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ATORVASTATIN, AN HMG-COA REDUCTASE INHIBITOR AND EFFECTIVE LIPID-REGULATING AGENT. PART III.[1a,b] SYNTHESES OF [$^2H_5$]-, [$^{13}C_8$], AND [$^{13}C_7$,^{15}N]ATORVASTATIN AND THEIR APPLICATION IN METABOLIC AND PHARMACOKINETIC STUDIES.

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SUMMARY

Three stable-isotope-labeled forms of atorvastatin, [$^2H_5$], [$^{13}C_8$], and [$^{13}C_7$,^{15}N], were synthesized. They were utilized primarily for metabolic mapping and pharmacokinetic studies of bioequivalence. Analytical assays were performed by using LC/MS/MS methodologies.[2]

Keywords: [$^2H_5$]-, [$^{13}C_8$]-, and [$^{13}C_7$,^{15}N]atorvastatin, synthesis, metabolite mapping, bioequivalence study, HMG-CoA reductase inhibitor, lipid-regulating agents.

INTRODUCTION

In the preceding papers, two ^{14}C-labeled forms of atorvastatin (Lipitor®; CI-981), a recently-approved and highly effective lipid-regulating agent and a member of the class of HMG-CoA reductase inhibitors, were presented.[1a,b] During the development of this medicinal agent, three stable-isotope-labeled forms of this medicinal agent were also synthesized and extensively utilized for metabolic mapping and pharmacokinetic studies of bioequivalence. The quality of these studies was significantly enhanced over that achievable with the ^{14}C-labeled forms.[3]

The use of stable-labeled analogs offers unique advantages,[3,4] particularly in instances where ethical concerns restrict the use of radioactivity. The incorporation of a stable-label into a drug dose for mass spectrometric study, i.e., isotopic dilution mass spectrometry, has rapidly become a widely accepted technique to aid in the identification of drug metabolites.[5] The metabolites are thereby readily recognized by an artificial isotope-cluster, with a pattern determined by mass difference and abundance of the stable-label.[3b] Moreover, the use of stable labeled compound as an internal standard for mass spectral quantitative assays offers significant improvements in the accuracy and precision of the assay over the use of a structural analog as internal standard.

This paper will present the syntheses of the [$^2H_5$]-, [$^{13}C_8$]-, and [$^{13}C_7$,^{15}N]atorvastatins, providing the M+5, M+8, and M+8 isotopic forms,[2] respectively. The utility of these labeled analogs is illustrated with the application of the M+5 form in a metabolic disposition study of bile-cannulated rat, to aid metabolite detection and identification. The M+5 was also used in pharmacokinetic studies of

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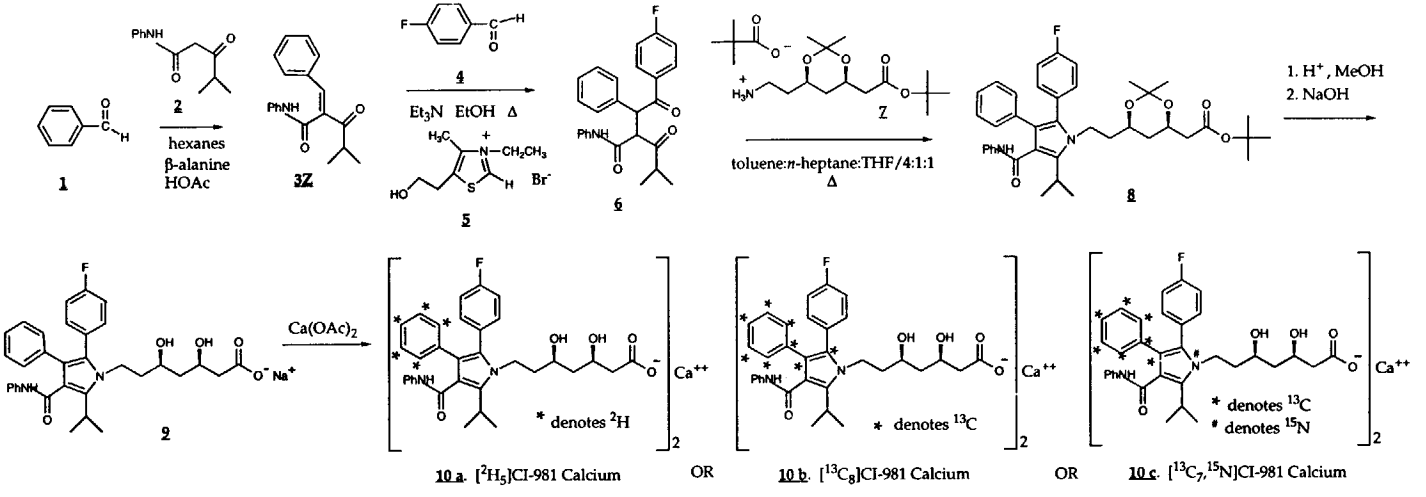
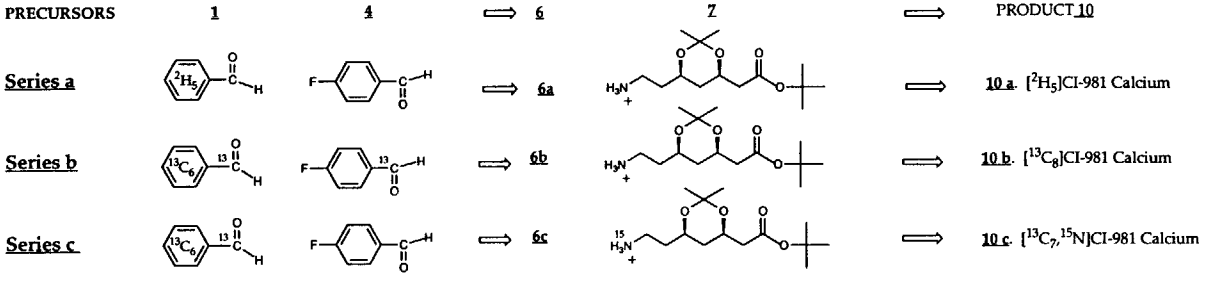
SCHEME 1. Synthetic Sequences for Three Stable-Isotope-Labeled Atorvastatins (CI-981) (10a, 10b, and 10c).

