METABOLISM OF CERIVASTATIN BY HUMAN LIVER MICROSONES IN VITRO

Characterization of Primary Metabolic Pathways and of Cytochrome P450 Isozymes involved

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ABSTRACT:

Biotransformation of cerivastatin, a new cholesterol-lowering drug, by human liver microsomes was investigated using the 14C-labeled drug. Metabolite profiles were established by HPLC and structures of metabolites were elucidated. Two metabolic pathways were equally important, demethylation of the benzylic methyl ether and hydroxylation at one methyl group of the 6-isopropyl substituent. The product of combined hydroxylation and demethylation was observed as a minor metabolite. During sample preparation the lactone forms of both primary metabolites were isolated in small amounts. Detailed structural analysis by NMR and LC-ESI-MS showed that hydroxylation occurred with high regio- and stereoselectivity. The proposed structures were confirmed by chemical synthesis of enantiomerically pure reference compounds. Microsomes from a human lymphoblastoid AhH-1 cell line, stably expressing CYP 3A4, catalyzed the demethylation reaction. Upon incubation of cerivastatin with human liver microsomes in the presence of the specific CYP 3A inhibitor TAO, both hydroxylation and demethylation were considerably reduced. This indicates that CYP 3A enzymes play a major role in cerivastatin metabolism.

Cerivastatin, sodium (E)-(+-)(3R,5S)-7-[4-(4-fluorophenyl)-2,6-disopropyl-5-(methoxymethyl)-pyrid-3-yl]-3,5-dihydroxyhept-6-enoate (BAY w 6228), is a highly potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA)1 reductase (EC 1.1.1.34) and is currently under clinical development for the treatment of hypercholesterolemia (1).

This paper describes detailed structural analysis as well as independent chemical synthesis of cerivastatin metabolites formed in vitro by human liver microsomes. Furthermore, cytochrome P450 3A4 (CYP3A4)1 is identified as a primary enzyme involved in oxidative cerivastatin metabolism.

Materials and Methods

Radiolabeled Compound and Reagents. For in vitro metabolism studies, sodium (E)-(+-)(3R,5S)-7-[4-(4-fluorophenyl)-2,6-disopropyl-5-(methoxymethyl)-pyrid-3-yl]-3,5-dihydroxy-[7,14C]hept-6-enoate (114C)cerivastatin1 with a specific activity of 2.2 MBq/mg was used. The radiochemical purity was at least 95% when determined by HPLC.

All reagents and chemicals used for analytical or HPLC grade and purchased from E. Merck, Darmstadt, Germany. All reagents and chemicals used for chemical synthesis were of synthetic grade and, with the exception of potassium monomethyl malonate (Fluka), methyl isobutanoylacetate (Wacker Chemie), and (R)-5-carbomethoxy-4-tert.butyldimethylsilyloxy-2-oxopentylphosphonate 9 (Ube Industries Ltd.), purchased from Aldrich-Chemie, Steinheim, Germany.

Chemical Syntheses. The synthesis of desmethylcerivastatin (metabolite M-1 reference) has been described previously [2]. Metabolite M-23 reference was synthesized as outlined in scheme 1. Owing to partial racemization, which is supposed to occur during formation of the TBDPS-oxypentanoate precursor 2, all following intermediates contained the corresponding diastereomers (structures not shown). Separation was performed on the last but one step and the enantio-merically pure ester 12 was subsequently hydrolyzed to the final product. The synthesis of M-24 reference followed the same synthetic strategy, the TBDMS-group was used for the protection of the 5-hydroxymethyl function (scheme 2).

(R)-3-t-Butyldiphenylsilyloxy-2-methylpropionic acid 1. In a 40-liter vessel equipped with a stirrer, 2667.0 g (22.60 mol) methyl (R)-(+-)-3-hydroxy-2-methylpropionate (EGA, Steinheim, Germany) was dissolved in 14 liters DMF p.a. After the addition of 6768.8 g (24.65 mol) TBDPSCI, 3376.4 g (49.65 mol) imidazole, and 10 g 4-dimethylaminopyridine, the reaction temperature rose to 45°C. The reaction mixture was stirred with cooling to room temperature for 16 hr until the reaction was complete. The mixture was then poured into 75 liters water and washed with 2 × 20 liters EtOAc and the combined organic phases were washed with 2 × 10 liters water, dried over Na2SO4, and concentrated, yielding 8928 g (110%) of a crude oil of methyl (R)-3-t-butyldiphenylsilyloxy-2-methylpropionate.

