APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1979, p. 693-696
0099-2240/79/04-0693/04$02.00/0

Vol. 37, No. 4

Production of 3-Acetoxyscirpene-4,15-Diol from Anguidine (4,15-Diacetoxyscirpene-3-ol) by *Fusarium oxysporum* f.sp. *vasinfectum*

C. A. CLARIDGE* AND HENRY SCHMITZ

Research Division, Bristol Laboratories, Division of Bristol-Myers Company, Syracuse, New York 13201

Received for publication 19 January 1979

Growing cells of *Fusarium oxysporum* f.sp. *vasinfectum* (ATCC 7808) formed 3-acetoxyscirpene-4,15-diol from anguidine (4,15-diacetoxyscirpene-3-ol) by way of the intermediates triacetoxyscirpene, 3,4-diacetoxyscirpene-15-ol and 3,15-diacetoxyscirpene-4-ol. The new 3-acetoxy analog was found to be less active than anguidine and the two other monoacetoxy derivatives when tested against a series of fungal strains and against HeLa cells in vitro.

We have been studying the transformation of the antitumor antibiotic anguidine by both microbiological and chemical means, seeking one or more derivatives having enhanced in vivo chemotherapeutic properties. One such series of derivatives already has been prepared, and their antifungal and cytotoxic activities have been determined (4). This series contained triacetoxyscirpene, scirpenetriol, the three diacetoxyscirpenols, and two of the monoacetoxyscirpenediols, namely, 4-acetoxyscirpene-3,5-diol and 15-acetoxyscirpene-3,4-diol. We were unable to obtain the hitherto unknown 3-acetoxyscirpene-4,15-diol either by treatment of scirpenetriol with *Mucor mucedo* or of 3,15-diacetoxyscirpene-4-ol with *Streptomyces griseus*, two of the organisms previously reported to transform anguidine to the 4- and 15-acetoxy analogs (4).

The present paper describes experiments with growing cells of *Fusarium oxysporum* f.sp. *vasinfectum* (ATCC 7808) which form 3-acetoxyscirpene-4,15-diol (VII) from anguidine (IV) by way of intermediates triacetoxyscirpene (I), 3,4-diacetoxyscirpene-15-ol (III), and 3,15-diacetoxyscirpene-4-ol (II). The activity of the 3-acetoxy derivative in both tissue culture cytotoxicity and antifungal tests is reported.

MATERIALS AND METHODS

Organism and culture conditions. *Fusarium oxysporum* f.sp. *vasinfectum* was obtained from the American Type Culture Collection (ATCC 7808) and maintained on slants of yeast malt extract agar (Difco Laboratories). The culture was grown first in a vegetative medium consisting of glucose (3%), lactose (1%), corn steep liquor (3%, vol/vol), ammonium sulfate 0.2%, monobasic potassium phosphate 0.05%, and calcium carbonate (0.5%; pH 6.0) for 3 days at 27°C on a rotary shaker at 250 rpm. This culture was blended and used at 10% (vol/vol) as an inoculum for the following medium: Staclipse J corn starch (7%; A. E. Staley Manufacturing Co.), Pharmamedia (3%; Traders Oil Mill Co.), washed brewer's yeast (1.5%; Amber Laboratories), calcium carbonate (0.5%), and dibasic potassium phosphate (0.25%; pH 7.0). The cells were grown on a rotary shaker at 250 rpm for 2 days at 27°C.

Compounds tested for bioconversion were dissolved in 0.3 ml of dimethylformamide and added to the cells (25 ml in a 125-ml Erlenmeyer flask) such that the final concentration was 0.5 mg/ml. The culture flasks were reincubated as before and were sampled at the various noted times.

Detection of transformed products. At each sampling period, 1 ml of growth was extracted with 0.5 ml of chloroform. After a brief centrifugation to break the emulsion, 40 μl of the solvent phase was spotted on Analtech silica gel GF thin-layer chromatography plates which were developed in ethyl acetate-toluene (3:1). Starting material and transformed zones were detected with a spray of either alkaline potassium permanganate or 7% phosphomolybdic acid in ethanol. R_f values of the compounds are given in Table 1 (see also Fig. 1).

The recovery and purification of the suspected intermediates from broth samples was accomplished by an initial chloroform extraction followed by column chromatography. As an example, the reaction from a total of 10 liters of fermentation broth to which 0.5 mg of anguidine per ml had been added was extracted with 2 liters of chloroform. This extract was concentrated to an oil, taken up in 10 ml of benzene, and placed on a column (2.5 by 80 cm) of silica gel (Grace Chemical Co.). The column was washed with benzene and eluted with benzene containing increasing amounts of methanol. At concentrations of 4 to 5% methanol, thin-layer chromatography of the eulate showed a single zone. This eluate was dried, and the product was crystallized from ethyl acetate-ether.

