DIHYDROMEVINOLIN, A POTENT HYPOCHOLESTEROLEMIC METABOLITE PRODUCED BY *ASPERGILLUS TERREUS*

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A new, potent hypocholesterolemic agent is produced by cultures of *Aspergillus terreus*. The isolation of the compound and its characterization as 4a,5-dihydromevinolin containing a *trans*-fused octahydro-naphthalene system are described. Comparative data for dihydromevinolin and mevinolin in three biological assays are given: *in vitro* inhibition of HMG-CoA reductase, inhibition of sterol synthesis in cell cultures, and inhibition of cholesterol synthesis *in vivo* in rats.

There is considerable current interest in the discovery of hypocholesterolemic agents whose mode of action is the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase, EC 1.1.1. 34). Fermentation sources have yielded potent inhibitors. The first of these to be described was ML-236B (compactin). It was isolated as an antifungal agent from *Penicillium brevicompactum* and called compactin by BROWN *et al.*[1] Its isolation from *Penicillium citrinum* and hypocholesterolemic properties were described by ENDO and his associates[2,3,4] under the designation ML-236B. The clinical efficacy of ML-236B in lowering cholesterol has also been reported.[5]

Recently, a more potent relative of ML-236B was isolated in our laboratory[6] from *Aspergillus terreus*. We named it mevinolin. The same compound was isolated by ENDO[7] from *Monascus ruber* and designated monacolin K. The structure of this metabolite without stereochemistry was disclosed by ENDO[8] and described completely by ourselves including absolute configuration.[6] We now report the isolation, structure and some of the pharmacological properties of still another highly potent inhibitor of HMG-CoA reductase. This new inhibitor, which is a dihydro analog of mevinolin, both *in vitro* and *in vivo* is comparable to mevinolin in activity. The discovery in our laboratories of dihydrocompactin in *Penicillium citrinum* is described in an accompanying communication[9].

Fermentation and Isolation

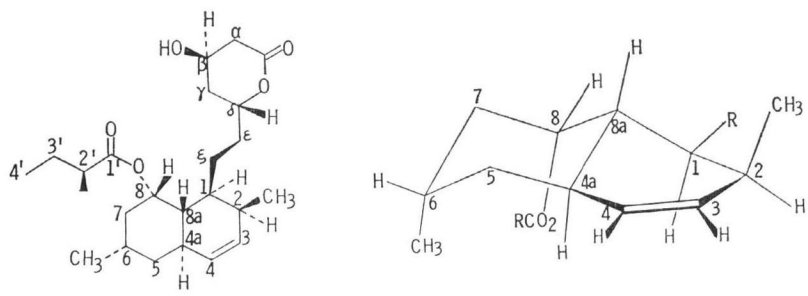
The same strain of *Aspergillus terreus* ATCC 20542 which was used to produce mevinolin yielded this new product as a minor metabolite. As described earlier,[6] the fermentation was conducted aerobically at 28°C for six days (pH 7.4) in a medium containing dextrose 45 g/liter, peptonized milk 24 g/liter, yeast extract 2.5 g/liter and polyglycol P 2000 2.5 ml/liter. Ethyl acetate extracts of acidified (pH 5) broth were dried over sodium chloride, treated with ammonia gas and concentrated to a small volume. Addition of further ammonia and dilution with acetone resulted in a precipitate of crude ammonium mevinolate which was trimethylsilylated with *bis*-trimethylsilyl-trifluoroacetamide in dimethylformamide (BSTFA - DMF, 1: 1) and analyzed by GC/MS (3% OV-25 on Chromosorb WHP, 2 mm ID, 3 ft., 30 ml He/minute, 230°C). The tris-trimethylsilyl (tris-TMS) derivative of mevinolinic acid emerged with

