BIOSYNTHESIS OF MONACOLINS: CONVERSION OF MONACOLIN L TO MONACOLIN J BY A MONOOXYGENASE OF *MONASCUS RUBER*

DAISUKE KOMAGATA†, HIDEAKI SHIMADA, SHIGEO MURAKAWA and AKIRA ENDO

Department of Agricultural and Biological Chemistry, Tokyo Noko University, 3-5-8 Saiwaicho, Fuchu-shi, Tokyo 183, Japan

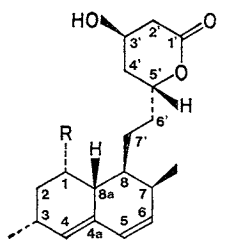
(Received for publication October 17, 1988)

The microbial metabolites monacolins J and L are specific inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, the rate-limiting enzyme in cholesterol synthesis. The producing strain *Monascus ruber* M 4681 was found to convert exogenously added monacolin L to J. In this hydroxylation reaction $^{18}O_2$ was incorporated into monacolin L, giving [^{18}O]-monacolin J. The cell-free extracts of *M. ruber* quantitatively hydroxylated monacolin L to J, and molecular oxygen was required for the hydroxylation. The enzyme was located in the microsomal fraction and specific for NADPH. The enzyme activity was inhibited by metyrapone, carbon monoxide, sulfhydryl reagents and cytochrome c. The results indicate that monacolin L is the precursor of monacolin J, and that a monooxygenase is involved in this reaction.

Monacolin K (mevinolin)[1] and its related compounds, monacolins J, L[2], M[3] and X[4] (Fig. 1) are secondary metabolites of *Monascus ruber*. These compounds specifically inhibit 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis[5]. These compounds are effective in lowering plasma cholesterol levels in various mammalian species including man, and are thereby effective in the therapy of hypercholesterolemia[5]. Monacolin K has recently been introduced into commercial use in several countries.

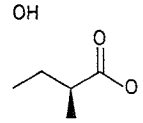
Biosynthesis of monacolin K has been studied by several groups, which indicated that the main skeleton of monacolin K is a polyketide of acetate origin[6,7]. Growth experiments of *M. ruber* with ^{14}C-labeled monacolin J or L suggested that both compounds are the precursors of monacolin K[6].

Fig. 1. Structures of lactone forms of monacolin K (mevinolin) related compounds.



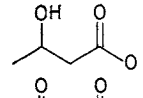
Monacolin J R= OH

Monacolin K (mevinolin) R=

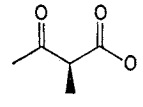


Monacolin L R= H

Monacolin M R=



Monacolin X R=



† Present address: Pharmaceutical Research Laboratory, Pharmaceuticals Division, Nippon Kayaku Co., Ltd., 3-31-12 Shimo, Kita-ku, Tokyo 115, Japan.

This communication describes that *M. ruber* hydroxylates monacolin L to J by a monooxygenase system involving cytochrome P-450.

Materials and Methods

Microbial Strain and Growth

M. ruber M 4681 was employed in the present study. This strain was grown aerobically at 25°C for 5 days in a medium containing glycerol 7%, glucose 3%, meat extract 3%, Polypeptone 0.8%, $NaNO_3$ 0.2% and $MgSO_4 \cdot 7H_2O$ 0.1%. This preculture was transferred (inoculum size 5%) into the same medium and grown aerobically at 25°C for 2 to 3 days.

Preparation of Cell-free Extracts and Subcellular Fractions

The culture broth (200 ml) grown under the above conditions for 2 days was filtered and the resulting mycelia were washed with 50 mM potassium phosphate buffer, pH 8.3, containing 2 mM EDTA. All the following procedures were carried out at 0~4°C.

The wet mycelia (3.4 g) obtained were ground to a smooth paste in a mortar containing 6.8 g of alumina and 1.7 ml of 50 mM potassium phosphate buffer, pH 8.3, containing 1 mM dithiothreitol, 2 mM EDTA and 250 mM sucrose (buffer A). The resulting paste was mixed with 13.6 ml of buffer A supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at $1,500 \times g$ for 10 minutes. The resulting supernatant (cell-free extract) was fractionated by successive centrifugation for 15 minutes at $12,000 \times g$, followed by 90 minutes at $105,000 \times g$. Pellets obtained by these centrifugations were resuspended in buffer A supplemented with 1 mM PMSF, and stored at −80°C until use.

