ISOLATION AND BIOSYNTHESIS OF 3α-HYDROXY-3,5-DIHYDROMONACOLIN L

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3α-Hydroxy-3,5-dihydromonacolin L acid (acid form), a new compound related to monacolin K (mevinolin), was isolated from the culture broth of a strain of *Monascus ruber*. The structure of the compound was determined by a combination of physical techniques. 4a,5-Dihydromonacolin L was converted to 3α-hydroxy-3,5-dihydromonacolin L by a cell-free extract of *M. ruber* in the presence of molecular oxygen. The results demonstrate that the former is the direct precursor in the biosynthesis of the latter.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the major rate-limiting enzyme in cholesterol synthesis. Potent inhibitors of HMG-CoA reductase have been isolated from *Monascus ruber*, which include the monacolins J (**4**), K (mevinolin), L (**3**), M, and X and 4a,5-dihydromonacolin L (**2**) (Fig. 1)[1~6]. Monacolin K has been on the market as a hypocholesterolemic drug since 1987.

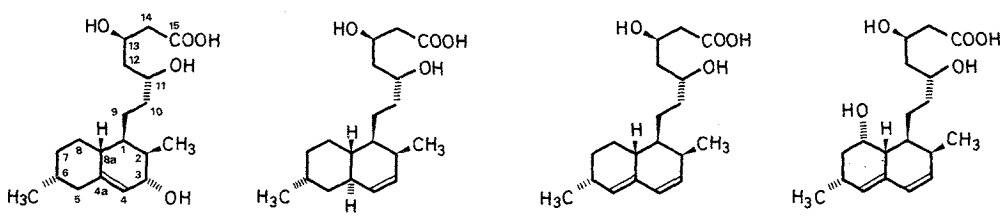
The present communication describes the isolation and biosynthesis of 3α-hydroxy-3,5-dihydromonacolin L (**1**), a new metabolite of the monacolin family.

Materials and Methods

Fermentation and Isolation of 3α-Hydroxy-3,5-dihydromonacolin L (1)

M. ruber J-199 was grown aerobically at 25°C for 11 days in a medium (15 liters) containing glycerol 7%, glucose 3%, meat extract 3%, peptone 0.8%, $NaNO_3$ 0.2% and $MgSO_4 \cdot 7H_2O$ 0.1% using a 30-liter fermenter. Under these growth conditions, *M. ruber* J-199 produced ~20 μg/ml of **1**, which was detected by monitoring the UV absorbance at 200 nm in HPLC. The culture filtrate obtained (11.7 liters) was adjusted to pH 10 with NaOH and passed through a column of Diaion HP-20 (6.5×43 cm) packed with water. The column was washed with water (4.2 liters) and then developed with a mixture of acetonitrile - water (2:3, 2 liters). The eluate containing **1** (300 ml) was concentrated to 200 ml under reduced pressure to give 19 g of solid material. Half of the concentrated solution (100 ml) was adjusted to pH 3 with H_3PO_4 and extracted three times with 100 ml of ethyl acetate. The solvent layers were mixed with first 100 ml and then

Fig. 1. 3α-Hydroxy-3,5-dihydromonacolin L (**1**), 4a,5-dihydromonacolin L (**2**), monacolin L (**3**) and monacolin J (**4**).



50 ml of water at pH 7.5 (adjusted with NaOH). The aqueous layers were pooled, adjusted to pH 10.0 with NaOH and then applied to a column of Diaion HP-20SS (3.0 × 25 cm). After washing with 900 ml of water, the column was developed with a 2-liter linear gradient of 0 to 20% acetone. The active eluate obtained (560 ml, containing 2.4 g of oily residue) was adjusted to pH 3 with H_3PO_4 and extracted with first 560 ml and then 280 ml of ethyl acetate. The combined extract was concentrated to 140 ml under reduced pressure, giving 1,650 mg of oily residue, which was dissolved in 7 ml of ethanol and passed through a column of Supelclean (LC-18, 1 ml, Supelco Inc., U.S.A.). The column was washed with water and the active eluate (18 ml containing 1,600 mg of solid) was pooled. Part of the material (250 mg) was submitted to HPLC in a column of Silica ODS (1.03 × 30 cm), using a solvent system of acetonitrile - 0.1% H_3PO_4 (35 : 65). Fractions containing **1** were pooled and applied to the same column, which was developed with a mixture of acetonitrile and 0.1% H_3PO_4 (30 : 70). The active eluate (200 ml) was extracted with 300 ml of ethyl acetate. The solvent layer was concentrated to 2 ml under reduced pressure and dissolved in 2 ml of water. The mixture was brought to pH 7.5 with NaOH. The aqueous layer (3 ml) was pooled and lyophilized, giving 46.8 mg of **1**.

