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

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**Abstract**—Toxic metabolites are produced by the mold *Fusarium nivale* when grown at cool temperatures. The most abundant toxin is a butenolide characterized as 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone (I). Isolation of (I) was guided by a rabbit-skin or mouse bioassay. Also isolated were lesser amounts of a sesquiterpenoid (8-(3-methylbutyryloxy)-diacetoxyscirpenol). *F. nivale* was isolated from tall fescue hay toxic to cattle and may be implicated in such toxicity.

## INTRODUCTION

TALL fescue toxicity is a term that describes different maladies occasionally seen in cattle grazing on tall fescue grass. Symptoms may vary from loss of weight to sloughing of gangrenous extremities of the leg or tail.[1-3] The toxic factor(s) in fresh forage is retained in the hay and may be extracted from it into 80:20 ethanol:water. Such extracts produce the typical fescue foot syndrome when fed to cattle.[3]

Mycotoxins were suspected as the cause of fescue foot because of the sporadic occurrence of the disease and its similarity to ergotism. Available evidence indicates that neither ergot nor the alkaloids of tall fescue are causative factors.[3] Since some alkaloids of tall fescue, notably festucine,[4] could not be detected in hay samples infected with *Stemphylium*, evidently fungi can alter the metabolism of the plant.[5]

Because uncommon fungi could not be associated with toxicity of fescue hay, we turned our attention to the more common molds. Using subsurface hemorrhage of rabbit skin as a guide, we reported the isolation of several toxin-producing molds of tall fescue.[6] One isolate, tentatively identified as *Fusarium nivale*, was selected for further investigation.

* Part of this work has been described briefly in Ref. 7. While this manuscript was being prepared, E. P. White published an independent isolation of compound I from another *Fusarium* species: *J. Chem. Soc.* (*C*), 346 (1967).

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[1] Lameness, usually of the hindquarters, followed by gangrene of the extremities is called fescue foot. Although serious to the animal, fescue foot is not so important economically as the failure to make expected weight gains.

[2] S. G. YATES, *Econ. Botany* **16**, 295 (1962).

[3] D. R. JACOBSON, W. M. MILLER, D. M. SEATH, S. G. YATES, H. L. TOOKEY and I. A. WOLFF, *J. Dairy Sci.* **46**, 416 (1963).

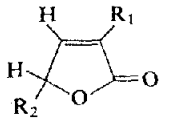
[4] S. G. YATES and H. L. TOOKEY, *Australian J. Chem.* **18**, 53 (1965).

[5] S. G. YATES, *J. Chromatog.* **12**, 423 (1963).

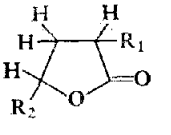
[6] A. C. KEYL, J. C. LEWIS, J. J. ELLIS, S. G. YATES and H. L. TOOKEY, *Mycopathol. Mycol. Appl.* **31**, 327 (1967).

Our strain of *F. nivale*, NRRL–3249, grown at 15° produces yellow and red pigments in Sabouraud's agar; the aerial mycelium is floccose and white at first, but becomes light yellowish buff to light pinkish buff with age. Cultures which are about 3 weeks old produce materials that are toxic to the skin of the rabbit. However, certain *F. nivale* isolates from the parent strain appear to lose their ability to produce a toxic extract through repeated transfers on agar slants incubated at room temperature. This response sometimes occurs after only one transfer at room temperature and may reflect the heterokaryotic nature of the parent strain. Thus far, such losses have been accompanied by a change in pigment production; i.e. in the cultures producing lesser amounts of toxins, the pigments are a dark reddish brown and the colony appears tan. Ordinarily, extracts of a culture of *F. nivale* growing on Sabouraud's agar at 15° or at temperatures alternated between 7° and 15° or 7° and 20° produce a positive rabbit skin test. Extracts of cultures grown at room temperatures, however, do not give a positive skin response.

