MICROBIAL TRANSFORMATIONS OF NATURAL ANTITUMOR AGENTS. 23. CONVERSION OF WITHAFERIN-A TO 12β- AND 15β-HYDROXY DERIVATIVES OF WITHAFERIN-A.

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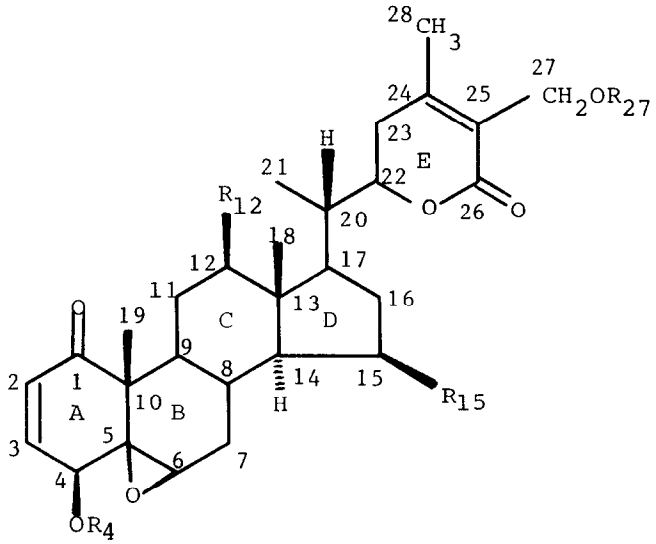
ABSTRACT

Microbial transformation experiments were conducted with the antitumor lactone withaferin-A. Cunninghamella elegans NRRL 1393 transformed withaferin-A (1a) to 15β-hydroxywithaferin-A (2a) and 12β-hydroxywithaferin-A (3a). The hydroxylated metabolites were isolated by solvent extraction and were purified by column and thin-layer chromatography. Structures of the hydroxylated metabolites were determined by proton- and carbon-13 NMR, IR and mass spectral analyses, and by the preparation of acylated derivatives. Compounds 2a and 3a inhibited the growth and biochemical functions of in vitro grown P-388 lymphocytic leukemic cells.

INTRODUCTION

The withanolides are a group of growth inhibitory steroidal lactones (1-3) of interest as potential antitumor agents. Withaferin-A (1a) is an abundant and widely occurring member of the withanolides which was isolated from Withania somnifera Dunn (4), Acnistus arborescens (5) Physalis viscosa (6), and Acnistus breviflorus Griseb.(7). The structurally related withanolide-E has been selected by the National Cancer Institute for further development as an antitumor drug (8). The demonstrated interest in this compound and other withanolides as potential antitumor agents prompted further investigations into the preparation of interesting analogs of withaferin-A by microbiological transformations. This report describes the preparation of withanolides 2a and 3a from withaferin-A by Cunninghamella elegans NRRL 1393.



Compound	R_4	R_{12}	R_{15}	R_{27}
1a	H	H	H	H
1b	$COCH_3$	H	H	$COCH_3$
2a	H	H	OH	H
2b	$COCH_3$	H	OH	$COCH_3$
2c	$COCH_3$	H	$OCOCH_3$	$COCH_3$
3a	H	OH	H	H
3b	$COCH_3$	OH	H	$COCH_3$
3c	$COCH_3$	$OCOCH_3$	H	$COCH_3$

Figure 1. The structures of withaferin-A (1a), 15β-hydroxywithaferin-A (2a), and 12β-hydroxy-withaferin-A (3a) and their respective acetates.

EXPERIMENTAL & RESULTS

Infrared spectra were taken in KBr discus in a Beckmann 4240 instrument. Uncorrected melting points were obtained in open-ended capillaries. Mass spectra were obtained on an AEI MS 902 instrument using a direct inlet probe at 200° at an electron energy of 70 ev. High resolution mass spectra and fast atom bombardment (FAB) spectra were provided through the Massachusetts Institute of Technology, Department of Chemistry, Cambridge Mass. Proton and Carbon-13 NMR spectra were measured on FX90Q Bruker (22.3 MHz) and Varian XL-200 (proton NMR at 200 MHz; carbon-13 NMR at 50.3 MHz) instruments operating in the FT mode. Tetramethylsilane was used as an internal standard in deuteriochloroform ($CDCl_3$) or dimethylsulfoxide-d_6 (DMSO-d_6) solution. Spectral assignments were generally based on comparisons with reported carbon-13 NMR assignments (6,9) for 1a and

1c; and by invoking well-known effects on chemical shift assignments for steroids (10).