Incorporation of $^{18}O_2$ into Monacolin L

The culture broth (100 ml) grown under the above conditions for 3 days was centrifuged, and washed with 100 mM potassium phosphate buffer, pH 7.0. The washed mycelia were suspended in the same buffer (90 ml). Three test tubes (3×20 cm) containing 27 ml of the cell suspension and 3 mg of monacolin L acid form (3 ml) were incubated at 25°C with shaking in the atmospheres of air, $^{18}O_2$ (or O_2) and N_2, respectively. After 24 hours, each suspension was filtered, and the resulting mycelia were mixed with 10 ml of acetone. Both the filtrate and the acetone fraction were combined and evaporated *in vacuo*. The resulting aqueous solution was adjusted to pH 2 with TFA, and extracted with an equal volume of EtOAc 3 times. The solvent layers were pooled, dehydrated with sodium sulfate, and then dried *in vacuo*.

The resultant residue (~70 mg) was submitted to HPLC on a Silica ODS column (Nihon Bunko) by using a solvent mixture of acetonitrile - water (55 : 45), and the eluate fractions containing monacolin J were pooled. The mixture was extracted with an equal volume of chloroform and the solvent layer was pooled, dehydrated with anhydrous sodium sulfate, and then dried *in vacuo*. The residue (~1 mg) was submitted to HPLC on a silica gel column (Nihon Bunko) by using a solvent mixture of dichloromethane - 2-propanol (95 : 5), giving ~0.1 mg of purified monacolin J.

The electron impact (EI)-MS of monacolin J was determined on a Shimadzu LKB 9,000 instrument: *m/z* 320 (M^+), 302 (M−18), 284 (M−36), 269 (M−51), 224 (M−96), 198 (M−122), 172 (M−148), 159 (M−161, 100%) and 157 (M−163); exact mass calculated for $C_{19}H_{28}O_4$: 320.1988. Found 320. Monacolin J labeled with $^{18}O_2$ was analyzed under the same conditions as the unlabled sample.

Hydroxylation of Monacolin L by Subcellular Fractions

The reaction mixture, containing (in a total volume of 1.0 ml) 100 mM potassium phosphate buffer, pH 7.0, 1 mM dithiothreitol, 1 mM NADPH, NADPH-recycling system (10 mM glucose 6-phosphate and 1 U of glucose-6-phosphate dehydrogenase), 0.33 mM monacolin L acid form and an appropriate amount of cell-free extracts or subcellular fractions, was incubated at 25°C with shaking. To determine the cofactor requirement, the same reaction mixture without NADPH and NADPH-recycling system was incubated in the presence or absence of different cofactor(s). After 24 hours incubation, the reaction was stopped by adding 2 drops of TFA, and the mixture was extracted with an equal

volume of EtOAc (3 times). Monacolin J in the extract was analyzed by HPLC as described above.

Other Methods

NADPH-cytochrome c reductase was assayed by measuring the increase in absorbance at 550 nm as described by STROBEL and DIGNAM[8], except that 1 mM KCN was supplemented to the reaction mixture. Protein was determined as described by LOWRY *et al.*[9] using bovine serum albumin as the standard.

Chemicals

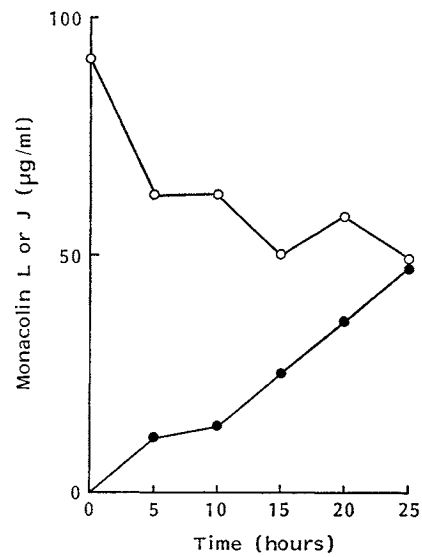
Oxygen 18 (98.6 atom% ^{18}O) was obtained Gif-Sur-Yvette (France). NADH, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd. Metyrapone, PMSF, cytochrome c from horse heart (Type VI) and bovine serum albumin were from Sigma Chemical Company. Carbon monoxide was from Gasukuro Kogyo Inc. Monacolins J and L were obtained as described previously[2]. These compounds were converted to their respective acid forms (sodium salts) by saponification prior to use.

Results

Incorporation of ^{18}O into Monacolin L

Fig. 2 shows the conversion of monacolin L to J by *M. ruber* in the atmosphere of air. After 24 hours, 45 μg/ml of monacolin J was produced, while 40 μg/ml of monacolin L was consumed during this period of time. No detectable monacolin J was formed when *M. ruber* was incubated with no exogenously added monacolin L. When incubated in the atmosphere of O_2, 45.7 μg/ml of monacolin J was produced, while only 7.2 μg/ml of monacolin J was formed in N_2. These results suggested the requirement of molecular oxygen for the conversion of monacolin L to J.