a retention time of about five minutes. The minor component, by peak area amounting to approximately 8% of the major component, emerged at 4.5 minutes and could be identified mass spectrometrically as the tris-TMS derivative of a dihydromevinolinic acid. The compound was isolated preparatively as follows: One liter of whole broth was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was concentrated to dryness and the residue (5.5 g) chromatographed on methanol-equilibrated LH-20 (4 liters) using methanol as eluant. The fractions having HMG-CoA inhibitory activity were combined and concentrated to dryness. The residue (1.2 g) was refluxed for $5\frac{1}{2}$ hours in toluene to convert hydroxyacids to lactones. Solvent was removed *in vacuo* and the residue chromatographed at 40°C on a 1.4 cm ID × 183 cm long column filled with Bondapak C_{18}, using 0.05 M ammonium phosphate pH 3 - acetonitrile (45: 55) as eluant. Mevinolin and dihydromevinolin appeared in elution volumes of 0.6 ~ 1.2 liters and 1.2 ~ 1.8 liters, respectively. The fractions containing dihydromevinolin were concentrated *in vacuo* to *ca.* 1/2 of their original volume and extracted with dichloromethane. The extract was concentrated to dryness yielding 58.7 mg of over 90% pure dihydromevinolin. Final purification was achieved by preparative reverse-phase high-performance liquid chromatography on a 9 mm ID × 50 cm long ODS-3 column using the last mentioned eluant at 10 ml per minute. Fractions eluting between 24 and 27 minutes after each of 11 injections of *ca.* 5 mg each were combined and treated as above, yielding 54.2 mg dihydromevinolin which showed (at 200 nm) only one component by analytical HPLC.

Structure Determination

The tris-trimethylsilyl derivative of the compound showed a molecular ion peak at *m/z* 640 (*m/z* 667 for the tris-$^2H_9$-TMS analog) and characteristic fragment ion peaks at *m/z* 625, 550, 538, 535, 523, 460, 448, 445, 433, 358 and 343 representing the losses of methylbutyric acid and two moieties of trimethylsilanol. The underivatized product, recrystallized from acetonitrile (m.p. 131 ~ 132°C; $[\alpha]_D^{25}$ +148.6° (*c* 0.52, CH_3CN)), showed a molecular ion peak at *m/z* 406.2706 corresponding to the elemental composition $C_{24}H_{38}O_5$ (calcd. 406.2719) which was confirmed by combustion analysis (calcd.: C, 70.90; H, 9.42. Found: C, 70.87; H, 9.45%). An abundant fragment of *m/z* 304 is again attributed to the loss of methylbutyric acid in a MACLAFFERTY rearrangement. Most of the remaining fragmentation pattern is analogous to that described earlier[6] for mevinolin. Ultraviolet spectra show maximum absorption at 205 nm. Infrared spectra ($CHCl_3$) show broad ester and lactone carbonyl absorption at 1715 cm^{-1}. ^1H NMR resonances, which are listed in Table 1, establish the structure as that of 4a,5-dihydromevinolin with *trans*-fused rings (Fig. 1).

Fig. 1. Structure and conformation of 4a,5-dihydromevinolin.



Resonances previously[6]* assigned to the δ-lactone and 2-methylbutyryl ester moieties of mevinolin are clearly present and identified in Table 1. Irradiation of the olefinic signal at δ 5.65 (H3) collapses the H4 doublet at δ 5.38 to a broad singlet, sharpens the multiplet for H2 at δ 2.30 and establishes the allylic coupling of ~2.5 Hz to H4a at δ 2.48. Irradiation of H4a or H8 collapses the doublet of triplets at δ 1.19 to a broad doublet (J=11 Hz) or a triplet (J=11 Hz), respectively. The signal may, therefore, be assigned to H8a. The large coupling of 11 Hz between H4a and H8a establishes the ring junction as *trans* with diaxial protons. A small (3 Hz) and large (12 Hz) coupling of H4a to the protons at C5 (at about δ 1.28 and 1.56) and small couplings (3 Hz) of both protons at C7 to H8 at δ 5.21 argue strongly in favor of a chair conformation for ring A with the ester group at C8 in the axial orientation. The position of the methyl group at C6 can be approached either from H4a or H8 and be shown to have the axial orientation by irradiation of the doublet at δ 1.08 which reduces the multiplet at δ 2.06 (H6) to a broad triplet (J=4 Hz).