Withaferin-A. Withaferin-A (1a) was isolated from the leaves of Withania somnifera Dunn. (11,12). The compound was fully characterized (melting point, proton- and carbon-13 NMR, mass spectrum, elemental analysis, and acetate derivatives) prior to use in these experiments.

Chromatography. Thin-layer chromatography was performed on non-activated silica gel (Silufol UV 254, Kavalier, CSSR) plates which were developed in ethyl acetate/acetone 2/1. Plates were visualized by spraying with a p-anisaldehyde reagent (p-anisaldehyde 0.5 g dissolved in a mixture of 60% $HClO_4$:Acetone:H_2O 1:2:8) followed by heating at 140° to yield characteristic colors for withaferin-A and derivatives. Typical mobilities and colors for each compound were: withaferin-A (Rf 0.44,pink/red), 12B-hydroxywithaferin-A (2a, Rf 0.32, blue/violet), 15B-hydroxywithaferin-A (3a, Rf 0.21, blue/gray).

Fermentation Methods. Cunninghamella elegans (NRRL 1393) was stored in sealed, cotton plugged tubes at 4° on Sabouraud Maltose agar slants. The organism was grown in a two-stage fermentation procedure (12) in a soybean meal-glucose medium of the following compositon: soybean meal 5 g, glucose 20 g, K_2HPO_4 5 g, NaCl 5 g, yeast extract 5 g, distilled water 1,000 ml. The medium was adjusted to pH 7.0 before being autoclaved at 121° for 15 minutes. Small scale fermentations were conducted in 125 ml cotton-plugged Erlenmeyer flasks holding 25 ml of medium on a rotary shaker operating at 250 rpm at 27°. Larger scale incubations were performed under the same conditions in 500 ml flasks holding 100 ml of medium. Withaferin-A substrate was added to 24-hr old second-stage cultures as a 10% solution in dimethylformamide (DMF) to a final medium concentration of 0.5 mg/ml. Samples (4 ml) of substrate-containing incubations were taken at various time intervals, extracted with 1 ml of ethyl acetate, and 30 ul of the extracts were examined for metabolites by TLC.

Conversion of Withaferin-A to 2a and 3a by C. elegans. A total of 1.0 gm of withaferin-A in 10 ml of DMF was evenly distributed into each of 20 - 500 ml flasks containing 100 ml of 24-hr old stage-two cultures, and the formation of metabolites was followed by TLC analysis. After 120 hrs, the fermentation beers were combined and exhaustively extracted with ethyl acetate. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. The resulting solids were dissolved in a minimum volume of ethyl acetate, applied to a silica gel column (12) and eluted with ethyl acetate in 10 ml fractions. Metabolites were finally purified by TLC and crystalized from ethyl acetate to yield the analytical samples of 3a (250 mg) and 2a (320 mg).

The analytical sample of 3a provided the following physical data: mp 117 - 120°; IR (cm^{-1}) 3420, 2940, 2865, 1675, 1453, 1270, 1193, 1165, 1110 and 660; mass spectrum, Fast Atom Bombardment (glycerol) m/e (% relative abundance) MH^+ 487 (51%), 469 (83), 451 (78), 433 (51); high resolution electron impact spectrum 468.2505 (17%, calculated for $C_{28}H_{36}O_6$ 468.2512), 450 (8.21), 424 (28.52), 422 (23.07), 406 (19.17), 345 (11.34), 327 (17.32), 281 (48.97), 170 (44.97), 166 (55.5), 141 (75), 124 (100) - a mass spectral fragmentation pathway is suggested in Figure 2; proton-NMR ($CDCl_3$) - presented in Table 1; carbon-13 NMR presented in Table 2.