The toxic factors were extracted from agar-plate cultures with ether, 80:20 ethanol: water, or dichloromethane. From moldy grain or liquid media the factors were extracted with ethyl acetate. Extracts of liquid media contain three toxins: the butenolide (I);[7] an unknown toxin (III), and a sesquiterpenoid (IV). The ratio of their respective abundance



| | $R_1$ | $R_2$ |
|---|---|---|
| (I) | H | $NHCOCH_3$ |



| | $R_1$ | $R_2$ |
|---|---|---|
| (IIa) | H | $NHCOCH_3$ |
| (IIb) | $NHCOCH_3$ | H |

was 87:8:3. The most toxic compound, (IV), was produced in higher proportion when the mold grew on wheat and was, therefore, finally isolated from this source. The butenolide (I) was isolated in large quantities from liquid medium by fractional crystallization.

Compound (I) was obtained in milligram quantities by preparative-scale extraction of agar-plate cultures with dichloromethane. The yield of crude (I) was about 29 mg/plate.

Crude (I) was recrystallized from ethyl acetate-cyclohexane to give a white solid melting at 116·5° to 118·5°. The elemental analysis and molecular weight were consistent with the empirical formula $C_6H_7NO_3$.

The $\Delta^{\alpha,\beta}$-butenolide ring was indicated by the presence of a pair of characteristic bands[8,9] (1760 and 1790 $cm^{-1}$) in the i.r. spectrum of (I).[7] $\beta$-Angelica lactone, an $\alpha,\beta$-unsaturated butenolide, has two bands in the C=O stretching region at 1760 and 1780 $cm^{-1}$. $\alpha$-Angelica lactone, the $\beta,\gamma$-unsaturated isomer, has but a single band at 1795 $cm^{-1}$. Moreover, if (I) is hydrogenated to form (IIa), then the pair of bands at 1760 and 1790 $cm^{-1}$ are no longer detected in the i.r. spectrum. In their place a single band is seen at 1775 $cm^{-1}$. $\gamma$-Valerolactone also absorbs at 1775 $cm^{-1}$.[8] In the NMR spectrum of (IIa) (Fig. 1) the $\alpha$- and $\beta$-methylene

[7] S. G. YATES, H. L. TOOKEY, J. J. ELLIS and H. J. BURKHARDT, *Tetrahedron Letters* No. 7, 621 (1967).

[8] The two bands are probably the result of Fermi resonance. The C=O stretching band interacts with an overtone of a band about one-half its frequency (perhaps the C—H deformation of the $\alpha$-carbon). See R. N. JONES, C. L. ANGELL, T. ITO, and R. J. D. SMITH, *Can. J. Chem.* **37**, 2007 (1959).

[9] R. P. M. BOND, T. CAIRNS, J. D. CONNOLLY, G. EGLINTON, and K. H. OVERTON, *J. Chem. Soc.* 3958 (1965).