Compounds for bioconversion. Anguidine was obtained by fermentation in the Bristol-Myers Industrial Division Pilot Plant, using *Fusarium* sp. C-37410. Scirpenetriol and 15-acetoxyscirpene-3,4-diol were

prepared through chemical transformation of anguidine by established procedures (5, 6). 3,4-Diacetoxyscirpene-15-ol, 4-acetoxyscirpene-3,15-diol, and triacetoxyscirpene were prepared by the microbiological transformation of anguidine as previously outlined (4).

The intermediates and products of the transformation with *F. oxysporum*, 3,4-diacetoxyscirpene-15-ol, 3,15-diacetoxyscirpene-4-ol and 3-acetoxyscirpene-4,15-diol were isolated from large-scale transformations and identified by comparative thin-layer chromatography employing marker compounds of established identity.

Tissue culture cytotoxicity tests. Doses required to cause a 50% decrease in net protein production (TD_{50}s)using HeLa cell cultures were obtained by following published procedures (2).

Physical and chemical analyses. Proton magnetic resonance spectra were measured with a Varian Associates HA-100 spectrometer; chemical shifts are expressed in parts per million (δ) downfield from the internal standard tetramethylsilane. Infrared spectra were obtained by using a Beckman model 4240 diffraction grating spectrometer. Optical rotations were determined with a Perkin-Elmer 241 polarimeter.

RESULTS

During the process of screening organisms for the conversion of anguidine, we noticed a new metabolite from *F. oxysporum* f. sp. *vasinfectum*. This material had a lower R_f value than anguidine in the thin-layer chromatographic system employed, but cochromatography with suspected intermediates 4-acetoxyscirpene-3,15-diol and 15-acetoxyscirpene-3,4-diol showed it to be different. When the reaction was scaled up, the new product was isolated in a 25% yield (mp, 174 to 175°C) and characterized as 3-acetoxyscirpene-4,15-diol, based on its infrared and proton magnetic resonance spectra. Infrared absorption peaks υ_{max}^{KBr} were at 3,550, 3,500, 3,430, 2,970, 2,440, 1,730, 1,255, and 1,060 cm^{-1}. The proton magnetic resonance spectrum showed signals similar to those given by anguidine (4), except for the proton at C3 at 4.9 ppm (shifted from 5.15) and a singlet for CH_3CO at 2.2 ppm (compared to two peaks at 2.06 and 2.15 ppm for anguidine). The $[\alpha]_D^{22}$ was −11.7° (c = 0.875, chloroform) and +37.4° (c = 1, acetone).

Analysis; calculated for $C_{17}H_{24}O_6$: C, 62.95: H, 7.45. Found: C, 62.46; H, 7.47.

These data identify the product as 3-acetoxyscirpene-4,15-diol.

3-Acetoxyscirpene-4,15-diol was acetylated in pyridine with acetic anhydride. The acetate had the same R_f in thin-layer chromatography and identical proton magnetic resonance spectrum and optical rotation to that of authentic triacetoxyscirpene.

With anguidine as substrate, the conversion to 3-acetoxyscirpene-4,15-diol is complete within 48 to 72 h. Examination of this reaction at 6 h has revealed the first new product visible on thin-layer chromatograms to be triacetoxyscirpene. By 10 h, the triacetoxyscirpene has disappeared and the 3,4 and 3,15 analogs are visible. To isolate these intermediates, we stopped a fermentation 10 h after the addition of the anguidine substrate by extraction of the broth with methylene chloride. This extract was dried to an oil, taken up in ether, and then absorbed on silica gel (Grace Chemical Co.). The dried silica gel was placed on top of a column (1 by 20 cm) of fresh silica and then eluted first with a mixture of ether–Skellysolve B (1:1) followed by ether and then with chloroform containing increasing amounts of methanol.