The analytical sample of metabolite 2a provided the following

physical data: mp 270 - 274°; IR cm^{-1} 3420, 2970, 2940, 2880, 1675, 1453, 1395, 1315, 1223, 1200, 1187, 1133, 1039, 1020, 800, 610; proton NMR ($CDCl_3$) presented in Table 1; carbon-13 NMR presented in Table 2. Metabolite 2a was directly compared with an authentic sample of a compound previously identified (12) as 14α-hydroxywithaferin-A and found identical by melting point, mixture melting point, IR, proton and carbon-13 NMR, TLC and mass spectra. However, the availability of carbon-13 NMR spectral data in the present study strongly indicated that the original structural assignment for 14α-hydroxywithaferin-A was in error.

Preparation of Derivatives. Withaferin-A (1a) and metabolites 2a and 3a were acetylated using standard procedures (12). Withaferin-A provided a diacetate (1b) while metabolite 3a provided a 1:1 mixture of the diacetate 3b and triacetate 3c. Metabolite 2a provided a mixture (2.3/1) of diacetate 2b and triacetate 2c. These compounds were purified by preparative layer TLC.

Trichloroacetylcarbamoyl derivatives of withaferin-A and the metabolites were prepared directly in NMR tubes by the addition of a slight excess of trichloroacetylisocyanate (TAI, Merck Darmstadt, West Germany) (13, 14). Derivatives are formed immediately by this method and the resulting compounds were characterized only by their proton NMR spectra. Withaferin-A formed a bis-trichloroacetylcarbamoyl derivative while metabolites formed di- and triacylated compounds.

Inhibition of P388 Leukemic Cells by Withanolides. One ml of a suspension of P388 lymphocytic leukemic cells (3×10^6 cells ml^{-1}) obtained from the intraperitoneal cavity of mice (15) on the seventh day after tumor implantation was placed into each of several test tubes. After a preincubation period of 1 hr at 37°, various withanolides (100 μg in 10 μl dimethylsulfoxide) were added together with one of the following radiolabeled substrates: $8^{14}C$-adenine (0.766 μg, 13 KBq.μg^{-1}), $U^{14}C$-L-valine (1.12 μg, 5.9 KBq. μg^{-1}), $2^{14}C$-thymidine (11.2 μg, 7.14 KBq.μg^{-1}) and $U^{14}C$-uridine (0.26 μg, 25.4 KBq.μg^{-1}). Control incubations consisted of cells plus individual radiolabeled precursor. Mixtures were incubated for 1 hr at 37° and were terminated by the addition of 1 ml of 5% trichloroacetic acid. The inhibitory effects of the withanolides on the uptake of biochemical precursors were determined as previously described (15).

DISCUSSION

Microbial transformations are being utilized as a general means of preparing difficult-to-synthesize derivatives of structurally complex antitumor compounds such as withaferin-A (12, 16). An earlier study identified Cunninghamella elegans (NRRL 1393) as a microorganism capable of accumulating metabolites of withaferin-A (12). In the present study, C. elegans converted withaferin-A into a mixture of three metabolites. The least polar compound (TLC) was produced in only trace amount while two other metabolites were produced in 25% (3a) and 32% (2a) yields

Table 1. ^{1}H-NMR Spectral data for withaferin-A (1a), 15β-hydroxywithaferin-A (2a), 12β-hydroxywithaferin-A (3a) and acetylated derivatives. Spectra were obtained in deuteriochloroform at 200 MHz. Chemical shifts are indicated in ppm. Coupling constants (a - i) are given in Hz.

Proton	Multiplicity	Chemical Shifts (ppm) in Compound						
		1a	2a	2b	2c	3a	3b	3c
H(2)[a]	d	6.21	6.22	6.26	6.26	6.20	6.25	6.26
H(3)[b]	dd	6.94	6.95	7.05	7.03	6.96	7.05	7.05
H(4)[c]	d	3.77	3.77	4.67	4.72	3.78	4.70	4.67
H(6)	bs	3.23	3.27	3.25	3.24	3.26	3.25	3.25
H(12)[d]	dd	----	----	----	----	3.38	3.34	4.57
H(15)[e]	bt	----	4.21	4.21	5.01	----	----	----
H(18)	s	0.71	0.98	0.98	0.93	0.72	0.72	0.82
H(19)	s	1.42	1.45	1.42	1.44	1.42	1.40	1.39
H(21)[f]	d	1.00	1.02	1.01	1.02	1.12	1.12	0.96
H(22)[g]	m	4.42	4.41	4.41	4.36	4.61	4.60	4.43
H(23)[h]	bdd	2.50	2.50	2.57	2.52	2.49	2.52	2.49
H(27)[i]	ABq	4.36	4.37	4.88	4.89	4.36	4.88	4.88
H(28)	bs	2.03	2.04	2.08	2.07	2.03	2.07	2.06
OCOCH$_3$	s	----	----	2.06	2.04	----	2.06	2.05
"	s	----	----	2.06	2.06	----	2.07	2.06
"	s	----	----	----	2.07	----	----	2.06

