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MICROBIAL TRANSFORMATIONS OF NATURAL ANTITUMOR AGENTS, 25. CONVERSIONS OF 3-KETOAPHIDICOLIN

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ABSTRACT.—Microbial transformation experiments were conducted using 3-ketoaphidicolin (**2**) as a starting material. Metabolites were isolated by solvent extraction and chromatography, and structures were elaborated by cmr and pmr spectroscopy, ms, and ir analyses. Several microorganisms provided metabolites in excellent yields, including 3-*epi*-aphidicolin (**4**), 6β-hydroxy-3-ketoaphidicolin (**5**), and 19-*nor*-16,17-dihydroxyaphidicolan-3-one (**6**). The last compound is formed *via* oxidation of the primary alcohol functional group at position 18 to the corresponding β-keto acid derivative which spontaneously decarboxylates. This reaction is analogous to the metabolic demethylation of sterol intermediates. Each metabolite was tested for antitumor activity in the P-388 leukemic test system, and in the 6C63 1 colon tumor model system. None of the compounds were active *in vivo*, and all were less active than aphidicolin in the *in vitro* P-388 test system.

3α,16,17,18-Tetrahydroxyaphidicolane (aphidicolin, **1**) is a novel antitumor diterpene derivative produced by a species of *Cephalosporium* (1) and of *Nigrosporum* (2). Microbiological transformations were successfully used to produce six major derivatives of aphidicolin (3). These included acylated, hydroxylated, epimerized, and dehydrogenated microbial products, all of which were obtained in good yield. One of the major metabolites described in our previous work was 16,17,18-trihydroxyaphidicolan-3-one (**2**). Because the 3-ketoaphidicolin analog could be prepared in high yield, further biotransformation studies were performed using **2** as substrate. This report describes the production, isolation, and characterization of metabolities formed from **2** by microbial transformation. (See Figure 1.)

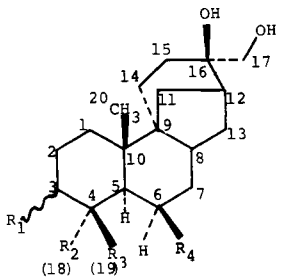

FIGURE 1. Structures of aphidicolin (**1**) and microbial transformation products.

Compound	R_1	R_2	R_3	R_4
1	α-OH	CH_2OH	CH_3	H
2	=O	CH_2OH	CH_3	H
3	α-OH	CH_2OH	CH_3	β-OH
4	β-OH	CH_2OH	CH_3	H
5	=O	CH_2OH	CH_3	β-OH
6	=O	CH_3	H	H
7	α-OH	COOH	CH_3	H

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were obtained in open-ended capillaries and are uncorrected. Ir spectra were obtained in KBr discs with a Beckman Model 4240 spectrophotometer. Nmr spectra were taken in pyridine-D_5, $CDCl_3$, or C_6D_6 using TMS as an internal standard. Pmr

and cmr spectra were taken on a Brüker HX-90E instrument, and low resolution mass spectra were obtained on a Hewlett-Packard 5985 gc/ms system with a direct inlet probe. High resolution mass spectra were obtained through the Midwest Center for Mass Spectrometry, The Chemistry Department, University of Nebraska, Lincoln, NE.

CHROMATOGRAPHY.—Chromatographic separations were accomplished as previously described (3). Open-column chromatography was performed on Baker 3405 silica gel, 40-140 mesh, and samples were generally applied to columns by preadsorption from solutions in MeOH or Me_2CO. Tlc was performed on 0.25 mm silica gel GF_{254} (Merck) plates developed in $CHCl_3$-EtOH (5:1).

FERMENTATION PROCEDURES.—A standard two-stage fermentation protocol was employed in all experiments (3) using a soybean meal-glucose culture medium. In general, organic substrates were added to 24-h-old, Stage II cultures, and incubations were continued until substrates were completely consumed or until maximum yields of metabolies were obtained.

