Biosynthesis of Acridone Alkaloids. A Cell-Free System from *Ruta graveolens* Cell Suspension Cultures

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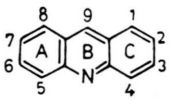
Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Ruta graveolens, Suspension Cultures, Cell-Free System, Acridone Biosynthesis, 1,3-Dihydroxy-N-methylacridone

A cell-free extract of *Ruta graveolens* cell suspension cultures, strain R-20, was prepared which synthesizes 1,3-dihydroxy-N-methylacridone from N-methylanthranilic acid and malonyl-coenzyme A. The reaction is strongly dependent on the presence of ATP. After adding of coenzyme A to the assay mixtures a second compound X2 is accumulated to a considerable extent. The structure of this substance has not yet been clarified. Apparently X2 is a precursor of 1,3-dihydroxy-N-methylacridone, the key intermediate in the pathway leading to more complex acridones.

Introduction

Acridone alkaloids are very weak bases which are yellow in colour. It is noteworthy that the first naturally occurring derivatives of acridine (**1**) were isolated after the Second World War in 1948 [1]. At present about 70 members of this particular alkaloid type are known. They have been found exclusively in twenty genera of the three main sub-families of the Rutaceae, namely the Rutoideae, Toddaliodeae and Aurantioideae.



1 Acridine

Recently acridones have been detected for the first time in the genus *Citrus* [2] and also the first isolation of a "binary" acridone alkaloid containing a C-C linkage from natural sources was reported [3].

Robinson [4] has postulated that acridones are biosynthetically derived from anthranilic acid and acetate *via* a polyketo-acid (Fig. 1). As potential intermediates in the biogenetic pathway aminobenzophenones have been discussed [5]. It is of special interest that aminobenzophenones are chemically easily converted into acridones [6], and that the co-occurrence of an N-methylaminobenzophenone and the corresponding acridone in the same plant was recorded (Fig. 2) [7]. The specific incorporation of anthranilic acid and N-methylanthranilic acid into various acridone alkaloids is well documented [8–11]. Feeding experiments using [^{13}C]acetate provided conclusive evidence that Ring C of rutacridone is acetate-derived [12].

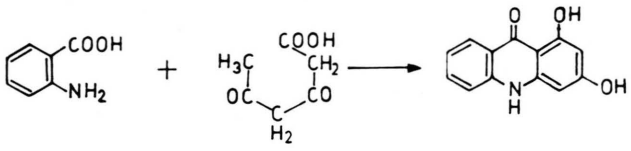


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Fig. 1. Biosynthesis of acridone alkaloids according to Robinson.

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Fig. 2. Constituents of *Teclea verdoorniana* and *Oricia suaveolens*.

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Some enzyme activities involved in acridone alkaloid biosynthesis were recently discovered [13, 14] using cell suspension cultures of Ruta graveolens as enzyme source. In this paper we wish to describe further attempts to elucidate the suggested pathway of acridone formation at the cell-free level.

Experimental

Plant material

The mainly rutacridone producing cell culture, strain R-20, of the common rue (*Ruta graveolens* L.) was employed throughout these studies. A modified medium of Murashige and Skoog (MS) [15] which did not contain plant growth hormones was used for cultivation. It was composed of MS inorganic medium supplemented with sucrose (30.0 g/l), meso-inositol (80.0 mg/l), nicotinic acid (0.5 mg/l), thiamine · HCl (0.1 mg/l), pyridoxine · HCl (0.1 mg/l), Ca-panthotenate (5 mg/l) and was designated as M20 medium. Cell suspension cultures of cell line R-20 were propagated in the dark at 27 °C on rotary shakers at 80 rpm. in 250-ml erlenmeyer flasks containing 40 ml medium. After 10 d of cultivation the cells were harvested by filtration washed with distilled water, frozen at −25 °C and subsequently lyophilized.

Buffer

For enzyme preparation and in the assay mixtures a 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5 mM EDTA, 0.5 mM glutathion (reduced), 2 mM mercaptoethanol and 10% glycerol was used.

Chemicals

[^{14}COOH]Anthranilic acid and N-methyl-[^{14}COOH]anthranilic acid (7.93 mCi/mmol) were prepared according to [16] and [11], respectively.

1,3-Dihydroxy-N-methylacridone (**12**) was synthesized according to Hughes and Ritchie [17], mp 285–290 °C, UV in ethanol [λ_{max}, nm (log ε)] 242 (sh. 4.89), 249 (5.02), 255 (5.11), 262 (5.19), 268 (5.14), 295 (4.18), 318 (3.87), 394 (3.81).

