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BIOSYNTHESIS OF WITHANOLIDES IN *ACNISTUS BREVIFLORUS*

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Key Word Index—*Acnistus breviflorus*; Solanaceae; withaferin A; withanolide; biosynthesis.

Abstract—Administration of [2-^{14}C]mevalonolactone to excised leaves of *Acnistus breviflorus* produced labelled withaferin A and jaborosalactone A. The former was degraded leading to the isolation of glyceric acid from C-25–C-27 of the withanolide. These carbons represented only 2% of the total radioactivity of withaferin A. The relative radioactivity of these carbons indicated that C-26 is directly derived from C-2 of mevalonolactone suggesting that the 25-pro-*R*-methyl group of cholesterol or any other sterol intermediate had been oxidized to form the lactone ring of the withanolide. The total radioactivity value found for C-25–C-27 was much lower than the expected 20% of the total value for the withanolide indicating that the side chain of the sterol precursor had been partially cleaved during the biosynthetic process.

INTRODUCTION

The withanolides are a group of C-28 steroidal lactones structurally related to withaferin A (**1**) and jaborosalactone D (**2**). Studies carried out on *Acnistus breviflorus* by Lavie [1], Bukovits and Gros [2], and Veleiro *et al.* [3] have shown that this plant contains different withanolides depending upon its origin. The plants grown in Argentina are characterized by the presence of withanolides without a hydroxyl group at C-4 (e.g. jaborosalactone A, **3**) and the absence of C-17 and C-20 hydroxylated derivatives.

Lockley *et al.* [4] reported biosynthetic experiments in *Withania somnifera* with [2-^{14}C]mevalonolactone and 24-[28-^{3}H]methylenecholesterol and proposed the latter compound as a sterol precursor of withanolides although no degradations were carried out on these compounds to determine the positions in which the label could have been incorporated. On the other hand, Vande Velde *et al.* [5] have communicated the presence in *W. coagulans* of related compounds and made the hypothesis, without confirmation, that these products could act as precursors of withanolides.

If it is assumed that cholesterol or any other related 24-alkylated sterol is in the biosynthetic pathway to withanolides, a further step would involve the oxidation of one of the prochiral methyl groups (C-26 or C-27) to a carboxyl group giving rise to the lactone ring characteristic of these compounds.

We report the investigation of the stereospecifity of this oxidation by administration of [2-^{14}C]mevalonolactone to excised leaves of *A. breviflorus* followed by chemical degradation of the labelled withaferin A which allowed the isolation and radioactivity determination of C-25–C-27 of the withanolide.

RESULTS

Leaves of *A. breviflorus* were cut and their stems immediately immersed in an aqueous solution of [2-^{14}C]mevalonolactone. The radioactive withanolides were extracted as described by Lockley *et al.* [4] and withaferin A and jaborosalactone A were isolated by prep. TLC. Further purification of the two main withanolides was accomplished independently by prep. TLC on silica gel using a mixture of ethyl acetate–hexane–*iso*-propanol (30:3:2) which we have demonstrated to be satisfactory for HPLC separation of these compounds on silica gel [6]. The radioactive withaferin A was diluted with unlabelled product and recrystallized to constant specific activity. The chemical and radiochemical purity of the undiluted withaferin A was confirmed by ^{1}H NMR spectroscopy and by prep. HPLC where the radioactivity co-eluted with the withaferin A peak. The radioactive jaborosalactone A was purified by prep. HPLC and its chemical and radiochemical purity confirmed as above.

The radioactive withaferin A was subjected to degradation as shown in Fig. 1. Treatment of **1** with methanol–sodium acetate followed by acetylation with acetic anhydride–pyridine afforded 2,3-dihydro-3-methoxy-2,27-diacetoxywithaferin A (**4b**). This was ozonized and the resulting ozonide was reduced with zinc in acetic acid. Mild alkaline hydrolysis of the reduced product yielded the β-hydroxyketone (**5**) and glyceric acid. The latter was cleaved with sodium periodate giving formaldehyde from C-27, collected as its dimedone derivative, carbon dioxide from C-26, collected as barium carbonate, and formic acid from C-25, collected as the volatile acid fraction. The specific activities of withaferin A and of its degradation products are indicated in Table 1.

DISCUSSION

From Table 1 it is evident that C-26 was 10 times more radioactive than C-27. Moreover, C-25 and C-27 had comparable radioactivity values which indicated a small amount of scrambling of the label. These results strongly support the conclusion that C-26 derives from C-2 of mevalonolactone and, considering that it has been established that this carbon is stereospecifically incorpor-