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\[ H-NMR \ (DMSO-d_6): \ \delta = 0.98 \ (s, \ 9H, \ t-Bu), \ 1.10 \ (d, \ 3H, \ CH_3), \ 2.74 \ (m, \ 1H, \ CH), \ 3.64 \ (s, \ 3H, \ OCH_3), \ 3.78 \ (d, \ 2H, \ OCH_2), \ 7.47 \ (m, \ 6H, \ Ar), \ 7.61 \ (m, \ 4H, \ Ar) \ ppm. \]

A solution of 4464 g (11.3 mol) of this crude product in 27.5 liters THF was heated under reflux (internal temperature 65°C) with 5.65 liters (11.3 mol) 2 N NaOH for 46 hr in a 40-liter vessel equipped with a stirrer. The THF was removed by distillation on a rotary evaporator and the residue was diluted with 5 liters water and 3 liters CH₂Cl₂ and adjusted to pH 4 with 15% HCl. The phases were separated, the aqueous phase was washed with 3 liters CH₂Cl₂, and the combined organic phases were dried over Na₂SO₄ and concentrated to an oil, yielding 3930 g (100%) of crude 1.

\[ H-NMR \ (DMSO-d_6): \ \delta = 1.00 \ (s, \ 9H, \ t-Bu), \ 1.08 \ (d, \ 3H, \ CH_3), \ 2.60 \ (m, \ 1H, \ CH), \ 3.74 \ (m, \ 2H, \ OCH_2), \ 7.43 \ (m, \ 6H, \ Ar), \ 7.61 \ (m, \ 4H, \ Ar), \ 12.26 \ (s, \ 1H, \ COOH) \ ppm. \]

Methyl (R)-5-t-butyldiphenylsilyloxy-4-methyl-3-oxopentanoate 2.

To a solution of 1927.5 g (3.77 mol) 1 (67%) in 13 liters THF 744.4 g (4.59 mol) N,N′-carbonyldiimidazole was added at room temperature. The reaction mixture was stirred for 1 hr at room temperature and for 1 hr under reflux. After cooling to room temperature this solution A was used without further purification in the next step.

In a 40-liter vessel equipped with a stirrer 1258.3 g (8.06 mol) of potassium monomethyl malonate was suspended in 12.4 liters CH₃CN at 0°C. After the addition of 1124.5 ml (8.06 mol) triethylamine and

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**SCHEME 1.** Synthesis of metabolite M-23 reference.

Reagents: (a) TBDPSCl; (b) NaOH; (c) N,N carbonyldiimidazole; (d) CH₃OOCCH₂COOK, MgCl₂, Et₃N; (e) NaOCH₃; (f) CH₃COONH₄, CH₃COOH; (g) ammonium cerium (IV)nitrate; (h) DIBAL; (i) NaH, CH₃J; (k) LiAlH₄; (l) Al₂O₃ PCC; (m) K₂CO₃; (n) HCl; (o) Et₃B, NaBH₄; (p) chromatography; (q) NaOH.
847.1 g (8.92 mol) anhydrous MgCl₂, the mixture was stirred for 5 hr at room temperature. The reaction solution was added with 10 liters water, and the mixture was stirred for 16 hr at room temperature, diluted with 20 liters EtOAc, and adjusted to pH 4 with 15% HCl. The organic phase was separated, washed with 3 × 1.5 liters EtOAc, and neutralized by washing with 2 × 3 liters saturated NaHCO₃ solution, dried over Na₂SO₄, and concentrated to an oil, giving 2.015 kg (72%) of 4 as a mixture of 2 diastereomers.

1 H-NMR (CDCl₃): δ = 0.94 –1.35 (several d, 9H, CH₃), 1.13 (several s, 9H, t-Bu), 3.59, 3.63, 3.64, 3.67 (4s, 6H, 2 OCH₃), 3.73 – 4.30 (complex region, 4H, CH, OCH₂), 4.99 and 5.03 (2s, 1H, H₄), 6.80 –7.78 (complex region, 14H, Ar) ppm.

A solution of 561 g (0.51 mol) of crude 5 (purity 57%) in 1.7 liters toluene p.a. was processed with 2.5 liters of MeOH and treated in portions with 104 g (1.92 mol) NaOCH₃, which lead to slight warming. The mixture was stirred at room temperature for 1.5 hr (HPLC monitoring), treated with 480 g (6.25 mol) ammonium acetate, and 1.65 liters of 10% KI solution, washed with 3 × 3 liters EtOAc, and the combined organic phases were washed with 5 liters water, dried over Na₂SO₄, and concentrated to an oil, giving 2.015 kg (72%) of 4 as a mixture of 2 diastereomers.

1 H-NMR (CDCl₃): δ = 0.94 –1.35 (several d, 9H, CH₃), 1.13 (several s, 9H, t-Bu), 3.59, 3.63, 3.64, 3.67 (4s, 6H, 2 OCH₃), 3.73 – 4.30 (complex region, 4H, CH, OCH₂), 4.99 and 5.03 (2s, 1H, H₄), 6.80 –7.78 (complex region, 14H, Ar) ppm.