[a] $J_{2,3}$=10.0; [b] $J_{3,2}$=10.0, $J_{3,4}$=6.0; [c] $J_{4,3}$=6.0; [d] $J_{12,11}$=10.8 and 4.5; [e] $J_{15,14}$= 6.2, $J_{15,16}$=6.2 and cca 0; [f] $J_{21,20}$= 6.5; [g] $J_{22,23}$=13 and 3.5, $J_{22,20}$=3.5; [h] $J_{23,23}$=17.8, $J_{23,22}$=13; [i] $J_{27,27}$=12.0.

Table 2. ^{13}C-NMR Spectral data for withaferin-A (1a), 15β-hydroxy-withaferin-A (2a), 12β-hydroxywithaferin-A (3a) and acetylated derivatives. Spectra were obtained in deuteriochloroform at 50.3 MHz.

Carbon	Chemical Shift in ppm For Compound 1a*	2a	2c	3a	3c
C(1)	202.3s	202.1s	201.2s	202.2s	201.2s
C(2)	132.3d	132.5d	133.7d	132.0d	134.0d
C(3)	142.5d	142.2d	139.8d	142.7d	140.0d
C(4)	69.8d	69.9d	72.4d	69.7d	71.9d
C(5)	63.9s	64.2s	61.0s	63.7s	61.0s
C(6)	61.7d	61.9d	60.2d	62.3d	60.4d
C(7)	29.8t	30.6t	30.2t	27.8t	27.8t
C(8)	31.1d	25.7d	25.8d	32.6d	30.8d
C(9)	44.0d	44.4d	44.4d	43.0d	42.7d
C(10)	47.8s	47.9s	48.0s	47.6s	47.8s
C(11)	21.8t	21.7t	21.3t	32.0t	28.6t
C(12)	39.2t	39.8t	40.2t	77.0d	79.1d
C(13)	42.5s	42.2s	42.7s	47.3s	46.6s
C(14)	56.0d	60.5d	57.8d	54.2d	54.4d
C(15)	24.2t	69.8d	71.7d	23.4t	23.7t
C(16)	27.2t	40.6t	38.2t	26.8t	27.5t
C(17)	51.8d	52.2d	51.9d	52.5d	52.8d
C(18)	11.6q	14.3q	13.3q	7.5q	8.0q
C(19)	17.0q	17.2q	15.9q	17.2q	15.4q
C(20)	38.7d	38.6d	38.3d	37.8d	37.9d
C(21)	13.3q	13.5q	13.5q	14.6q	14.5q
C(22)	78.7d	78.6d	77.8d	79.4d	78.4d
C(23)	29.8t	30.0t	30.1t	30.8t	30.8t
C(24)	153.5s	153.1s	156.6s	153.4s	157.0s
C(25)	125.6s	125.7s	122.0s	125.5s	121.9s
C(26)	167.0s	166.9s	165.1s	167.0s	165.1s
C(27)	57.0t	57.2t	58.7t	59.2t	58.0t
C(28)	20.0q	20.1q	20.5q	20.0q	20.8q
-O-C=O	----	----	170.0s	----	170.0s
"	----	----	170.8s	----	170.4s
"	----	----	170.9s	----	170.8s
CH_3-	----	----	20.7q	----	20.7q
"	----	----	20.8q	----	20.8q
"	----	----	21.3q	----	21.9q

*Chemical shift data for 1a was taken from Pelletier et al., (6), and assignments for carbons 12 and 16 have been corrected.

respectively.