A. General Reaction Scheme (Labels shown only at the final products 10a, 10b, and 10c).



B. The appropriate labeled precursors in the three synthetic series a, b, and c.



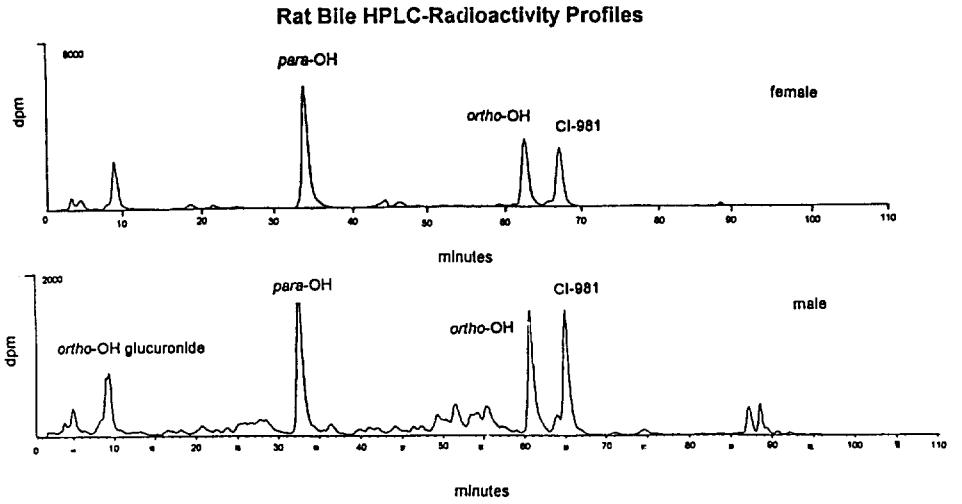


Fig. 1. GRADIENT HPLC RADIOACTIVITY PROFILES of CI-981 and metabolites from male and female rat biles, Protocol 93-085: 40 μCi dose, 10 mg CI-981 (d_5, d_0, ^{14}C mixture)/kg, 4 - 8 hour.

BioSil ODS-5s (150 x 4.6 mm), Brownlee RP-18 Spheri-5, 5μ guard cartridge. MeCN-0.1 M NH_4OAc, pH4.0 (adjusted with acetic acid):25% MeCN; 75:25 to 20:80.

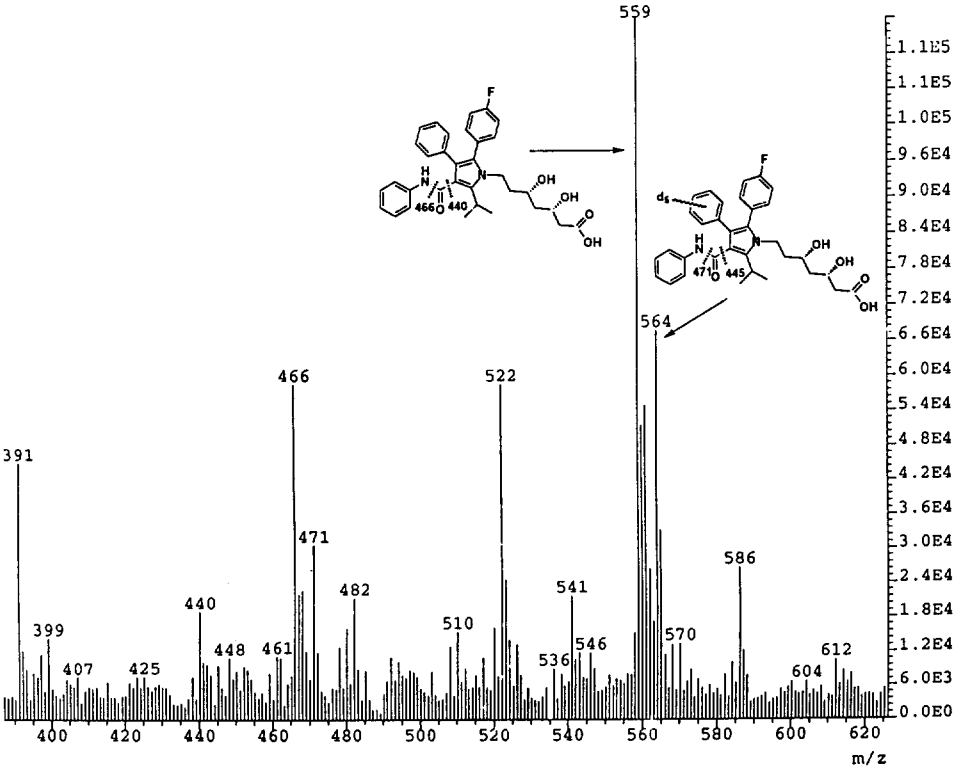


Fig. 2. LSIMS POSITIVE ION MASS SPECTRA of HPLC fraction at retention time of 65 minutes: $[M + H]^+$ of m/z 559/564 (CI-981).

