Biosynthesis of Sepedonin by Cell Free Extract of Sepedonium Chrysospermum

Biosynthesis of Sepedonin by Cell-Free Extract of *Sepedonium Chrysospermum*

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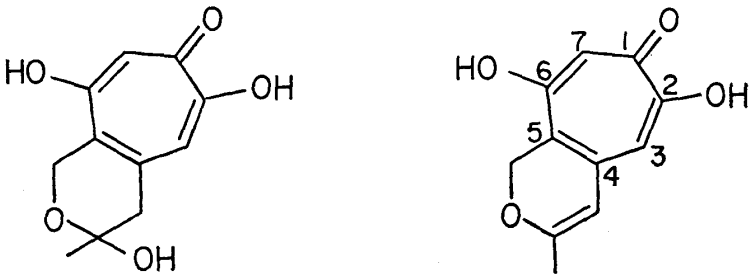
(Received February 7, 1974)

Synopsis

A supernatant obtained by centrifugation at 20,000 g from homogenate of mycelium of *Sepedonium chrysospermum* was proved to be capable of incorporating acetyl-CoA, malonyl-CoA and methionine into sepedonin. This biosynthesis required O_2 in the atmosphere and a cofactor such as NADH, NADPH or $NADP^+$, among which $NADP^+$ was proved to be most effective. Incorporation of methionine into sepedonin was enhanced by addition of vitamin B_{12} and suppressed by ethionine or iodoacetamide.

I. Introduction

The fungal tropolones such as stipitatic acid[1] and puberulonic acid[2] were proved to be constructed with four acetate units and one C_1 unit from the inspection of labeling patterns of specimens obtained by isotopic studies of biosynthesis. Recently the tropolone ring of sepedonin (Chart I) was also found to be formed



Sepedonin Anhydrosepedonin

Chart I.

in a similar manner[3,4]. The pathway of C_1 unit insertion was suggested by Bentley *et al*[5] to be similar to the rearrangement of methylmalonate to succinate

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(1) R. Bentley, J. Biol. Chem., **238** (1963), 1895.
(2) J.H. Richards and L.D. Ferretti, Biochem. Biophys. Res. Comm., **2** (1960), 107.
(3) S. Takenaka and S. Seto, Agr. Biol. Chem. (Japan), **35** (1971), 862.
(4) A.G. McInnes, D.G. Smith and L.G. Vining, Chem. Commun., (1971), 325.
(5) R. Bentley and P.M. Zwitkowits, J. Amer. Chem. Soc., **89** (1967), 681.

catalyzed by vitamin B_{12}, and Scott *et al*[6] proposed a pathway *via* 3-methylorsellinic acid for the biogenesis of stipitatonic acid by use of a culture of *Penicillium stipitatum*. Many studies on cell-free syntheses of acetogenides have been carried out[7~12], while nothing has been reported on sepedonin synthesis, and therefore we carried out the studies by use of a cell-free preparation from the mycelial mat of *Sepedonium chrysospermum* in order to clarify biosynthetic pathways of tropolones and obtained the results described below.

II. Materials and methods

Sepedonium chrysospermum Ag 39 was kindly supplied by Dr. L.C. Vining (Atlantic Regional Laboratory, Halifax, Canada). [Methyl-^{14}C] methionine and [2-^{14}C] acetate were purchased from New England Nuclear Corp. Vitamin B_{12} was kind gift from Sankyo Co. Ltd. Acetyl-CoA and malonyl-CoA were prepared by the methods as reported[13,14]. Other chemicals were obtained from commercial sources.

The mycelial mat of the fungus grown in Czapek-Dox medium for 14 days at 27°C was washed with 0.05M phosphate buffer (pH 7.2), squeezed dry and kept at –20°C. The mat (20 g) was ground in a mortar with sea sand (40 g) and 40 ml of the same phosphate buffer containing dithiothreitol (1 mmole) and EDTA (1 mmole) at 4°C and its supernatant obtained by centrifugation at 20,000 *g* for 30 min was used as a crude enzyme preparation. Protein concentration was estimated by the measurement of optical density at 280 nm.