Table 1. ^{1}H NMR assignments for dihydromevinolin.*

Assignment**	δ ppm (Hz)
$H\alpha_e$	2.59 ddd (1H, J=1.5, 4, 18)
$H\alpha_a$	2.72 dd (1H, J=5, 18)
$H\beta$	4.34 ddt (1H, J=4, 5, 4)
$H\gamma_e$	1.95 m (1H)
$H\gamma_a$	~1.64 obsc
$H\delta$	4.60 m (1H)
H1	~1.66 obsc
H2	2.30 m (1H)
C2-CH_3	0.83 d (3H, J=7)
H3	5.65 ddd (1H, J=2.5, 4.5, 10)
H4	5.38 brd (1H, J=10)
$H4_a$	2.48 vbrt (1H, $J\simeq$12)
$H5_a$	~1.28 obsc
$H5_e$	1.56 m (1H)
H6	2.06 m (1H)
C6-CH_3	1.08 d (3H, J=7)
$H7_a$	~1.66 obsc
$H7_e$	~1.66 obsc
H8	5.21 dt (1H, J=2.5)
$H8_a$	1.19 dt (1H, J=2.5, 11, 11)
H2′	2.34 m (1H)
C2′-CH_3	1.11 d (3H, J=7)
H3′	~1.48 obsc
	~1.66 obsc
H4′(CH_3)	0.90 t (3H, J=7)

* Spectra were recorded in $CDCl_3$ at 50°C; chemical shifts are in δ ppm downfield of internal TMS; coupling constants in Hertz are given in brackets. Abbreviations: s=singlet, d=doublet, t=triplet, m=multiplet, v=very, br=broad, obsc=obscured (overlapping signals).

** Subscripts a and e refer to axial and equatorial carbon-proton bonds, respectively.

Dreiding models reveal that, with ring A in a chair conformation, ring B can assume either a flattened half-chair as shown in Fig. 1, or a less stable boat conformation. The large coupling of 11 Hz between H1 and H8a as in mevinolin readily argues in favor of the half-chair conformation as also found in the solid state by X-ray diffraction (see below). Coupling between H4 and H4a is not immediately apparent as H4 appears as only a slightly broadened doublet at δ 5.38. Irradiation at this frequency, however, results in sharpening of the broad triplet for H4a at δ 2.48, which is consistent with a dihedral angle of close to 90° between the two protons. For the same reason an appreciable allylic coupling of 2.5 Hz is observed between H3 and H4a. In this conformation the coupling constant of 4.5 Hz between H2 and H3 is consistent with a pseudo-axial orientation of the methyl group at C2. This is confirmed by the narrow signal obtained for H2 at δ 2.30 on irradiation of the methyl doublet at δ 0.83 and by the lack of allylic coupling between H2 and H4. The chemical shifts

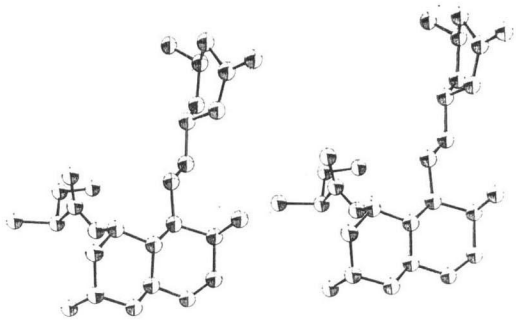
* Chemical Abstract rules require opposite directions of numbering carbons 1 to 8 in mevinolin and mevinolinic acid (open lactone) and related structures. The numbering system used in reference 6 strictly applies only to mevinolinic acid. Although in the present paper we discuss the structure of dihydromevinolin rather than dihydromevinolinic acid, we have retained the acid numbering system for clarity.

for H2 and the methyl group at C2 differ little from those observed for mevinolin. This evidence for the decalin ring system and the similar chemical shift and coupling constant data for the methylbutyryl and δ-lactone moieties argue strongly in favor of the same relative stereochemistry in dihydromevinolin as in mevinolin.

Vigorous alkaline hydrolysis followed by acidification and relactonization leads to the C8-alcohol whose ^{1}H NMR spectra lack signals for the methylbutyryl group and show the signal of H8 moved upfield to δ 4.16. The rate of ester hydrolysis is noticeably slower than for mevinolin, a fact which is most likely attributable to 1,3-diaxial interactions with the methyl group at C6 and the hydrogens at C1 and C4a. These interactions are in part relieved in mevinolin by the absence of H1 and the conformational change due to the 4a,5-double bond.