PRODUCTION OF 3-KETOAPHIDICOLIN (**2**) BY *CHAETOMIUM FUNICULUM*.—*C. funiculum*, strain MR 141, was grown in 3.5 liters of soybean meal-glucose medium in a 5-liter fermentor (New Brunswick Scientific Co., Edison, NJ), which was stirred at 300 rpm while 1.2 liters of air was sparged into the medium per minute. Incubations were conducted at 24°, and the 10% inoculum for fermentors was obtained from 72-h stage I cultures grown in 1-liter DeLong culture flasks. Aphidicolin (**1**) (2.0 g) was dissolved in 40 ml of dimethylformamide DMF) and added to the 24-h-old culture. After incubating for 3-4 days, tlc and hplc analyses indicated that aphidicolin (Rf 0.55) was converted to a mixture of 3-ketoaphidicolin (**2**) (Rf 0.65, 85% yield) and 3-epiaphidicolin (**4**) (Rf 0.50, 15% yield). The incubation was terminated at this time, and cells were separated from the culture medium by filtration through cheesecloth. Cells were extracted exhaustively with MeOH, and the culture filtrate with EtOAc-*n*-BuOH (9:1). The chromatographically similar extracts were combined, adsorbed onto silica gel, and purified over 300 g of silica gel in a column 4.0×46 cm. Elution was carried out by stepwise variation of solvents beginning with $CHCl_3$ and ending with $CHCl_3$-EtOH (4:1). Similar fractions from this column were combined to give 1.0 g of 3-ketoaphidicolin. Compounds produced in this incubation were completely identical (mp, ir, pmr, and ms) to authentic samples identified in our earlier work (3).

ENHANCED PRODUCTION OF 3-EPIAPHIDICOLIN (**4**) FROM APHIDICOLIN UNDER REDUCING FERMENTATION CONDITIONS.—Aphidicolin (2.0 g) was added to 3.5 liters of Stage II *C. funiculum* culture in a 5-liter fermentor, and incubations were conducted as described in the previous experiment. After 4 days of incubation with substrate, air flow to the fermentor was stopped, and the agitation rate was reduced to 100 rpm. Under these conditions, the fermentor was incubated for an additional 24 h, at which time the ratios of various metabolites present in the incubation mixture were estimated to be 3-ketoaphidicolin (50%), 3-epiaphidicolin (30%), and aphidicolin (20%). Metabolites were isolated using silica gel column chromatography as previously described to afford 3-ketoaphidicolin (902 mg), aphidicolin (110 mg), and 3-epiaphidicolin (580 mg). All compounds were compared and found completely identical to authentic samples (3).

PRODUCTION OF 6β-HYDROXY-3-KETOAPHIDICOLIN (**5**) FROM **2** BY *STREPTOMYCES PUNIPALUS*, STRAIN 3529.—3-Ketoaphidicolin (400 mg) was dissolved in 8.0 ml of DMF and distributed evenly among four 1-liter, 24-h, stage II culture flasks, each holding 200 ml of *S. punipalus* culture. Incubations were continued for 3 days when the conversion of substrate to metabolites was maximal, leaving approximately 25% unreacted **2**. The reaction mixture was harvested by filtration through cheesecloth, and the culture filtrate was extracted exhaustively with EtOAc-*n*-BuOH (3:2). The extract was concentrated and adsorbed onto silica gel and applied to a 130 g (3.0×36 cm) column of silica gel slurry-packed in $CHCl_3$. Elution was accomplished with $CHCl_3$-EtOH mixtures beginning with (15:1) and ending with (3:2), while 20-ml fractions were collected at a flow rate of 2 ml/min. Chromatographically similar fractions were combined, and the first metabolite (80 mg, Rf 0.45) was crystallized from EtOAc-hexanes to give the following physical properties: mp 140-141°; ir (KBr disc) 3430 (broad OH), 2933, 2866, 1681 (carbonyl), 1036 cm^{-1}; pmr (pyridine-D_5) see Table 1; cmr (pyridine D_5) see Table 2; high resolution ms, *m/z* 321.2070 (M-CH_2OH for $C_{19}H_{29}O_4$, 18%-calcd 321.2067), 291.1966 ($C_{18}H_{27}O_3$ 100%), 275.2002 ($C_{18}H_{27}O_2$, 7.6%), and 273.1853 ($C_{18}H_{25}O_2$, 9.1%). Two minor metabolites formed in this experiment were not investigated since they were formed only in trace amounts.