MS: *m/e* 241 (M^+), 226, 213, 212, 198, 184, 170.

1-Hydroxy-3-methoxy-N-methylacridone (**14**) was prepared synthetically [17] or isolated from Ruta graveolens callus cultures, strain R-4, mp 173–175 °C, UV in ethanol [λ_{max}, nm (log ε)] 242 (sh. 4.18), 249 (5.02), 255 (5.11), 262 (5.19), 268 (5.14), 295 (4.18), 318 (3.87), 394 (3.81).

MS: *m/e* 255 (M^+), 226, 212, 197, 182.

The above-mentioned acridones were acetylated according to Rastogi *et al.* [18] or [19]. 25 mg **12** in 2 ml of a mixture of dry pyridine/acetic anhydride (1:1) were heated at 110 °C for 4 h. Three substances were separated on TLC plates PF_{254} (Merck) using solvent system I, benzene/ethyl acetate (8:2). For further purification the substances were separately chromatographed in solvent II, chloroform/ethyl acetate/acetone (8:1:1). It could be identified: **12** R_f 0.29 solvent I, R_f 0.37 solvent II; 1-hydroxy-3-acetoxy-N-methylacridone, R_f 0.49 solvent I, R_f 0.69 solvent II, m.p. 210–211 °C.

MS: *m/e* 283 (M^+), 255, 241, 226, 213, 198, 184;

1,3-diacetoxy-N-methylacridone, R_f 0,25 solvent I, R_f 0.54 solvent II, m.p. 196–198 °C.

MS: *m/e* 325 (M^+), 297, 283, 255, 241, 226, 213, 212, 184.

Acetylation of **14** afforded 1-Acetoxy-3-methoxy-N-methylacridone. Both compounds were separated by TLC in solvent I and solvent III, benzene/ethyl acetate (9:1). The latter was identified by m.p. 217–219 °C.

MS: *m/e* 297 (M^+), 255, 226, 197.

Acridones substituted in 1-position showed a strong blue fluorescence under UV light.

[2-^{14}C]Malonyl-coenzyme A (58 mCi/mmol) was provided by Amersham. Unlabelled malonyl-CoA and coenzyme A came from Serva Heidelberg. ATP was obtained from Boehringer, Mannheim.

Preparation of cell-free extracts

All operations were carried out at 4 °C. Lyophilized cells (2 g) 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. The homogenate was centrifuged at 15000 × *g* for 30 min. Alternatively the Polyclar AT was omitted and the supernatant after centrifugation was stirred with 1 g Dowex 1 × 4 ion exchange resin equilibrated in the above Tris-HCl buffer. The resin was stirred for 20 min and filtered off. The enzyme-containing solution obtained either after the treatment with Polyclar AT or Dowex was brought to 80% saturation by addition of solid $(NH_4)_2SO_4$ under stirring and was then centrifuged at 15000 × *g*. The sediment was suspended in 5 ml buffer and

chromatographed on a Sephadex G-25 column. Elution was performed with Tris-HCl buffer pH 7.5 and the protein fraction used for enzyme assays.

Enzyme assay

Standard assay conditions were as follows: Assay A contained in a total volume of 0.5 ml: 20 nmol [2-^{14}C]malonyl-coenzyme A (2.55×10^5 dpm), 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). Some assays were performed in the presence of 0.25 μmol coenzyme A.

Assay B contained in 0.5 ml: 0.1 μmol N-methyl-[^{14}COOH]-anthranilic acid (1.76×10^6 dpm), 20 nmol malonyl-coenzyme A, 2.5 μmol ATP, 2.5 μmol $MgCl_2$, 1 mg protein, 50 μmol/Tris-HCl buffer (pH 7.5). Some assay mixtures contained 0.25 μmol coenzyme A. The reaction was stopped by adding of 1 ml ethanol and by heating the mixture in a water-bath.

Incubations were carried out at 32 °C for 2 h.