On the basis of all physical comparisons, metabolite 2a was the same as the previously described 14α-hydroxywithaferin-A (12). The ^{13}C-NMR spectral properties of withaferin-A and other withanolides have been described recently in the literature (6, 9). The availability of this powerful spectral tool in the present study simplified assignments of the metabolite structures, and enabled a correction in the structure of metabolite 2a as 15β-hydroxywithaferin-A. In general, chemical shift assignments for withaferin-A are as reported by Pelletier et al., (16) except for signals for carbon atoms 12 and 16. These have been assigned as 39.2 ppm and 27.2 ppm respectively on the basis of comparisons of the chemical shifts of similar carbon positions on other steroids (10) and for other withanolides (9).

Identification of Metabolite 2a as 15β-hydroxywithaferin-A. Spectral data for metabolite 2a were reported earlier (12), and the structure of the metabolite was assigned as 14α-hydroxywithaferin-A. The structural assignment was based on four major criteria including the magnitude of the observed anisotropic effect of the introduced hydroxyl group on the C-18 angular methyl group protons; the low yield of a triacetate derivative which suggested that the introduced hydroxyl group was 3^{o} and in a sterically hindered location; mass spectral fragmentation patterns which indicated the presence of a hydroxyl oxygen functional group in the metabolite; and on previously noted fermentation-type reactions for Cunninghamella species. Neither the mass spectral data, nor the ^{1}H-NMR spectral data (12) could distinguish between a 14α-hydroxyl or 15β-hydroxyl substituent in the metabolite structure.

^{1}H-NMR Spectral comparisons between withaferin-A and the metabolite

and various derivatives provided new information in the present work. Major notable differences in the spectra of withaferin-A (1a) and the metabolite (2a) were in the presence of a new carbinol proton signal at 4.21 ppm in the metabolite, and in the chemical shift of the C-18 angular methyl group proton signal which occurs at 0.71 ppm in withaferin A and at 0.98 ppm in the metabolite. No equivalent downfield shift was observed for protons of the angular methyl group at position 19. Such angular methyl group deshielding effects are associated with the presence of hydroxyl groups at specific positions about the steroid nucleus (17 - 20). Only 14α- or 15β-hydroxylation of withaferin-A could cause a sufficiently large shift in the position of the C-18 methyl group proton signal (0.27 ppm). Similar arguments have been used in the location of the position of hydroxylation of Reichstein's Substance S by microorganisms (21). Other ^{1}H-NMR spectral data are listed in Table 1. This includes the clear observation of two and three acetyl-methyl group signals for di-(2b) and triacetate (2c) derivatives of 2a.

^{13}C-NMR Spectral data for withaferin-A (1a) and the metabolite (2a) and the metabolite triacetate (2c) are presented in Table 2. Chemical shifts of the metabolite would indicate that a methylene signal originally at 24.2 ppm in withaferin-A was shifted to 69.8 ppm where it occurs as a methine doublet signal. This result fixes the position of hydroxylation specifically at 15-C of withaferin-A. Support for the assignment of C-15 as the hydroxylation site is evident from the downfield shifts induced in adjacent carbon atoms 14 (4.5 ppm) and 16 (13.4 ppm) and on 18 (2.7 ppm). The upfield shift of carbon 8 (5.4 ppm) is consistent with the expected gamma effect. In addition, the combined shifts of all carbon atoms in rings C and D of the metabolite correspond very well to values expected for a 15β-hydroxylated withaferin-A derivative (10).

These findings together with the selective anisotropic deshielding effect on C-18 methyl group protons in the ^{1}H-NMR spectrum of 2a (12) allows for the identification of 2a as 15β-hydroxywithaferin-A.

Identification of Metabolite 3a as 12β-Hydroxywithaferin-A. Mass spectral fragmentation patterns for the withanolides have been described before (12). In general, withanolides provide weakly intense ions at higher mass by electron impact mass spectrometry. Fast atom bombardment mass spectrometry provided a protonated molecular ion at m/e 487 for $C_{28}H_{38}O_7$ indicating that C. elegans had introduced an oxygen atom into the structure of withaferin-A. High resolution electron impact mass spectra gave the highest mass at m/e 468.2505 for $C_{28}H_{36}O_6$ for an ion derived by loss of water from the molecular ion. Other major ions observed in the high resolution mass spectrum of the metabolite are shown in Figure 2. The presence of an additional hydroxyl group in the metabolite structure was confirmed in the mass spectrum of the triacetate derivative (3c) which gave a molecular ion of m/e 612 for $C_{34}H_{44}O_{10}$.

