances by the C-4 protons show that $J_{3,4}$ is almost identical to the long range 2–4 coupling.

The corresponding doublet splittings of these bands in the spectrum of D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone show that it has a single proton located at C-4. This hydrogen must give rise to the doublet-split triplet at $\delta = 6{\cdot}57$ because of the 1·7 Hz triplet splitting. The $J = 9{\cdot}2$ Hz doublet splitting of this multiplet shows in turn that the carbon is bonded to the amide nitrogen.

4-Acetamido-4-hydroxy-2-butenoic acid-γ-lactone has an asymmetric center located at C-4. The synthetic product can be assumed and has been proven by ORD measurements to represent a D,L-mixture. The natural material was originally thought to be optically active but has been proven to be optically inactive. It is completely identical with the synthetic compound as shown by ORD measurement, m.p., mixed m.p., and spectral data. Both compounds were optically inactive and equally toxic to mice. The lethal doses LD_{50} were about 42 ± 1·2 mg/kg.

EXPERIMENTAL

Physical. UV absorption spectra were obtained with a Cary Model 15 recording spectrophotometer.[4] The instrument was flushed with N_2 when spectra were recorded below 210 mμ. IR spectra were obtained with a Beckman Model IR-7 or Cary Model 90 Spectrophotometer. PMR spectra were recorded either with a Varian HR-100 spectrometer equipped with a field-frequency lock, or with a Varian A-60 spectrometer equipped with a Technical Measurement Corporation 1024-channel analyzer and a 5 kc computer of average transients (CAT). ORD measurements were taken with a Cary Model 60 spectrophotometer. High resolution mass spectral data were obtained with a Consolidated Electrodynamics Corporation Model 21-110B double focusing mass spectrometer, and the low resolution spectrum (Fig. 1) was obtained on a Bendix Time-of-Flight mass spectrometer, Model 12.

Chromatography. TLC was performed on silica gel G (Merck) coated plates for all compounds. Components were detected by spraying the dried chromatoplates with a mixture of 1 ml anisaldehyde, 10 ml AcOH, 85 ml MeOH, and 5 ml conc H_2SO_4 followed by heating to 140° for 10–20 min. Components were located by preparative TLC by precutting 4 cm strips on both ends of the 20 × 40 cm plates and breaking them off after application of sample and separation of the mixture. These strips were developed for the visualization of zones and were realigned with the rest of the plate. Lines connecting identical zones were drawn over the unsprayed portion of the plate (32 cm).

Biotests. The lethal doses (LD_{50}) were determined using 10 mice per dose and computed according to the log-probit method of Miller and Tainter.[5] Mice used were 24–26 g, white Webster strain, female. D,L-4-Acetamido-4-hydroxy-2-butenoic acid-γ-lactone was dissolved in water and injected intraperitoneally in amounts between 0·1 and 0·5 ml/mouse.

D,L-4-*Acetamido-4-hydroxy-2-butenoic acid-γ-lactone.* 4-Bromo-4-hydroxy-2-butenoic acid-γ-lactone (16·3 g, prepared from furan according to Elming and Clauson-Kaas),[2] acetamide (11·8 g), and dry $CHCl_3$ (150 ml) were heated under reflux for 3 hr. A solid was separated and washed twice with $CHCl_3$ (20 ml). The filtrates and washings were combined and evaporated to give an oil (12 ml) to which 17 ml of water was added. The lower phase (7·0 ml, 8·5 g) was separated and washed twice with water (7 ml). The washings were added to the upper phase and evaporated *in vacuo* at a bath temp of 70° leaving 10·8 g of a semi-solid residue. The lower phase, after washing, was distilled *in vacuo*. 6·54 g of unreacted starting material (4-bromo-4-hydroxy-2-butenoic acid-γ-lactone) b.p. 2 mm = 68–70°; $n_D^{25} = 1{\cdot}5352$ was reclaimed. The semi-solid residue was triturated with four portions of AcOEt (100 ml each), decanted, and the solvent evaporated *in vacuo* leaving solid I (2·67 g). The remaining material (8·1 g) was taken up in water and the total volume, adjusted to 15 ml, was extracted 12 times with 15 ml AcOEt. The combined AcOEt extracts (150 ml) were dried over anhyd Na_2SO_4 (5 g), and the solvent evaporated to dryness yielding II (1·03 g). Solids I and II were combined (3·70 g) and dissolved in hot AcOEt (40 ml). AcOEt washed charcoal (1·0 g, Darco G 60) was added, the soln was stirred for 30 min, and filtered. After addition of pentane (80 ml), crystallization yielded crude D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone (2·65 g). This material was recrystallized from 7 ml acetone. The crystals were isolated and washed with cold acetone (2 ml, −50°) and pentane (10 ml); yield, 1·51 g, m.p. 114–116°. An additional 240 mg of the same material was isolated by concentration of the mother liquor. Total yield of D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone: 1·75 g = 20·8%. For elemental and spectral analysis, two further