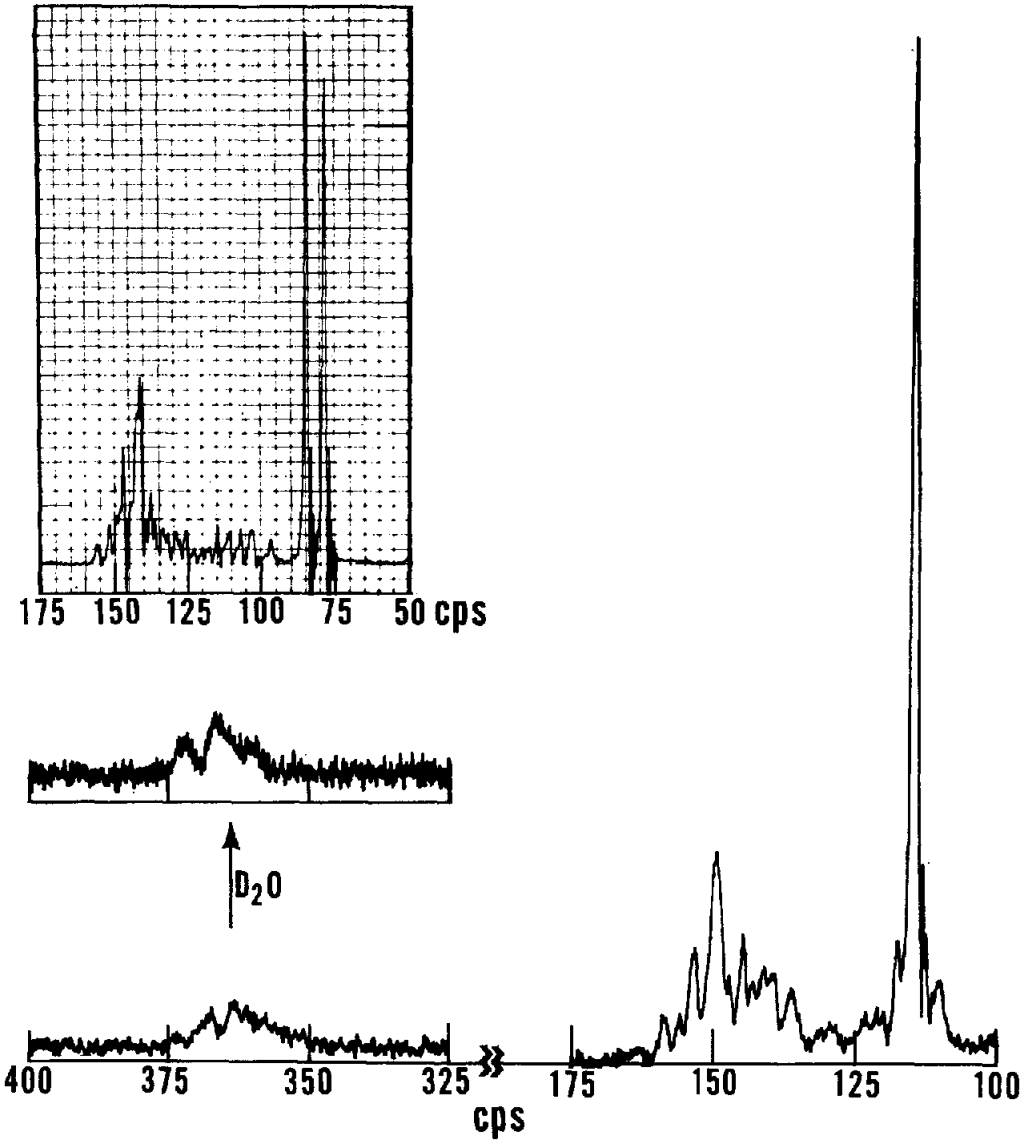



FIG. 1. A 60 MCPS NMR SPECTRUM OF THE HYDROGENATED DERIVATIVE (IIa) OF 4-ACETAMIDO-4-HYDROXY-2-BUTENOIC ACID-$\gamma$-LACTONE (I) SHOWING EFFECT OF $D_2O$ EXCHANGE; INSET SHOWS THE NMR SPECTRUM OF $\gamma$-VALEROLACTONE (REPRODUCED WITH THE PERMISSION OF THE PUBLISHER[10(a)]); CPS FROM TMS.

protons form a complex pattern, centered at 150 c/s, which is strikingly similar to that in the published spectrum of $\gamma$-valerolactone.[10(a)]

The high molecular extinction coefficient in the u.v. spectrum of (I) ($\lambda_{max}$ 202, $\epsilon = 11{,}300$) shows that the double bond is conjugated with the carbonyl.[10(b)] The spectra of the closely

[10(a)]R. M. SILVERSTEIN and G. C. BASSLER, *Spectrometric Identification of Organic Compounds*, p. 130; (b) p. 93; (c), p. 65; (d), p. 107. Wiley, New York (1964).

related angelica lactones demonstrate this point: $\beta$-angelica lactone ($\alpha,\beta$-unsaturated) has an absorption maximum at 203 nm, $\epsilon = 15{,}200$, whereas $\alpha$-angelica lactone ($\beta,\gamma$-unsaturated) has much weaker absorption, $\lambda_{max} = 217$ nm, $\epsilon = 2000$. If the double bond were not in the $\alpha,\beta$-position, hydrogenation of (I) should not have given a saturated lactone. Hydrogenation of $\beta,\gamma$-unsaturated butenolides usually is accompanied by hydrogenolysis to form the corresponding desoxy acid.[11,12]