Fig. 2. Time course of the conversion of monacolin L to J by resting mycelia of *Monascus ruber*.
○ Monacolin L, ● monacolin J.



The washed-cell suspension of *M. ruber* (27 ml) and 3 mg (3 ml) of monacolin L were incubated with shaking at 25°C in the atmosphere of air. One-ml aliquots were withdrawn at different time intervals and monacolins L and J were assayed. Experimental details are described in Materials and Methods.

The quantity (120 μg) of [^{18}O]monacolin J obtained proved to be too small for ^{13}C NMR detection of isotope shifts and was therefore analyzed by MS. Table 1 shows the EI-MS data for [^{18}O]monacolin J. The peak at *m/z* 320 (M^+ for unlabeled monacolin J) was very low and a prominent peak was observed at *m/z* 322 (M+2). These results indicate that one atom of ^{18}O is incorporated into a monacolin L molecule to form [^{18}O]monacolin J, possibly by the action of a monooxygenase. Peaks in the mass spectrum at *m/z* 302 and 284 can be readily explained by the loss of $H_2{}^{18}O$ and H_2O from the molecular ion (M+2) indicating that ^{18}O is incorporated into C-1 or C-3′ of monacolin J. Furthermore the hydroxylation was inhibited by anoxia. These results suggest that ^{18}O is incorporated into the C-1 hydroxyl group in monacolin J.

Table 1. MS data for ^{18}O-labeled monacolin J.

m/z		Relative abundance (%)[a]	Empirical formula	Fragment lost
Measured	Calcd			
322	322.2031	5.6	$C_{19}H_{28}O_3{}^{18}O$	
320	320.1988	1.3	$C_{19}H_{28}O_4$	
302	302.1883	11.6	$C_{19}H_{26}O_3$	$H_2{}^{18}O$
284	284.1777	20.6	$C_{19}H_{24}O_2$	$H_2{}^{18}O$, H_2O
269	269.1542	9.1	$C_{18}H_{21}O_2$	$H_2{}^{18}O$, H_2O, CH_3
224	224.1566	27.3	$C_{17}H_{20}$	$H_2{}^{18}O$, H_2O, $C_2H_4O_2$
198	198.1409	22.3	$C_{15}H_{18}$	$H_2{}^{18}O$, H_2O, $C_2H_4O_2$, C_2H_2
172	172.1253	35.0	$C_{13}H_{16}$	$H_2{}^{18}O$, H_2O, $C_2H_4O_2$, $2C_2H_2$
159	159.1174	100	$C_{12}H_{15}$	$H_2{}^{18}O$, H_2O, $C_3H_5O_2$, $2C_2H_2$
157	157.1018	92.8	$C_{12}H_{13}$	$H_2{}^{18}O$, H_2O, $C_2H_4O_2$, $2C_2H_2$, CH_3

[a] The base peak of this spectrum was observed at m/z 159 as 100%.

Table 2. Monacolin L hydroxylase and NADPH-cytochrome c reductase activities in different subcellular fractions.

Subcellular fraction	Monacolin L hydroxylase			NADPH-cytochrome c reductase specific activity[c]
	Total activity[a]	Specific activity[b]	Yield (%)	
1,500×g supernate	11.54	83.0	100	17.4
12,000×g supernate	7.73	67.8	67.0	12.9
12,000×g pellet	ND	ND	—	5.1
105,000×g supernate	0.31	3.8	2.7	14.7
105,000×g pellet	7.33	207	63.5	32.2

[a] Data are expressed as nmol/minute.
[b] Data are expressed as pmol/minute/mg of protein.
[c] Data are expressed as nmol/minute/mg of protein.
ND: Not detectable.

Subcellular Localization of Monacolin L Hydroxylase

The cell-free extract of *M. ruber* was able to hydroxylate monacolin L to J quantitatively. This activity was not detected when the extracts were heat-treated at 100°C for 2 minutes or the reaction was carried out in an atmosphere of N_2 (data not shown).

Most of the hydroxylase activity was associated with the 105,000×g pellet fraction, which also contained the highest specific activity of NADPH-cytochrome c reductase of all the subcellular fractions (Table 2). These results indicate that the enzyme involved in the conversion of monacolin L to J is localized in the microsomal fraction.