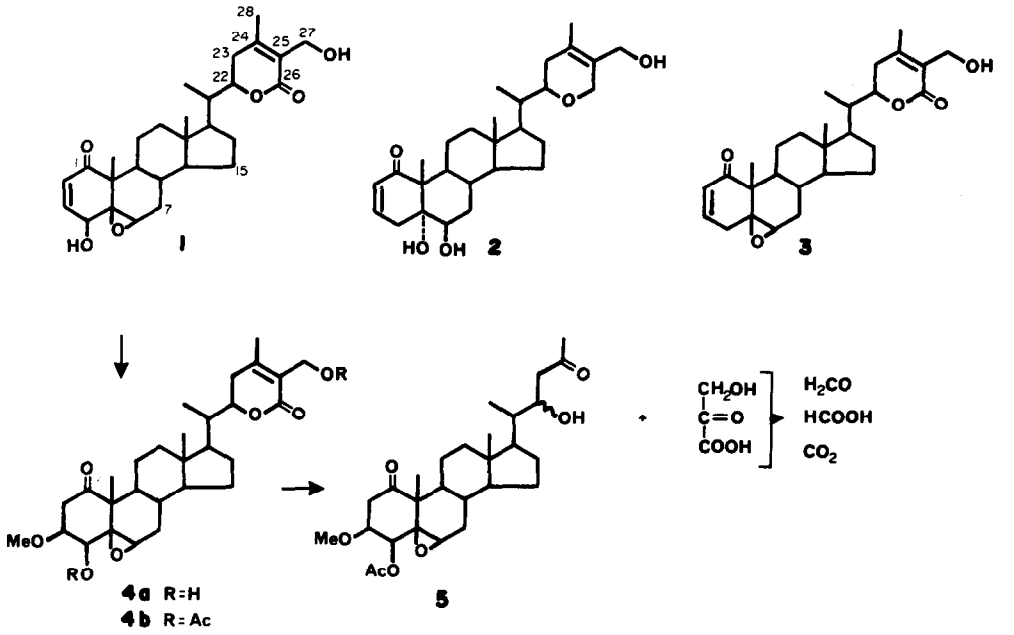

Fig. 1. Main withanolides from *A. breviflorus* (1, 2 and 3) and degradation of labelled 1.

ated into the pro-*R*-methyl (C-26) of several sterols (sitosterol, 24-methylenecholesterol, stigmasterol, α-spinasterol) [7], this methyl group appears to be the one that is oxidized to the carboxylic C-26 of the withanolides.

A further analysis of the results presented in Table 1, indicated that the amount of radioactivity in the glyceric acid was much lower than the predicted 20% of the total radioactivity of withaferin A, considering that the biosynthetic pathway from [2-^{14}C]mevalonolactone to withanolides through cholesterol would label C-1, C-7, C-15, C-22 and C-26. This suggests that there may be a major alternative route for the biosynthesis of withanolides in *A. breviflorus* in which the side chain of the sterol precursor is cleaved with loss of C-26. The point along the side chain at which this cleavage takes place still remains to be established.

Table 1. Specific activity values of withaferin A and its degradation products

Compound	Sp. act. (dpm/mmol)	Relative sp. act. (%)
Withaferin A (1)	6.20×10^6	100
2,3-Dihydro-3-methoxywithaferin A diacetate (4b)	6.18×10^6	99.7
β-Hydroxyketone (5)	6.06×10^6	97.7
CO_2 (from C-26)	9.9×10^4	1.6
H_2CO (from C-27)	1.0×10^4	0.16
HCO_2H (from C-25)	$1.3 \times 10^{4*}$	*ca* 0.20

*Estimated value from the total radioactivity of the volatile acid fraction.

Lockley *et al.* [4] reported the incorporation of 24-[28-^{3}H]methylenecholesterol into withanolides and isolated the tritium-labelled compounds suggesting that the C-28 of the precursor had been retained. However, this result should be considered with caution as the incorporation could have originated via the minor pathway with oxidation of C-26 to a carboxylic group or may even be explained by a tritium migration taking into account that no degradations were performed on the isolated withanolides to confirm that the label was still at C-28. The latter argument must be considered in view of the results obtained by the same authors with 24-[28-^{3}H]methylcholesta-5,24-dien-3β-ol where an extensive loss of tritium from the sterol was observed upon its reisolation from the plant.

The cleavage and rebuilding of a lactonic side chain is not an unusual phenomenon in the biosynthesis of steroidal cardiotonic products, for similar mechanisms are involved in the cardenolide [8, 9] and plant bufadienolide [10] biosynthetic processes where the cholesterol side chain is cleaved to a pregnane intermediate which condenses afterwards at C-20 with acetate or oxaloacetate to form the respective butenolide and bufadienolide rings.

EXPERIMENTAL

General. ^{1}H NMR and ^{13}C NMR spectra were recorded at 100 and 25.2 MHz, respectively in $CDCl_3$ with TMS as int. standard. Radioactivity was measured by liquid scintillation counting. [2-^{14}C]Mevalonolactone (53 mCi/mmol) was purchased from New England Nuclear.