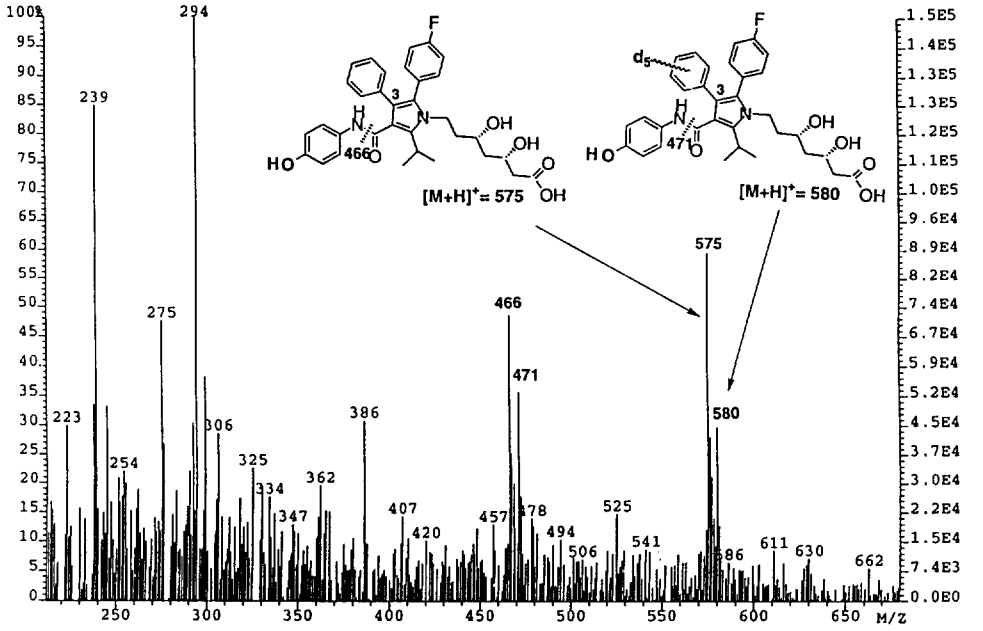


Fig. 3. LSIMS POSITIVE ION MASS SPECTRA of HPLC fraction at retention time of 35 minutes: $[M + H]^+$ of m/z 575/580.

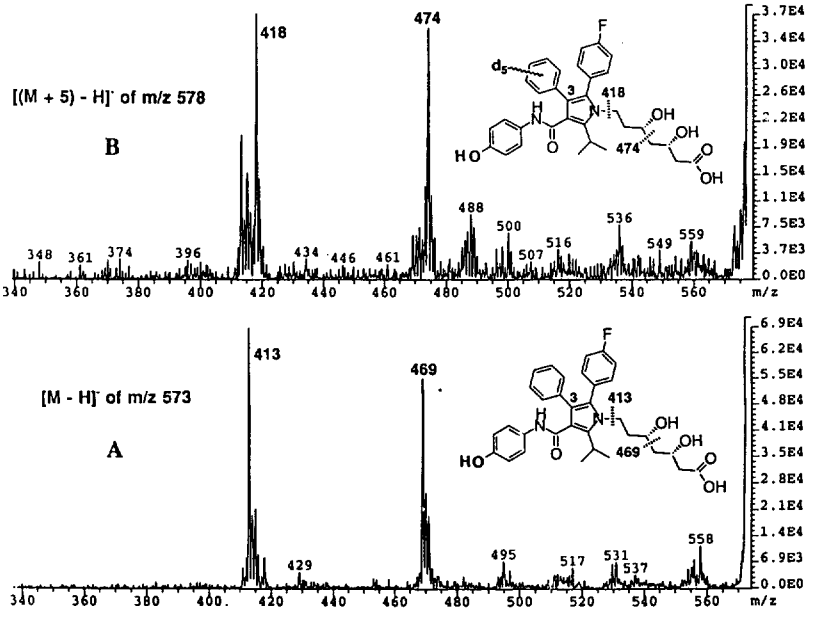
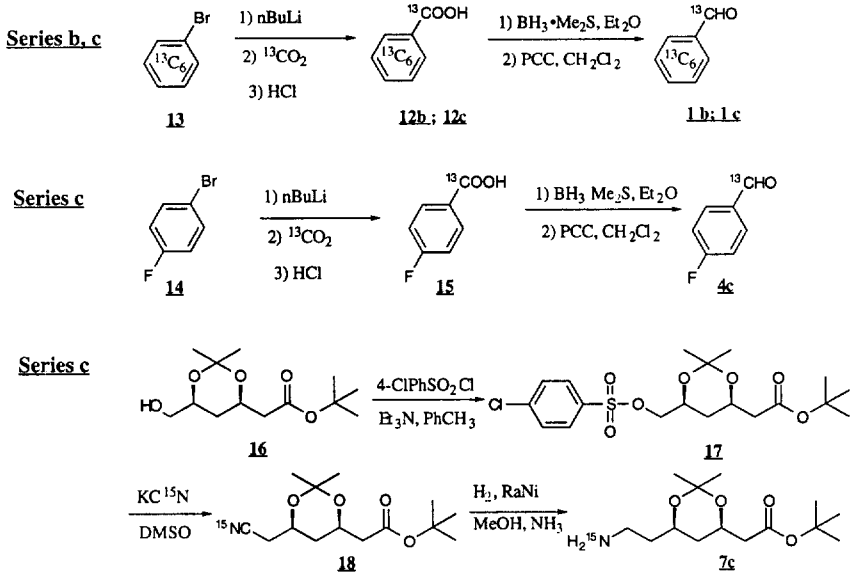


Fig. 4. LSIMS NEGATIVE ION LINKED SCAN B/E MS/MS SPECTRA of HPLC fraction at retention time of 35 minutes: Panel A, $[M - H]^+$ of m/z 573; Panel B, $[(M+5) - H]^-$ of m/z 573. Identification of PD 142542, *p*-hydroxy CI-981.

SCHEME 2. Synthetic Schemes for Selected Stable-Isotope-Labeled Precursors



bioequivalence. The M+8 analogs were used as internal standards for the development and validation of the bioanalytical assay used for the pharmacokinetic studies and were integral to the development of robust and rugged LC/MS/MS assays.

RESULTS AND DISCUSSION

The syntheses of the three stable-isotope-labeled atorvastatin are based on the methodology for the ring-labeled [^{14}C]atorvastain.[1a,6] The general reaction scheme is shown in Scheme 1 - part A, where various precursors and reagents **1** to **9** are shown in unlabeled forms and the final product from each of the three series of reactions, **10a**, **10b**, and **10c**, are shown with the proper labels. Each of them (**10a to c**) was derived from the appropriate combination of various precursors **1**, **4**, **7** (Scheme 1 - part B). The syntheses of individual labeled precursors **1b** (or **1c**), **4c**, and **7c**, are shown in Scheme 2.