The presence of the acetamido group in (I) was indicated by its i.r. spectrum.[7] The band at 1705 $cm^{-1}$ is consistent with the C=O stretch of an amide.[10(c),13] Bands at 3440 and 3340 $cm^{-1}$ are in the range for the free and hydrogen-bonded N—H stretching bands of a monosubstituted amide.[10(c)] The NMR spectra of both (I[7]) and (IIa) (Fig. 1) contain peaks (118 c/s and 116 c/s, respectively) which are in agreement with the methyl group of acetamide.[10(d)] Acetamide was detected on both polar and nonpolar gas-liquid chromatography (GLC) columns following alkaline hydrolysis of (I). The weak u.v. absorption of (IIa) ($\lambda_{max}$ 201 nm, $\epsilon = 2{,}700$) is also indicative of an amide.[14] A positional isomer 2-acetamido-4-hydroxybutyric acid-$\gamma$-lactone (IIb) has a $\lambda_{max}$ at 201 nm, $\epsilon = 3{,}080$.

The acetamido group must be in the $\gamma$-position of the $\alpha,\beta$-butenolide to agree with the NMR spectrum of (I)[7]. The following additional evidence supports this positional assignment. The $\alpha$-acetamido derivative of butyrolactone (IIb) was prepared. Compound (IIb) differed from (IIa) with respect to melting point, i.r. spectrum, and response to Tollens reagent. Compound (IIa) gives a positive Tollens test since it has a potential aldehyde on the $\gamma$-carbon, whereas (IIb) does not.

With the presence of a chiral center at the $\gamma$-carbon we were surprised to find (I) had no optical rotation between 600 and 290 m$\mu$. Since some butenolides are known to isomerize quite easily,[15] perhaps we racemized (I) during its isolation and purification. A similar anomaly in optical activity has been reported for another $\Delta^{\alpha,\beta}$-butenolide (ancepsenolide).[16]

The pure butenolide (I) produces a positive rabbit-skin response (30 mg suspended in 5 ml of olive oil) but is not as toxic as an equivalent amount of a dichloromethane extract. Compound (I) is toxic to mice, $LD_{50}$ $43{\cdot}6 \pm 1{\cdot}24$ mg/kg (ip). A crude preparation of (I) was also tested for inhibitory activity against a number of bacteria, yeasts, and molds. Eleven of 14 bacteria and 2 of 3 molds were markedly inhibited at a concentration of 10 mg/ml. Only slight activity was detected at 1 mg/ml and none at 0·1 mg/ml.[17]

Compound (III), another of the toxins present in an ethyl acetate extract of liquid media, has not yet been identified, but possesses the same order of toxicity as the butenolide (I).

The third toxin (IV) was isolated from *F. nivale* grown on red winter wheat. The yield of (IV) was about 14 mg/kg. Final crystallization from cyclohexane yielded a white solid, m.p. 150–151°, $[\alpha]_D^{24} = -50°$ (*c* 0·16, cyclohexane). Its elemental analysis and molecular weight were in agreement with the empirical formula $C_{24}H_{34}O_9$. The $LD_{50}$ of (IV) in mice was $3{\cdot}04 \pm 0{\cdot}14$ mg/kg (ip). During the process of characterization we learned that a similar

[11] W. A. JACOBS and A. B. SCOTT, *J. Biol. Chem.* **87**, 601 (1930).
[12] W. A. JACOBS and A. B. SCOTT, *J. Biol. Chem.* **93**, 139 (1931).
[13] The electron withdrawing effect of the butenolide ring shifts this band to the upper end of the amide range.
[14] H. G. LEEMANN, K. STICH, J. GMÜNDER and A. LINDENMANN, *Helv. Chim. Acta* **46**, 1148 (1963).
[15] R. FILLER, E. J. PIASEK and L. H. MARK, *J. Org. Chem.* **26**, 2659 (1961).
[16] F. J. SCHMITZ, K. W. KRAUS, L. S. CIERESZKO, D. H. STIFFORD and A. J. WEINHEIMER, *Tetrahedron Letters* **1**, 97 (1966).
[17] Useful antibiotics are usually inhibitory at cencentrations of 0·1 to 0·01 mg/ml or less. However, microbial inhibition might be useful for measuring amounts of (I) in concentrations of 10 mg/ml or above. Private communication with L. A. LINDENFELSER, Northern Regional Research Laboratory.

compound (8-(3-methylbutyryloxy)-diacetoxyscirpenol) had been isolated from cultures of *F. tricinctum*.[18] Direct comparison of the two mold metabolites by mixed melting, optical rotation, and i.r. spectroscopy indicated they are identical.

