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Biosynthesis of acridone alkaloids

Formation of rutacridone by cell-free extracts of *Ruta graveolens* cell suspension cultures

Walter Maier, Brigitte Schumann and Detlef Gröger

Institute of Plant Biochemistry, Academy of Sciences of the GDR, DDR-4050 Halle (Saale), Weinberg 3, GDR

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Microsomes prepared either by ultracentrifugation or $MgCl_2$ precipitation from cultured *Ruta graveolens* cells catalyze the condensation of 1,3-dihydroxy-*N*-methylacridone and isopentenylpyrophosphate or dimethylallylpyrophosphate. In the presence of NADPH and oxygen rutacridone was identified as reaction product. By omission of NADPH glycocitrine-II is accumulated. The results suggest that at first a prenylated acridone is formed which in turn is cyclized giving the dihydrofuran part of rutacridone.

Microsome; Acridone biosynthesis; Dihydrofuran ring formation; (*Ruta graveolens*)

1. INTRODUCTION

Acridone alkaloids are found in about 20 genera of the Rutaceae family of higher plants. The anthranilic acid-derived acridones and furoquinolines occur frequently concomitantly in various rutaceous species. A number of furoacridones including rutacridone (**7**) has been found in *Ruta graveolens* L. Their possible biosynthetic relationship was recently discussed [1]. Cell-free extracts of *R. graveolens* cell suspension cultures are capable to synthesize **1** from *N*-methylanthranilic acid and malonyl-CoA [2,3]. **1** is apparently the key intermediate in the pathway leading to more complex acridones e.g. furo- and pyranoacridones. Radioactive **7** could be isolated after feeding of ^{14}C-labelled mevalonic acid to *R. graveolens* cell suspension cultures [4]. These results did not show unambiguously the origin of the isopropylidenedihydrofuran part of **7**. Here we now report on the prenylation of **1** and cyclization leading to rutacridone by cell-free preparations of *R. graveolens* cell suspension cultures.

2. MATERIALS AND METHODS

2.1. *Plant cell culture and enzyme preparation*

Cell suspension cultures of an alkaloid-producing *R. graveolens* cell line (R-20) were grown for 8–10 days in the dark at 27°C and then harvested as described [5].

Correspondence address: D. Gröger, Institute of Plant Biochemistry, Academy of Sciences of the GDR, DDR-4050 Halle (Saale), Weinberg 3, GDR

Four g of lyophilized cells were intensively ground in a mortar with dry ice and 90 ml Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 2 mM dithioerythritol and 10% glycerol. The homogenate was centrifuged at 20000 × *g* for 30 min. The supernatant (crude enzyme extract) was removed and subjected to highspeed centrifugation (1 h, 200000 × *g*). The pellet (microsomal fraction) was resuspended in Tris-HCl buffer (pH 7.8) and gently homogenized at 0°C.

Alternatively a microsomal fraction was prepared by Mg^{2+} precipitation [6] with some modifications: magnesium chloride (1.2 mM dissolved in water) was slowly added under stirring to the crude enzyme extract to reach a 180 mM final concentration.

2.2. *Incubations and analytical procedures*

The assay system contained in 0.5 ml total volume: 300 μg protein (microsomal pellet) or 500 μg protein (crude enzyme extract), 0.13 μmol 1,3-dihydroxy-*N*-methylacridone, 2 μmol NADPH, 0.5 μmol $MnSO_4 \cdot H_2O$, 25 μl [1-^{14}C]isopentenylpyrophosphate (IPP) solution (1.4 × 10^5 dpm) = Assay A or 10 μl [1-^{3}H]dimethylallylpyrophosphate (DMAPP) solution (1.32 nmol = 10000 dpm) = Assay B and 1 mM Tris-HCl buffer (pH 7.8) ad 0.5 ml.

