L-669,262, A POTENT HMG-CoA REDUCTASE INHIBITOR

HENRY JOSHUA, MICHAEL S. SCHWARTZ and KENNETH E. WILSON

Merck Sharp & Dohme Research Laboratories, Inc.,
P. O. Box 2000, R80Y-340,
Rahway, N.J. 07065, U.S.A.

(Received for publication September 14, 1990)

The microbial transformation of simvastatin (MK-733) by *Nocardia autotrophica* subspecies *amethystina* yielded *iso*-simvastatin-6-one as a minor component. This transformation product is a dienone and is one of the more potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase found to date.

Simvastatin (**I**)[1] as well as its homologs mevinolin (**II**)[2] and compactin (**III**)[3] are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are useful as hypocholesteremic agents. The generation of a variety of products has been reported through the microbial hydroxylation of **I**[4], **II**[4,5] and **III**[5,6].

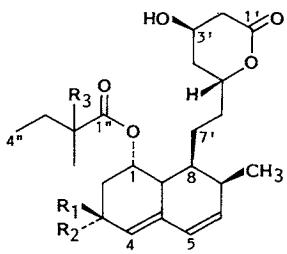
When the sodium salt of **I** in its hydroxy acid form was added to a growing culture of *Nocardia autotrophica* subspecies *amethystina* the corresponding salts of 3β-hydroxymethyl-3α-desmethylsimvastatin (**IV**) and 3β-carboxy-3α-desmethylsimvastatin (**V**) were produced[4]. Additional compounds isolated in low yields from this microbial transformation reaction were the salts of 3α-hydroxymethyl-3α-desmethylsimvastatin (**VI**)[4] and *iso*-simvastatin-6-one (**VII**)[7]. Compound **VII** showed increased HMG-CoA reductase inhibiting activity when compared to other compounds in this series.

This paper reports the isolation, characterization and some biological properties of compound **VII**.

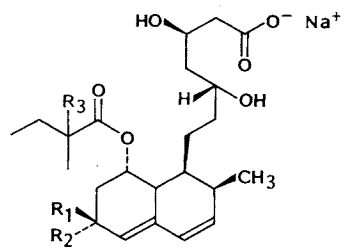
Results

Bioconversion and Isolation

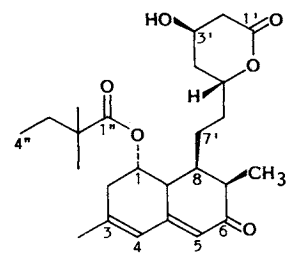
The microbial hydroxylation of simvastatin (**I**), sodium salt of the hydroxy acid form, with *N.*



Lactone



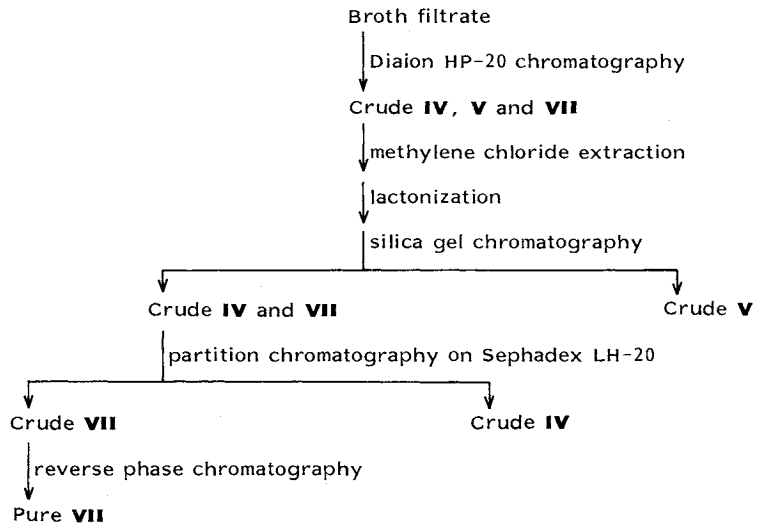
Hydroxy acid



L-669,262 (**VII**)

