Synthesis of 1,3-dihydroxy-*N*-methylacridone by cell-free extracts of *Ruta graveolens* cell suspension cultures

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Cell-free extracts of *Ruta graveolens* cell suspension cultures obtained after $(NH_4)_2SO_4$ precipitation and gel filtration on Sephadex G-25 catalyze the condensation of *N*-methylanthranilic acid and malonyl-CoA in the presence of ATP. The reaction product has been identified as 1,3-dihydroxy-*N*-methylacridone. This alkaloid **6** is apparently the key intermediate in the pathway leading to more complex acridone alkaloids.

Ruta graveolens *Cell-free extract* *Biosynthesis* *1,3-Dihydroxy-*N*-methylacridone*

1. INTRODUCTION

The acridone alkaloids which are yellow in colour comprise a group of secondary metabolites found solely in the Rutaceae family of higher plants. Robinson [1] has postulated that acridones are biosynthetically derived from anthranilic acid and a polyketo-acid. Radiolabelled anthranilic acid [2,3] and *N*-methylanthranilic acid [3,4] were incorporated in various acridone alkaloids. Feeding experiments using [^{13}C]acetate provided conclusive evidence that a polyketide is involved in acridone alkaloid biosynthesis [5]. An *N*-methyltransferase catalyzing the formation of *N*-methylanthranilic acid (**1**) was found in cell suspension cultures of *Ruta graveolens* L. [6]. Furthermore ATP-dependent activation of **1** was demonstrated in *Ruta* cells [7]. Here we report for the first time the cell-free synthesis of an acridone alkaloid.

2. MATERIALS AND METHODS

2.1. *Plant cell culture and enzyme preparation*

Cell suspension cultures of an acridone alkaloid-producing *R. graveolens* cell line were grown for days and then harvested as described [6]. 2 g of lyophilized cells were intensively ground in a mortar with dry ice in the presence of 1 g Polyclar AT and subsequently suspended in 50 ml Tris-HCl buffer (pH 7.5) [7]. The homogenate was centrifuged at 15000 × *g* for 30 min. The supernatant was brought to 80% saturation by the addition of solid $(NH_4)_2SO_4$ whilst stirring, and was then centrifuged at 15000 × *g*. The sediment was suspended in 5 ml buffer (pH 7.5) and chromatographed on a Sephadex G-25 column. Elution was performed with Tris-HCl buffer (pH 7.5) and the protein fractions used for enzyme assays.

2.2. *Assay for acridone synthetase*

Standard assay conditions (table 1) were as follows: assay A contained in a total volume of 0.5 ml: 20 nmol [2-^{14}C]malonyl-CoA (2.55×10^5 dpm) (**3**), 0.5 μmol *N*-methylanthranilic acid, 2.5 μmol ATP, 2.5 μmol $MgCl_2$, 1 mg protein and 50 μmol Tris-HCl buffer (pH 7.5). Assay B contained in 0.5 ml: 0.1 μmol *N*-methyl[*carboxyl*-^{14}C]-anthranilic acid (1.76×10^6 dpm), 20 nmol malonyl-CoA, 2.5 μmol ATP, 2.5 μmol $MgCl_2$, 1 mg protein, 50 μmol Tris-HCl buffer (pH 7.5). Incubations were carried out at 32°C for 2 h. The reaction was stopped by adding 100 μg 1,3-dihydroxy-*N*-methylacridone (**6**) in 1 ml

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Table 1

Enzymatic synthesis of 1,3-dihydroxy-*N*-methylacridone using various incubation mixtures by cell-free extracts of *R. graveolens* cells

Assay and radioactive substrate	Assay conditions	Radioactivity incorporated alkaloid (dpm)
Assay A: [2-^{14}C]-malonyl-CoA	complete	11470
	minus *N*-methyl-anthranilic acid	300
	minus ATP	310
	boiled enzyme	420
Assay B: *N*-methyl-[*carboxyl*-^{14}C]-anthranilic acid	complete	13450
	minus malonyl-CoA	770
	minus ATP	450
	boiled enzymes	400

ethanol. After extraction with $CHCl_3$, the alkaloid fraction was chromatographed on silica gel PF_{254} plates, which were prepared with 0.5% KOH solution using solvent system I, benzene/ethyl acetate (6:4). After elution of the alkaloid zone an aliquot was used for quantitation by measuring the extinction at 400 nm and another aliquot was used to measure the radioactivity by scintillation spectrometry (Tricarb, Packard Instruments).

2.3. *Product identification*

To identify the labelled acridone the extracts of several assay mixtures were combined and chromatographed in different solvent systems: I; II, chloroform/ethanol (8:2) and III, toluene/ethyl acetate/formic acid (85%) (5:4:1). The radioactive zones were recorded with a thin layer scanner (Berthold, Wildbad).

For further identification of the enzyme reaction product 10 assay A mixtures were collected, combined and diluted with 10 mg non-labelled **6**. The alkaloid fraction was chromatographed in solvent system I and rechromatographed in II and III. After the last thin-layer chromatography (TLC) again 10 mg 1,3-dihydroxy-*N*-methylacridone were added and twice crystallized from ethanol.