PRODUCTION OF 10-*NOR*-16,17-DIHYDROXYAPHIDICOLAN-3-ONE (**6**) FROM **2** BY *TRICHOTHECIUM ROSEUM*, STRAIN UI 320.—A total of 1.8 g of 3-ketoaphidicolin (**2**) was dissolved in 36 ml of DMF and divided evenly among 18, 1-liter DeLong culture flasks containing 200 ml each of 24-h-old, stage II cultures of *T. roseum*. Incubations were continued for ten more days after substrate addition when conversions of **2** to **6** (Rf 0.8) had progressed to approximately 50% yield (tlc estimate). The incubation was terminated, and solids were removed from the culture medium by centrifugation at 4000×g for

TABLE 1. Pmr Spectral Data for Aphidicolin and Its Microbial Transformation Products

Compound	Proton[a]					
	H-3[b]	H-17[c]	H-18[c]	H-19[d]	H-20[d]	Misc.
1[e]	3.96	3.76	3.88, 3.54 dd (10 Hz)	0.78	1.02	—
2[e]	—	3.78	4.08, 3.62 dd (10 Hz)	0.95	1.02	—
3[e]	4.13	3.82	3.95	1.52	1.63	4.35[b]
4[e]	3.58	3.77	4.32, 4.03	1.06	1.06	—
5[e]	—	3.64	4.40, 3.87 dd (9 Hz)	1.62[h]	1.67[h]	4.70[b]
6[e]	—	3.84	—	1.10 (d 6 Hz)	1.05	—
6[f]	—	3.48	—	1.00 (d 6 Hz)	1.17	—
6[g]	—	3.25	—	1.07 (d 7 Hz)	1.28	—

[a]60 MHz spectra, shifts given in parts per million from TMS. Unmarked signals are singlets. Figures in parentheses are coupling constants.
[b]Broad Signal.
[c]Two proton intensity.
[d]Three proton intensity
[e]Spectrum in pyridine-d_5.
[f]Spectrum in $CDCl_3$.
[g]Spectrum in C_6D_6.
[h]Signal assignments may be interchangeable.

TABLE 2. Cmr Spectral Data for Aphidicolin and Microbial Transformation Products[a]

Carbon	Compound					
	1	**2**	**3**	**4**	**5**	**6**
1	27.2t	31.4t	29.2t	32.5t	33.2t	28.4t
2	27.2t	37.1t	27.3t	28.5[b]t	36.8[c]t	34.2t
3	76.1d	217.1	78.1d	73.4d	216.1	211.8
4	40.8	53.3	41.8	43.3	54.5	45.8d
5	33.9d	38.6d	37.3d	38.7d	42.5d	45.8d
6	23.4t	25.2[b]t	68.4d	23.7t	68.4d	24.4t
7	27.2t	26.4t	37.6t	27.0t	36.6[c]t	28.4t
8	40.3d	40.5d	34.2d	40.3d	34.1d	40.1d
9	49.5	48.7	49.6	49.3	49.1	47.7
10	40.0	39.2	41.1	40.0	40.0	39.5
11[b]	33.2t	33.3t	33.7t	33.7t	33.4[b]t	33.5t
12	41.9d	41.8d	42.2d	42.0d	41.9d	41.7d
13[b]	31.6t	31.4t	31.7t	31.6t	31.1t	31.1t
14	25.4t	25.6[b]t	25.4t	25.3t	25.0t	26.1t
15	28.9t	28.7t	28.8t	28.9[b]t	29.0t	28.4t
16	74.2	74.1	74.3	74.2	74.2	74.1
17	68.1t	68.3[c]t	68.3t	68.3t	68.2t	68.2t
18	71.1t	68.7[c]t	72.4t	67.8t	67.5t	—
19	17.9q	18.6q	20.7q	13.2q	20.9q	12.4[b]q
20	15.3q	14.9q	18.5q	15.6q	18.5q	13.0[b]q

[a]Spectra were obtained at 22.635 MHz in pyridine-d_5, and chemical shifts are given in ppm downfield from TMS. Unmarked signals are singlets.
[b,c]Values within any vertical column may be interchanged.