	R_1	R_2	R_3
Simvastatin (**I**)	H	CH_3	CH_3
Mevinolin (**II**)	H	CH_3	H
Compactin (**III**)	H	H	H
IV	CH_2OH	H	CH_3
V	COOH	H	CH_3
VI	H	CH_2OH	CH_3

Fig. 1. Isolation of L-669,262 (**VII**).



autotrophica subspecies *amethystina* (ATCC 35204) yielded a number of oxidation products (**IV**, **V**, and **VI**). An additional product which was isolated in low yield was *iso*-simvastatin-6-one (**VII**) which has a dienone moiety. The isolation of **VII**, which is shown schematically in Fig. 1 and which is described in greater detail in the Experimental section, involved adsorption and desorption on a resin column, extraction into organic solvent, lactonization, silica gel chromatography and partition chromatography on Sephadex LH-20 followed by preparative reverse phase chromatography. A total of 10 mg of pure **VII** (lactone) was isolated from a reaction starting with 400 g of **I**.

Structure of **VII** (Lactone)

The isolated bioconversion product **VII** exhibits a molecular ion by HRFAB-MS at *m/z* 432.2433, corresponding to a molecular formula of $C_{25}H_{36}O_6$ (calcd *m/z* 432.2511), and therefore has one additional oxygen and two fewer hydrogens than does simvastatin (**I**). EI-MS fragment ions at *m/z* (base) 316.1664 ($M^+ - C_6H_{11}O_2$, calcd *m/z* 316.1674) and at *m/z* 173.0962 ($M^+ - C_6H_{11}O_2 - C_7H_{11}O_3$, calcd *m/z* 173.0966), are consistent with losses of a 2,2-dimethylbutanoate residue and a (tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl)ethyl residue and strongly indicate that the structural differences are solely limited to the central bicyclic moiety. The UV spectrum of **VII** in methanol displays a single absorption maximum at 290 nm (ε 21,900). The MS and UV data together indicate that the diene moiety of **I** has been converted to a dienone. Supportive of this, the IR spectrum of **VII** displays strong C=O stretching bands at 1722 and 1660 cm^{-1} and strong C=C stretching bands at 1630 and 1596 cm^{-1}.

The structure of **VII** was established straightforwardly from ^{13}C NMR data and 1D and 2D (COSY, *J*-resolved) ^1H NMR experiments. Of particular interest, the proton spectrum in C_6D_6 exhibited a vinyl methyl group (br s) at 1.45 ppm and a methyl doublet (J=7 Hz) at 1.00 ppm, in addition to the methyls of the 2,2-dimethylbutanoyl side chain. The vinyl methyl is long-range coupled to an olefinic proton at 5.76 ppm (br s), which in turn shows long-range coupling to the methylene protons 2-H_A (*ca.* 2.10 ppm) and 2-H_B (2.23 ppm). This supports the locations of the vinylic proton and methyl in ring A as shown in **VII**. The carbonyl of the dienone is positioned at C-6 since the methyl doublet at 1.00 ppm is coupled to 7-H (2.69 ppm), which appears as a quartet of doublets (J=4 and 7 Hz) due to additional coupling only

to 8-H (*ca.* 2.05 ppm). The predicted UV λ_{max} of the dienone in **VII** is in agreement with the observed value.

Selected proton assignments relevant to the above structural discussion are listed in Table 1. Additional NMR data are summarized under Experimental.

Table 1. ^{1}H NMR (C_6D_6) assignments for L-669,262.

Proton	Chemical shift (δ), multiplicity[a]
1-H	5.29 q (2.5)
2-H_A	2.10 obscured
2-H_B	2.23 br dd (19, 2)
3-CH_3	1.45 br s
4-H	5.76 br s
5-H	5.93 br s
7-H	2.69 dq (7, 4)
7-CH_3	1.00 d (7)
8-H	~2.05 m
8a-H	2.15 m

[a] Spectra were recorded at 300 MHz and chemical shifts are reported with respect to TMS at 0 ppm, using the C_6D_6 solvent line at $\delta_H = 7.15$ ppm as internal reference. Coupling constants (J = Hz) are reported in parentheses.