Conversion of 4a,5-Dihydromonacolin L (**2**) to 3α-Hydroxy-3,5-dihydromonacolin L (**1**)

M. ruber J-199 was grown aerobically at 25°C for 4 days in the medium described above. The resulting mycelial pellet was washed with 50 mM potassium phosphate buffer, pH 8.3, containing 2 mM EDTA. The wet mycelia (1.5 g) obtained were ground to a smooth paste at 0°C in a mortar with 3 g of alumina and 8 μl of a solution containing 100 mM dithiothreitol (DTT), 100 mM phenylmethylsulfonyl fluoride and 10 mM leupeptin (PFL solution). The resulting paste was mixed with 5.3 ml of 50 mM potassium phosphate buffer containing 2 mM EDTA, 0.25 M sucrose and 50 μl of PFL solution, and then centrifuged at 1,500 × *g* for 20 minutes. The resultant supernatant (cell-free extract) contained 15 ~ 25 mg protein per ml.

A mixture (5 ml) containing 100 mM potassium phosphate buffer, pH 7.0, NADPH 1 mM, DTT 1 mM, 4a,5-dihydromonacolin L (**2**) 0.3 mM and 15 ~ 30 mg protein of freshly prepared cell-free extract was incubated at 25°C with gentle shaking. After 180 minutes of incubation, aliquots (1.5 ml) of the mixture were extracted twice with ethyl acetate at pH 3 and the solvent layer was assayed for **1**, **2**, **3** and **4** by HPLC on a column of Silica ODS. In the experiments indicated, **2** was replaced by **1** in the reaction mixture.

Other Methods

Monacolin J (**4**), monacolin L (**3**) and 4a,5-dihydromonacolin L (**2**) were prepared as described previously[3,4]. Determination of **3** and **4** was done according to the absorbance at 237 nm, while **1** and **2** were determined at 200 nm. HMG-CoA reductase was assayed as described previously[7]. Protein was determined according to the method of BRADFORD using bovine serum albumin as a standard[8].

Results

3α-Hydroxy-3,5-dihydromonacolin L (**1**) showed no UV spectrum characteristic of the conjugated double bonds of the fused ring system. It was very unstable and converted quantitatively to monacolin L (**3**) when kept at pH 2 and 25°C for 10 hours.

The mass spectral and ^{13}C NMR data for the sodium salt of **1** established $C_{19}H_{31}O_5Na$ as the molecular formula. Significant peaks were seen in the SI mass spectrum at *m/z* 385 $(M-H+2Na)^+$, 363 $(M+Na)^+$, 323 $(M-H_2O+H)^+$ and 281 $(M-CH_3COOH+H)^+$. ^{13}C NMR data of **1** (Table 1) revealed the presence of 19 carbon atoms including one carbonyl carbon at 179.4 ppm, one olefinic methine at 121.9 ppm, one olefinic quaternary carbon at 141.0 ppm, 3 oxycarbons at 68.4, 70.4 and 70.5 ppm and 2 methyl carbons at 11.6 and 16.9 ppm. Major signals of the ^1H NMR spectrum of **1** (Table 1) were observed at δ 5.38 (1H, br d, J = 4.6 Hz, 4-H), 4.11 (1H, m, 13-H), 3.77 (1H, br dd, 3-H and 1H, m, 11-H), 0.80 (3H, d, J = 6.8 Hz, 2-CH_3) and 0.92 (3H, d, J = 6.8 Hz, 6-CH_3). The ^1H-^1H and ^{13}C-^1H COSY of **1** supported the structure of the 3-hydroxy 4-ene decaline system in **1**. Irradiation of the 2-CH_3 showed an enhance-

Table 1. ^{13}C and ^{1}H NMR data of **1** (Na salt)[a].