We do not know at present if *F. nivale* or its metabolites are related to fescue foot or any other form of fescue toxicity. We plan to determine their involvement by controlled testing in cattle as soon as sufficient quantities are available.

## EXPERIMENTAL

All melting points were taken on a Kofler hot stage.[19] I.R. spectra were obtained with a Perkin–Elmer 337, Beckman IR 7, or Cary 90 spectrophotometer. Since only very small samples of (IV) were available, their i.r. spectra were secured by attenuated total reflectance (2 mm KRS-5 plate in a Wilks, Model 9, internal reflection optical bench mounted on a Beckman IR 8 spectrophotometer). The sample area was flushed with dry air. The u.v. spectra were made on a Beckman DK-2 or Cary, Model 15, recording spectrophotometer purged with nitrogen. The NMR spectra were obtained with a Varian A-60 spectrometer. The optical rotation was measured with a Model 60 Cary recording spectropolarimeter. The molecular weight was determined either by mass spectrometry[20] or by vapor pressure osmometry with a Mechrolab 301A (F & M Scientific Division of Hewlett Packard). GLC data were obtained on an F & M 810 gas chromatograph equipped with a flame-ionization detector. The nonpolar column (20% Apiezon L on AW DMCS-treated 100–200 mesh chromosorb W) was programmed from 130° to 220° at a rate of 4°/min; the polar column (5% LAC-2-R 446 on the same support) was programmed from 100° to 195° at a rate of 4°/min. Both columns were 3 m × 3 mm copper tubing. The carrier gas was helium; inlet temperature 290°; detector temperature 350°. The angelica lactones were analyzed on a Burrell Kromo-Tog K-5 gas chromatograph equipped with 2 m × 6 mm glass columns packed with 15% LAC-2-R 446 on celite 545 and operated iso-thermally at 206°.

### *Handling of* Fusarium nivale *Cultures*

The strain of *F. nivale* (NRRL-3249) used in this study was isolated from a sample of fescue hay toxic to cattle and was preserved by lyophilization.

Spore suspension inocula for agar-plate cultures were prepared by growing *F. nivale* on hay infusion agar[21] slants at 25° for 3–5 days, followed by incubation at 7° until used. Spores from the agar slants were streaked over the surface of Sabouraud's agar[22] in petri plates (*ca.* 25 ml per plate) and the inoculated plates were incubated at 15° for 3–4 weeks.

Inoculum (*F. nivale*) for the liquid culture medium was grown at room temperature on Sabouraud's maltose agar slants. A suspension from ten mature slants was used to inoculate thirty Fernbach flasks containing 400 ml of liquid Sabouraud's maltose medium. The flasks were incubated at 3° in the dark. Maximum toxicity, as verified by a weekly mouse bioassay, was reached after 20–30 weeks

*F. nivale* inoculum for the wheat cultures was grown on slants also, Fernbach flasks containing 1 kg of red winter wheat and 500 ml of water each were autoclaved and inoculated with a spore suspension of *F. nivale*. The flasks were incubated at 3° in the dark for 10–12 weeks until maximum toxicity was reached as verified by a weekly mouse bioassay.

### *Rabbit Skin Bioassay*

Olive oil solutions or suspensions of *F. nivale* extracts were painted on the dehaired skin of a rabbit's back at 2-hr intervals (five times daily). The rabbit was prevented from licking his back during the time the suspensions were applied. Only subsurface hemorrhage[6] was taken as a positive response; the hemorrhage usually appeared by the morning of the third day of testing.

### *Mouse Bioassay*

The lethal doses ($LD_{50}$) in mice were determined using ten mice per dose and were computed according to the log-probit method of Miller and Tainter.[23] Mice used were 24–26 g, white Webster strain, females.

[18] J. R. Bamburg and F. M. Strong, private communication. See also *Abstr. Papers*, No. A-93, **152nd** Meeting, *Am. Chem. Soc.*, September, 1966, and No. Q-70, 154th Meeting, September, 1967.