Biological Properties

In one study it was found that compound **VII** was approximately six to seven times as active as compound **I** in inhibiting rat liver HMG-CoA reductase activity with IC_{50} values of 0.10 and 0.66 ng/ml, respectively. The IC_{50}'s in the HMG-CoA reductase assay were determined by the method of ALBERTS *et al.*[2].

Compound **VII** was also compared with compounds **I** and **II** in respect to their ability to inhibit cholesterol synthesis in the rat and to lower plasma cholesterol in the dog. In both of these systems compound **VII** was significantly more active than either **I** or **II** (Y. S. CHAO *et al.*; manuscript in preparation).

Discussion

The reverse phase HPLC analysis of a microbial hydroxylation reaction of **I** (UV λ_{max} nm 237) indicated the presence of a component **VII** that exhibited a ratio of inhibitory activity in the HMG-CoA reductase assay to UV absorbance (237 nm) much greater (~10×) than the ratio found for other compounds in this series.

Due to the very similar behavior of **IV** and **VII** in preparative chromatography on silica gel and reverse phase chromatography these two compounds could not be separated preparatively by these methods. Partition chromatography on Sephadex LH-20 using an eluent of hexane-toluene-methanol (3:1:1) afforded a separation of **IV** from **VII**. The latter compound was then further purified by preparative reverse phase HPLC.

The significant increase of HMG-CoA reductase inhibition on a weight basis of **VII** when compared to **I** was surprising for the following reasons: 6α-hydroxy-*iso* **II** was reported[5] to be less active than parent **II** and similarly 6-hydroxy-*iso* **III** was reported[8] to have no HMG-CoA reductase effect. Thus the rearrangement of the double bonds to the *iso* position and the hydroxylation of C-6 lowers or destroys the biological activity in this series. It was therefore very surprising to find that **VII**, which has a C-6-oxo group, has not reduced but superior HMG-CoA reductase inhibitory activity compared to **I**.

Experimental

HRFAB-MS data was obtained on the [M+Cs] ion on a Finnigan-MAT MAT90 spectrometer using CsI clusters as internal reference. HREI-MS data was obtained on a Finnigan-MAT MAT212 instrument using perfluorokerosene as internal reference. NMR results were obtained at 300 MHz for proton and at 75 MHz for carbon on a Varian XL300 spectrometer. Spectra were reported with respect to TMS at 0 ppm, using the solvent lines of perdeuterobenzene ($\delta_H = 7.15$ ppm) and dideutero-dichloromethane ($\delta_C = 53.8$ ppm) as internal reference. UV spectra were recorded on a Beckman Model DU70 spectrophotometer. IR spectra were recorded by multiple internal reflectance of sample films deposited on a ZnSe crystal using a Perkin-Elmer Model 1750 FT-IR spectrophotometer.

Fermentation

To a growing fermentation of *N. autotrophica* subspecies *amethystina* ATCC 35204, MA 6180 in 500 liter media containing glucose, Hycase (Sheffield), beef extract and corn steep liquor at 10, 2, 1 and 3 g/liter, respectively, was added 100 g **I** as the sodium salt of the hydroxy acid form. When **I** was depleted, as indicated by HPLC analysis in *ca.* 24 hours, an additional 100 g **I** was added. This was repeated to achieve a total addition of 400 g **I**.

Isolation

The filtered broth was charged to a 100-liter Diaion HP-20 column and the resin was eluted successively with 400 liters each of 60% and 90% aqueous methanol taking 20 liter fractions. Fractions 7 through 9 from the 90% aqueous methanol elution contained a mixture of bioconversion products **IV**, **V** and **VII** in their hydroxy acid forms. The pooled fractions were concentrated in a rotary evaporator to 1.4 liters. The aqueous concentrate was acidified to pH 3.5 with 1 N H_3PO_4 and extracted twice with methylene chloride (1.4- and 0.4-liter, respectively). The combined methylene chloride extract was concentrated to dryness and the residue was taken up in 1 liter methyl isobutyl ketone. One hundred μl of TFA was added and the mixture refluxed for 80 minutes causing lactonization of the bioconversion products.