The incubation mixture for the assay of sepedonin formation is as follows: a reaction mixture (1 ml) contained 0.2 ml (protein 0.5 mg) of the enzyme solution, acetyl-CoA (50 nmoles. 2.2×10^5 dpm when acetyl-CoA was used as a radioactive substrate), malonyl-CoA (100 nmoles), L-methionine (50 nmoles. 3.1×10^5 dpm when methionine was used as a radioactive substrate), NADH (1 μmole) and potassium phosphate buffer (pH 7.2, 1 μmole).

Each run of incubation was carried out at 37°C for 30 min., the reaction was stopped by addition of 0.2 ml of 5% methanolic KOH and the mixture was warmed at 40°C for 30 min. Then the mixture was acidified to pH 1 with conc. HCl and heated on a water bath for 1 hr to convert sepedonin into anhydrosepedonin (Chart I). Because sepedonin showed a tailing on TLC, it was converted to anhydrosepedonin which showed no tailing for the quantitative determination.

(6) A.I. Scott, H. Guilford and Eun Lee, J. Amer. Chem. Soc., **93** (1971), 3534.
(7) S. Gatenbeck and S. Hermodsson, Acta Chem. Scand., **19** (1965), 65.
(8) S.W. Tanenbaum and E.W. Bassett, Biochem. Biophys. Acta, **59** (1962), 524.
(9) R.J. Light, J. Biol. Chem., **242** (1967), 1880.
(10) G.M. Gaucher and M.G. Shepherd, Biochem. Biophys. Res. Commun., **32** (1968), 664.
(11) S. Gatenbeck, P.O. Eriksson and Y. Hansson, Acta Chem. Scand., **23** (1969), 699.
(12) R.J. Light and B. Buslig, Arch. Biochem. Biophys., **122** (1967), 494.
(13) E.J. Simon and D. Shemin, J. Amer. Chem. Soc., **75** (1953), 2520.
(14) E.G. Trams and R.O. Brady, J. Amer. Chem. Soc., **82** (1960), 2972.

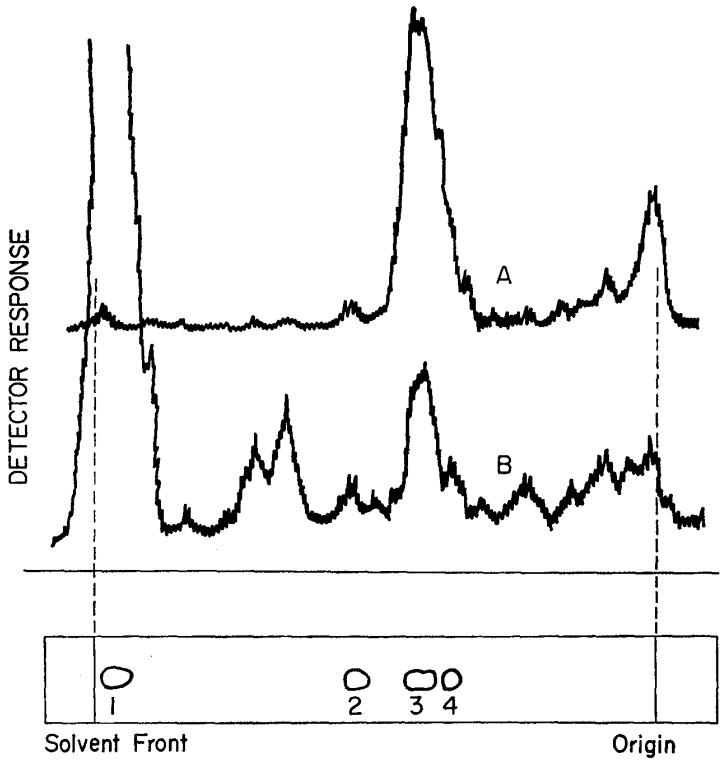

Fig. I. Thin-layer radiochromatograms of the products. The incubation condition and the treatment of the products are described in the text. A: Result obtained when [methyl-^{14}C]-methionine was used as a radioactive substrate. B: Result obtained when [2-^{14}C] acetyl-CoA was used as a radioactive substrate. Lower portion: Spots of the references. 1, fatty acid; 2, 4-hydroxy-6-methyl-2-pyrone; 3, anhydrosepedonin; 4,6-acetonyl-4-hydroxy-2-pyrone.