[19] Reference to a company or a product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

[20] Mass spectral data were supplied by W. H. McFadden, Western Regional Research Laboratory.

[21] Hay infusion agar consists of an aqueous extract from 50-g tall fescue hay, 2 g $K_2HPO_4$, 20 g agar, and 1000 ml tap water; pH is adjusted to 6·0–6·5.

[22] Sabouraud's agar consists of 40 g Bacto-dextrose, 10 g peptone, 20 g agar, and 1000 ml tap water.

[23] L. C. Miller, and M. L. Tainter, *Proc. Soc. Exp. Biol. Med.* **57**, 261 (1944).

Culture filtrates were bioassayed by intraperitoneal injection in amounts up to 1·0 ml/mouse. Solids containing the toxins were dissolved in water and injected in amounts between 0·1 and 0·5 ml/mouse. Samples not soluble in water were dissolved in acetone and mixed with 1,2-propanediol. The acetone was completely evaporated and not more than 0·15 ml of propanediol was injected per mouse.

*Extraction of* F. nivale *cultures*

Agar from the petri plates was placed on filter paper and diced. The paper and agar were extracted for at least 3 hr with ether, 80:20 ethanol:water, or dichloromethane in a Soxhlet extractor. After extracting with dichloromethane the extract was concentrated at 30° under reduced pressure to about 25 ml (per agar plate). The relative amounts of (I) in the sample could be estimated by the i.r. bands at 1760 and 1790 $cm^{-1}$. If the concentrate was to be tested by the rabbit skin bioassay, 10 ml of olive oil was added to the concentrate equivalent of one petri plate, and the remainder of the solvent was removed by bubbling dry nitrogen through the solution at room temperature. Any solids were dispersed in the oil by stirring or by means of a 40-Kc sonic oscillator. The yield of dichloromethane-soluble material was about 50 to 70 mg/plate.

The liquid-medium cultures were harvested after 23 weeks. However, it was determined from remaining flasks that the toxicity unexpectedly continued to increase and reached a maximum 6 weeks later. At 23 weeks toxicity was only 40 per cent of that maximum. The cultures were filtered and the mycelium was washed with water. The filtrate was exhaustively extracted with ethyl acetate; then the latter was filtered through a pad of anhydrous $Na_2SO_4$ and evaporated to dryness under reduced pressure at 50°. The residue from thirty Fernbach flasks amounted to 24 g (*ca.* 0·2 per cent). This residue contains three toxins which can be separated by TLC on silica gel G. The approximate $R_f$ values in ethyl acetate:hexane (3:1) were: 0·21 for the butenolide (I); 0·57 for an unknown toxin (III), and 0·63 for the sesquiterpenoid (IV).

The red winter wheat cultures grown in Fernbach flasks were extracted with ethyl acetate. A large fritted glass funnel (12 l. volume) was provided with a stopcock. Material from six flasks was emptied into the funnel and 6 l. of ethyl acetate added. This was allowed to soak for 30 min, then drained off. This procedure was repeated seven times with each batch. The ethyl acetate was concentrated 56-fold and exhaustively extracted with water. The ethyl acetate phase was evaporated under reduced pressure to an oil which contained $\frac{5}{6}$ of the total toxicity. From forty-two flasks, 248 g of residue was obtained.

*4-Acetamido-4-Hydroxy-2-Butenoic Acid-γ-Lactone (I) A*

Agar-plate cultures of *F. nivale* were extracted with dichloromethane. As the solvent was removed, compound (I) began to crystallize. The solvent was completely removed and the residue taken up in ethyl acetate. The resulting solution was treated with activated charcoal (Darco G-60) and filtered through a pad of Celite (Johns-Manville). The filtrate was concentrated on a steam bath and cyclohexane added until the solution became turbid. Upon slow cooling, crystals appeared. They were separated and washed with a cold mixture of ethyl acetate:cyclohexane, m.p. 116–117·5°. Recrystallization from ethyl acetate:cyclohexane gave the following:

| Fraction | Weight (g) | m.p. (°C) |
|---|---|---|
| 1 | 0·234 | 115·5–117·5 |
| 2 | 0·066 | 117·5–119·5 |
| 3 | 0·020 | 116·5–119·0 |