Methyl 6-[(S)-(t-butyldiphenylsilyloxymethyl)ethyl]-4-(4-fluorophenyl)-5-hydroxymethyl-2-isopropylpyridine-3-carboxylate 6. A solution of 561 g (0.51 mol) of crude 5 (purity 57%) in 1.7 liters toluene p.a. was processed with 2.5 liters of MeOH and treated in portions with 104 g (1.92 mol) NaOCH₃, which lead to slight warming. The mixture was stirred at room temperature for 1.5 hr (HPLC monitoring), treated with 480 g (6.25 mol) ammonium acetate, and 1.65 liters of 10% KI solution, washed with 3 × 3 liters EtOAc, and the combined organic phases were washed with 5 liters water, dried over Na₂SO₄, and concentrated to an oil, giving 2.015 kg (72%) of 4 as a mixture of 2 diastereomers.

1 H-NMR (CDCl₃): δ = 0.94 –1.35 (several d, 9H, CH₃), 1.13 (several s, 9H, t-Bu), 3.59, 3.63, 3.64, 3.67 (4s, 6H, 2 OCH₃), 3.73 – 4.30 (complex region, 4H, CH, OCH₂), 4.99 and 5.03 (2s, 1H, H₄), 6.80 –7.78 (complex region, 14H, Ar) ppm.
THF was heated to boiling and a solution of 125 g (0.209 mol) of a suspension of 9.35 g (0.313 mol) NaH (80%) in 500 ml anhydrous (4-fluorophenyl)-2-isopropyl-5-methoxymethylpyridine was added and the precipitate was removed by filtration under a water atmosphere and 3 concentrated HCl was added. The solution was then extracted with 3 EtOAc, and passed through kieselguhr under suction to remove flocculations, which hindered the phase separation. The combined organic phases were washed with 2 saturated brine, dried over NaSO4, and concentrated in vacuo. The residue was chromatographed on silica with PE/EtOAc 9:1 and 8:2, affording 124.9 g (43%) of 6 as an oil.

1-H-NMR (CDCl3): δ = 0.95 (s, 9H, t-Bu), 1.17 (d, 3H, CH3), 1.25 (2d, 6H, CH3), 1.30 (d, 3H, CH3), 1.35 (sept., 1H, CH), 1.35 (3H, OCH3), 1.38–1.40 (m, 2H, CH2), 1.42 (d, 3H, CH3), 2.48 (2H, CH2), 2.51 (m, 2H, CH2), 3.18 (3H, OCH3), 3.32 (m, 2H, CH2OH), 3.69 (s, 3H, OCH3), 3.88 (d, 2H, CH2), and 4.35 (1H, CH2OSi) ppm.

Methyl (E)-(3R,5S)-7-{4-(4-fluorophenyl)-6-(S)-(hydroxymethyl)-2-isopropyl-5-methoxymethylpyridin-3-y}-3-hydroxy-5-oxohept-6-enoate. A mixture of 600 ml anhydrous THF, 240 ml anhydrous methanol and 130 ml 1 N HCl was stirred for 4 days at room temperature (TLC monitoring). Then 1000 ml CH2Cl2 was added and the solution was washed with 2 × 500 ml saturated NaHCO3 solution. The organic phase was dried over Na2SO4 and concentrated in vacuo. The residue was chromatographed on silica with EtOAc/PE 1:1 to afford 59.2 g (91%) of 11.

1-H-NMR (CDCl3): δ = 1.28 (2d, 6H, CH3), 1.42 (d, 3H, CH3), 2.48 (2H, CH2), 2.61 (m, 2H, CH2), 3.20 (s, 3H, OCH3), 3.28 (sept., 1H, CH), 3.32 (m, 2H, CH2OH), 3.71 (3H, OCH3), 3.88 (m, 1H, CH2-CHO), 4.0 – 4.2 (m, 2H, CH2-O), 4.41 (m, 1H, CH-OH), 5.90 (d, 1H, =CH), 7.0 – 7.2 (4H, Ar), 7.45 (d, 1H, =CH) ppm.

Methyl (E)-(3R)-7-(4-(fluorophenyl)-6-(S)-(4-fluorophenyl)-2-isopropyl-5-methoxymethylpyridin-3-y)-3-oxopentylphosphonate. A mixture of 82.5 g (0.12 mol) 10 in 1170 ml anhydrous methanol and 243.2 ml (0.24 mol) of a 1 M Et3B solution in THF was stirred for 1 hr at room temperature. After cooling to −75°C (internal temperature, acetone/dry ice bath), 59.2 g (0.12 mol) 11 dissolved in 150 ml anhydrous THF was added. After 30 min at −75°C, 6.9 g (0.18 mol) NaBH4 was added in portions and stirring was continued for further 3 hr at −75°C. The cooling bath was removed and 100 ml of saturated NH4Cl solution was added dropwise at 0°C. Then 700 ml water and 500 ml EtOAc were added and the aqueous phase was separated and extracted with 2 × 200 ml EtOAc. The combined organic phases were washed with 400 ml saturated brine, dried over Na2SO4, and concentrated in vacuo. The residue was dissolved in 500 ml methanol and concentrated again on the rotary evaporator. The procedure was repeated 5 times and the residue was finally chromatographed on silica with EtOAc/PE 1:1. The fractions containing the product were concentrated to give 50.5 g crude product, which was chromatographed again on silica yielding 33.9 g (58%) of 12 as a mixture of diastereomers (de = 59%, HPLC).