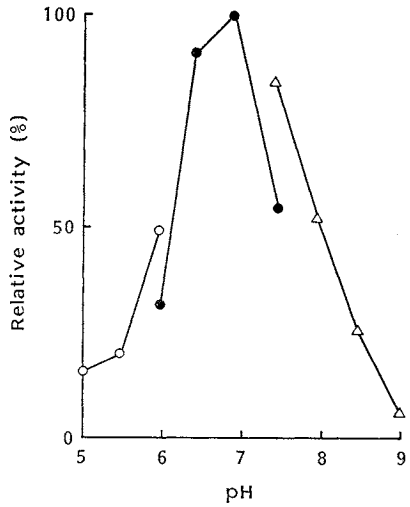
Properties of the Hydroxylating Enzyme

The optimum pH for the monacolin L hydroxylase activity of microsomal preparations was found to be around 7 (Fig. 3).

The microsomal preparations showed no detectable hydroxylase activity in the absence of cofactors. However, remarkable activity was observed on the addition of NADPH (Table 3). The stimulation by NADPH was not replaced by NADH or ascorbate. Addition of both NADPH-recycling system and NADPH to the reaction system stimulated slightly the hydroxylase activity, as compared to that of NADPH alone.

Fig. 3. Effect of pH on the monacolin L hydroxylase activity of *Monascus ruber*.

The enzyme activity was assayed as described in Meterials and Methods using 0.57 mg protein/ml of microsomal fraction, except that the pH of the reaction mixture was varied as indicated, using 0.2 M citrate-phosphate buffer, pH 5.0~6.0 (○), 0.2 M potassium phosphate buffer, pH 6.0~7.5 (●), and 0.2 M Tris-HCl buffer, pH 7.5~9.0 (△).



The hydroxylase activity of microsomal preparations was inhibited by the cytochrome P-450 inhibitors, metyrapone and CO, and the sulfhydryl reagents, *p*-chloromercuribenzoic acid (PCMB) and *N*-ethylmaleimide (Table 4). Cytochrome c also inhibited the enzyme activity but KCN and NaN_3 were not inhibitory.

Table 3. Requirements for cofactors in monacolin L hydroxylation[a].

Cofactor(s)	Relative activity[b]
None	0
NADPH (1 mM)	100
NADH (1 mM)	0
Ascorbate (1 mM)	0
NADPH (1 mM)+NADPH-recycling system	112
NADPH (1 mM)+NADH (1 mM)	102

[a] The reactions were carried out as described in Materials and Methods using 0.57 mg protein/ml of microsomal fraction.

[b] Relative to the value (86.3 pmol/minute/mg of protein) for NADPH.

Table 4. Effects of some metabolic inhibitors on monacolin L hydroxylation activity[a].

Inhibitor	Relative activity[b]
None (control)	100
Metyrapone (1 mM)	45.2
CO (bubbled for 2 minutes)	15.1
KCN (1 mM)	100
NaN_3 (1 mM)	95.0
Cytochrome c (0.1 mM)	58.5
PCMB (1 mM)	3.8
N-Ethylmaleimide (1 mM)	13.3

[a] The reactions were carried out as described in Materials and Methods using 0.57 mg protein/ml of microsomal fraction.

[b] Relative to the control value (96.8 pmol/minute/mg of protein).

Discussion

In the present experiments, *M. ruber* is shown to incorporate ^{18}O into monacolin L, giving [^{18}O]-monacolin J (Table 1). In this hydroxylation reaction, molecular oxygen was shown to be involved. However, it is not ruled out that $H_2{}^{18}O$ derived from $^{18}O_2$ may be involved. If all the $^{18}O_2$ (~100 ml) used in the tracer experiments described was metabolized to $H_2{}^{18}O$ (~9.8 mmol), $H_2{}^{18}O/H_2O$ ratio might be less than 0.006, because the total amount of water in the reaction mixture was ~30 ml (1.67 mol). From the mass spectral data (Table 1), the ratio of intensities of [^{18}O]monacolin J/[^{16}O]-monacolin J (*m/z* 322/320) peaks was 4.3. These results demonstrate that more than 80% of the oxygen incorporated into the metabolite was drived from $^{18}O_2$ but not from $H_2{}^{18}O$. They also rule out the above-mentioned possibility to give [*hydroxyl*-3′-^{18}O]monacolin J as the result from exchange reaction between the hydroxyl groups of monacolin J or L and $H_2{}^{18}O$.