The eluted materials were identified as triacetoxyscirpene, 3,4-diacetoxyscirpene-15-ol, and anguidine by cochromatography with known standards in thin-layer chromatographic systems, ethyl acetate–toluene (3:1) and toluene–

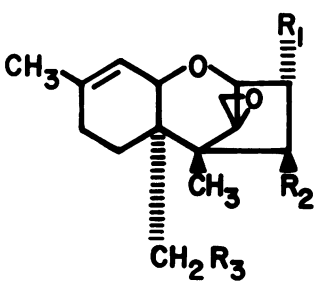

FIG. 1. *Structure of anguidine and analogs.*

TABLE 1. *Composition and R_f values of compounds shown in Fig. 1*[a]

Compound	Name	R_1	R_2	R_3	R_f
I	Triacetoxyscirpene	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$	0.9
II	3,15-Diacetoxyscirpene-4-ol	$OCOCH_3$	OH	$OCOCH_3$	0.7
III	3,4-Diacetoxyscirpene-15-ol	$OCOCH_3$	$OCOCH_3$	OH	0.82
IV	4,15-Diacetoxyscirpene-3-ol (anguidine)	OH	$OCOCH_3$	$OCOCH_3$	0.65
V	15-Acetoxyscirpene-3,4-diol	OH	OH	$OCOCH_3$	0.25
VI	4-Acetoxyscirpene-3,15-diol	OH	$OCOCH_3$	OH	0.5
VII	3-Acetoxyscirpene-4,15-diol	$OCOCH_3$	OH	OH	0.4
VIII	Scirpenetriol	OH	OH	OH	0.2

[a] The solvent system used was ethyl acetate–toluene (3:1).

methanol (84:16). The diacetates were obtained crystalline, and their identities were confirmed by melting point and proton magnetic resonance spectra.

A second experiment treated in the same manner yielded a third diacetate, 3,15-diacetoxyscirpene-4-ol, in addition to the other two previously isolated. This compound was identified by comparative thin-layer chromatography in the two systems listed, using an authentic standard. This compound had been previously noted in small-scale time study reactions.

Biological properties. We have previously reported on the biological properties of some of these anguidine derivatives (3, 4; C. A. Claridge, H. Schmitz, and W. T. Bradner, Cancer Chemother. Pharmacol., in press). The results of the comparison of anguidine and the three monoacetoxy analogs in a limited antifungal screen are recorded in Table 2. Anguidine has been reported to have some antifungal activity (1), but neither it nor the monoacetoxy analogs show activity comparable to that of the known agents, amphotericin B and nystatin, included as controls.

The tissue culture cytotoxicity measurements comparing the three monoacetoxy analogs to anguidine are given in Table 3. As has been noted previously (4), anguidine and the 15-acetoxy derivative have very similar TD_{50}s, whereas the 4-acetoxy analog is less active, and 3-acetoxyscirpene-4,15-diol is more than 100-fold less active than anguidine. This greatly decreased level of activity of the 3-acetoxy derivative compared to the other monoacetoxy analogs and to anguidine is also seen in the in vivo P-388 and L-1210 lymphatic leukemia tests in mice (Claridge et al., in press).

DISCUSSION

The finding that cells of *F. oxysporum* f.sp. *vasinfectum* remove the C4 and C15 acetoxy functions and add a C3 acetoxy group was surprising but could possibly be explained by one or more of three pathways: (i) anguidine (4,15-diacetoxyscirpene-3-ol) → triacetoxyscirpene → 3,4-diacetoxyscirpene-15-ol → 3-acetoxyscirpene-4,15-diol; (ii) anguidine → triacetoxyscirpene → 3,15-diacetoxyscirpene-4-ol → 3-acetoxyscirpene-4,15-diol; and/or (iii) anguidine → 4-acetoxyscirpene-3,15-diol or 15-acetoxyscirpene-3,4-diol → scirpenetriol → 3-acetoxyscirpene-4,15-diol.

In the first pathways, cells of *F. oxysporum* f.sp. *vasinfectum* should produce the 3-acetoxy derivative from triacetoxyscirpene and the 3,4-diacetoxy compound. This in fact is the case, for if these compounds are added to the growing cells, then the 3-acetoxyscirpene-4,15-diol is formed. If the second pathway is operative, then the intermediate 3,15-diacetoxyscirpene-4-ol will lead to the 3-acetoxy derivative when added to the cells. This reaction also occurs. The final product was not isolated in these studies, but appeared on thin-layer chromatograms in a coincident spot with authentic 3-acetoxyscirpene-4,15-diol. The third mechanism can be eliminated, since added 15-acetoxyscirpenol and scirpenetriol remained unreacted in the presence of the *F. oxysporum* cells.

