Formation of 1,3-Dihydroxy-N-methylacridone from N-Methylanthraniloyl-CoA and Malonyl-CoA by Cell-Free Extracts of *Ruta graveolens*

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N-Methylanthraniloyl-CoA was synthesized *via* N-succinimidyl N-methylanthranilate and subsequent transesterification with CoA-SH. This compound was characterized by LSIMS and NMR data. An enzyme preparation from cell suspension cultures of *Ruta graveolens* catalyzed the formation of 1,3-dihydroxy-N-methylacridone from N-methylanthraniloyl-CoA and malonyl-CoA with a pH optimum of 7.5.

Introduction

Cell-free extracts of *Ruta graveolens* cell suspension cultures catalyze the condensation of N-methylanthranilic acid and malonyl-CoA in the presence of ATP and Mg^{2+} [1, 2]. After addition of CoA-SH to the incubation mixture an inhibitory effect on alkaloid formation was observed as was previously described for the chalcone synthase reaction [3, 4]. It may be assumed that the incorporation of N-methylanthranilic acid proceeds *via* the corresponding CoA thiol ester. Activation of N-methylanthranilic acid in the presence of hydroxylamine was described earlier but the exact mechanism of this reaction has not been clarified *e.g.* a specific N-methylanthranilate: CoA ligase was not detected [5].

In this communication we report for the first time a chemical synthesis of N-methylanthraniloyl-CoA (**1**) and its role as primer molecule for the formation of 1,3-dihydroxy-N-methylacridone.

Materials and Methods

Mass spectra were recorded on an AMD 402; 70 eV EIMS and LSIMS experiments were performed. – ^{1}H NMR: Bruker AC 300, calibration according to ref. [6].

Abbreviations: EIMS, Electron impact mass spectrometry; LSIMS, Liquid secondary ion mass spectrometry.

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Chemicals

Coenzyme A, free acid was obtained from Boehringer, Mannheim. Malonyl-CoA came from Serva, Heidelberg. [2-^{14}C]Malonyl-CoA was from Amersham-Buchler, Braunschweig. All other chemicals were of analytical grade.

Preparation of N-succinimidyl N-methylanthranilate

N-methylanthranilic acid (10 mmol, 1.5 g) and N-hydroxysuccinimide (10 mmol, 1.2 g) were dissolved in 100 ml absolute $CHCl_3$. After addition of dicyclohexyl carbodiimide (11 mmol, 2.5 g) the mixture was kept at room temperature for 21 h with stirring. The dicyclohexylurea was then filtered off and the organic layer evaporated *in vacuo*. The residue was dissolved in 50 ml ethylacetate and the solution extracted 3-times with saturated 1 M sodium bicarbonate solution and 3-times with water. The organic phase was dried (Na_2SO_4), filtered, and evaporated *in vacuo*. The residue was dissolved in a few ml $CHCl_3$, and the ester purified by column chromatography (silica gel, 20 × 400 mm solvent; $CHCl_3$). Fractions containing the ester were pooled, concentrated *in vacuo* and the ester crystallized by addition of light petrol as yellow needles, m.p. 151–154 °C in 80% yield. MS (70 eV): *m/z* 248 (17%, M^+), 134 (100, M-succinimidyl), 116 (13), 106 (9), 91 (9), 77 (14).

Preparation of N-methylanthraniloyl coenzyme A (**1**)

All steps were carried out under N_2 atmosphere in the dark at 4 °C. N-Succinimidyl N-methylanthranilate (248 mg) was dissolved in 30 ml acetone

and the solution diluted with 30 ml distilled water. Subsequently CoA (200 mg) and $NaHCO_3$ (212 mg) were added and the mixture stirred for 24 h. The yellow mixture was acidified with 2 M formic acid, the acetone removed *in vacuo*, and the aqueous phase exhaustively extracted with ethylacetate. The aqueous phase was poured on an ion-exchange column (DEAE-Sepharose, 20 × 160 mm, equilibrated with 0.1 M HCOOH). The column was washed with 0.1 M HCOOH until no UV-absorbing material was detectable.