1-H-NMR (CDCl3): δ = 1.25 (2d, 6H, CH3), 1.40 (m, 2H, CH2), 1.43 (3H, CH3), 2.41 (m, 2H, CH2), 3.18 (s, 3H, OCH3), 3.2 – 3.4 (2H, CH, and CH2-CHO), 3.71 (s, 3H, OCH3), 3.85 (m, 2H, CH2-CHO), 4.0 – 4.2 (m, 3H, CH2-OH, and CH-OH), 4.32 (m, 1H, CH-OH), 5.28 (dd, 1H, =CH), 6.31 (d, 1H, =CH), 7.0 – 7.2 (4H, Ar) ppm.

For isolation of the pure diastereomer by preparative chiral HPLC, 30 g of this mixture was dissolved in 160 ml ethanol and diluted with 640 ml n-heptane. A total of 940 portions of 0.8 ml (30 mg) each were injected onto the HPLC column every 15 min with an autoinjector, and 13 fractions were collected with the aid of a fraction collector.
<table>
<thead>
<tr>
<th>Protons</th>
<th>Cerivastatin</th>
<th>M-1</th>
<th>M-23</th>
<th>M-24</th>
</tr>
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<tr>
<td></td>
<td>δ (ppm)</td>
<td>Integral</td>
<td>Multiplicity</td>
<td>Coupling Const. (Hz)</td>
</tr>
<tr>
<td>CH₃ (at CH-C2')</td>
<td>1.24</td>
<td>6</td>
<td>d</td>
<td>6.7</td>
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<tr>
<td>CH₃ (at CH-C6')</td>
<td>1.29</td>
<td>6</td>
<td>d</td>
<td>6.7</td>
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<tr>
<td>CH₂-OH (at CH-C6')</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CH (at C2')</td>
<td>3.36</td>
<td>1</td>
<td>septet</td>
<td>6.7</td>
</tr>
<tr>
<td>CH (at C6')</td>
<td>3.42</td>
<td>1</td>
<td>septet</td>
<td>6.7</td>
</tr>
<tr>
<td>CH₂-OCH₃ (at C5')</td>
<td>4.08</td>
<td>2</td>
<td>AB</td>
<td>10.3</td>
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<tr>
<td>H-2a</td>
<td>2.26</td>
<td>1</td>
<td>dd</td>
<td>15.3; 4.4</td>
</tr>
<tr>
<td>H-2b</td>
<td>2.17</td>
<td>1</td>
<td>dd</td>
<td>15.3; 8.0</td>
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<tr>
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<td>3.74</td>
<td>1</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>H-4a</td>
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<td>1</td>
<td>ddd</td>
<td>13.8; 9.1; 7.2</td>
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<td>H-4b</td>
<td>1.27</td>
<td>1</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>H-5</td>
<td>4.17</td>
<td>1</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>H-6</td>
<td>5.33</td>
<td>1</td>
<td>dd</td>
<td>16.1; 6.2</td>
</tr>
<tr>
<td>H-7</td>
<td>6.29</td>
<td>1</td>
<td>dd</td>
<td>16.1; 1.2</td>
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<tr>
<td>Aromatic</td>
<td>7.10–7.18</td>
<td>4</td>
<td>m</td>
<td>—</td>
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</table>

*a* Solvent methanol-d₄, CD₃OD calibrated at δ = 3.30 ppm.

*b* Numbering used as assigned in scheme 3.

*c* Centered at.
with peak/time control. After a purity check of these fractions by HPLC, fractions 1–6 (pure diastereomer 12) and 7–8 (mixture of diastereomers) were combined accordingly. The solvent was removed by distillation in vacuo. The mixed fractions were again separated analogously.

Finally 16.9 g pure 12 (de = 99.2%, 76% yield as referred to crude 12) were obtained.

To remove colored impurities, 16.9 g separated 12 was chromatographed on silica with EtOAc/PE 1:1, yielding 15.9 g 12 as colorless oil.

Sodium (E)-7-{(S)-[t-butyldiphenylsilyloxy(methyl)ethyl]-2-isopropyl-5-methoxymethylpyrid-3-yl}-3,5-dihydroxyhept-6-enoate 13. To a solution of 10.6 g (21.7 mmol) 12 in 150 ml THF, 238.5 ml of a 0.1 N NaOH solution was added at room temperature. After 1 hr at room temperature, the THF was removed on a rotary evaporator and the aqueous residue was freeze dried, affording 10.7 g of the residue which was triturated with sufficient CH₂Cl₂. The filtrate was concentrated in vacuo and dried, yielding 26.56 g (79.5%) of the title compound as an oil.