Solvent was removed from the lactonization reaction mixture and the residue taken up in 200 ml methylene chloride. The methylene chloride solution was charged to a column of 2.5 kg silica gel (EM Science SX 0143U-3) which had been slurry packed and equilibrated in methylene chloride. The column was eluted with 20 liter portions of methylene chloride, methylene chloride - ethyl acetate (8 : 2), methylene chloride - ethyl acetate (6 : 4), methylene chloride - ethyl acetate (4 : 6), and ethyl acetate - acetic acid (99 : 1). Each eluate was collected in 6 approximately equal fractions. Compound **IV** (lactone form, 30 g by HPLC) eluted in fractions 2 ~ 6 of the 4 : 6, methylene chloride - ethyl acetate elution with some tailing into the 99 : 1, ethyl acetate - acetic acid eluate. Compound **VII** co-eluted with **IV**. Compound **V** (lactone form, 11 g) eluted in fractions 3 ~ 6 of the 99 : 1, ethyl acetate - acetic acid eluent. Solvent was removed from the pooled heart cuts containing **IV** and **VII** *i.e.* fractions 3 and 4 of the 4 : 6, methylene chloride - ethyl acetate elution, leaving 12.3 g residue. This residue was dissolved in a mixture of 50 ml toluene and 5 ml methanol and charged to a column containing 6 liters Sephadex LH-20 equilibrated with 3 : 1 : 1, hexane - toluene - methanol. The column was eluted at 100 ml/minute with the 3 : 1 : 1 mixture. A forecut of 3 liters was collected followed by 20 cuts of 300 ml each. The eluent was then changed to 2 : 1 : 1, hexane - toluene - methanol and elution continued collecting 300 ml cuts.

Analysis of the cuts by HPLC at 237 nm and 293 nm showed that compound **IV** (lactone) eluted in cuts 18 ~ 30 and that compound **VII** (lactone) eluted in cuts 11 ~ 17. Solvent was removed from 200 ml of cut No. 14 leaving 67 mg residue. This residue was triturated with 1 ml acetonitrile and the soluble components charged to a 21.4 mm i.d. × 25 cm C18 column (Rainin Dynamax C18 cat No. 83-221-c) and eluted at 10 ml/minute with 58 : 42, water - acetonitrile taking 1.5 minute fractions.

Enzymatic analysis of the cuts indicated that the area of greatest HMG-CoA reductase inhibition centered on cut 21. Cut 21 was concentrated leaving 10 mg of residue consisting of pure **VII**.

^{1}H NMR of **VII** (C_6D_6): δ 5.93 (1H, br s, 5-H), 5.76 (1H, br s, 4-H), 5.29 (1H, q, J=2.5 Hz, 1-H), 4.56 (1H, m, 5'-H), 3.84 (1H, quintet, J=4 Hz, 3'-H), 2.69 (1H, qd, $J_{7,7\text{-}CH_3}$=7.5 Hz, $J_{7,8}$=4 Hz, 7-H), 2.51 (1H, br d, $J_{2'A,2'B}$=17 Hz, 2'-H_B), 2.35 (1H, dd, $J_{2'A,2'B}$=17 Hz, $J_{2'A,3'}$=4.5 Hz, 2'-H_A), 2.23 (1H, br dd, $J_{2A,2B}$=19 Hz, $J_{1,2B}$=2 Hz, 2-H_B), 2.15 (1H, m, partially obscured, 8a-H), 2.10 (1H, obscured, 2-H_A), 2.05 (1H, m, 8-H), *ca.* 1.58 (1H, obscured, 4'-H_B), 1.30 ~ 1.44 (2H, m, 3''-CH_2), 1.45 (3H, br s, 3-CH_3), 1.21 (1H, ddd, $J_{4'A,4'B}$=14 Hz, $J_{4'A,5'}$=11 Hz, $J_{3',4'A}$=2.5 Hz, 4'-H_A), 1.05 (6H, s, 2''-$(CH_3)_2$), 1.00 (3H, d, $J_{7,7\text{-}CH_3}$=7 Hz, 7-CH_3), 0.72 (3H, t, $J_{3'',4''}$=7.5 Hz, 4''-CH_3).