The ^{1}H-NMR spectrum of 3a contained all of the expected proton signals for rings A and E of withaferin-A plus their respective substituents; and for methyl groups at positions 18, 19 and 21. The presence of three hydroxyl groups in the structure of metabolite 3a was confirmed by ^{1}H-NMR analysis of the trichloroacetylcarbamoyl derivative (13, 14) which displayed three-NH signals; and by analysis of the DMSO-d_6 spectrum of 3a which revealed two secondary and one primary alcohol functional groups. The chemical shifts of angular methyl group protons were unchanged in 3a relative to withaferin-A. This lack of an anisotropic effect by a hydroxyl group on a steroid nucleus could only occur if the hydroxyl group was found at positions 7, 12, 15 or 16 (17 - 20).

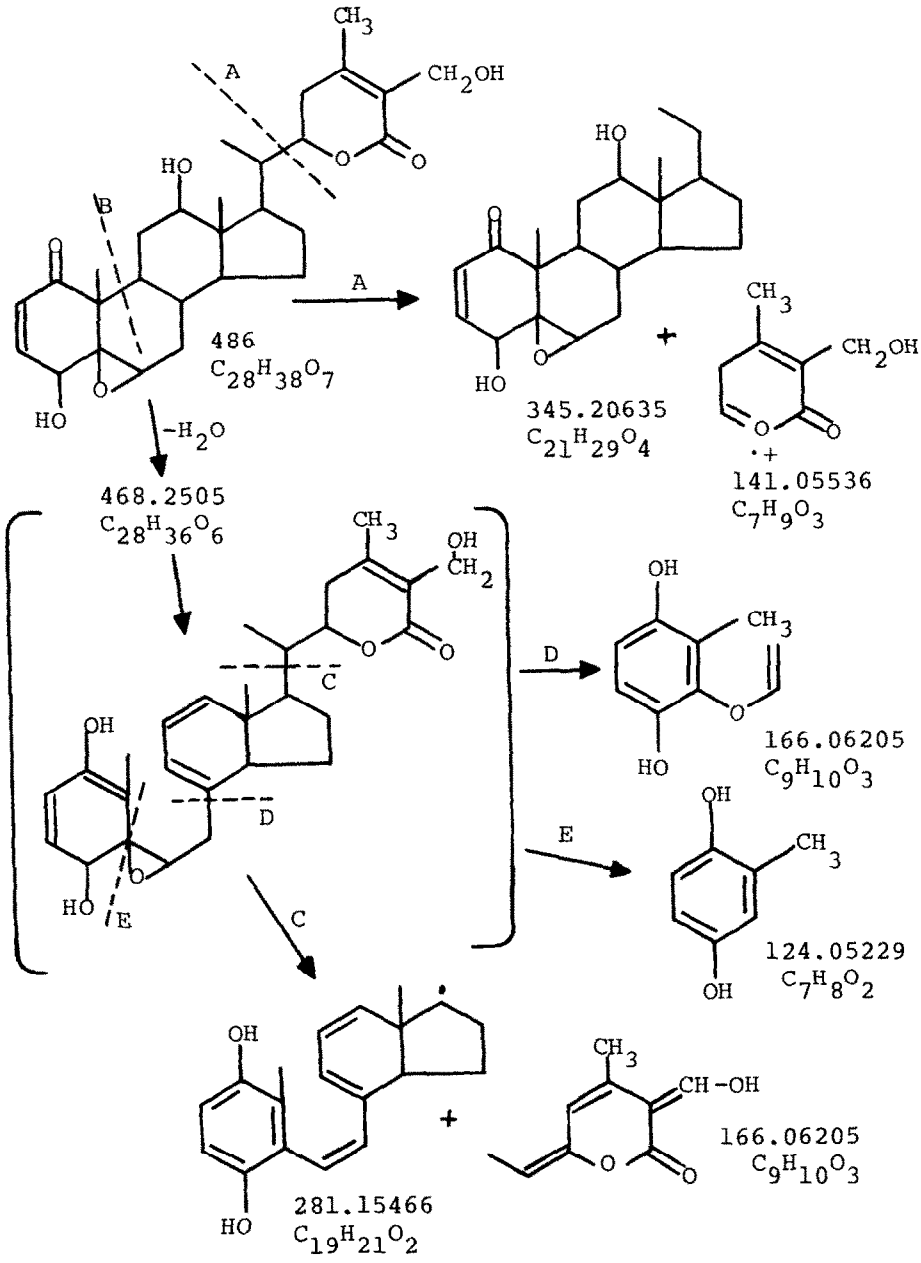


Figure 2. High resolution mass spectral fragmentation pathway for 12B-hydroxywithaferin-A (3a).

The metabolite spectrum contained a new one-proton carbinol proton signal which occurred as a doublet of doublets (J = 10.8 and 4.5) at 3.38 ppm. This indicated that the hydroxyl group was introduced into a methylene position, and the measured coupling constants would indicate that the carbinol proton is split only by two adjacent methylene protons. This situation could only occur if the hydroxyl group was located at position 12. The magnitude of the coupling constants would also suggest that the orientation of the hydroxyl group is 12-beta (18). Other proton signals of the metabolite 3a which differed from withaferin-A were at positions 21 and 22 which were deshielded by 0.12 and 0.19 ppm respectively. This observed anisotropic effect could only be explained by a 12-beta orientation of the hydroxyl group in the metabolite.

^{13}C-NMR Spectral data for 3a are presented in Table 2. An examination of the chemical shifts of all carbons in the metabolite and their multiplicities indicates that a methylene group at 39.2 ppm in withaferin-A was replaced by a methine-doublet signal at 77.0 ppm. This result clearly fixes the position of the introduced hydroxyl group at position 12. The resonance at 77.0 ppm is also in agreement with a 12-beta orientation for a hydroxy-methine