¹H-NMR (CDCl₃): δ = 0.5 (s, 3H, CH₂Si), -0.1 (s, 3H, CH₃Si), 0.87 and 0.89 (2s, 18H, t-Bu), 1.2 - 1.3 (3d, 6H, CH₂), 3.51 (s, 3H, OCH₃), 3.51 – 3.6 (m, 1H, CH₂OSi), 3.6 - 3.75 (m, 1H, C₂H₅O), 3.8 – 4.6 (m, 1H, C₂H₅O), 4.52 (dd, 1H, CH₂OSi), 4.73 (d, 1H, CHOSi), 5.90 (d, 1H, CHO) ppm.

Methyl (E)-(3R)-7-{[(S)-(hydroxymethyl)ethyl]-2-isopropyl-5-hydroxymethyl-2-isopropyl-pyrid-3-yl}-3,5-dihydroxyhept-6-enoate 15. A solution of 2.4 g (6.38 mmol) dimethyl (R)-5-carbomethoxy-4-t-butyldimethylsilyloxy-2-oxypropylphosphonate 9, 0.875 g (6.33 mmol) K₂CO₃ and 0.09 ml water in 18.8 ml isopropanol was stirred for 1 hr at room temperature. Then 3.3 g (8.83 mmol) aldehyde (previous step) suspended in 18.8 ml isopropanol was added. After stirring for 5 days at room temperature (TLC monitoring), 100 ml water was added and the reaction mixture was extracted with 3 × 100 ml EtOAc. The combined EtOAc phases were washed with saturated brine, dried over Na₂SO₄, and concentrated in vacuo. The residue was chromatographed on silica with EtOAc/PE 5:95, giving 3.6 g (79.4%) of 15.

¹H-NMR (CDCl₃): δ = 0.5, 0.1, and 0.2 (3s, 12H, CH₂Si), 0.87, and 0.9 (3s, 27H, t-Bu), 1.15 - 1.35 (m, 9H, 3CH₃), 2.42 (m, 2H, CH₂), 2.6 (d, 2H, CH₂), 3.28 (sept., 1H, CH), 3.65 (m, 3H, OCH₃), 3.8 - 4.2 (m, 2H, CH₂O, and 1H, CH₂OSi), 4.52 (dd, 1H, CH₂OsI), 4.73 (d, 1H, CHOSI), 5.90 (d, 1H, =CH), 6.9 – 7.65 (m, 14H, Ar, and 1H, =CH) ppm.

Methyl (E)-(3R)-7-{[(S)-(fluoroaryl)ethyl]-2-isopropyl-5-hydroxymethyl-2-isopropyl-3,5-dihydroxyhept-6-enoate 16. A solution of 3.6 g (3.8 mmol) 15 in 63 ml anhydrous methanol and 7 ml 1N HCl was stirred for 5 days at room temperature (TLC monitoring). Then 100 ml CH₂Cl₂ was added and the solution was washed with 2 × 100 ml saturated NaHCO₃ solution. The organic phase was dried over Na₂SO₄ and concentrated in vacuo. The residue was chromatographed on silica with EtOAc/PE 6:4 to afford 1.4 g (77.9%) of 16.

¹H-NMR (CDCl₃): δ = 1.2 - 1.45 (3d, 9H, CH₃), 2.48 (m, 2H, CH₂), 2.6 (m, 2H, CH₂), 3.2 - 3.6 (m, 1H, CH₂), 3.7 (s, 3H, OCH₃), 3.8 - 4.6 (m, 1H, CH₂CH₂), 4.2 (m, 2H, CH₂O, and 1H, CH₂OSi), 5.92 (d, 1H, =CH), 7.0 – 7.4 (m, 4H, Ar, and 1H, =CH) ppm.

Methyl (E)-(3R,5S)-7-{[(S)-(fluoroaryl)ethyl]-5-hydroxymethyl-2-isopropyl-3,5-dihydroxyhept-6-enoate 17. A mixture of 24 mg anhydrous THF, 6 ml anhydrous methanol, and 5.9 ml (5.9 mmol) of a 1 M Et₄NBr solution in THF was stirred for 1 hr at room temperature. After cooling to −75°C (internal temperature, acetone/dry ice bath), 1.4 g (2.96 mmol) 16, dissolved in 20 ml anhydrous THF was added. After 30 min at −75°C, 168 mg (4.44 mmol) NaBH₄ was added in portions and stirring was continued for a further 3 hr at −75°C. The cooling bath was removed and 100 ml of saturated NH₄Cl solution was added dropwise at 0°C. Then 100 ml water and 100 ml EtOAc were added and the aqueous phase was separated and extracted with 2 × 100 ml EtOAc. The combined organic phases were washed with 50 ml saturated brine, dried over Na₂SO₄, and concentrated in vacuo. The residue was dissolved in 100 ml methanol and concentrated again on the rotary evaporator. This procedure was repeated 5 times and the residue was finally chromatographed on silica with EtOAc/PE 6:4 yielding 1.16 g (82.5%) of the crude title compound as a mixture of diastereomers (de = 59%, HPLC).
Ten µg [14C]cerivastatin was incubated for 3 hr at 37°C with 0.5 mg microsomal protein in 1 ml HEPES buffer, supplemented with a NADH and NADPH generating system as described above. An incubation with control microsomes (native AH-1 cells) was performed in parallel. The reaction was initiated by adding ice cold microsomes to prewarmed buffer, containing substrate and the cofactors. After initial mixing of the microsomes, samples were taken at 0, 0.5, 1, 2, and 3 hr (1 ml per sample) and further processed as described above.