The CoA ester was eluted with a gradient by mixing 0.1 M formic acid and 2 M sodium formiate pH 3.5 and 10 ml fractions were collected. The fractions containing the thioester (No. 20–26) were collected and desalted by passage through a column of Dowex 50 WX4 (H^+, 20 × 180 mm). The eluate was lyophilized yielding 126 mg (57%) of N-methylanthraniloyl-CoA. The product was identified by MS, NMR, the hydroxamate test and alkaline hydrolysis. MS (LSIMS): *m/z* 901 (23%, $[M + H^+]$, 508 (11), 428 (7), 394 (19), 282 (5), 167 (7), 136 (45), 134 (100, $CH_3-NH-C_6H_4-\overset{+}{C}=O$); ^1H NMR 300 MHz, D_2O: δ 0.81 s, 3H (CH_3); 0.93 s, 3H (CH_3); 2.48 t ($J = 6.3$ Hz), 2H (CH_2); 3.05 s, 3H ($N-CH_3$); 3.22 t (6.3), 2H (CH_2); *ca.* 3.48, m overlapped, 4H (2 × CH_2); 3.62 dd (9.9/4.1), 1H (CH); 3.86 dd (9.8/4.5), 1H (CH); 4.30 br s, 2H (CH_2); 4.60 br s 1H (CH); 6.15 d (5.7), 1H (CH); 7.42 d (7.8, 1H (H-3); 7.43 t (7.8), 1H (H-5); 7.72 t (7.8), 1H (H-4); 8.10 d (7.8), 1H (H-6); 8.37 s, 1H (CH); 8.63 s, 1H (CH).

Preparation of enzyme extracts

The cultivation of the acridone alkaloid-producing *R. graveolens* cell line R-20 has been described earlier [2]. The crude enzyme was prepared according to [1, 2]. General procedure: Lyophilized cells (7.0 g) were thoroughly ground in a mortar with dry ice in the presence of 1 g Polyclar AT and subsequently suspended in 70 ml 0.1 M Tris-HCl buffer pH 7.5 (unless otherwise stated) containing 0.5 mM EDTA, 2 M mercaptoethanol and 10% glycerol. The homogenate was centrifuged at 15000 × *g* for 30 min and the supernatant used for enzyme assay.

Enzyme assays

Assay A contained in a total volume of 0.5 ml: 20 nmol [2-^{14}C]malonyl-coenzyme A (7.33 KBq), 50 nmol N-methylanthraniloyl-CoA, 1 mg protein and 300 µl Tris × HCl buffer pH 7.5 (unless otherwise stated).

Assay B contained in a total volume of 0.5 ml: 20 nmol [2-^{14}C]malonyl-coenzyme A (7.3 KBq), 0.5 µmol N-methylanthranilic acid, 2.5 µmol ATP, 2.5 µmol $MgCl_2$, 1 mg protein and 300 µl Tris-HCl buffer pH 7.5.

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

Analytical procedures

Isolation and identification of the enzyme reaction product (1,3-dihydroxy-N-methylacridone) were performed as described [2].

Protein concentrations were determined according to Bradford [7] using bovine serum albumin as standard.

Results and Discussion

It has been postulated [8], that the incorporation of anthranilic acid and/or N-methyl-anthranilic acid into acridone alkaloids may proceed *via* the corresponding CoA thiol esters as the activated acyl moiety. In order to test this hypothesis we decided to prepare the CoA-derivative of N-methylanthranilic acid (**1**). Recently, 2-aminobenzoyl-CoA was obtained by enzymatic synthesis using a coenzyme A ligase from a *Pseudomonas* strain, but the attempted chemical synthesis of this compound was not successful [6].

Various methods are known for the chemical synthesis of acyl-CoA thioesters [9]. In our hands the N-succinimidyl ester of N-methylanthranilic acid proved to be especially useful as activated intermediate to prepare N-methylanthraniloyl-CoA. The crystallized succinimidyl-derivative was obtained in good yield and was used for a transacylation step with CoA-SH giving the desired thioester.