The application of the stable isotopes in metabolic studies is illustrated with an example in which a mixture of [$^2H_5$], [$^2H_0$], and [^{14}C]CI-981,[7a] the latter in trace amount, was administered to rats, and the metabolic profiles in biliary excretions were examined by LC-ESI-MS. The identification of the major radioactive components in the HPLC chromatogram (Fig. 1) was performed by tandem mass spectrometry. The major metabolites identified include a glururonide of *ortho*-OH atorvastatin, *para*- and *ortho*-hydroxylated products, and ß-oxidized atorvastatin (t_r = 87 min, male) (Fig. 2; d = ^{2}H).[7b]

The detection and identification of drug metabolites were significantly aided by the presence of the $^2H_5$ (or d_5) isotope, as illustrated with the *para*-OH metabolite (Fig. 3 and 4; d = ^{2}H). First, the detection of potential drug metabolites was facilitated by searching for the characteristic molecular ion [d_0]/[d_5] (M/M+5) cluster (575, 580, Fig. 3). Subsequently, the structures of the drug metabolites were determined by using collisionally activated decomposition[8a] (CAD) MS/MS of the molecular ions of the unlabelled and stable isotope in the cluster. Thus [M-H]$^-$ of m/z 573 of the unlabeled molecular ion gave rise to characteristic fragmentation peaks at 413 and 469, and correspondingly, the 578 peak of the $^2H_5$ molecular ion led to the characteristic fragmentation peaks 418 and 474.

The other metabolites in Fig 1 were identified in a similar manner. Although the choice and position of isotope could result in the loss of label by either exchange or metabolism, no loss of label was observed during the screening of radioactive metabolite fractions in the present study.

This technique of metabolic mapping shows a great deal of promise for use in human disposition studies.[7a]

The [M+5] stable labeled atorvastatin was also used for a bioequivalence study. Therein, unlabeled atorvastatin in tablet form is co-administered with a solution of the [M+5] atorvastatin **10a**. The [$^{13}C_8$] or [$^{13}C_7$,^{15}N]-labeled compound, **10b** or **10c** respectively, was used as an internal standard for the quantitative assay.

EXPERIMENTAL

General. All synthesized labeled compounds were identified with authentic unlabeled reference compounds[6] by TLC and/or HPLC as a minimum.

TLC analyses were performed with Silica Gel 60 F_{254} precoated plates by EM Science. HPLC was performed with Alltech Econosil C18 10μ, 4.6 mm x 250 mm analytical columns. NMR spectra were obtained with a Varian Gemini 200 NMR spectrometer [^{1}H (200 MHz) and ^{13}C (50 MHz)] or a Varian Unity Plus 400 spectrometer [^{1}H (400 MHz) and ^{13}C (100 MHz)]. δ values are given in ppm. The letter "e" after a δ value denotes enhanced ^{13}C signal from a labeled atom. For non-metabolic studies, mass spectra (MS) were mostly obtained with a Micromass PlatformLC operating under APCI conditions,[2] using acetonitrile:water/4:1 as solvent, probe temperature of 450 °C, and cone voltage of 15V, and *m/z* values are reported for the positive (AP+) and the negative (AP-) modes. Some data were also obtained with a VG Trios 2A or Trios 1000 spectrometer (CI+ mode with methane or 1% ammonia in methane). Melting points were obtained with a Thomas Hoover capillary melting point apparatus with silicone oil bath and were uncorrected.

[$^2H_5$]Benzaldehyde (98 atom %) was purchased from ICON Services, Summit, NJ. Barium[^{13}C]-carbonate (99%), was purchased from Cambridge Isotope Laboratories, Andover, MA. Potassium [^{15}N]cyanide (99%) was purchased from Isotec, Miamisburg, OH. 4-Fluorobromobenzene, 4-chlorobenzenesulfonyl chloride, *n*-butyllithium (2.5 *M* in hexanes) and borane methylsulfide complex were purchased from Aldrich Chemical Co., Milwaukee, WI. 1,1-Dimethylethyl(4R-cis)-6-hydroxymethyl-2,2-dimethyl-1,3-dioxane-4-acetate (**16**) was provided by Parke-Davis Chemical Developmemt, Holland, MI.

[$^{13}C_7$]Benzaldehyde (1b or 1c) was prepared from **12b** (or **12c**) according to the procedure for **4c**. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 9.99 (dd, J_{1H-1H} = 23 Hz, J_{1H-13C} = 180 Hz, 1H), 7.85 (dm, J_{1H-13C} = 159 Hz, 2H), 7.57 (dm, J_{1H-13C} = 161 Hz, 3H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 129 (m), 135 (m), 192 (d, J = 53 Hz).

4-Methyl-3-oxo-N-phenyl-2-([$^2H_5$]phenylmethylene)pentamide (3a), (benzylidene-isobutyrylacetanilide; BIBEA). The reaction was run in a 250-mL two-necked round-bottomed flask equipped with an electric motor-driven mechanical stirrer and an oil bath. The flask was fitted with a Dean-Stark trap and condenser, and the entire apparatus was maintained under nitrogen. The flask was charged with isobutyrylacetanilide (**2**) (4.67 g, 18.36 mmol), [$^2H_5$]benzaldehyde (**1**) (2 g, 18 mmol), β-alanine (433 mg, 4.86 mmol), hexanes (65 mL), and glacial acetic acid (432 mg, 7.2 mmol), and then the contents were heated under reflux for 28 h, resulting in a slurry. The crude solid product of **3Z**/**3E**[1a] was filtered and dried by air suction to give 5.25 g (97% yield) of material. TLC (hexanes: CH_2Cl_2:EtOAc/2:2:1) showed **3Z** as the major product.