Position	^{13}C (ppm)	^{1}H (ppm)	Position	^{13}C (ppm)	^{1}H (ppm)
C-1	36.1	1.5~1.8[b](1H, m)[c]	C-9	24.8	1.3~1.5[b](2H, m)
C-2	39.7	1.5~1.8[b](1H, m)	C-10	35.7	1.47 (2H, m)
C-3	70.4	3.77 (1H, br dd, J=3.4, 4.6)	C-11	70.5	3.77 (1H, m)
C-4	121.9	5.38 (1H, br d, J=4.6)	C-12	43.8	1.5~1.8[b](2H)
C-4a	141.0	—	C-13	68.4	4.11 (1H, m)
C-5	41.4	2.24 (1H, br dd, J=5.0, 13.6), 1.96 (1H, br d, J=13.6)	C-14	44.5	2.37 (1H, dd, J=4.4, 14.8), 2.27 (1H, dd, J=8.0, 14.8)
C-6	29.2	2.03 (1H, m)	C-15	179.4	—
C-7	31.9	1.5~1.8[b](2H)	2-CH_3	11.6	0.80 (3H, d, J=6.8)
C-8	27.2	1.5~1.8[b](2H)	6-CH_3	16.9	0.92 (3H, d, J=6.8)
C-8a	41.1	1.5~1.8[b](1H)			

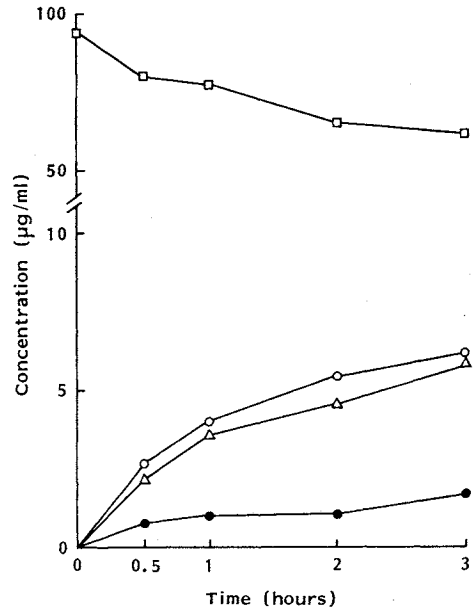
[a] ^{13}C NMR (100 MHz) and ^{1}H NMR (400 mHz) spectra were measured in CD_3OD at 25°C. TMS was used as an internal reference.
[b] Overlapping multiplets.
[c] Proton number, multiplicity and coupling constants in Hz are indicated in parentheses.

ment of 7.6% to 8a-H and 18.1% to 3-H in NOE difference spectroscopy, respectively. Thus configuration of the 3-hydroxy could be assigned as α. The oxygen-bearing methine proton (3-H) was coupled to 2-H and 4-H with J=3.4 and 4.6 Hz, respectively. These results were comparable with those of phenacyloxy derivative of 3α-hydroxy-3,5-dihydromonacolin L[9] and 3α,5β-dihydroxy ML-236B[10].

When **2** was incubated with a cell-free extract of *M. ruber* in an atmosphere of air, **1**, **3**, and **4** were detected as products. The result of a typical experiment are shown in Fig. 2, which demonstrated that the three products were formed at the expense of **2**. No detectable products were formed when the cell-free extract was incubated without added **2**. When incubated in an atmosphere of N_2, production of the three products was inhibited by over 80%, indicating that molecular oxygen was required for the conversion of **2** to **1**, **3** and **4**. However, these results did not show the precursor-product relationship among the three compounds. No detectable amounts of **2**, **3** and **4** were produced when **1** was incubated with a cell-free extract of *M. ruber* under various conditions.

Fig. 2. Conversion of **2** into **1**, **3** and **4** by cell-free extract of *Monascus ruber*.

□ **2**, ● **1**, △ **3**, ○ **4**.



Discussion

The present experiments have demonstrated that **1** is present in the culture broth of *M. ruber*. The presence of **1** in the culture broth of *Aspergillus terreus* was suggested by TREIBER *et al.*, who isolated its phenacyl ester[9].

The structures of **1** and **2** suggest that **1** is derived from **2** by hydroxylation in the presence of O_2

which is analogous to the conversion of **3** to **4**[11]. It is unlikely that **3** is directly derived from **2**, since O_2 is required for the formation of both **1** and **3**. It is reasonable to assume that **2** is first converted to **1**, which is dehydrated to **3**, although exogenously added **1** was not converted to **3** by cell-free extract of *M. ruber*. The data that **1** can be converted spontaneously to **3** under acidic conditions also support this hypothesis.

Acknowledgments

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