N-Methylanthraniloyl-CoA was identified by the hydroxamate assay, alkaline hydrolysis and, definitely by LSIMS and ^1H NMR. In the MS especially indicative were the fragments at *m/z* 901 $(M + H)^+$ and *m/z* 134 ($CH_3-NH-C_6H_4-\overset{+}{C}=O$). The ^1H NMR spectrum of N-methylanthraniloyl-CoA closely resembles that of 2-aminobenzoyl-CoA [6] except that in the first compound the signals from the four aromatic protons of the an-

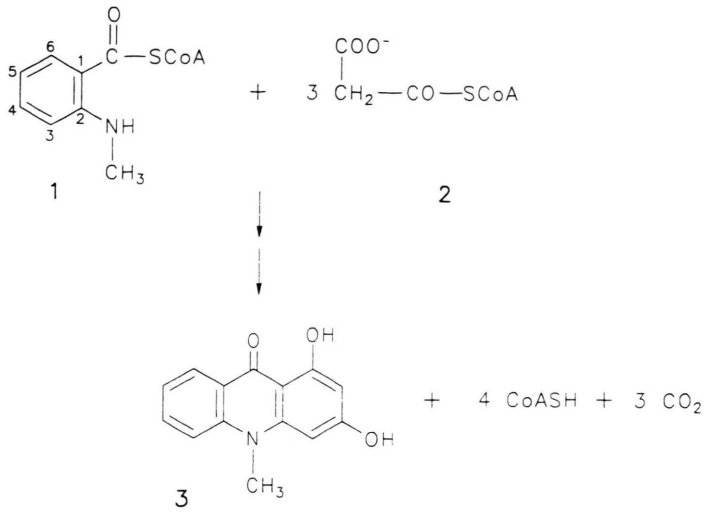

Fig. 1. Hypothetical scheme for the formation of an acridone alkaloid from N-methylanthraniloyl-CoA and malonyl-CoA.

thranilate moiety are shifted downfield and that it shows in addition the $N-CH_3$ signal at 3.05 ppm.

The results of incubations of an enzyme preparation from *Ruta graveolens* cells with various substrates are summarized in Table I. The protein extract catalyzed the condensation of N-methylanthraniloyl-CoA and [2-^{14}C]malonyl-CoA (**2**) (assay A) forming radioactive 1,3-dihydroxy-N-methylacridone (**3**). No cofactors were required for acridone synthesis. Optimal enzyme activity was found at pH 7.5. Dithiothreitol and Mg^{2+} did not affect the enzyme activity. The same reaction product was found in assay B which contained N-methylanthranilic acid and malonyl-CoA as substrates [1, 2]. 1,3-Dihydroxy-N-methylacridone was not formed in the absence of ATP. It is conceivable that a small amount of free CoA-SH is initially found in the crude extract upon enzymatic transacylation or hydrolysis of malonyl-CoA and that this amount is sufficient to serve as substrate for a N-methylanthranilate: CoA ligase. A similar situation was observed in the case of the enzymatic synthesis of naringenin and bis-noryangonin [10]. Other well-known examples in higher plants of chain elongation of a primer molecule by acetate *via* malonyl-CoA are the flavonoid and stilbene biosynthesis. In the case of the chalcone and resveratrol synthases the acyl acceptor which is activated by coenzyme A is *p*-coumaric acid [11].

We are now pursuing the identification of a specific N-methylanthranilate: CoA ligase as well as the purification of acridone synthase.

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

Assay	pH	Specific activity of the acridone synthase [pmol **3**/mg protein/h)
Assay A: with N-methyl-anthraniloyl-CoA	7.0	150
	7.3	171
	7.5	190
	7.8	155
	8.0	150
	boiled enzyme	–
Assay B: with N-methyl-anthranilic acid, ATP and Mg^{2+}	7.5	128

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