A solution of the crude product in 15 mL of CH_2Cl_2 was washed with 5 mL of water and added to a column containing 50 g of silica gel which had been packed in hexanes and washed with 40 mL of CH_2Cl_2/hexanes (1/1). The column was first developed with 150 mL of CH_2Cl_2 to give a fraction, followed by 3% EtOAc in CH_2Cl_2 with the collection of 10-mL fractions for a total of 85 fractions. Fractions 1 to 34 and 35 to 70 were combined into two larger fractions. All fractions apparently contained **3Z** as the major product. Repeated fractional crystallization from CH_2Cl_2:hexanes/1:6.5 (approx. 13 mL/g of product) resulted in 1.1 g of pure **3Z** and 2.1 g of slightly less pure material. M.p. 144.5-146 °C (unlabeled reference 147.5-149.5 °C). MS ($C_{19}H_{14}{}^2H_5NO_2$, mol wt 298) *m/z*: (AP+) 299.2, (AP-) 297.1.

^{1}H NMR (400 MHz, DMSO-d_6[2]): δ 0.97 (d, J = 7.1 Hz, 0.24H, from **3E**), 1.05 (d, J = 6.8 Hz, 5.76H), 7.04 (t, J = 7.5 Hz, 1H), 7.28 (t, J = 7.8 Hz, 2H), 7.55 (d, J = 8.0 Hz, 2H), 7.66 (s, 1H), 10.40 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.56, 35.22, 120.04, 124.33, 129.29, 133.65, 136.91, 138.94, 139.15, 166.25, 202.31.

The [$^{13}C_8$] analogs 3b (or 3c) were prepared in 93% yield by similar procedures from **1b** (or **1c**) (Scheme 1 - part B). MS *m/z*: (CI+) 301, 283, 208, 94. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.21 (d, J = 7 Hz, 6H), 1.63 (s, 1H), 3.37 (heptet, J = 7Hz, 1H), 7.40 (m, 6H). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ130.1 (m), 140.2 (m), 140.3, 141.3, 141.5.

4-Fluoro[α-^{13}C]benzaldehyde (4c). To a solution of 4-fluoro[α-^{13}C]benzoic acid (**15**) (1.83 g, 13.0 mmol) in Et_2O (30 mL) was added borane/dimethyl sulfide (1.3 mL, 13.7 mmol) over 15 min. The reaction mixture was heated at reflux for 75 min. After cooling, the solvent was evaporated *in vacuo* and the residue was redissolved in CH_2Cl_2 (10 mL). To the solution was added a suspension of pyridinium chlorochromate (3.1 g, 14.4 mmol) in CH_2Cl_2 (25 mL) over 20 min. The mixture was heated at reflux for 90 min. The reaction mixture was diluted with Et_2O (50 mL) and passed through a short column of Florisil eluting with additional Et_2O (50 mL). The solvent was removed *in vacuo*, and the resulting liquid (1.235 g) was vacuum-distilled to give **4c** (850 mg, 6.797 mmol, 52% yield). The material was used immediately in the next step because of its instabililty. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.16 (t, J = 8.43 Hz, 2H), 7.82 - 7.91 (m, 2H), 9.92 (d, $J_{^1H\text{-}^{13}C}$ = 175 Hz, 1H). 13C-NMR (50 MHz, $CDCl_3$): δ 116.1, 116.5, 132.1, 132.3, 190.5e.

(±) 4-Fluoro-α-(2-methyl-1-oxopropyl)-γ-oxo-N-phenyl-β-[$^2H_5$]phenylbenzenebutaneamide (6a). A mixture of pure **3Z** (500 mg, 1.68 mmol) and 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide (**5**) (110 mg, 0.42 mmol) was placed in a 15-mL two-necked flask, fitted with a Teflon-lined septum, a magnetic stirrer and a condenser through which either vacuum or argon atmosphere could be maintained with a Firestone valve. The reaction flask was repeatedly evacuated and filled with argon. Absolute EtOH (0.17 mL), Et_3N (190 mg, 1.88 mmol), and *p*-fluorobenzaldehyde (**4**) (208 mg, 1.68 mmol) were added. The mixture was heated at 71 °C for 1 h to give a clear viscous liquid, and then at 60 °C for 12 h. To the resulting mass of solid was added *i*-PrOH (4 mL), and the mixture was heated at 70 °C with stirring under argon for 5 min and then at room temperature for 2 h. The mixture was filtered and washed with isopropyl alcohol and dried *in vacuo* to give 388 mg (55% yield) of white crystals. HPLC [(MeCN:THF:MeOH/3:2:1):water/55:45] showed a chemical purity of 98.45% with no desfluoro or difluoro analogs present.

Another run on the same scale was carried out similarly with nearly identical results. The total yield for the two runs was 752 mg of **6a**, m.p. 203.5 - 204.5 °C (unlabeled reference, 206.5 - 207 °C). MS ($C_{26}H_{19}{}^2H_5FNO_3$, mol wt 422) *m/z*: (AP+) 423.2, (AP-) 421.1.

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.88 (d, J = 6.6 Hz, 3H), 1.11 (d, J = 7.1 Hz, 3H), 2.84 (septet, J = 6.8 Hz, 1H), 4.81 (d, J = 11.0 Hz, 1H), 5.37 (d, J = 11.0 Hz, 1H), 6.96 (t, J = 7.2 Hz, 1H), 7.14 - 7.26 (m, 6H), 8.05 - 8.09 (m, 2H), 10.14 (s, 1H). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.09 (d, J = 6.8 Hz, 3H), 1.17 (d, J = 6.8 Hz, 3H), 1.52 (s, 1H), 2.92 (heptet, J = 6.8 Hz, 1H), 4.50 (d, J = 10.7 Hz, 1H), 5.29 (d, J = 10.7 Hz, 1H), 6.95 - 7.21 (m, 7H), 7.89 - 7.93 (m, 2H).