Incubations were carried out at 36°C for 1 h. The reaction was stopped by adding 200 μg rutacridone in 2 ml ethanol. After extraction with $CHCl_3$, the alkaloid fraction was chromatographed on silica gel PF_{254} plates using the following solvent systems (by vol.): I, toluene/ethyl acetate/NH_4OH solution (25%) (40:10:1) and subsequently rechromatographed in II, $CHCl_3$/MeOH (95:5) and III, *n*-hexane/ether (1:1). After elution of the alkaloid an aliquot was used for quantitation by measuring the extinction at 400 nm and another aliquot was used to count the radioactivity.

Protein concentrations were determined according to Bradford [7].

2.3. *Product identification*

To identify the labelled acridones extracts of several assay mixtures were combined and chromatographed in solvent systems I (R_F for rutacridone = 0.65; R_F for glycocitrine-II = 0.24), II (R_F for rutacridone 0.89; R_F for glycocitrine-II 0.46) and III (R_F for **7** = 0.3; R_F for **4** = 0.33). The radioactive zones were recorded with a thin-layer scanner (Berthold, Wildbad).

For further identification of the enzyme reaction product 15 assay A mixtures were combined and diluted with 3 mg **7**. The alkaloid

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fraction was chromatographed in solvent system I and rechromatographed in II and III. After the last TLC again 10.4 mg rutacridone were added and three times crystallized from ethanol.

2.4. *Chemicals*

NADPH was obtained from Boehringer, Mannheim and [1-^{14}C]isopentenylpyrophosphate (IPP) from Amersham. [1-^{3}H]Dimethylallylpyrophosphate was a gift from Dr R. Welle, Freiburg. Rutacridone and 1,3-dihydroxy-*N*-methylacridone synthesized according to Hughes and Ritchie [8] were kindly provided by Dr A. Baumert, Halle, Saale. Glycocitrine-II was a gift from Professor H. Furukawa, Tempaku, Nagoya.

3. RESULTS AND DISCUSSION

Recently [2,3] we have identified 1,3-dihydroxy-*N*-methylacridone as key intermediate in the pathway leading to more complex acridones. After incubation of a crude extract of *R. graveolens* suspension cultures with **1** and [1-^{14}C]isopentenylpyrophosphate in the presence of NADPH and Mn^{2+}, a radioactive product was detected by TLC and radioscanning which was identical with **7** in three different solvent systems. Subsequently we found that microsomes prepared from *R. graveolens* cells catalyzed the prenylation of **1** and the subsequent cyclization forming a dihydrofuran ring. No qualitative difference in catalytic activity was detectable between microsomes isolated by $MgCl_2$ precipitation and those isolated by centrifugation. For routine experiments we used microsomal fractions obtained by ultracentrifugation. The extent of rutacridone formation after incubation for 60 min was proportional to the protein concentration up to at least 0.4 mg of the microsomal fraction. The supernatant from the microsomal pellet did not catalyze this reaction. To demonstrate unequivocally that cell-free extracts of *R. graveolens* tissue cultures catalyze the formation of rutacridone, the reaction product of a larger incubation (assay A) was purified to constant specific radioactivity (Table I). The formation of rutacridone required molecular oxygen and was dependent on NADPH and Mn^{2+} ions (Table II). Maximum enzyme activity was obtained only in the presence of Mn^{2+} and could be replaced partially by Mg^{2+}. By omission of **1** in the assay A mixture formation of **7** at a reduced rate was observed. We assume that **1** is present in trace amounts in microsomes. So far we could definitely isolate rutacridone from microsomes.