Analytical procedures

After termination of the enzyme reaction 0.1 mg 1,3-dihydroxy-N-methylacridone or in some cases a mixture of each 0.1 mg **12** and **14** was added to the assay mixture. The alkaloid fraction was extracted with $CHCl_3$ and chromatographed on silica gel PF_{254} (Merck, Darmstadt) plates, which were prepared with 0.5% KOH solution using solvent IV, 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 applied for counting radioactivity by scintillation spectrometry (Tricarb, Packard Instruments). Furthermore, the labelled alkaloids were separated by TLC using solvent V, chloroform/ethanol (8:2), and solvent VI, toluene/ethyl acetate/formic acid (85%) (5:4:1). The radioactive zones were also recorded with a thinlayer scanner (Berthold, Wildbad).

HPLC analysis was performed using a Milichrom OB4 apparatus (Academy of Sciences USSR) according to Baram *et al.* [20], fitted with a Silica RP 18 (5 μm) column (2 × 62 mm) (Serva, Heidelberg). The elution was performed with CH_3OH/H_2O (75:25, v/v) flow-rate: 100 μl · min^{-1}, detection at 270 nm.

Protein concentrations were determined according to Bradford [21] using bovine serum albumin as standard.

Results

For these studies *Ruta graveolens* cell-line R-20 was used which accumulates as main alkaloid rutacridone. Crude cell-free extracts of lyophilized cells were incubated with malonyl-CoA and N-methylanthranilic acid in the presence of ATP. Compounds interfering with the assay could be removed either by addition of Polyclar AT or by treatment of the protein solution with Dowex 1. At the beginning we added to the assay mixtures coenzyme A assuming the activation of **7** proceeds via the corresponding CoA ester [22]. Under these conditions we observed two radioactive products which were designated as compound X1 and compound X2 (Fig. 3) (Table I).

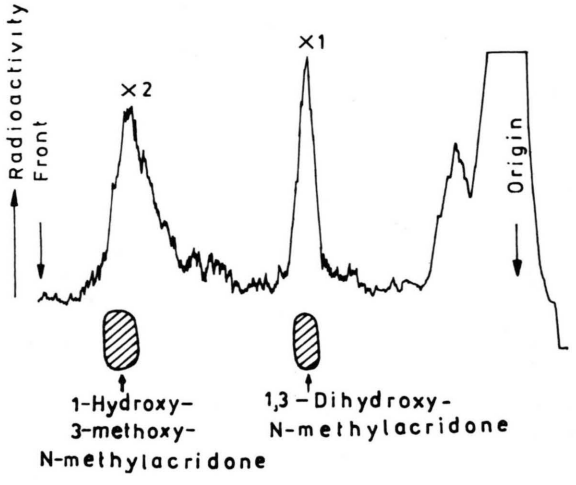

Fig. 3. Metabolism of [2-^{14}C]malonyl-CoA by cell-free extracts of *Ruta graveolens*. TLchromatogram of assay mixture A (10-fold) containing coenzyme A. Development in solvent system IV, benzene/ethyl acetate (6:4).

Table I. Incorporation of labelled putative acridone precursors into various compounds by cell-free extracts of *Ruta graveolens* cells.

Assay conditions	Radioactivity* [dpm] incorporated into compound X1**	compound X2
Assay A	8520	930
Assay A + CoA	7380	7450
Assay B	13000	955
Assay B + CoA	8440	13600

* Radioactivity of control experiments were deducted.
** Compound X1 has been identified as 1,3-dihydroxy-N-methylacridone.

Identical results were achieved either with labelled malonyl-CoA (assay A) or with labelled **7** (assay B).

This is the evidence that both precursors (**7** and **9**) are incorporated into the compounds X1 and X2. Compound X1 was eluted and cochromatographed with 1,3-dihydroxy-N-methyl-acridone (**12**) in 3 different solvent systems. Compound X1 had in all chromatographic systems (IV–VI) the same R_f value as **12**. Subsequently compound X1, obtained after a larger incubation (assay A, 10 fold), was diluted with **12** as carrier, rechromatographed in three different solvent systems and recrystallized twice from different solvents. Constant specific radioactivity was reached by this procedure. For further characterization enzymatically formed compound X1 using assay B was diluted with 20 mg **12** and acetylated by heating in a mixture of dry pyridine/acetic anhydride. Besides **12,** two acetylated products were obtained, namely 1-hydroxy-3-acetoxy-N-methylacridone and 1,3-diacetoxy-N-methylacridone. These three products showed the same specific radioactivity [23]. Now we were concerned about the identification of compound X2. In all TLC solvent systems so far tested X2 exhibited the same or nearly the same R_f value (Fig. 3) as 1-hydroxy-3-methoxy-N-methylacridone. During cocrystallization of compound X2 with **14** as carrier most of the radioactivity was lost. Furthermore X2 was acetylated giving only one radioactive product, which was not identical with 1-acetoxy-3-methoxy-N-methylacridone (Fig. 4).