The resulting solution was extracted with ether (5 ml each, 3 times), the extract was concentrated under a reduced pressure at room temperature, and the residue was redissolved in a small amount of methanol and subjected to TLC (Merck DC-Fertigplatten Kieselgel) with authentic anhydrosepedonin and some other compounds as references, using a solvent system $CHCl_3$-CH_3COOH-CF_3COOH (160: 40:15, by vol). Spots of anhydrosepedonin and other reference compounds were visualized by exposing the developed plate to iodine vapor, and radioactive regions were detected by a thin-layer radiochromatogram scanner. In case of quantitative determination of anhydrosepedonin, the plate was allowed to stand until the stain of the spot dissapeared, and a section of the plate which corresponds to the spot was scraped into a counting vial and its radioactivity was determined in toluene scintillator in the presence of Cab-O-Sil (the product of Cabot Corporation, Boston).

III. Results and discussion

Thin-layer radiochromatograms of the products obtained under standard incubation condition are shown in Fig. I. When [methyl-^{14}C] methionine was used as a radioactive substrate, a strong signal of radioactivity was observed at a spot

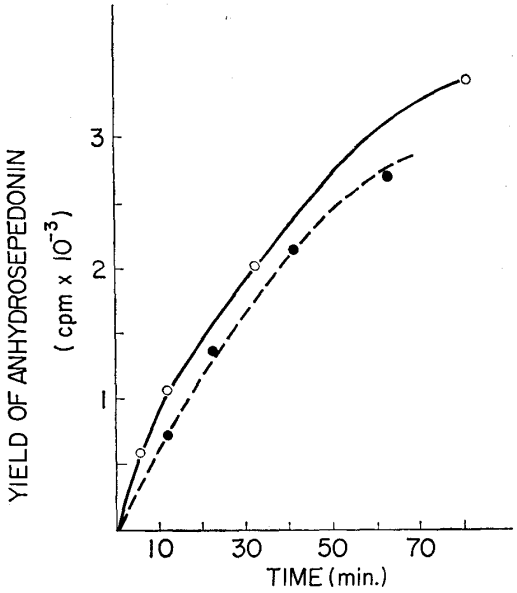

Fig. II. Time course of the formation of anhydrosepedonin. The incubation condition and the treatment of the products are descried in the text. On each experiment, the spot which corresponds to anhydrosepedonin was scraped into a counting vial and its radioactivity was determined in toluene scintillator in the presence of Cab-O-Sil. •—• [methyl-^{14}C] methionine was used as radioactive substrate. o—o [2-^{14}C] acetyl-CoA was used as radioactive substrate.

of anhydrosepedonin. When [2-^{14}C] acetyl-CoA was used as a radioactive substrate, in addition to a very strong signal which corresponds to a spot of fatty acids some other signals appeared, and one of them coincided with the spot obtained in the case of radioactive methionine incorporation. In order to confirm that this spot is that of anhydrosepedonin, the spots of the products obtained by fifty runs of incubation with [2-^{14}C] acetyl-CoA were collected and extracted with methanol. The extract was mixed with 50 mg of pure non-radioactive anhydrosepedonin and recrystallized from methanol four times to give a product with constant specific radioactivity (1,700 cpm/mg). The time course of the formation of anhydrosepedonin is shown in Fig. II.