Single crystal X-ray diffraction studies confirmed the stereochemical and conformational assignments. Crystals of **1** from methanol were found to have $P2_12_12_1$ symmetry with a=10.358(1) Å, b=18.692(3) Å and c=24.937(3) Å for Z=8. Of the 3666 unique reflections measured, 2569 were observed ($I \geq 3\sigma$ I). The structure was solved by using a multi-solution tangent formula approach[10] and refined by using least squares techniques.[11] The final unweighted residual is 0.088. Fig. 2 gives a perspective drawing of dihydromevinolin[12]*. The absolute stereochemistry of all chiral centers of mevinolin has been determined previously[6].

Fig. 2. ORTEP View of dihydromevinolin.



Biological Activity

The inhibition of HMG-CoA reductase *in vitro* by dihydromevinolin and mevinolin, assayed as previously described,[6] is shown in Table 2. The concentration of dihydromevinolin required for 50% inhibition of the enzyme was calculated from the data to be 2.7 nM, compared to 2.0 nM for mevinolin.

Table 3 shows that both dihydromevinolin and mevinolin are potent inhibitors of sterol synthesis from ^{14}C-acetate in mouse L-M cells (grown as previously described[13]) with IC_{50} of 22.7 and 33.6 nM, respectively. At the concentration tested, neither fatty acid synthesis from ^{14}C-acetate nor sterol synthesis from ^{3}H-mevalonate was affected by either compound.

The inhibition of cholesterol synthesis in rats, measured as previously described,[6] is reported in Table 4. Dihydromevinolin and mevinolin both effectively inhibit cholesterol synthesis from ^{14}C-acetate in this acute assay.

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* Tables containing the atomic coordinates and temperature parameters can be obtained from the authors (J. P. S.).

Table 2. Effect of dihydromevinolin and mevinolin on rat liver HMG-CoA reductase activity.

Inhibitor (ng/ml)	Dihydromevinolin* (% of control)	Mevinolin* (% of control)
None	100	100
0.5	66.6	64.2
1.0	48.9	49.0
2.5	30.5	31.1
5.0		23.3
10.0	17.1	

* Added as the sodium salt.

Table 3. Effect of dihydromevinolin and mevinolin on desmosterol synthesis in L-M cells.*

Inhibitor (ng/ml)	Dihydromevinolin (% of control)**	Mevinolin (% of control)
0	100.0	100.0
0.5	99.8	91.4
1.0	82.1	95.1
2.0	69.8	81.6
5.0	57.3	63.1
10.0	40.1	48.3
100.0	19.7	24.1

* Grown as previously described[13] in serum and lipid-free medium. Cells were incubated in the presence or absence of different levels of inhibitors in 25 cm^2 Corning tissue culture flasks with ^{14}C-acetate and ^{3}H-mevalonate for 3 hours. The reaction was terminated by removal of the medium, washing with saline, and the addition of KOH. After removing an aliquot for protein determination the remainder of the sample was extracted with petroleum ether. Lipid fractions were isolated by TLC on silica gel using petroleum ether, diethyl ether, acetic acid (75 : 25 : 1).

** The sterol and fatty acid regions were scraped off and counted in a liquid scintillation counter. Percent inhibition figures are averages of two determinations each.

Table 4. Effect of dihydromevinolin and mevinolin on cholesterol synthesis in the rat.*

Inhibitor (ng/kg)	Cholesterol synthesis (% decrease)	
	Dihydromevinolin	Mevinolin
0.15	38	46
0.60	51	71
1.20	70	83

* Plasma cholesterol synthesis assayed as previously described[6] with ^{14}C-acetate. Rats were dosed with compound dissolved in 5% Emulphor in saliva by stomach tube. Control animals received vehicle. Each determination is based on an average decrease in the rate of synthesis in ten rats compared to the average synthesis rate in ten untreated animals. All decreases are highly significant ($P<0.001$).

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