In another approach the alkaloid fractions of 10 assay B mixtures were chromatographically (solvent system I) purified and diluted with 20 mg 1,3-dihydroxy-*N*-methylacridone. The alkaloid was dissolved in a mixture of dry pyridine/acetic anhydride (1:1) and heated at 110°C for 4 h. Three substances were separated on an alkaline TLC PF_{254} plate using solvent IV, benzene/ethyl acetate (8:2). After elution the substances were separately chromatographed in V, chloroform/ethyl acetate/acetone (8:1:1) and identified as **6**, 1-hydroxy-3-acetoxy-*N*-methylacridone and 1,3-diacetoxy-*N*-methylacridone.

2.4. *Chemicals*

ATP was obtained from Boehringer, Mannheim, and [2-^{14}C]malonyl-CoA from Amersham. Unlabelled malonyl-CoA was from Serva, Heidelberg.

Compounds prepared as substrate and as reference material were identified by m.p., UV, IR and mass spectrometry (MS). *N*-Methyl-[*carboxyl*-^{14}C]anthranilic acid was prepared as described in [4]. 1,3-Dihydroxy-*N*-methylacridone was synthesized according to Hughes and Ritchie [8]. The acetylation of the latter compound was performed by a standard procedure [9,10].

3. RESULTS AND DISCUSSION

Recently [7] we have shown the ATP-dependent activation of *N*-methylanthranilic acid in *Ruta* cells which proceeds presumably via the corresponding acyl adenylate. During the course of acridone biosynthesis activated **1** should react with malonyl-CoA. After incubation of a partially purified enzyme preparation of *R. graveolens* suspension cultures with [2-^{14}C]malonyl-CoA and *N*-methylanthranilic acid a radioactive product was detected by TLC and radioscanning which was identical with 1,3-dihydroxy-*N*-methylacridone (**6**) in 3 different solvent systems. To demonstrate unequivocally that cell-free extracts of *R. graveolens* tissue cultures catalyze the formation of the acridone alkaloid **6** the reaction product of a larger incubation (assay A) was purified to constant specific radioactivity (table 2). For further characterization of the enzymatically synthesized alkaloid a larger incubation (assay B) was diluted with unlabelled **6** and acetylated. Three compounds could be identified as: (a) 1-hydroxy-3-acetoxy-*N*-methylacridone, (b) 1,3-diacetoxy-*N*-

Table 2

Purification of the enzymatically formed acridone alkaloid by cell-free extracts of *R. graveolens* cells

Treatment[a]	Radioactivity in 1,3-dihydroxy-*N*-methyl-acridone (dpm/mmol)
(a) TLC in solvent I	2.36×10^6
(b) TLC in solvent II	2.42×10^6
(c) TLC in solvent III	2.29×10^6
(d) After TLC separation of (c) and dilution with 10 mg **6**	8.29×10^5
(e) 1st crystallization	8.38×10^5
(f) 2nd crystallization	8.30×10^5

[a] The extract of 10 assay A mixtures was diluted with 10 mg 1,3-dihydroxy-*N*-methylacridone

methylacridone and (c) starting material **6**, which gave the following values of specific radioactivity: (a) 4.95×10^5, (b) 5.30×10^5, and (c) 4.94×10^5 dpm/mmol. 1,3-Dihydroxy-*N*-methylacridone and the corresponding acetylated derivatives showed the same specific radioactivity. Additional evidence for the specific incorporation of *N*-methylanthranilic acid and malonyl-CoA into ring A and ring C, respectively, is presented in table 1. No alkaloid was detected when *N*-methylanthranilic acid was omitted from the assay A mixture and malonyl-CoA from assay B mixture, respectively. Under our assay conditions the reaction was strongly dependent on the presence of ATP. Obviously this is due to the fact that **2** has to be synthesized in situ. When **2** becomes available the requirements of cofactors for the condensation of **2** and **3** should be further investigated.

Based on the above and our previous work [5–7] using cell cultures of *R. graveolens* we are now able to formulate the biosynthesis of an acridone alkaloid as depicted in fig.1. The enzymatically catalyzed primary condensation product between *N*-methylanthranilic acid and malonyl-CoA proved to be 1,3-dihydroxy-*N*-methylacridone. This alkaloid **6** may be regarded as the key intermediate in the pathway which leads to the structurally more complex acridone alkaloids.

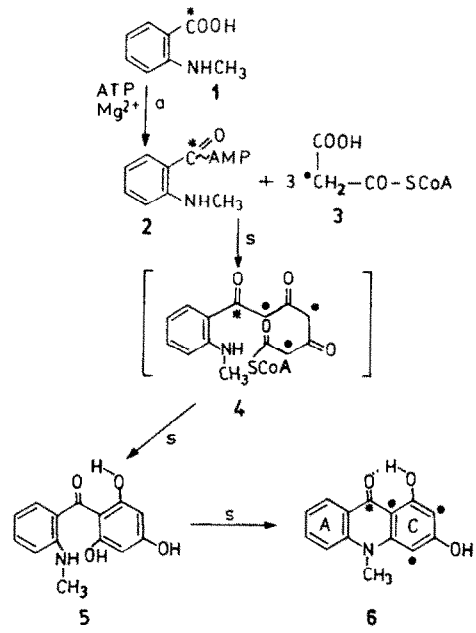

Fig.1. Scheme for the enzymatic synthesis of an acridone alkaloid from malonyl-CoA and *N*-methylanthranilic acid. (a) Reaction catalyzed by an *N*-methylanthranilic acid activating enzyme, (s) reaction catalyzed by an 'acridone synthetase'.

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