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MICROBIAL TRANSFORMATION OF 2,3-DIHYDRO-3-METHOXYWITHAFERIN-A BY *CUNNINGHAMELLA ELEGANS*

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Key Word Index—*Cunninghamella elegans*; 2,3-dihydro-3-methoxywithaferin-A; 2,3-dihydro-12β-hydroxy-3-methoxywithaferin-A; biotransformation; cytotoxic effect; leukemia P388.

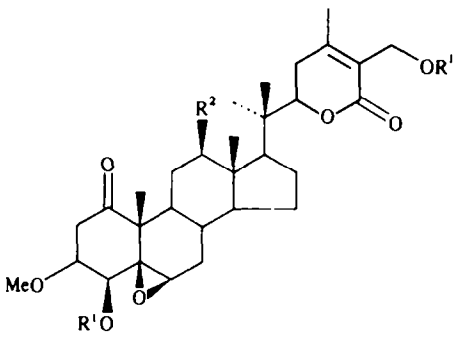
Abstract—*Cunninghamella elegans* (NRRL 1393) transformed 2,3-dihydro-3-methoxywithaferin-A to 2,3-dihydro-12β-hydroxy-3-methoxywithaferin-A. Both compounds inhibited the growth and biochemical functions of *in vitro* grown P388 lympholeukemic cells.

INTRODUCTION

Withaferin-A (**5**), the natural steroidal lactone isolated from various Solanaceae plants [1–4], was transformed with *Cunninghamella elegans* to 15β- and 12β-hydroxywithaferin-A [5, 6]. Both these substances inhibited the growth of P388 and S-180 cells with higher activity as did the parent compound **5** [5, 7]. For the cytotoxic activity the Δ^2-double bond is also important as was confirmed by the lower activity of 2,3-dihydrowithaferin-A on cells of leukemia P388 [7]. In this connection, the question arose about the products of the biotransformation of the analogue of 2,3-dihydrowithaferin-A, namely 2,3-dihydro-3-methoxywithaferin-A (**1**) and about their biological activity. This paper reports results relevant to this problem.

RESULTS AND DISCUSSION

Cunninghamella elegans transformed **1** into two metabolites, the major product was the compound **3**, the molecular formula of which differed from that of the parent compound **1** in the number of oxygen atoms only. In the mass spectrum of **3** the peak at *m/z* 500 corresponded with the fragment $[M-18]^+$. The new hydroxyl group, as deduced from ^{1}H NMR spectroscopy (Table 1), was attached to the secondary carbon, whose proton appeared as a *dd* at δ3.40, which after acetylation shifted to 4.57. It is known that the hydroxyl groups bound in the steroidal skeleton influence the position of signals of protons of the angular methyl groups H-18 and H-19 [8, 9]. In withanolides this effect can be extended also to signals of the protons H-21 and H-22. In the spectrum of 15β-hydroxywithaferin-A, when compared with that of withaferin A (**5**), there was a significant shift of H-18 (+0.27 ppm). In the spectrum of 12β-hydroxywithaferin-A, while the positions of H-18 and H-19 were practically unchanged, shifts were observed in the position of protons H-21 (+0.12 ppm) and H-22 (+0.19 ppm). Similar shifts were observed also in the spectrum of compound **3**, compared with that of spectrum



	R¹	R²
1	H	H
2	Ac	H
3	H	OH
4	Ac	OAc

1: H-18 (±0.00 ppm), H-19 (+0.03 ppm), H-21 (+0.17 ppm), H-22 (+0.22 ppm). These results indicated that the new metabolite **3** was the 2,3-dihydro-3-methoxy-12β-hydroxywithaferin-A. This presumption was confirmed by a synthetic route whereby 12β-hydroxywithaferin-A, prepared previously [6], was converted to compound **3** by addition of methanol according to the procedure described by Kupchan *et al.* [10].

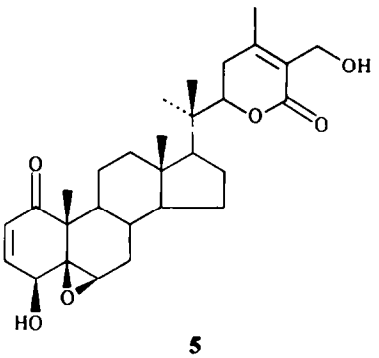
The minor metabolite of 2,3-dihydro-3-methoxywithaferin-A was not isolated in a sufficiently pure state, but we assume that this compound is 2,3-dihydro-15β-hydroxy-3-methoxywithaferin-A.