functional group on a steroid nucleus (10). Other carbon signals which move considerably in the spectrum of the metabolite relative to withaferin-A are a methyl signal from 11.6 to 7.5 ppm (a gamma effect); a quaternary carbon from 42.5 ppm to 47.3 ppm (a beta effect); and the C-11 methylene resonance from 21.8 ppm to 32.0 ppm in the metabolite spectrum (a beta effect). These results are in complete agreement with the structure of 12β-hydroxywithaferin-A for metabolite 3a.

Inhibition of P388 Cells. The abilities of withaferin-A (1a),

15β-hydroxywithaferin-A (2a) and 12β-hydroxywithaferin-A (3a) to inhibit the uptake of nucleic acid and protein precursors was determined using in vitro grown P388 lymphocytic leukemia cells (15). A comparison of the inhibitory effects of these compounds is presented in Table 3.

Table 3. Effect of withaferin-A (1a), 15β-hydroxywithaferin-A (2a) and 12β-hydroxywithaferin-A (3a) on the incorporation of nucleic acid and protein precursors into P388 cells.

Precursor Added	*Percent Inhibition of Incorporation by 1a	2a	3a
8^{14}C-Adenine	78.3	65.7	72.6
U^{14}C-L-Valine	48.8	65.2	17.0
U^{14}C-Uridine	85.1	58.6	28.1
2^{14}C-Thymidine	62.4	49.9	78.7

*Inhibitory activities are all measured at 100 μg/ml of the respective withanolide.

Both metabolites retained inhibitory activity in the in vitro assay. The 15β-hydroxywithaferin-A (2a) derivative was slightly less active than withaferin-A in preventing the uptake of nucleic acid precursors and slightly more active than the parent compound in preventing valine uptake. The 12β-hydroxywithaferin-A (3a) derivative retained the ability to inhibit uptake of adenine and thymidine by P388 cells, but had little significant inhibitory activity on the uptake of uridine and valine. These differences in activities suggest that subtle structural changes may result in significant differences in the level or type of activity displayed by withanolides. Detailed studies on the relationship between the structure and cytotoxicity of withaferin-A, its metabolites and various chemical derivatives with the P388 in vitro system is the subject of a separate report.

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