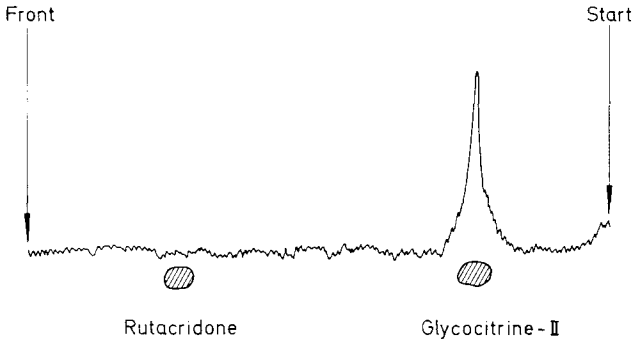

Fig. 1. Radioscan of TLC on silica gel with solvent system I from an incubation mixture of assay A without NADPH.

Table I

Purification of the enzymatically formed acridone alkaloid by microsomes of *R. graveolens* cells

Treatment[a]	Radioactivity in rutacridone (dpm/mmol)
(a) TLC in solvent I	1.95×10^7
(b) TLC in solvent II	1.97×10^7
(c) TLC in solvent III	1.96×10^7
(d) After TLC separation of (c) and dilution with 10.4 mg **7** and crystallization	3.75×10^6
(e) 2nd crystallization	3.85×10^6
(f) 3rd crystallization	3.73×10^6

[a] The extract of 15 assay A mixtures was diluted with 3 mg rutacridone

Table II

Enzymatic synthesis of rutacridone using various incubation mixtures by microsomal preparations of *R. graveolens* cells

Assay A (using microsomes)	Radioactivity in rutacridone (dpm/mg)
Complete	8.6×10^4
Minus NADPH	4.8×10^3
Minus Mg^{2+}	3.1×10^3
Minus 1,3-dihydroxy-*N*-methylacridone	1.9×10^4
Boiled enzyme	2.9×10^3
Removal of oxygen[a]	4.1×10^3

[a] Assay A plus 20 μmol glucose, 46 U glucose oxidase and 50 U catalase

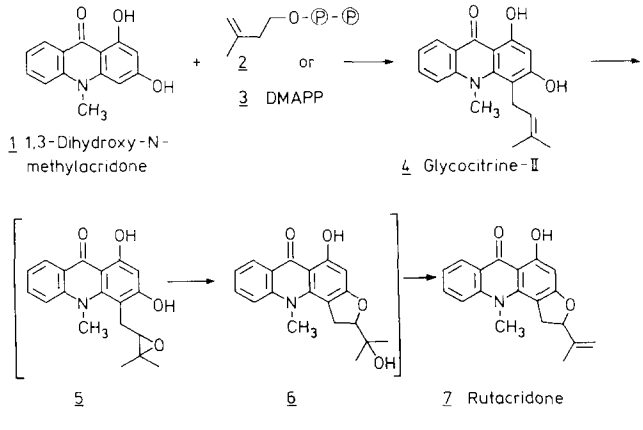

Fig. 2. Hypothetical reaction sequence for conversion of 1,3-dihydroxy-*N*-methylacridone into rutacridone by *Ruta graveolens* microsomes.

As substrate dimethylallylpyrophosphate was also used. About 10% of the radioactivity of [1-^{3}H]DMAPP was incorporated into **7**. By omission of NADPH in the assay A mixture another main compound was accumulated which comigrated (Fig. 1) with glycocitrine-II. Due to scarcity of material an unequivocal proof of the proposed structure is still lacking. Interestingly, **4** has been isolated from the root and stem bark of *Glycosmis citrifolia* [9] but has not yet been found in *R. graveolens*.

The first monoprenyl:aryl transferase in higher plants was found in *R. graveolens* [10]. This particle associated enzyme catalyzes the first step in furanocoumarin biosynthesis viz. the conversion of umbelliferon to demethylsuberosine. The cyclization of the latter compound is mediated in *Ammi visnaga* by a microsomal marmesin synthase [11]. This step is a cytochrome-P_{450}-dependent process. Based on our results and analogous to the biosynthesis of furanocoumarins the formation of dihydrofuranoacridones may be depicted as shown in Fig. 2. Compounds **5** and **6** are hypothetical intermediates.

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