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BIOSYNTHESIS OF WITHANOLIDES IN *ACNISTUS BREVIFLORUS*: BIOGENETIC RELATIONSHIPS AMONG THE MAIN WITHANOLIDES

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

Abstract—To excised leaves and 15-day-old seedlings of *Acnistus breviflorus* sodium [1-^{14}C]acetate, [2-^{14}C]mevalonolactone and [^{14}C-methyl]methionine were administered in separate experiments. From the absolute incorporation values of withaferin A (**1**), jaborosalactone A (**2**) and jaborosalactone D (**3**) isolated at different times after administration of the tracers, it was deduced that compound **2** is a precursor of both **1** and **3** and that the withanolides are later biodegraded to unknown products. Inoculation of [^{14}C]jaborosalactone A confirmed its transformation into **1** and **3**.

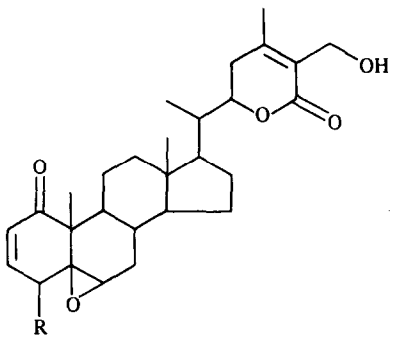
INTRODUCTION

In a previous paper [1] we have described the incorporation of [2-^{14}C]mevalonolactone into withaferin A (**1**) and jaborosalactone A (**2**) in *Acnistus breviflorus* plants. Degradation of **1** indicated that the biosynthetic pathway to these compounds in *A. breviflorus*, would involve the cleavage of the side chain of a precursor sterol [1]. It was of interest to determine the role of simple plausible precursors in the biosynthesis of these compounds as well as the biogenetic relationships among them. We report tracer studies carried out with sodium [1-^{14}C]acetate, [2-^{14}C]mevalonolactone, [^{14}C-methyl]methionine and [^{14}C]jaborosalactone A, the last obtained biosynthetically from the previous work with [2-^{14}C]mevalonolactone [1].

RESULTS

Previous results obtained by TLC analyses of seeds and seedlings of *A. breviflorus* had indicated that seeds as well as seedlings younger than 15 days were devoid of withanolides. Between 15 and 18 days the accumulation of withaferin A was observed and after day 18 jaborosalactone A and jaborosalactone D could be detected in minor amounts, considering a detection limit of about 300 ng/seedling. Therefore, comparative experiments using [2-^{14}C]mevalonolactone and [^{14}C-methyl]methionine were carried out on 15-day-old seedlings and on freshly cut leaves from adult plants as previously described [1]. When [2-^{14}C]mevalonolactone was administered to 15-day-old seedlings through the watering solution and the withanolides were isolated by preparative HPLC [2] after 72 hr, the absolute incorporation into withaferin A was between 3.4 and 3.9% while incorporations into jaborosalactone A and jaborosalactone D (**3**) were *ca* ten times smaller (Table 1). However, when the seedlings were left for a total of 144 hr after the administration of the tracer, only 2.5% absolute incorporation was observed in **1** and concomitantly a smaller incorporation was also observed in **2** and **3**.

Experiments carried out in isolated leaves fed with [2-^{14}C]mevalonolactone through the stems, indicated an initial rapid incorporation into jaborosalactone A (3.2% in 17 hr) followed by a slower incorporation into withaferin A (8.1% in 24 hr) (Table 2).



1 R = OH
2 R = H

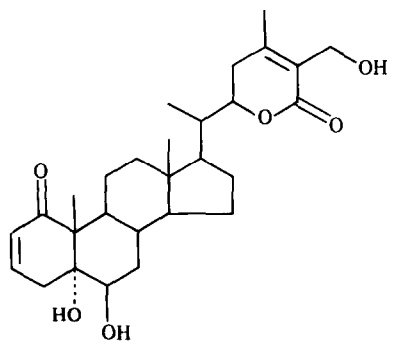


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Table 1. Tracer experiments in 15-day-old seedlings of *A. breviflorus*

Precursor	Amount fed (μg/plant)	Time (hr)	Withaferin A sp. act. (mCi/mmole)	Withaferin A abs. inc. (%)	Jaborosalactone A sp. act. (mCi/mmole)	Jaborosalactone A abs. inc. (%)	Jaborosalactone D sp. act. (mCi/mmole)	Jaborosalactone D abs. inc. (%)
[2-^{14}C]Mevalonolactone*	14	72	1.83	3.9	0.25	0.3	0.39	0.4
	14	144	0.66	2.1	0.14	0.2	0.20	0.3
[^{14}C-methyl]Methionine	26.5	48	0.09	0.03	0.05	0.01	0.14	0.03

*4.7 mCi/mmole.