**Chromatographic Methods.** Analytical HPLC was performed on a HP 1090 M liquid chromatograph with diode array detection (Hewlett-Packard, Waldbronn, Germany) and online radioactivity monitor Ramona®5 (Raytest, Straubenhardt, Germany), connected via A/D converter that transformed DPM values into mV units.

For metabolite profiling of microsomal incubates a 5 µm LiChrospher® RP8 column (250 × 4 mm) was eluted with a step gradient from 70% solvent A (0.1% aqueous TBAH, adjusted to pH 5 with 0.1% aqueous sodium hydroxide) to 100% solvent B (20% solvent A, 80% acetonitrile) within 130 min. The flow rate was 1.3 ml/min and the oven temperature was 40°C.

The diastereomeric purity of metabolites M-23 and M-24 was determined by chiral HPLC on a 5 µm-Chiral AGP® column (150 × 4 mm, ChromTec, Hägersten, Sweden). Isocratic elution employed a 66 mM phosphate buffer pH 5 (KH2PO4/Na2HPO4), containing 0.5% 2-propanol and a flow of 0.9 ml/min at room temperature. For this chiral HPLC assay M-23 and M-24 were separated from other metabolites by preceding achiral HPLC.

Preparative separation of the diastereomeric esters of metabolite M-23 and M-24 reference, respectively, was performed using two HPLC pumps 305 and 306, a fraction collector 201, an autoinjector model 231 × 6 (Gilson, Beltline-Middleton, WI), a UV detector SP100 (Spectra Physics, San Jose, CA) and a recorder 320D Servogor (Metrawatt, Nuernberg, Germany). A Chiralpak® AS column (250 × 20 mm, Daicel Chemical Ind., Tokyo, Japan) was eluted isocratically with n-heptane/ethanol 95:5 (M-23) or 88:12 (M-24) at a flow rate of 10 ml/min. UV detection was used at 230 nm, the oven temperature was 40°C. The diastereomeric purity of metabolites M-23 and M-24 was determined by chiral HPLC on a 5 µm-Chiral AGP® column (150 × 4 mm, ChromTec, Hägersten, Sweden). Isocratic elution employed a 66 mM phosphate buffer pH 5 (KH2PO4/Na2HPO4), containing 0.5% 2-propanol and a flow of 0.9 ml/min at room temperature. For this chiral HPLC assay M-23 and M-24 were separated from other metabolites by preceding achiral HPLC.

**Spectroscopic Methods.** Positive mode ESI mass spectra were recorded by LC-MS using an ABI 140B HPLC system (Applied Biosystems, Inc., CA) coupled with a PE/Sciex API III mass spectrometer (Perkin Elmer Sciex Instruments, Ontario, Canada). A 5

**TABLE 2**

<table>
<thead>
<tr>
<th>Individual Metabolites as Percentage of Total Metabolites</th>
<th>M-1</th>
<th>M-23</th>
<th>M-24</th>
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<td>Cerivastatin</td>
<td>95.6</td>
<td>2.4</td>
<td>2.1</td>
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<tr>
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<td>80.2</td>
<td>11.2</td>
<td>8.1</td>
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<tr>
<td></td>
<td>91.1</td>
<td>6.8</td>
<td>2.1</td>
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<tr>
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<td>72.3</td>
<td>19.7</td>
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<td>90.0</td>
<td>6.0</td>
<td>4.1</td>
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<td>82.7</td>
<td>10.2</td>
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<td></td>
<td>66.0</td>
<td>15.7</td>
<td>17.0</td>
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<td></td>
<td>34.3</td>
<td>27.1</td>
<td>29.1</td>
<td>7.8</td>
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</tbody>
</table>

* Experimental conditions were as described in Materials and Methods.

* Arithmetic means of duplicate incubations are given.