The monacolin L hydroxylase was localized in the microsomal fraction (Table 2) and was a NADPH-dependent monooxygenase (Table 3). This enzyme can be assumed to be monacolin L 1-monooxygenase (monacolin L, NADPH: Oxygen, oxidoreductase (1-hydroxylating)). The microsomal hydroxylase activity was significantly inhibited by CO, metyrapone, PCMB and *N*-ethylmaleimide (Table 4), and the findings may indicate the involvement of a cytochrome P-450 system. The

inhibition of the hydroxylase by cytochrome c and the presence of high levels of NADPH-cytochrome c reductase activity in the microsomal fraction suggest this possibility.

Various hydroxylase systems have been found in microsomal fractions from different microorganisms and mammals[10~16]. The involvement of cytochrome P-450 and NADPH (or NADH)-cytochrome P-450 (c) reductase has been suggested to be involved in these hydroxylase systems.

References

1) ENDO, A.: Monacolin K, a new hypocholesterolemic agent produced by a *Monascus* species. J. Antibiotics 32: 852~854, 1979
2) ENDO, A.; K. HASUMI & S. NEGISHI: Monacolins J and L, new inhibitors of cholesterol biosynthesis produced by *Monascus ruber*. J. Antibiotics 38: 420~422, 1985
3) ENDO, A.; D. KOMAGATA & H. SHIMADA: Monacolin M, a new inhibitor of cholesterol biosynthesis. J. Antibiotics 39: 1670~1673, 1986
4) ENDO, A.; K. HASUMI, T. NAKAMURA, M. KUNISHIMA & M. MASUDA: Dihydromonacolin L and monacolin X, new metabolites those inhibit cholesterol biosynthesis. J. Antibiotics 38: 321~327, 1985
5) ENDO, A.: Compactin (ML-236B) and related compounds as potential cholesterol-lowering agents that inhibit HMG-CoA reductase. J. Med. Chem. 28: 401~405, 1985
6) ENDO, A.; Y. NEGISHI, T. IWASHITA, K. MIZUKAWA & M. HIRAMA: Biosynthesis of ML-236B (compactin) and monacolin K. J. Antibiotics 38: 444~448, 1985
7) MOORE, R. N.; G. BIGAM, J. K. CHAN, A. M. HOGG, T. T. NAKASHIMA & J. C. VEDERAS: Biosynthesis of the hypocholesterolemic agent mevinolin by *Aspergillus terreus*. Determination of the origin of carbon, hydrogen, and oxygen atoms by ^{13}C NMR and mass spectrometry. J. Am. Chem. Soc. 107: 3694~3701, 1985
8) STROBEL, H. W. & J. D. DIGNAM: Purification and properties of NADPH-cytochrome P-450 reductase. Methods Enzymol. 52: 89~96, 1978
9) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265~275, 1951
10) BJÖRKHEM, I.; H. DANIELSSON & K. WIKVALL: 7α-Hydroxylation of cholesterol by reconstituted systems from rat liver microsomes. Biochem. Biophys. Res. Commun. 61: 934~941, 1974
11) CERNIGLIA, C. E. & D. T. GIBSON: Metabolism of naphthalene by cell extracts of *Cunninghamella elegans*. Arch. Biochem. Biophys. 186: 121~127, 1978
12) KOMINAMI, S.; H. OCHI, Y. KOBAYASHI & S. TAKEMORI: Studies on the steroid hydroxylation system in adrenal cortex microsomes. Purification and characterization of cytochrome P-450 specific for steroid C-21 hydroxylation. J. Biol. Chem. 255: 3386~3394, 1980
13) JAYANTHI, C. R.; P. MADYASTHA & K. M. MADYASTHA: Microsomal 11α-hydroxylation of progesteron in *Aspergillus ochraceus*: Part I: Characterization of the hydroxylase system. Biochem. Biophys. Res. Commun. 106: 1262~1268, 1982
14) GHOSH, D. K.; D. DUTTA, T. B. SAMANTA & A. K. MISHRA: Microsomal benzo(a)pyrene hydroxylase in *Aspergillus ochraceus* TS: Assay and characterization of the enzyme system. Biochem. Biophys. Res. Commun. 113: 497~505, 1983
15) ČREŠNAR, B.; K. BRESKVAR & T. HUDNIK-PLEVNIK: Resolution and reconstitution of the NADPH-cytochrome c (P-450) reductase induced by progesterone in *Rhizopus nigricans*. Biochem. Biophys. Res. Commun. 133: 1057~1063, 1985
16) SHOUN, H.; Y. SUDO & T. BEPPU: Subterminal hydroxylation of fatty acids by a cytochrome P-450-dependent enzyme system from a fungus, *Fusarium oxysporum*. J. Biochem. 97: 755~763, 1985