Total yield was 52·3 per cent of the dichloromethane extract or approximately 0·1 per cent of the total culture, assuming 25 ml of medium per plate. Fraction 1 was used for determining spectral and analytical data. (Found: C, 51·2; H. 4·8; N, 10·1; molecular weight in acetone 138. $C_6H_7NO_3$ requires: C, 51·1; H, 5·0; N, 9·9%; molecular weight 141). The i.r. spectrum[7] of (I) was made from a 0·5 per cent solution in chloroform; the u.v. spectrum was from methanol (0·0134 g/l.), $\lambda_{max}$ 202 nm, $\epsilon$, 11,300. A 16 per cent solution in deuteroacetonitrile was used for the previously reported NMR spectrum.[7] The optical rotation of (I) was 0·0° between between 600 and 290 nm. (*c* 2·6, acetonitrile). A once-crystallized sample from another preparation did show slight negative optical rotation although this rotation has not been definitely assigned to (I).

*4-Acetamido-4-Hydroxy-2-Butenoic Acid-γ-Lactone (I) B*

The ethyl acetate residue (24 g) from the liquid medium cultures was dissolved in hot ethyl acetate and Darco G-60 charcoal was added. The solution was filtered, and the volume reduced to 200 ml. Slow cooling in a water bath overnight yielded crystals. The total yield was 14·05 g of crude (I) This material was dissolved in acetone, refluxed, filtered, and the solution slowly cooled. The crystals were separated, washed with cold

acetone, and dried. This compound (12·31 g) was dissolved in 160 ml acetone, and addition of 1320 ml pentane gave 11·07 g of crystalline butenolide, m.p. 113–115°. The yield was about 1·0 g/l. of culture filtrate. Two further crystallizations from ethyl acetate and acetone yielded material of m.p. 115–116·5°; the optical rotation was 0·0° from 600 to 280 nm (*c.* 2·0, water). The i.r. spectrum of (I) from liquid cultures by ATR was identical with that of (I) isolated from agar-plate cultures. The $LD_{50}$ (ip) of (I) in mice was $43{\cdot}6 \pm 1{\cdot}24$ mg/kg.

*Alkaline Hydrolysis*

A sample of (I) weighing 0·0196 g was dissolved in *N* NaOH. Air was bubbled through the solution at room temperature for 3½ hr. An ether extract of the acidified reaction mixture yielded 5·4 mg of material; 3·2 mg of this was recovered as methyl esters after treatment with diazomethane.[24] GLC of the product gave evidence of acetamide on both polar and nonpolar columns, but methyl esters of 4-carbon dibasic acids were not detected.

4-*Acetamido*-4-*Hydroxy-Butyric Acid*-γ-*Lactone* (*IIa*)

Compound (I) was hydrogenated in ethyl acetate at room temperature and atmospheric pressure with 10% Pd on C (Matheson, Coleman, and Bell) as a catalyst. The uptake of $H_2$ was 82 per cent of theory. The dihydro derivative (IIa) was recrystallized twice from ethyl acetate-cyclohexane, m.p. 146–147°. (Found: C, 50·2; H, 6·3; N, 9·8. $C_6H_9NO_3$ requires: C, 50·3; H, 6·4; N, 9·8). The i.r. spectrum (saturated chloroform solution, *ca.* 0·5%) showed bands at 3445 (amide N—H), 3350 (amide N—H), 1775 (lactone C=O), 1700 $cm^{-1}$ (amide C=O); u.v. (methanol, 0·0302 g/l.) $\lambda_{max}$ 201 nm, $\epsilon$ 2700. NMR spectra were obtained with a $CD_3CN$ solution (10 per cent solute). The amide proton was exchanged with $D_2O$. Compound (IIa) gave a positive Tollens test.