* Not detected.
HPLC was performed as described under Materials and Methods with radioactivity detection.

\[ \mu \text{-Supelcosil} \text{® LC-18 (250 × 2.1 mm) column was eluted with a step gradient from 50\% 10 mM ammoniumacetate to 90\% acetonitrile over a period of 30 min. The column effluent (250 µl/min) was connected to the Ionspray® interface, with a splitting ratio of 1:10.} \]

1H-NMR spectra of isolated metabolites and reference compounds were recorded at 500 MHz on a Bruker AMX 500 (Bruker, Rheinstetten, Germany) using methanol-d4 (99.96\% deuterium incorporation) as solvent. The two-dimensional NOESY spectrum (Nuclear Overhauser Effect Spectroscopy) was performed with a mixing time of 600 ms.

**Isolation and Purification of Metabolites.** Approximately 20 ml incubation mixture with human liver microsomes was separated by solid phase extraction on a 20 ml Isolute® E18 cartridge into 3 fractions (recovery 80\%) using 0.1\% aqueous trifluoroacetic acid/acetonitrile gradient elution. From fractions 1 and 2 single metabolites were isolated by HPLC on a 7μm-Nucleosil® C18 column (250 × 8 mm) using 0.1\% aqueous trifluoroacetic acid/acetonitrile gradient elution. Fractions containing metabolites M-1, M-22, M-23, M-24, and M-31 were combined and submitted to spectroscopic analysis.

**Results**

**Metabolite Profiles.** Incubation of [14C]cerivastatin with four different batches of human liver microsomes for 60 min resulted in qualitatively similar, but quantitatively different metabolite profiles (table 2). Two major metabolites were equally important, the demethylated drug M-1 and metabolite M-23, hydroxylated at one methyl group of the 6-isopropyl substituent. In microsomal batches 1–3 total drug turnover was similar (17–28% after 60 min), whereas in batch no. 4 metabolism was more extensive (66%). Besides M-1 and M-23, considerable amounts of metabolite M-24, comprising both biotransformations, were formed by batch no. 4, in particular when incubated on a larger scale for 5 hr (fig 1).

**Structure Elucidation of Metabolites.** The ESI mass spectra as well as the 1H-NMR spectra of isolated and purified metabolites M-1, M-23, and M-24 were identical with those of the synthetic reference compounds (tables 1 and 3). Identity was also proven by chromatographic comparison using achiral, and - for M-23 and M-24 - also using chiral HPLC. Minor metabolites M-22 and M-31 were identified by their ESI mass spectra (table 3).

All mass spectra comprise [M+H]+ ions, which are in accordance with the proposed structures.

The 1H-NMR spectrum of metabolite M-1 was highly similar to the spectrum of cerivastatin (table 1). Essential differences were the lack of the methylether group and the downfield shift of the -CH2O- AB spin system to δ = 4.32 ppm. By means of a NOESY (Nuclear Overhauser Effect Spectroscopy) experiment (scheme 4, the short distances found in the NOESY spectrum are indicated by arrows) it was shown that the upfield absorbing 6H doublet at δ = 1.23 ppm represented the isopropyl methyl groups in C2' position, whereas the doublet at δ = 1.31 ppm represented the C6' isopropyl moiety.

The 1H-NMR spectrum of metabolite M-23 comprised two important differences as compared with the cerivastatin spectrum: one downfield absorbing methyl group had disappeared and a new 2H doublet at δ = 3.88 ppm, typical for a CH2OH group indicated hydroxylation at this methyl group. Based on the NOESY results for metabolite M-1, it was concluded that the 6'-isopropyl moiety was functionalized. This structure proposal was in agreement with the molecular weight (475 dalton) determined by mass spectrometry and was confirmed by the independent synthesis.

The 1H-NMR spectrum of metabolite M-24 was very similar to that of metabolite M-23 (table 1). In the mass spectrum the molecular weight difference of 14 dalton, as compared with M-23 (table 3), indicated metabolic demethylation. Accordingly, the -CH2O- AB spin system was shifted to δ = 4.33 ppm. The new CH2OH group resulting from the hydroxylation of the 6'-isopropyl moiety appeared as an ABX spin system centered at δ = 3.87 ppm.

The hydroxylation leading to metabolite M-23 created a new chiral center in the cerivastatin molecule. As the stereochemistry of the dihydroxyheptenoic acid side chain is fixed, two diastereomeric metabolite structures were theoretically conceivable. In the NMR spectrum of the isolated M-23 sample, no additional signal splitting was observed.

### TABLE 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cerivastatin</th>
<th>M-1</th>
<th>M-23</th>
<th>M-24</th>
<th>M-22</th>
<th>M-31</th>
</tr>
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<tbody>
<tr>
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<td>446</td>
<td>476</td>
<td>462</td>
<td>428</td>
<td>458</td>
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<tr>
<td>Ion</td>
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<td>[M + H]+</td>
<td>[M + H]+</td>
<td>[M + H]+</td>
<td>[M + H]+</td>
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</tr>
</tbody>
</table>

**Fig. 1. Profile of metabolites formed from [14C]cerivastatin by human liver microsomes on a 20 ml scale after 5 hr.**
provided evidence for the presence of diastereomers. Using a chiral HPLC column, a diastereomeric excess (de) of 93% was determined for the isolated M-23 (fig. 2). The reference sample used for this assay was a synthetic mixture of both diastereomers.