15 min. The resulting pellets were extracted once with MeOH, and then exhaustively with EtOAc-*n*-BuOH (9:1), and the same solvent mixture was used to extract the clear supernatant solutions. The extract from the supernatant was adsorbed onto 20 g of silica gel and separated on a 120 g silica gel column (3.0×33 cm) using 200 ml of $CHCl_3$ and then $CHCl_3$-EtOH (97:3), while 20 ml fractions were collected at a flow rate of 2 ml/min. The metabolite **6** (450 mg) was obtained from fractions 51-100, and the analytical sample was crystalized as needles from EtOAc-hexanes to give the following physical properties: mp 127-128°; ir 3360 (broad OH), 2930, 2860, 1700 (carbonyl), 1033 cm^{-1}; pmr (pyridine-D_5, $CDCl_3$, C_6D_6) see Table 1; cmr (pyridine-D_5) see Table 2; high resolution ms 275.2017 (M-CH_2OH for $C_{18}H_{27}O_2$, 100%, calcd 275.2012) and 257.1910 ($C_{18}H_{25}O$, 6.1%).

An additional 100 mg of **6** was obtained from the centrifuged solids extract by similar column chromatography, to provide a combined yield of 34%.

DISCUSSION

Microbiological transformations have been particularly useful in preparing analogs of naturally occurring antitumor compounds of widely differing structure (4). Our previous work with aphidicolin resulted in the production of numerous metabolites of the parent diterpene compound (3). Organic reactions catalyzed by microbial enzymes with aphidicolin included: hydroxylation, acetylation, oxidation of alcohols to ketone and carboxylic acid, and reduction of a ketone. Reactions were highly regiospecific, a feature common to enzymes used as reagents in organic synthesis. The major products obtained in our previous work included 3α-16,17-trihydroxyaphidicolan-18-oate (aphidicolin-18-carboxylic acid (**7**), 3-ketoaphidicolin (**2**), and 6β-hydroxyaphidicolin (**3**). In the present study, these three compounds were examined as potential substrates for microorganisms which were known to perform various metabolic transformation with aphidicolin.

Of all compounds examined, metabolites were formed in good yield only from 3-ketoaphidicolin (**2**). No metabolic transformations were observed with 6-hydroxyaphidicolin or aphidicolin-carboxylic acid. The major microbial metabolites formed were isolated by solvent extraction, purified by column chromatography or hplc, and subjected to spectral and physical analysis. Each metabolite was also evaluated by both *in vivo* and *in vitro* antitumor testing.

It became necessary to obtain larger amounts of 3-ketoaphidicolin to be used as a substrate in further biotransformation work. This compound was ultimately prepared in good yield from aphidicolin in stirred tank fermentors. While 3-ketoaphidicolin was the major metabolite formed, small amounts of 3-epiaphidicolin (**4**) were also obtained. Yields of 3-epiaphidicolin were increased by using a unique biotransformation procedure. This involved incubating aphidicolin with *C. funiculum* until maximal yields of 3-ketoaphidicolin were obtained. At this point, oxygen supplies to the biotransforming culture were shut off, and the fermetor agitation rate was reduced. Under the reducing atmosphere so established, 3-epiaphidicolin and aphidicolin yields were greatly enhanced.