recrystallizations from AcOEt and acetone yielded material, m.p. 115–116·5°; m.m.p. (isolated D,L-4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone) 115–116·5°; λ_{max} 189·5 mμ (ε 18,960); i.p. LD_{50} 42·4 ± 1·24 mg/kg or 1·06 ± 0·041 mg/animal of 25 g body wt; UV, IR, MS, and NMR spectra were identical with those obtained for the natural compound (Fig. 1). ORD measurements of a 2% soln in water showed no deviation from 0 between 280 and 600 mμ. (Found: C, 51·10; H, 4·97; N, 9·97. $C_6H_7O_3N$ requires: C, 51·07; H, 5·00; N, 9·92%.)

cis-*Formyl acrylic acid A.* 4-Bromo-4-hydroxy-2-butenoic acid-γ-lactone (1·0 g) was suspended in water (5 ml) and heated for 10 min. The soln was extracted 15 times with 9 ml portions of ether. The combined ether extracts were dried and evaporated leaving 285 mg of residue. This was redissolved in ether (1·0 ml) and 500 mg aluminum oxide, activity V, was added to adsorb all the dissolved material. The aluminum oxide was dried in the open air and added to the top of a 0·9 × 9 cm column of aluminum oxide, activity V, in heptane. Pure *cis*-formyl acrylic acid was eluted with CCl_4 (100 ml), yield 96 mg, m.p. 127°, λ_{max} 199 mμ (H_2O).

cis-*Formyl acrylic acid B.* D,L-Acetamido-4-hydroxy-2-butenoic acid-γ-lactone (1·0 g) was dissolved in 78 ml 0·1N NaOH and left for 16 hr at room temp. The reaction mixture was acidified with 1N HCl to pH 1·3 and extracted 15 times with 100 ml portions of ether and the acid isolated as described under A, yield 86 mg, m.p. 127°.

cis-*Formyl acrylic acid phenylhydrazone. cis*-Formyl acrylic acid (100 mg) was dissolved in water(1·0 ml) and 0·6 ml phenylhydrazone in 2 ml glacial AcOH and 5 ml water were added. The yellow ppt was filtered off, washed with water, and dried, yield 80 mg of *cis*-formyl acrylic acid phenylhydrazone. Crystallization from acetone–water gave 67 mg; m.p. 159–160°; m.m.p. (*trans*-formyl acrylic acid phenylhydrazone) 145–147°.

trans-*Formyl acrylic acid phenylhydrazone.* This phenylhydrazone was prepared by purification of the mixture formerly believed to be *cis*-formyl acrylic acid phenylhydrazone, m.p. 147–148° by Elming and Clauson-Kaas.[2] The material (1·0 g) was dissolved in acetone (4 ml) and separated into two components by preparative TLC on four 40 cm plates. A mixture of 47 ml hexane, 50 ml ether, and 2 ml AcOH was used for development. The lower component represented the *trans*-phenylhydrazone (R_f value about 0·5), the higher one the *cis*-phenylhydrazone (R_f value about 0·7). The lower zone was scraped off and eluted with acetone. Recrystallization from acetone–water yielded *trans*-formyl acrylic acid phenylhydrazone (120 mg), m.p. 157–158°.

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