Based on the configuration of the starting materials used for reference synthesis, the absolute configuration at the new chiral center of M-23 was assigned to be (S)-(hydroxymethyl)ethyl.

The 1H-NMR spectrum of metabolite M-24 resembled the characteristic features of both, M-1 and M-23 (table 1). Like M-23, M-24 was formed with high stereoselectivity, for the isolated sample a de of 78% was determined by chiral HPLC. The new chiral center exhibits the (S)-configuration, as inferred from the stereochemistry of the synthetic starting materials.

The lactones of main metabolites M-1 and M-23, M-22 and M-31, respectively, were not found in the original microsomal incubation mixtures. They were most likely formed in small amounts from their open hydroxy acid precursors during the workup process. Lactonization of cerivastatin and metabolites occurs preferentially under acidic conditions, as used for preparative HPLC.

**Cytochrome P450 specific in vitro metabolism.** [14C]Cerivastatin was incubated with microsomes from 11 human B lymphoblastoid AHH-1 cell lines, which stably express single human CYP isozymes (3). CYP 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6Met, 2D6Val, and 2E1 did not metabolize the drug to a measurable extent. With microsomes from cell line no. M107r, expressing human CYP 3A4, a 2% turnover was achieved within 2 hr. The only product detected was the demethylated metabolite M-1.

To further substantiate the extent to which CYP 3A enzymes are involved in the metabolism of cerivastatin, specific inhibition experiments were performed. Human liver microsomes from batch 4, which exhibited the highest cerivastatin turnover, were used. Incubations were performed with and without the addition of TAO, a highly selective suicide inhibitor of CYP 3A enzymes (4, 5). Following a 20 min preincubation with TAO a higher inhibitory effect was achieved than without preincubation (fig. 3). After 3 hr 45% of [14C]cerivastatin remained unmetabolized whereas in the control experiment without TAO only 18% was left. The amounts of metabolites M-1, M-23, and M-24 formed decreased to a similar extent under the influence of the inhibitor (table 4). After preincubation with TAO 73% of M-1, 82% of M-23, and 31% of M-24 were formed after 3 hr as compared with the control experiments.

**Discussion**

In human liver microsomes cerivastatin is subject to two primary biotransformation reactions. Demethylylation of the benzylic methyl-
ether leads to metabolite M-1, whereas hydroxylation of one methyl group in the 6'-isopropyl moiety furnishes metabolite M-23. Metabolite M-24 comprises the combination of both primary reactions (scheme 5). Lactonization of M-1 and M-23 to M-22, and M-31, respectively, most likely occurred during sample workup. The hydroxylation reaction was found to be highly stereoselective, producing the 3R,5S,1'S-enantiomers of the primary metabolite M-23 as well as of its demethylated analogue M-24.

The absolute configuration of these single enantiomers was determined by comparison with the synthetic reference compounds. Although each synthesis started from an enantiomerically pure precursor, racemization on one of the following steps afforded both diastereomeric products. Racemization was not complete, thus the configuration of the major diastereomer had to be identical to the starting material. This conclusion was confirmed by the finding that the opposite diastereomeric ratio was obtained for the final products when the starting material with opposite configuration was used (data not shown). Chromatographic comparison unequivocally showed that the isolated metabolites M-23 and M-24 were identical with the main component in the respective reference mixture.

The incubations of cerivastatin with microsomes from single CYP isozyme expressing cell lines suggest that only the demethylation pathway, but not the hydroxylation pathway is mediated by CYP 3A4. In the inhibition experiments, the selective CYP3A4 inhibitor TAO was used in a concentration (approximately 5 μM) which has been shown to exert the maximum inhibitory effect on CYP 3A4 in human liver microsomes (5). Using testosterone 6β-hydroxylation as a specific probe reaction, CYP 3A4 activity was reduced to less than 30% of control. Virtually no inhibitory effect was observed for reactions mediated by CYP 1A2, 2C9, 2D6, or 2E1 (5).

Cerivastatin metabolism was reduced by TAO to 56% of control after 30 min of incubation. After 3 hr the relative turnover had increased to 67% of the control value, indicating that despite the suicide destruction of CYP 3A4 by TAO other enzymes are able to metabolize the drug. Moreover, contrary to the CYP 3A4 expressing cell line, TAO inhibition equally affects both metabolic pathways, hydroxylation and demethylation. The formation of metabolites M-1 and M-23 was inhibited to similar extents over a 3-hr reaction period. A possible explanation for these findings is that an enzyme other than CYP 3A4 is responsible for the hydroxylation pathway to M-23 and that this enzyme is inhibited by TAO and also demethylates cerivastatin to M-1. The characterization of this enzyme, as well as its clinical importance, e.g., for drug interaction studies, will be the subject of future investigations.

References