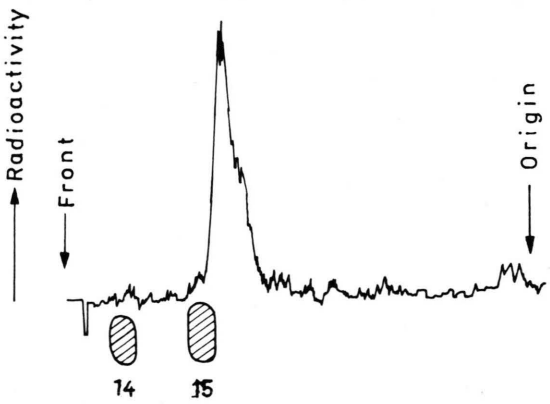

Fig. 4. Distribution of radioactivity after acetylation of compound X2. Reference substances: **14** = 1-Hydroxy-3-methoxy-N-methylacridone, **15** = 1-acetoxy-3-methoxy-N-methylacridone, silicagel PF_{254} plate, solvent system VI, benzene/ethyl acetate (8:2).

In another approach a purified extract of assay mixture A was analyzed by HPLC (Fig. 5). Under these conditions X1 and X2 have the same retention time as **12** and **14**, respectively.

1-Hydroxy-N-methylacridone was eluted after 5.4 min and is therefore not identical with compound X2. Despite the fact that compound X2 cochromatographs with **14** by HPLC analysis, its structure has not yet been elucidated. Substantial amounts of compound X2 were only accumulated in the presence of coenzyme A (Table II). In a given concentration of 250 nmol CoA per assay the relation between X1 and X2 varied considerably in different experiments. Later on we could demonstrate that the activation of **7** is not dependent on coenzyme A but rather pro-

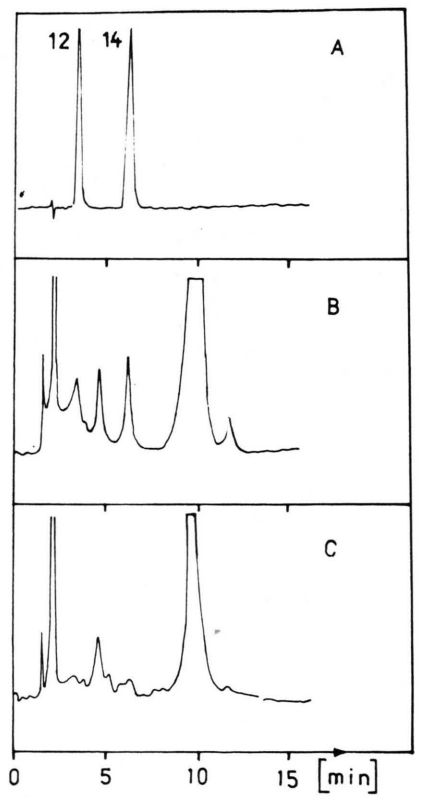

Fig. 5. HPLC diagram at 270 nm of a purified extract of ten assay mixtures A, containing coenzyme A. The chloroform extract was evaporated to dryness and the residue dissolved in 1 ml ethanol. 4 µl of this extract were injected. A, reference substances: **12** = 1,3-hydroxy-3-methoxy-N-methylacridone (r_t = 3.41 min, k' = 1.16), **14** = 1-hydroxy-3-methoxy-N-methylacridone (r_t = 6.17 min, k' = 2.89); B, extract of assay mixture A; C, control experiment: standard incubation mixture as in (B), but without malonylcoenzyme A.

Table II. The effect of CoA concentrations in various incubation mixtures on product formation by cell-free extracts of *R. graveolens* cells, strain R-20.

Assay conditions	Radioactivity* [dpm] incorporated into compound X1	compound X2
Assay A	n.d.	1250
plus 25 nmol CoA	13650	1420
plus 50 nmol CoA	12810	1365
plus 100 nmol CoA	7310	2320
plus 250 nmol CoA	8380	3830
Assay B	11500	50
plus 25 nmol CoA	14240	1550
plus 50 nmol CoA	12350	2725
plus 100 nmol CoA	9350	3720
plus 250 nmol CoA	9650	7000

n.d. = not determined.
* = Radioactivity of control experiments were deducted.

ceeds via the corresponding acyladenylate or acylphosphate. In a number of incubations CoA was therefore omitted from the assay mixtures. It was shown that N-methylanthranilic acid and malonyl-CoA label ring A and ring C, respectively, of 1,3-dihydroxy-N-methylacridone [23].