^{13}C NMR (100 MHz, DMSO-d_6): δ 17.91, 18.80, 51.70, 62.95, 115.71, 115.93, 119.68, 123.96, 128.65, 131.64, 131.74, 132.19, 134.87, 138.04, 163.71, 164.97, 166.22, 196.45, 208.05. ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.02, 18.47, 41.20, 54.50, 64.17, 115.56, 115.78, 120.24, 124.89, 128.89, 131.58, 131.67, 132.17, 135.08, 136.75, 164.41, 165.13, 166.94, 196.28, 209.82.

The [$^{13}C_8$] and [$^{13}C_7$] analogs (6b, 6c, respectively) were prepared by similar procedures as for **6a** above from **3b** (26% yield) and **3c** (25% yield), respectively (Scheme 1 - part B).

Compound **6b**. MS *m/z*: (CI+) 426, 408, 354, 333, 307, 289, 138, 124, 120, 119, 93. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.15 (d, J = 6 Hz, 3H), 1.22 (d, J = 6 Hz, 3H), 1.63 (s, 1H), 2.99 (q, J = 6 Hz, 1H), 4.55 (m, 1H), 5.36 (dm, $J_{^1H\text{-}^{13}C}$ = 134 Hz, $J_{^1H\text{-}^1H}$ = 5 Hz, 1H), 7.14 (m, 6H), 7.22 (m, 4H), 7.47 (m, 2H), 8.00 (m, 2H). ^{13}C-NMR ($CDCl_3$): δ 196e (d, J = 39 Hz), 134e (m), 129, 54e (d, J = 41 Hz).

Compound **6c**. MS *m/z*: (CI+) 425, 407, 353, 332, 306, 288, 138, 123, 120, 119, 93. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.15 (d, J = 6 Hz, 3H), 1.23 (d, J = 6 Hz, 3H), 1.55 (s, 1H), 2.99 (q, J = 6Hz, 1H), 4.53 (m, 1H), 5.34 (dm, $J_{^1H,^{13}C}$ = 119 Hz, 1H), 7.21 (m, 7H), 7.27 (dm, $J_{^1H\text{-}^{13}C}$ = 157 Hz, 5H), 7.99 (dd, J = 3, 4 Hz, 2H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 54 (d, J = 40 Hz), 128e (m), 135e (m).

1,1-Dimethylethyl(4R-cis)-6-(2-[^{15}N]aminoethyl)-2,2-dimethyl-1,3-dioxane-4-acetate (7c). A mixture of **18** (1.32 g, 4.90 mmol), Raney nickel (1.0 g) and CH_3OH saturated with NH_3 (100 mL) was shaken under a hydrogen atmosphere (50 psi) for 17 h at 30°C. Filtration followed by evaporation of the filtrate gave 1,1-dimethylethyl(4R-cis)-6-(2-[^{15}N]aminoethyl)-2,2-dimethyl-1,3-dioxane-4-acetate (1.48 g, >100 % yield) as a pale blue viscous oil. ^{1}H-NMR ($CDCl_3$): δ 1.25 (m, 4H), 1.40 (s, 12H), 1.55 (s, 2H), 2.22 (dd, 2H), 2.38 (d, 2H), 4.00 (m, 1H, 4.20 (m, 2H). MS *m/z* (CI+) 275, 259, 219, 201, 189, 161, 143, 131. This material was used directly in the next step (reaction with **6c**).

[4R-cis]-1,1-Dimethylethyl-6-[2[2-(4-fluorophenyl)-5-(1-methylethyl)-3-[$^2H_5$]phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-yl]ethyl]-2,2-dimethyl-1,3-dioxane-4-acetate (8a). A screw-cap tube (VWR #60826-199, 1.5 cm x 9.0 cm) with a Teflon-lined cap was used as the reaction vessel. Compound 6a (300 mg, 0.71 mmol) and the pivalate salt of [4R-cis]-1,1-dimethylethyl-6-(2-aminoethyl)-2,2-dimethyl-1,3-dioxane-4-acetate (7a) (267 mg, 0.71 mmol)[6b] were added to the argon- flushed reaction vessel, followed by 5 mL of toluene:*n*-heptane:THF/4:1:1. The reaction mixture was heated at 85 °C for 16 h. TLC (CH_2Cl_2:EtOAc/96:4, and n-pentane:ether/2:1) showed the presence of product (**8a**) and some starting material (**6a**) . The reaction was repeated using 350 mg (0.83 mmol) of **6a**, 312 mg (0.83 mmol) of **7a** and 4 mL of the solvent mix. The two reaction mixtures, along with 3 mL of *t*-BuOMe, were combined and extracted, in sequence, with 2 mL of 1 N NaOH, 2 mL of water, 2 mL of 1 N citric acid, 2 mL of saturated $NaHCO_3$, and 2 mL of saturated NaCl. Each of the aqueous phases was extracted sequentially with 2 mL of *t*-BuOMe, and the combined organic phases were dried ($MgSO_4$) and evaporated to give crude **8a** (1.4 g). The crude product was dissolved in 2 mL of ether:hexanes/1:5 and added to a column of 9.4 g of silica gel packed in hexanes. The column was eluted with ether:hexanes /1:5, then ether:hexanes/1:4, and finally ether:hexanes/1:3. Most of the desired product (**8a**) was obtained during the ether:hexanes/1:4 elution, and those fractions were combined and evaporated to give **8a** (537 mg, 53% yield relative to **6a**). HPLC analysis (CH_3CN:H_2O/80:20; 254 nm) showed 98.13% chemical purity. M.p. 132-134 °C (crystals from CH_2Cl_2:hexanes). MS ($C_{40}H_{42}{}^2H_5FN_2O_5$, mol wt 659) *m/z*: (AP+), 660.4, (AP-), 658.4.