However, as yet unexplained is the conversion we have noted of the 4-acetoxyscirpene-3,15-diol to the 3-acetoxy derivative by these cells. Perhaps the 4-acetoxyscirpenol is formed earlier in the first or second pathway, for there is evidence that anguidine appears on the thin-layer chromatograms when the 4-acetoxy derivative is used as a substrate for these cells. Another possibility, not ruled out by these experiments, would be the conversion of 4-acetoxyscirpene-3,15-diol to 3,4-diacetoxyscirpene-15-ol, followed

TABLE 3. *Cytotoxic activity of monoacetoxyscirpenols*

Compound	Name[a]	HeLa cell culture TD_{50} (g/ml)[b]
V	15-Acetoxyscirpene-3,4-diol	0.004
VI	4-Acetoxyscirpene-3,15-diol	0.057
VII	3-Acetoxyscirpene-4,15-diol	>0.33
IV	4,15-Diacetoxyscirpene-3-ol	0.002

[a] See Table 1.
[b] See text for explanation of TD_{50}.

TABLE 2. *Antifungal activity of monoacetoxyscirpenols*

Organism	Minimal inhibitory concentration (μg/ml)					
	Amphotericin B	Nystatin	Anguidine	VII[a]	VI[a]	V[a]
Candida albicans A9540	0.25	2	63	125	63	63
Candida tropicalis A15051	0.5	2	125	125	125	125
Candida krusei A15052	0.5	4	125	125	126	63
Cryptococcus neoformans A15053	0.5	125	125	125	125	125
Trichophyton mentagrophytes A9870	4	16	125	125	125	125
Microsporum canis A9872	4	16	125	125	125	125

[a] See Table 1.

by conversion of the latter compound to the 3-acetoxy derivative.

To account for these compounds appearing as intermediates, a suggested scheme combining mechanisms i and ii is represented in Fig. 2. The formation of the 3-acetoxy analog from 4-acetoxyscirpene-3,15-diol may be explained by assuming that the *F. oxysporum* cells are capable of forming anguidine from the 4-acetoxy derivative, which then enters the pathway. Anguidine has been detected by thin-layer chromatography with 4-acetoxyscirpene-3,15-diol as substrate. The *F. oxysporum* culture apparently is not able to acetylate scirpenetriol nor the 15-acetoxy analog because these two compounds remain unchanged when mixed with the cells. The chance that these compounds are unreacted because of lack of permeability is, however, a slight possibility that should not be overlooked.

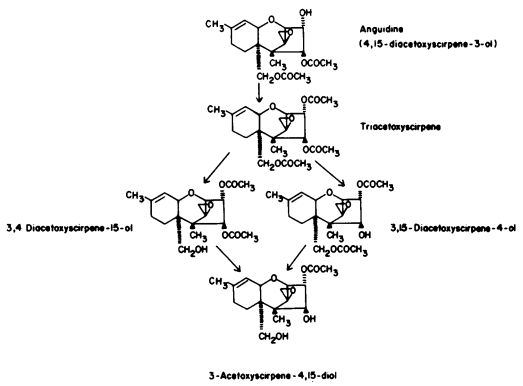

FIG. 2. *Formation of 3-acetoxyscirpene-4,15-diol from anguidine.*

ACKNOWLEDGMENTS

We thank A. Schlein for the cell culture experiments, M. Misiek for the minimal inhibitory concentration determinations, D. F. Whitehead for the proton magnetic resonance data, and J. Veitch and N. Billings for technical assistance.

This work was performed under Public Health Service contract NO1-CM-77138 from the National Cancer Institute.

LITERATURE CITED

1. **Bamburg, J. R., and F. M. Strong.** 1971. 12,13-Epoxytrichothecenes, p. 207-292. *In* S. Kadis, A. Ciegler, and S. Ajl (ed.), Microbial toxins, vol 7. Academic Press Inc., New York.
2. **Bradner, W. T., B. Heinemann, and A. Gourevitch.** 1967. Hedamycin, a new antitumor antibiotic, p. 613-618. Antimicrob. Agents Chemother. 1966.
3. **Claridge, C. A., W. T. Bradner, and H. Schmitz.** 1978. Antitumor activity of 15-acetoxyscirpenol. J. Antibiot. **31**:485-486.
4. **Claridge, C. A., and H. Schmitz.** 1978. Microbial and chemical transformations of some 12,13-epoxytrichothec-9,10-enes. Appl. Environ. Microbiol. **36**:63-67.
5. **Dawkins, A. W.** 1966. Phytotoxic compounds produced by *Fusarium equiseti*. Part II. The chemistry of diacetoxyscirpenol. J. Chem. Soc. C., p. 116-123.
6. **Sigg, H. P., R. Mauli, E. Fleury, and D. Hauser.** 1965. Die Konstitution von Diacetoxyscirpenol. Helv. Chim. Acta **48**:962-988.