Table 2. Tracer experiments in isolated leaves of *A. breviflorus*

Precursor	Amount fed (μg/leaf)	Time (hr)	Withaferin A sp. act. (mCi/mmole)	Withaferin A abs. inc. (%)	Jaborosalactone A sp. act. (mCi/mmole)	Jaborosalactone A abs. inc. (%)	Jaborosalactone D sp. act. (mCi/mmole)	Jaborosalactone D abs. inc. (%)
[2-^{14}C]Mevalonolactone*	27	17	3×10^{-2}	0.8	92×10^{-2}	3.2	—	—
	26.7	24	13×10^{-2}	8.1	64×10^{-2}	3.3	—	—
	26.3	48	14×10^{-2}	8.1	37×10^{-2}	3.5	—	—
[^{14}C-methyl]Methionine	20.5	15	17×10^{-3}	0.05	43×10^{-3}	0.09	—	—
	21.6	24	2×10^{-2}	0.11	27×10^{-3}	0.11	—	—
	16.5	48	3×10^{-2}	0.21	38×10^{-3}	0.20	—	0.05
Sodium [1-^{14}C]acetate	2100	24	14×10^{-4}	0.04	14×10^{-3}	0.52	—	—
	20000	48	59×10^{-4}	0.23	16×10^{-3}	0.67	—	0.05

*53 mCi/mmole.

Sodium [1-^{14}C]acetate was a much poorer precursor of withanolides in terms of absolute incorporation (Table 2) when administered to *A. breviflorus* leaves. The same was observed with [^{14}C-methyl]methionine both in seedlings and in isolated leaves (Tables 1 and 2).

Finally, [^{14}C]jaborosalactone A, obtained biosynthetically from [2-^{14}C]mevalonolactone [1], was fed to isolated leaves and to 15-day-old seedlings. The radioactive withanolides were isolated as above and purified by preparative HPLC. The absolute incorporations obtained are summarized in Table 3.

DISCUSSION

A comparison of the results obtained at different times after the administration of [2-^{14}C]mevalonolactone or [^{14}C-methyl]methionine to *A. breviflorus* leaves (Table 2) suggests that jaborosalactone A is biosynthesized prior to withaferin A. The lower absolute incorporations into withanolides in 15-day-old seedlings after 6 days when compared to those obtained after 3 days are indicative of a conversion of jaborosalactone A, jaborosalactone D and withaferin A into other compounds. The decrease of the specific activity of withaferin A by a factor of three may be explained as a dilution taking place as labelled withaferin A is consumed and replaced by unlabelled material once the administered [2-^{14}C]mevalonolactone has been consumed. Thus the mentioned withanolides cannot be considered as final products but rather as intermediates being converted to other withanolides and/or metabolized to non-withanolide compounds.

The role of jaborosalactone A as a plausible precursor of withaferin A and jaborosalactone D was confirmed by the feeding experiments summarized in Table 3. The fact that only 9% of the administered radioactivity was present in the mixture containing the main withanolides from isolated leaves and 6.5% in that from seedlings is in agreement with the above results that indicate an active metabolism of these compounds to non-withanolide components. In this experiment no jaborosalactone D was detected in the seedlings.

The previous results may be summarized as follows: jaborosalactone A would be produced initially and, in seedlings, does not accumulate until the 18th day being transformed in the meantime into withaferin A by hydroxylation at C-4. In isolated leaves, the administered [^{14}C]jaborosalactone A is diluted with endogenous jaborosalactone A and converted into withaferin A by

Table 3. Incorporation of [^{14}C]jaborosalactone A into withanolides by *A. breviflorus*

	Time (hr)	Absolute incorporation (%) Withaferin A	Jaborosalactone A	Jaborosalactone D
Leaves	24	2.0	5.6	1.4
Seedlings	72	3.1	3.4	—

hydroxylation at C-4 and into jaborosalactone D by hydrolytic opening of the epoxide ring.