Discussion

For the first time a cell-free system capable of synthesizing an acridone alkaloid has been obtained from *Ruta* cell cultures. The overall reaction must involve several enzymes. The strong dependence of the reaction on ATP is obviously due to the fact that **7** has to be "activated", probably as adenylate.

The first condensation product in acridone biosynthesis is 1,3-dihydroxy-N-methylacridone (**12**) which

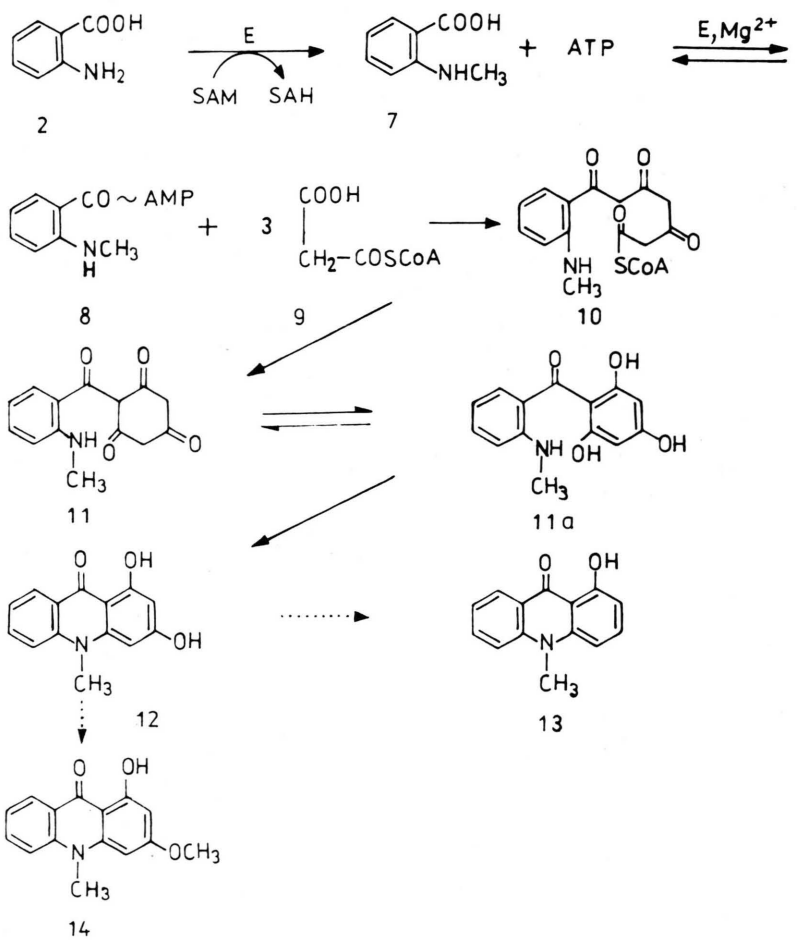

Fig. 6. Proposed biosynthetic pathway leading from anthranilic acid *via* "activated" N-methylanthranilic acid and malonyl-CoA to acridone alkaloids. E = enzyme, SAM = S-adenosyl-methionine, SAH = S-adenosyl-L-homocysteine.

serves apparently as key intermediate for the multitude of acridones. Surprisingly, **12** has not yet been detected in Nature. This means **12** is converted under *in vivo* conditions *in situ* to another more or less complex acridone. Still puzzling is the occurrence of compound X2.

This substance is only a minor compound under normal assay conditions but is accumulated to a remarkable extent in the presence of coenzyme A. According to our present knowledge X2 is not identical with **13** or **14**, which are rather simple transformation products of **12** and which are both found in *Ruta* cell cultures. It is tempting to speculate that compound X2 might be a precursor of 1,3-dihydroxy-N-methylacridone, presumably the corresponding aminobenzophenone (**11a**). Compound **11a** has neither been synthesized nor was it found in Nature. To test this hypothesis the conversion of compound X2 into an acridone alkaloid by chemical and enzymatic methods is currently investigated. Summarizing the data presented above and previous results we propose the reaction sequence of acridone biosynthesis starting from anthranilic acid as it is depicted in Fig. 6.

Acknowledgement

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