IDENTIFICATION OF 6β-HYDROXY-3-KETOAPHIDICOLIN **5**.—*S. punipalus* (NRRL 3529) was formerly used to produce 6β-hydroxyaphidicolin (**3**) (3), and this microorganism produced a new metabolite in 20% yield when incubated with 3-ketoaphidicolin. The high resolution mass spectrum gave an ion of highest mass at *m/z* 321 (M^+ -CH_2OH) for $C_{19}H_{29}O_4$. Aphidicolin and its metabolites always present an ion of highest mass which results from the loss of a -CH_2OH fragment. This result confirmed the introduction of an oxygen atom into the structure of 3-ketoaphidicolin. The presence of a strong carbonyl absorption at 1681 cm^{-1} confirmed the presence of the carbonyl functional group of the starting ketone, and this was verified by cmr spectroscopy. A new one-proton signal in the pmr spectrum (Table 1) at 4.70 ppm was consistent with the introduction of a hydroxy group at a methylene position. The cmr spec-

trum contained a new doublet signal at 68.4 ppm expected for a carbinol carbon methine and confirms that hydroxylation had occurred on one of eight possible methylene carbon atoms. As with 6β-hydroxylation of aphidicolin itself (3), protons of methyl groups at the 19- and 20-positions experienced downfield shifts of 0.67 and 0.65 ppm, respectively, in the pmr spectrum, and a similar effect was observed in the cmr spectrum where signals for C-19 and C-20 were shifted downfield by 2.3 and 3.6 ppm, respectively. As mentioned in our previous work with aphidicolin, the anisotropic effect observed in the pmr spectrum and the δ-synaxial effect observed in the cmr spectrum of the metabolite support the site of hydroxylation at either position 2β- or 6β (3). The cmr spectrum of **5** is readily explained by the presence of a 6β-hydroxyl group. Downfield shifts for C-1 (1.8 ppm), C-5 (3.9 ppm), C-6 (43.2 ppm), and C-7 (10.2 ppm) and an upfield shift for C-8 (6.4 ppm) are all consistent with the site of hydroxylation as 6β, and these results are different than those expected for 2β-hydroxylation (3). In addition, this microorganism was previously shown to hydroxylate aphidicolin itself at position 6β. Thus, the structure of the metabolite formed by *S. punipalus* from 3-ketoaphidicolin is 6β-hydroxy-3-ketoaphidicolin (**5**).

IDENTIFICATION OF 19-NOR-16,17-DIHYDROXYAPHIDICOLAN-3-ONE (**6**).—*T. roseum* (UI 320) provided a 30% yield of a relatively nonpolar metabolite of 3-ketoaphidicolin. This microorganism was earlier used to achieve regioselective oxidation of the 18-position of aphidicolin to form aphidicolin-18-carboxylic acid (**7**) (3). We thought that *T. roseum* might achieve the same oxidation reaction with 3-ketoaphidicolin, resulting in the formation of an unstable β-keto carboxylic acid derivative which could undergo decarboxylation. This type of reaction sequence finds striking parallel in the pathway involved in oxidative demethylation at the 4-position of sterols like lanosterol (5) which is outlined in Figure 2.

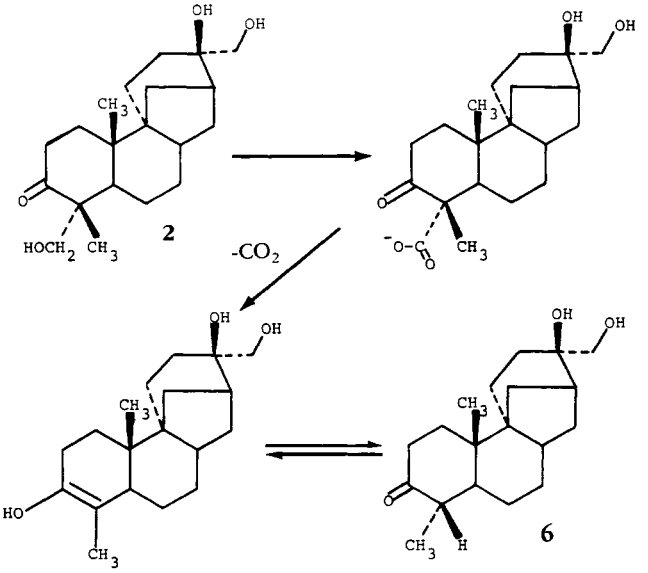

FIGURE 2. Proposed pathway for the formation of 19-*nor*-aphidicolin from 3-ketoaphidicolin (**2**) by *Trichothecium roseum*.