The supernatant of the enzyme preparation at 100,000 *g* centrifugation was less active than that at 20,000 g. The enzyme solution lost 90% of its activity on storage at 4°C for 24 hr, therefore, the freshly prepared solution was used on each experiment. NADH, NADPH or $NADP^+$ worked as a cofactor, among which $NADP^+$ was proved to be most effective (Table I). It was reported that in the presence of $NADP^+$ the activity of fatty acid synthetase was suppressed and a small amount of 4-hydroxy-6-methyl-2-pyrone was formed in place of fatty acids [15,16]. In our case, the substrates may have been more incorporated into sepedonin, presumably owing to the suppression of fatty acid synthesis by addition of

(15) D.J.H. Brock and K. Bloch, Biochem. Biophys. Res. Commun., **23** (1966), 775.
(16) J.D. Brodie, G. Wasson and J.W. Porter, J. Biol. Chem., **239** (1964), 1346.

TABLE I. COFACTORS AND INHIBITOR [Methyl-^{14}C] methionine was used as radioactive substrate. The experimental conditions and the treatment of the products are the same as the standard, except for the composition of the reaction mixture.

Reaction system	Yield of anhydrosepedonin (cpm)
Complete system	1,884
-NADH	693
-NADH +NADPH (1 μmole)	1,646
-NADH +NADP$^+$ (1 μmole)	2,974
+ICH_2CONH_2 (1 μmole)	264

TABLE II. MISCELLANEOUS EFFECTS [Methyl-^{14}C] methionine was used as a radioactive substrate.

	Yield of anhydrosepedonin (cpm)
Effect of O_2	
Control	1,653
O_2 (blowed on the surface)	1,721
N_2 (bubbled)	813
.....	
Effect of B_{12} and ethionine	
Complete system	1,616
+B_{12} (0.5 μmole)	3,562
+B_{21} (0.5 μmole) +NaCN (1 μmole)	1,994
+ethionine (1 μmole)	243

NADP$^+$. Iodoacetamide worked as an inhibitor, implying participation of SH enzyme (s) in the biosynthesis.

The effect of nitrogen bubbling through the incubation solution was serious and the tropolone formation was nearly a half of the control. This fact might suggest that O_2 in the atmosphere is used as oxygen atom of the carbonyl group at C-1 position of sepedonin, although the exact demonstration must await the experiment with $^{18}O_2$. Vitamin B_{12} enhanced the biosynthetic activity of the enzyme and its effect was lost by addition of NaCN. This result suggests that the formation of the seven-membered ring would occur through a rearrangement reaction in a manner similar to the isomerization of methylmalonate to succinate[17] as Bentley[5] and Tanenbaum[18] stated previously. Ethionine showed an inhibitory effect against the enzyme in coincidence with the result of the experiment with intact cell[19,20]. (Table II)

In order to confirm that pyrone derivatives such as 4-hydroxy-6-methyl-2-pyrone and 6-acetonyl-4-hydroxy-2-pyrone are not intermediates of the formation

(17) H.G. Wood, R.W. Kellermeyer, R. Stjernholm and S.H.G. Allen, Ann. N.Y. Acad. Sci., **112** (1964), 661.

(18) T.E. Acker, P.E. Brenneisen and S.W. Tanenbaum, J. Amer. Chem. Soc., **88** (1966), 834.

(19) R. Bentley, J.A. Ghaphery and J.G. Keil, Arch. Biochem. Biophys., **111** (1965), 80.

(20) R. Bentley and P.N. Zwitkowits, J. Amer. Chem. Soc., **89** (1967), 676.

of the tropolone[5,18,19,21] in the cell-free system, experiments were carried out with incubation mixtures of the control which contained [methyl-^{14}C] methionine but not acetyl- and malonyl- CoA's and of the test with pyrone derivatives. No significant amount of tropolones were formed in both runs of incubation, thus suggesting that these pyrone derivatives were not utilized as the direct precursor of the tropolone.

Acknowledgement

A part of the expenses for this work was supported by Grant-in-Aid from the Ministry of Education. We also thank to Sankyo Co. Ltd. who defrayed a part of the expenses.

(21) G.S. Marx and S.W. Tanenbaum, J. Amer. Chem. Soc., **90** (1968), 5302.