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.96 - 1.66 (m, 4H), 1.26 (s, 3H), 1.32 (s, 3H), 1.39 (s, 9H), 1.49 (d, H=7.1 Hz, 6H), 2.20 (dd, J = 6.4, 15.4 Hz, 2H), 2.35 (dd, J = 7.0, 15.2 Hz, 2H), 3.53 (heptet, J = 7.0 Hz, 1H), 3.62 - 3.82 (m, 1H), 3.74 - 3.82 (m, 1H), 4.00 - 4.15 (m, 2H), 6.81 (s, 1H), 6.93 - 7.17 (m, 9H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 19.63, 21.53, 21.69, 26.06, 28.06, 29.90, 35.97, 38.05, 40.83, 42.45, 65.88, 66.40, 80.64, 98.64, 115.19, 115.30, 115.40, 119.53, 121.71, 123.44, 128.25, 128.28, 128.62, 128.75, 133.10, 133.19, 134.45, 138.40, 141.51, 161.02, 163.48, 164.74, 170.11.

The [$^{13}C_8$] and [$^{13}C_7$,^{15}N] analogs (8b, 8c, respectively) were prepared by similar procedures from the condensation of **6b** with **7b** (47% yield), and of **6c** with **7c** (25% yield), respectively (Scheme 1 - part B).

Compound **8b**, m.p. 130-133 °C. MS (mol wt 662) *m/z*: (AP+) 663.3, (AP-) 661.3.

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.96 - 1.66 (m, 4H), 1.26 (s, 3H), 1.32 (s, 3H). 1.39 (s, 9H), 1.49 (d, J = 7.1 Hz, 6H), 2.20 (dd, J = 6.1, 15.4 Hz, 1H), 2.35 (dd, J = 7.0, 15.3 Hz, 1H), 3.53 (heptet, J = 7.1 Hz, 1H), 3.62 - 3.66 (m, 1H), 3.74 - 3.82 (m, 1H), 3.99 - 4.05 (m, 1H), 4.08 - 4.15 (m, 1H), 6.81 (s, 1H), 6.93 - 7.32 (m, 9H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 19.66, 21.56, 21.72, 26.09, 28.09, 29.93, 36.00, 38.08, 40.87, 42.49, 65.91, 66.44, 80.68, 98.67, 115.20, 115.24, 115.41, 115.46, 119.57, 121.09e, 121.69e, 121.80e, 122.39e, 123.47, 125.86e, 125.94e, 126.39e, 126.47e, 126.54e, 126.91e, 127.00e, 127.73e, 127.76e, 127.80e, 128.15e, 128.30e, 128.34e, 128.40e, 128.45e, 128.65,

128.82e, 128.90e, 128.94e, 129.14e, 129.15e, 129.17e, 129.92e, 130.23e, 130.49e, 130.57e, 131.08e, 133.78e - 133.91e (q, J = 4.6 Hz), 134.38e - 134.50e (m), 134.93e - 135.01e (m), 135.48 - 135.61e (q), 138.43, 161.05, 163.51, 170.15.

[R-(R*)]-2-(4-Fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-[$^2H_5$]phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid calcium salt (2:1) (10a). To a solution of **8a** (537 mg, 0.812 mmol) in 5 mL of MeOH, placed in a 15-mL conical flask, which had been flushed with argon and wrapped in aluminum foil to exclude light,[10] 1 N HCL (0.49 mL) was added incrementally with stirring. The mixture, initially containing a flocculent solid, was stirred in the dark . After 17 h the reaction was nearly complete (TLC, CH_2Cl_2:EtOAc/17:3). To the reaction mixture was added 1 N NaOH (1.3 mL), and stirring was continued for 20 h. TLC analysis showed that hydrolysis was nearly complete (CH_2Cl_2:MeOH/3:1). Water (12 mL) was added and the solution was washed three times with *t*-BuOMe (4-,4- and 2-mL portions). HPLC analysis [0.05 M citric acid(adjusted to pH 4.0 with NH_4OH):CH_3CN: THF:CH_3OH/51:21.6:14.4:13.0; 254 nm] of the aqueous phase showed the presence of **9a** in 98.83% chemical purity.

The aqueous solution of **9a** was partially evaporated, and then 2.9 mL of MeOH and 6 mL of THF were added. Aqueous calcium acetate (0.3M, 1.62 mL, 0.487 mmol) was added incrementally with stirring. The solvent was partially evaporated leaving an aqueous solution which was then extracted four times with EtOAc (8-, 4-, 4- and 2-mL portions). The combined organic phase was washed with a 0.06M aqueous calcium acetate solution and then evaporated to give a residue (448 mg), which was triturated in 8 mL of water overnight with stirring. The solid product was isolated by filtration, followed by a water rinse. It was dried under high vacuum for 18 h resulting in the final product, the calcium salt of [$^2H_5$]CI-981 (**10a**), 310 mg. The yield of **10a** was 65% relative to **8a**; the overall unoptimized yield relative to **2a** was approximately 6%. Chemical purity by HPLC [0.05 M citric acid (adjusted to pH 4.0 with NH_4OH):MeCN:THF:EtOH/51:21.6:14.4:13.0; 254 nm], 98.22%. M.p. 170 - 227 °C.[11] MS (mol wt of free acid form, $C_{33}H_{30}{}^2H_5FN_2O_5$, 563) *m/z*: (AP+) 564.3, (AP-) 562.3. [For comparison, MS *m/z* for unlabeled atorvastatin (**10a**, * = ^1H, mol wt of free acid form, 558): (AP+) 559.3, (AP-) 557.3].

^1H NMR (400 MHz, DMSO-d_6): δ1.17 (m, 1H), 1.31 (d, J = 6.8 Hz, 6H), 1.32 (m, 1H), 1.46 (m, 1H), 1.55 (m, 1H), 1.87 (dd, J = 8.1, 15.4 Hz, 1H), 2.00 (dd, J = 3.9, 15.1 Hz, 1H), 3.17 (septet, J = 6.8 Hz, 1H), 3.30 (br s, 6H, OH), 3.47 (br s, 1H), 3.69 (m, 2H), 3.89 (m, 1H), 6.92 (t, J = 7.2 Hz, 1H), 7.11-7.21 (m, 6H), 7.46 (d, J = 8.1 Hz, 2H), 9.79 (s, 1H). The spectrum was identical to that of unlabeled atorvastatin (**10a**, * = ^1H) except that the latter shows 5 more protons in the aromatic region at δ 6.91 - 7.07 (m).