^{13}C NMR of **VII** (CD_2Cl_2): δ 203.4, 177.6, 170.2, 154.9, 144.3, 124.5, 123.1, 76.0, 67.0, 63.1, 43.3, 42.7, 39.6, 39.0, 37.7, 36.8, 36.5, 33.4, 32.9, 24.9, 24.4, 24.3, 24.1, 10.6, 9.4.

Acknowledgments

We thank Dr. DAVID R. HOUCK for his initial observations, fermentation and analytical work, Dr. JERRY LIESCH and Mr. JACK L. SMITH for mass spectroscopic analysis and Dr. K. GBEWONYO, Ms. S. HONEYCUTT, Mr. J. J. KING

and Miss L. ZITANO for large scale fermentation support. We especially appreciate the encouragement given to us by Mr. A. W. ALBERTS.

References

1) HOFFMAN, W. F.; A. W. ALBERTS, P. S. ANDERSON, J. S. CHEN, R. L. SMITH & A. K. WILLARD: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. 4. Sidechain ester derivatives of mevinolin. J. Med. Chem. 29: 849~852, 1986
2) ALBERTS, A. W.; J. CHEN, G. KURON, V. HUNT, J. HUFF, C. HOFFMAN, J. ROTHROCK, M. LOPEZ, H. JOSHUA, E. HARRIS, A. PATCHETT, R. MONAGHAN, S. CURRIE, E. STAPLEY, G. ALBERS-SCHONBERG, O. HENSENS, J. HIRSCHFIELD, K. HOOGSTEEN, J. LIESCH & J. SPRINGER: Mevinolin: A highly-potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc. Natl. Acad. Sci. U.S.A. 77: 3957~3961, 1980
3) ENDO, A.; M. KURODA & Y. TSUJITA: ML-236A, ML-236B and ML-236C, new inhibitors of cholesterogenesis produced by *Penicillium citrinum*. J. Antibiotics 29: 1346~1348, 1976
4) INAMINE, E. S.; D. R. HOUCK, O. D. HENSENS, W. HALCZENKO, G. G. HARTMAN & R. L. SMITH (Merck): Novel HMG-CoA reductase inhibitors. Eur. Pat. Appl. 0 251 625, Jan. 7, 1988
5) SERIZAWA, N.; K. NAKAGAWA, K. HAMANO, Y. TSUJITA, A. TERAHARA & H. KUWANO: Microbial hydroxylation of ML-236B (compactin) and monacolin K (MB-530B). J. Antibiotics 36: 604~607, 1983
6) SERIZAWA, N.; K. NAKAGAWA, Y. TSUJITA, A. TERAHARA & H. KUWANO: 3α-Hydroxy-ML-236B (3α-hydroxy-compactin), microbial transformation product of ML-236B (compactin). J. Antibiotics 36: 608~610, 1983
7) JOSHUA, H.; K. E. WILSON, M. S. SCHWARTZ, T. J. LEE & G. E. STOKKER (Merck): 3-Keto HMG-CoA reductase inhibitors. U.S. 4,968,693, Nov. 6, 1990
8) PAN, H. Y.; D. EVERETT, S. M. SINGHVI, B. M. FRANTZ & D. A. WILLARD: Biotransformation profiles of pravastatin in man. Abstracts of Papers of National Meeting of American Federation for Clinical Research, Clinical Res. 37: 341-A, 1989