Feeding of tracer and isolation of labelled withanolides. Healthy leaves of *A. breviflorus* grown from seeds in our laboratory were excised and the stems immediately immersed in an aq. soln of [2-^{14}C]mevalonolactone (143 μCi). After 3 days, the leaves were

harvested and processed for the isolation of withanolides as described elsewhere [4]. The main withanolides, withaferin A (1) and jaborosalactone A (3), were isolated by prep. TLC (silica gel, CH_2Cl_2–MeOH, 14:1) and further purified by individual prep. TLC (silica gel, EtOAc–hexane–*iso*-PrOH, 30:3:2). Labelled withaferin A (5.6 mg, 3.85×10^8 dpm/mmol; absolute incorporation 2.9%) and jaborosalactone A (4.0 mg, 5.18×10^8 dpm/mmol; absolute incorporation 2.9%) had identical properties (HPLC, ^{1}H NMR) to those from authentic standards. The labelled withaferin A (2 mg) was diluted with unlabelled material (120 mg) and recrystallized to constant sp. act. (6.20×10^6 dpm/mmol).

Degradation of withaferin A. Labelled compound 1 (40 mg, 6.20×10^6 dpm/mmol) was added to a soln of NaOAc 0.05 M in MeOH (12 ml). The reaction mixture was heated under reflux for 9 hr and evaporated to dryness. The residue was triturated with CH_2Cl_2 and filtered. Evaporation of the solvent afforded 2,3-dihydro-3-methoxywithaferin A (**4a**). Treatment with Ac_2O–pyridine (1:1, 2 ml) for 16 hr at room temp. gave 2,3-dihydro-3-methoxywithaferin A diacetate (**4b**) (48 mg, 6.18×10^6 dpm/mmol). ^{1}H NMR: δ0.68 (3H, *s*, Me-18), 0.98 (3H, *d*, J = 6.5 Hz, Me-21), 1.28 (3H, *s*, Me-19), 2.08 (3H, *s*, Me-28), 2.09 (3H, *s*, $MeCO_2$), 2.70 (2H, *m*, H-2), 3.27 (1H, *br s*, H-6), 3.41 (3H, *s*, MeO), 3.60 (1H, *m*, H-3), 4.37 (1H, *dt*, J = 12, 4 Hz, H-22), 4.63 (1H, *d*, J = 2.5 Hz, H-4), 4.87 (2H, *s*, H-27).

The above compound was dissolved in 75 ml EtOAc–CH_2Cl_2 (1:1) and O_3 was bubbled through the soln at 0° for 90 min. The solvent was evaporated and the resulting ozonide treated with Zn dust (35 mg) and 50% aq. HOAc (10 ml). After 24 hr at room temp., 0.1 M NaOH was added to give pH 8 and the mixture stirred for a further 2 hr. The soln was acidified with dil. HCl to pH 5 and extracted with CH_2Cl_2. Evaporation of the solvent afforded 5 (32 mg, 6.06×10^6 dpm/mmol). ^{1}H NMR: δ0.68 (3H, *s*, Me-18), 0.98 (3H, *d*, J = 6.5 Hz, Me-21), 1.20 (3H, *s*, Me-19), 2.09 (3H, *s*, $MeCO_2$), 2.20 (3H, *s*, MeCO), 2.46 (2H, *br d*, J = 6 Hz, H-23), 2.80 (2H, *m*, H-2), 3.28 (1H, *br s*, H-6), 3.41 (3H, *s*, MeO), 3.62 (1H, *m*, H-3), 4.20 (1H, *m*, H-22), 4.63 (1H, *d*, J = 2.5 Hz, H-4). ^{13}C NMR: δ11.7 (C-18), 12.7 (C-21), 14.1 (C-19), 20.7 (C-11), 21.1 ($\underline{Me}CO_2$), 24.3 (C-15), 27.4 (C-12), 29.3 (C-25), 30.9 (C-7), 31.2 (C-8), 39.1 (C-16), 40.6 (C-2), 42.6 (C-13), 42.7 (C-20), 43.2 (C-9), 43.5 (C-23), 51.0 (C-10), 52.9 (C-17), 56.1 (C-14), 57.1 (MeO), 58.6 (C-6), 61.4 (C-5), 68.9 (C-22), 75.4 (C-4), 76.4 (C-3), 169.5 ($MeCO_2$), 208.6 (C-1), 210.3 (C-24).

The aq. layer was freeze-dried and the residue extracted exhaustively with Me_2CO. The residue obtained after evaporation of the solvent (containing glyceric acid identified by TLC and PC) was redissolved in 3 ml 0.1 M KPi buffer (pH 6) and treated with sodium periodate (120 mg). After 48 hr at room temp., N_2 was bubbled through the soln and the evolved CO_2 was collected as $BaCO_3$ (7.6 mg, 9.90×10^4 dpm/mmol). The remaining reaction mixture was adjusted to pH 6.5 and an aq. dimedone soln was added. The dimedone formaldehyde adduct was filtered and recrystallized from aq. MeOH (13.1 mg, 9514 dpm/mmol). The aq. soln was made basic with aq. NaOH, washed with CH_2Cl_2 which was discarded, its pH adjusted to 2 and then freeze-dried. The volatile acid fraction had a total radioactivity of 1100 dpm.

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