The new metabolite was isolated by solvent extraction and purified by column chromatography and displayed an apparent molecular ion at *m/z* 275. With aphidicolin and its metabolites, the ion of highest mass is always an M-CH_2OH fragment. Thus, the true molecular weight of the metabolite would be *m/z* 306 consistent for an empiri-

cal formula of $C_{19}H_{30}O_3$, and indicating the loss of a carbon atom from the structure of aphidicolin. The cmr spectrum revealed a total of 19 carbon atoms, the absence of the carbinol carbon at C-18, and the presence of a new methine carbon atom at 45.8 ppm assigned to the carbon at position 4 of the metabolite. In addition, one of the methyl group signals in the pmr spectrum occurred as a clear doublet, indicating that the methyl group was attached to a methine carbon atom. These data supported the structure of the metabolite as the 19-*nor*-3-ketoaphidicolin derivative (**6**).

The proposed decarboxylation mechanism would occur as shown in Figure 2, and it could lead to the formation of either a 4α- or 4β-methyl group in the metabolite. A 4α-methyl group would assume an equatorial configuration at position 4, while the corresponding 4β-isomer would exist in the less stable axial configuration. Since the methyl group occurs at an enolizable position, it would seem that the 4α-methyl isomer would predominate. The pmr spectrum of **6** provides evidence which supports the structure of the metabolite as the 4α-methyl isomer. The chemical shifts of methyl groups of **6** in $CDCl_3$ (Table 1) and 4α-methylandrostan-3-one (6) correspond closely. Comparison of the chemical shifts of the methyl group of **6** in $CDCl_3$ vs C_6D_6 reveals a downfield shift of 0.07 ppm. Axial methyl groups adjacent to carbonyl functional groups normally experience 0.2-0.3 ppm upfield shifts when examined in the two solvents mentioned (7). Thus, the structure of the metabolite formed from 3-keto-aphidicolin by *T. roseum* is assigned a **6**.

BIOLOGICAL ACTIVITY.—Each of the metabolites of 3-ketoaphidicolin was evaluated for possible antitumor activity vs. the 03C631 colon test system in the rat. None of the analogs was active in this system, while aphidicolin demonstrates clear-cut activity at the dosages examined.

An *in vitro* examination of the activities of all aphidicolins analogs was conducted using the P-388 antileukemic test system, and the results are shown in Table 3. The ED-50 dose given in micrograms per milliliter of incubate expresses the amount of compound required to inhibit the proliferation of P-388 cell growth by 50%. The most active compound in this test is aphidicolin. All other compounds are required in at least ten times the ED-50 concentration of aphidicolin to achieve a similar effect. Of the analogs examined, 3-ketoaphidicolin and aphidicolin-18-carboxylate were the next most active compounds. These results suggest that aphidicolin has highly specific structural features associated with P-388 *in vitro* activity, and that even slight structural changes such as those found in metabolites are sufficient to reduce activity.

TABLE 3. Effect of Aphidicolin and Its Metabolites on the Growth of P-388 Leukemic Cells *in vitro*

Compound	Concentration (μg/ml) Inhibiting 50% Growth of P-388 Cells (ED 30)
1	0.13
2	2.4
3	18.0
4	13.0
5	100.0
6	12.0
7	2.2

ACKNOWLEDGMENTS

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ERRATUM

T. Furuya, T. Yoshikawa, Y. Orihara, and H. Oda, *J. Nat. Prod.*, **47**, 70 (1984). The authors regret that the pages given for Literature Cited and an abbreviation in Table 3 were incorrect. The correct items are as follows:

1. T. Furuya, T. Yoshikawa, Y. Orihara, and H. Oda, *Planta Med.*, **48**, 83 (1983).
2. T. Furuya, T. Yoshikawa, T. Ishii, and K. Kajii, *Planta Med.*, **47**, 183 (1983).
3. T. Furuya, T. Yoshikawa, T. Ishii, and K. Kajii, *Planta Med.*, **47**, 200 (1983).

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