2-*Acetamido*-4-*Hydroxy-Butyric acid*-γ-*Lactone* (*IIb*)

One gram of α-amino-γ-butyrolactone hydrobromide (Aldrich) was treated with acetic anhydride.[25] The reaction product was dissolved in methanol and diluted with ethyl acetate. The portion soluble in ethyl acetate was diluted with cyclohexane as described for (I), but (IIb) did not crystallize. The solvent was removed. The resultant oil solidified after standing 2 weeks, m.p. 76–81·5° (literature value[25] 82°). Compound (IIb) was recrystallized from ethyl acetate: cyclohexane, m.p. 79–84°; $\lambda_{max}$ 201 nm, $\epsilon$ 3080. Further recrystallization from ethyl acetate at −16° yielded a solid with two melting forms (80·5–81·5° and 87·0–89·0°). The compound was triturated with ethyl acetate and recrystallized to yield a solid, m.p. 91·0–92·0°. This product gave a negative Tollens test. The i.r. spectrum in chloroform (0·25 per cent) showed bands at 3430 (amide N—H), 1780 (lactone C=O), and 1680 $cm^{-1}$ (amide C=O).

8-(3-*Methylbutyryloxy*)-*Diacetoxyscirpenol* (*IV*)

Two phases resulted when the 248 g of oil from the ethyl acetate extract of the red winter wheat cultures of *F. nivale* was partitioned between 950 ml of dioxane and 1250 ml water. The lower phase was filtered through charcoal and evaporated at reduced pressure and temperature not exceeding 50°. The residual oil was taken up in $CCl_4$ and filtered; then the filtrate was added to an alumina (activity IV) column (7·5 × 32 cm). The column was eluted with $CCl_4$, $CHCl_3$, ether, and then acetone at a flow rate of 30 ml/min. Compound (IV) was eluted in ether along with (III) and amounted to 5·7 g. This material was dissolved in acetone (25 ml) and water (50 ml) was added. The mixture was shaken and centrifuged after standing for 1 hr. The clear solution was then diluted with water (200 ml) and left standing at 4° until crystallization was complete. The crystals were dried over $P_2O_5$ and recrystallized from cyclohexane, m.p. 148–150°. Total yield was 0·59 g. Two further crystallizations gave a m.p. of 150–151°. $[\alpha]_D^{24} = -50°$ (*c* 0·16, cyclohexane). (Found: C, 61·8; H, 7·3; molecular weight by mass spectrometry 466. $C_{24}H_{34}O_9$ requires: C, 61·8; H, 7·4%; molecular weight 466.) The u.v. spectrum in cyclohexane showed $\lambda_{max}$ 187·5 nm, $\epsilon$ 13,900.

Compound (IV) isolated from liquid medium by preparative TLC and compound (IV) from the red winter wheat medium were identical. Both samples had the same $R_f$ values on TLC plates in several solvent systems—ethyl acetate: hexane (3:1); ethyl acetate: heptane (9:1); and ethyl acetate: ethanol (19:1). The i.r. spectra of compound (IV) from both sources were identical. Both had the same toxicity in the mouse bioassay.

Compound (IV) was compared to 8-(3-methylbutyryloxy)-diacetoxy-scirpenol supplied by J. R. Bamburg.[18] Their i.r. spectra were identical and mixed m.p. determinations showed no detectable depression.

α- *And* β-*Angelica Lactones*

The α- and β-lactones (K & K Laboratories) were analyzed by GLC. α-Angelica lactone was 93 per cent pure α-isomer. β-Angelica lactone was redistilled and by GLC analysis was 87 per cent pure β-isomer. The u.v. spectrum of β-angelica lactone in methanol (8 mg/l.) showed $\lambda_{max}$ 203 nm, $\epsilon$ 15,200; of α-angelica lactone (32·6 mg/l.) $\lambda_{max}$, 217 nm, $\epsilon$ 2000. The i.r. spectra in 0·25 per cent chloroform solutions were: β-angelica lactone 1760 and 1780 $cm^{-1}$ (C=O), α-angelica lactone 1795 $cm^{-1}$ (C=O).

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*Inhibitory Action of (I) Against Bacteria, Yeasts, and Molds*

A crude preparation of (I), m.p. 113–115°, was suspended in water (9·6 mg/ml), and the solution centrifuged to remove undissolved material. The clear supernatant (pH 4·2) was adjusted to pH 5·8 with 0·25 *N* NaOH. A standard disc agar assay method was used[26] to test the inhibitory action of (I). Tests were made at concentrations of approximately 10, 1, and 0·1 mg/ml.

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