Glotter *et al.* [3] have proposed the hypothesis that a 4-deoxy-5,6-unsaturated withanolide should be the precursor of 4-deoxy-5,6-epoxy withanolides as jaborosalactone A and, by a different pathway, of 4-hydroxy-5,6-epoxy withanolides such as withaferin A. This proposal was made at the time when it was believed that, except for *Physalis peruviana*, the plants which produced withanolides with the 4-hydroxy-5,6-epoxy system did not have withanolides of the type 4-deoxy-5,6-epoxy (jaborosalactone A). A similar pathway concerning the formation of withanolides in *Withania somnifera* has been recently proposed [4].

As in *A. breviflorus* we have found both types of withanolides, *i.e.* possessing 4-deoxy and 4-hydroxy-5,6-epoxy functions [5–7], and taking into account the present results, the proposed split pathway no longer holds, at least in the case of the mentioned plant where jaborosalactone A acts as a precursor of both jaborosalactone D and withaferin A. The hypothesis that a 4-deoxy-5,6-unsaturated compound acts as a precursor of jaborosalactone A cannot be discarded by these results.

The lower absolute incorporations obtained with sodium [1-^{14}C]acetate and with [^{14}C-methyl]methionine (see Tables 1 and 2) may be explained considering that these precursors are involved in a large number of biosynthetic pathways many of which are probably more active than those leading to the withanolides. The higher incorporation of [^{14}C-methyl]methionine into withanolides in isolated leaves when compared to 15-day-old seedlings indicates, as expected, a highly active metabolism of the precursor during the growing stage of the plant, probably involving protein synthesis. The incorporation of methionine into withanolides could involve the formation of a C_{28}-sterol that retains the C-28 methyl group upon conversion to the withanolides.

EXPERIMENTAL

Plant material and radiochemicals. Acnistus breviflorus seeds from Tucumán, Argentina were sterilized by immersion in 1% $Ca(ClO)_2$ soln for 2 min and germinated in Petri dishes containing a saline soln [8] in a growth chamber equipped with Sylvania Gro-Lux fluorescent lamps and forced air circulation. The temperature inside the chamber was kept constant at 25° with 12 hr photoperiods. Adult *A. breviflorus* plants were grown in soil in a greenhouse. [^{14}C-methyl]Methionine (56.5 mCi/mmole) and [2-^{14}C]-(3*RS*)-mevalonolactone (53 mCi/mmole) were supplied by Amersham International Ltd. and were diluted with unlabelled material as required. Sodium [1-^{14}C]acetate (0.05 mCi/mmole) was obtained from the Comisión Nacional de Energía Atómica, Argentina. [^{14}C]Jaborosalactone (5.2 $\times 10^8$ dpm/mmole) was obtained biosynthetically from [2-^{14}C]mevalonolactone as previously described [1]. Radioactivity was measured by liquid scintillation counting.

Feeding of tracers and isolation of labelled withanolides. Ten 15-day-old seedlings were immersed in an aqueous soln of the tracer (0.3 ml) as indicated in Table 1. Healthy leaves of the plants were excised and the stems immediately immersed in an aq. soln of the tracer (0.3 ml/leaf) as indicated in Table 2. After the appropriate time, the seedlings and the leaves were harvested, extracted with Et_2O at room temp for 24 hr and the residue obtained after filtration and evaporation of the solvent was fractionated by HPLC on silica gel as described elsewhere [2]. The fractions containing the withanolides were collected and assayed for radioactivity.

Feeding of [^{14}C]*jaborosalactone A and isolation of labelled withanolides.* [^{14}C]Jaborosalactone A (9.0×10^4 dpm) was suspended in water (0.25 ml) containing two drops of Tween 20 and the mixture was sonicated until a homogeneous suspension was obtained. Two freshly cut leaves were immersed in the suspension and harvested after 24 hr. Extraction and isolation of the withanolides were performed as above.

Ten 15-day-old seedlings were immersed in water (200 μl) and suspension of labelled jaborosalactone A (20 μl) was added. After 1 and 3 days seedlings were harvested and processed as above.

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