The changes in the ring A of the molecule of withaferin-A caused by the addition of methanol influenced the biotransformation of the substrate used. While *Cunninghamella elegans* transformed withaferin-A into 12β- and 15β-hydroxywithaferin-A in the ratio 2:3, the only metabolite isolated after the transformation of 2,3-

Table 1. ^{1}H NMR spectral data of compounds 1–4

Proton	Multiplicity	Chemical shifts (δ, ppm) in compounds 1	2	3	4
2a	*dd*	2.61	2.72	2.61	2.72
2b	*dd*	3.09	2.83	3.09	2.83
3	*ddd*	3.70	3.64	3.74	3.64
4	*d*	3.49	4.66	3.48	4.63
6	*s*	3.27	3.28	3.24	3.28
12	*dd*			3.40	4.57
18	*s*	0.68	0.67	0.68	0.82
19	*s*	1.29	1.28	1.32	1.30
21	*d*	0.98	0.96	1.15	0.97
22	*ddd*	4.38	4.41	4.60	4.43
23	*m*	2.49	2.52	2.49	2.48
27	*ABq*	4.08	4.83	4.06	4.81
OMe	*s*	3.33	3.41	3.35	3.41
Ac	*s*	—	2.06	—	2.04

J (Hz): 2a, 2b = 15.0; 2a, 3 = 6.0; 2b, 3 = 4.0; 3, 4 = 3.5; 12, 11 = 12.0 + 5.0; 21, 20 = 7.2; 22, 23 = 13.0 + 3.0; 23, 23 = 18.0; 22, 20 = 5.0; 27, 27 = 13.0.



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dihydro-3-methoxywithaferin-A with the same culture was 2,3-dihydro-12β-hydroxy-3-methoxywithaferin-A.

In *in vitro* experiments the new derivative 3 differed also in its cytotoxic effect from the parent compound 1. Substance 3 increased markedly the number of dead cells, their number increased with the time of cultivation (Table 2). Similar changes in biological effects were also found after the biotransformation of withaferin-A into 12β- and 15β-hydroxywithaferin-A, respectively.

EXPERIMENTAL

Substance 1, mp 241–243°, was prepared by the procedure described by Kupchan *et al.* [10]. (Found C, 68.38; H, 8.29. Calc. for $C_{28}H_{42}O_7$: C, 69.29; H, 8.42%.)

Cunninghamella elegans NRRL (1393) was cultivated on Sabouraud maltose agar and stored at 4°. In a two-stage fermentation procedure in soybean meal-glucose medium, the following composition was used (g/l): soybean meal 5, glucose 20, K_2HPO_4 5, NaCl 5, yeast extract 5, H_2O to 1 litre. The medium was adjusted to pH 7.0 before being autoclaved at 121° for 15 min. Small scale fermentation was performed in 100 ml Erlenmayer flasks holding 25 ml of medium on a rotary shaker operating at 220 rpm at 28°. Large scale fermentation was performed under the same conditions in 500 ml flasks holding 100 ml of the medium, which was inoculated with 15% of inoculum. Substrate 1, as a 10% soln in DMFA (30 mg/100 ml of the medium), was added to a 24 hr old second-stage cultivation. The formation of metabolites was followed by TLC (silica gel; $CHCl_3$–Me_2CO, 4:1), visualization by spraying with *p*-anisaldehyde reagent followed by heating to 120° [5]. After 80 hr, the fermentation liquors were combined, mycelium was filtered off and washed with 1000 ml of H_2O, which was combined with filtrate then extracted with $CHCl_3$, the extract dried over Na_2SO_4 and evaporated to dryness. The resulting solid (300 mg), dissolved in EtOAc, was separated on a silica gel column eluted with mixtures of EtOAc–Me_2CO (4:1–1:1). Metabolites were finally purified by TLC and recrystallized from $CHCl_3$–*n*-heptane (1:2). In this way there was obtained compound 3 (102 mg), mp 150° (dec.), M_r 518.65. (Found: C, 67.05; H, 8.20. $C_{29}H_{42}O_8$ requires C, 67.16; H, 8.16%.) UV λ_{max}^{MeOH} 217 mm (log ε 3.98); ^{1}H NMR (300 MHz, $CDCl_3$): see Table 1. Substance 3 was acetylated with Ac_2O–pyridine to yield triacetate 4, mp 190° (dec.), M_r 644.77. (Found: C, 65.03; H, 7.41. $C_{35}H_{48}O_{11}$ requires: C, 65.20; H, 7.50%.) ^{1}H NMR: see Table 1.

In the experiments designed for the study of the effects of the agents on the proliferation of P388 cells, the cells were resuspended in Eagle's medium supplemented with 10% of inactivated calf serum, 10 IU/ml of penicillin G (K-salt), and 100 μg/ml of streptomycin sulphate. One ml of the suspension contained *ca* 5×10^5 cells. The cells were cultivated at 37° and were counted after 24 and 48 hr of cultivation. The viable cells could be distinguished from the dead ones after staining with erythrosine soln.

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Table 2. *In vitro* inhibition of proliferation of P388 cells by 2,3-dihydro-3-methoxywithaferin-A (1) and 2,3-dihydro-12-hydroxy-3-methoxywithaferin-A (3)

Time (hr)	Number of P388 cells ($\times 10^3$) per ml: Control	1	3	
24	715 (133.7%)	642 (120.1%)	658 (123.2%)	total number of cells
	17 (2.3%)	204 (31.8%)	119 (18.0%)	number of dead cells
48	831 (155.6%)	552 (103.3%)	654 (122.4%)	total number of cells
	153 (18.4%)	283 (51.3%)	525 (80.3%)	number of dead cells

Number of cells at the beginning of experiments: $C_0 = 534 \times 10^3$/ml. Substances added in concentration 40 μg/ml.

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