^{13}C NMR (100 MHz, DMSO-d_6): δ 22.71, 22.75, 26.09, 41.31, 44.22, 44.34, 66.67, 66.74, 115.69, 115.90, 117.94, 119.86, 120.97, 123.39, 127.74, 128.85, 129.22, 133.77, 133.84, 135.16, 136.40, 139.90, 160.80, 163.23, 166.61, 178.32.

The [$^{13}C_8$] and [$^{13}C_7$,^{15}N] analogs (10b, 10c, respectively) were prepared by similar procedures from **8b** (85% yield) and **8c** (25% yield), respectively (Scheme 1).

Compound **10b**. M.p. approximately 150 - 220 °C. MS (mol wt of free acid form, 561) *m/z*: (AP+) 567.2, (AP-), 565.2.

^1H NMR (400 MHz, DMSO-d_6): δ1.19 (m, 1H), 1.31 (d, J = 6.6 Hz, 6H), 1.32 (m, 1H), 1.48 (m, 1H), 1.56 (m, 1H), 1.93(dd, J = 7.9, 15.0 Hz, 1H), 2.06 (dd, J = 3.8, 15.0 Hz, 1H), 3.17 (septet, J = 6.8 Hz, 1H), 3.48 (br s, 1H), 3.71 (br s, 2H), 3.89 (m, 1H), 6.92 (t, J = 7.3 Hz, 1H), 7.11 - 7.19 (m, 6H), 7.46 (d, J = 8.1 Hz, 2H), 9.78 (s, 1H).

^{13}C-NMR (100 MHz, DMSO-d_6): δ 22.75, 26.10, 41.29, 44.11, 44.19, 66.60, 115.70, 115.92, 119.86e, 120.31e, 120.93e, 121.02e, 121.63e, 123.38, 125.13e, 125.20e, 125.64e, 125.72e, 126.15e, 126.26e, 127.42e, 127.99e, 128.08e, 128.52e, 128.85e, 129.03e, 129.59e, 130.19e, 134.48e, 134.54e, 135.05e, 135.64e, 136.20e, 139.89, 160.80, 163.24, 166.59, 177.39.

Compound **10c**. MS (mol wt of free acid form, 561) *m/z*: (AP+) 567.3, (AP-) 565.3.

^1H NMR (400 MHz, DMSO-d_6): δ1.16 (m, 1H), 1.31 (d, J = 6.8 Hz, 6H), 1.32 (m, 1H), 1.46 (m, 1H), 1.56 (m, 1H), 1.85 (dd, J = 7.8, 15.1 Hz, 1H), 1.99 (dd, J = 4.2, 15.1 Hz, 1H), 3.17 (septet, J = 6.8 Hz, 1H), 3.47 (br s, 1H), 3.69 (br s, 2H), 3.90 (m, 1H), 6.92 (t, J = 7.3 Hz, 1H), 7.10 - 7.21 (m, 6H), 7.46 (d, J = 8.1 Hz, 2H), 9.79 (s, 1H).

^{13}C-NMR (100 MHz, DMSO-d_6): δ 22.71, 22.75, 26.09, 44.33, 66.67, 66.77, 115.68, 115.89, 119.86, 120.71e, 121.33e, 125.12e, 125.19e, 125.64e, 125.71e, 126.15e, 126.25e, 127.44e, 127.99e, 128.52e, 128.84, 129.03e, 129.59e, 130.19e, 134.46e, 134.56e, 135.08e, 135.17e, 135.63e, 136.18e, 136.25e, 139.90, 160.79, 163.23, 166.62, 177.97.

[$^{13}C_7$]Benzoic acid (12b or 12c) was prepared from $^{13}C_6$ bromobenzene according to the procedure for **15**. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.53 (dm, J_{1H-13C} = 167 Hz, 2H), 8.12 (dm, J_{1H-13C} = 160 Hz, 2H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 129e (m), 134e (m), 172e (m).

4-Fluoro[α-^{13}C]benzoic acid (15). A solution of 4-fluorobromobenzene (2.66 g, 15.2 mmol) in THF (53 mL) was cooled to -70°C (CO_2/*i*-PrOH) under a nitrogen atmosphere. A hexane solution of *n*-BuLi (6.1 mL, 15.2 mmol) was added over the course of 1 h maintaining the temperature below -70°C. The solution was stirred for 30 minutes. $^{13}CO_2$ was generated from $Ba^{13}CO_3$ (3.00 g, 15.2.mmol) and H_2SO_4 (25 mL) using standard vacuum-line techniques and transferred to the anion solution. The resulting solution was stirred at -70°C for 10 min then allowed to warm to room temperature over 16 h. Water (50 mL) was added to the reaction and the resulting mixture was extracted twice with Et_2O (50 mL). The aqueous layer was acidified with concentrated HCl, then extracted with CH_2Cl_2 (2 x 50 mL), and dried ($MgSO_4$). The solvent was removed *in vacuo* to give **15** as a white solid (1.86 g., 13.3 mmol, 87.4 % yield). MS (mol wt 141) *m/z*: (CI+) 142. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.07 (t, *o*-F, J = 8.7 Hz, 2H), 8.00-8.09 (m, *o*-^{13}COOH, 2H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 117.2, 117.6, 132.3, 132.5, 168.1e.

1,1-Dimethylethyl(4R-cis)-6-[^{15}N]cyanomethyl-2,2-dimethyl-1,3-dioxane-4-acetate (18). 4-Chlorobenzenesulfonyl chloride (3.14 g, 15.9 mmol) was dissolved in toluene (11 mL) and cooled in an ice bath. Et_3N (4.0 mL) was added. 1,1-Dimethylethyl(4R-cis)-6-hydroxymethyl-2,2-dimethyl-1,3-dioxane-4-acetate (**16**) (3.66 g, 14.1 mmol) in toluene (11mL) was added over 15 min. After 15 additional min, the ice bath was removed and the reaction stirred for 20 h at room temperature. The reaction solution was washed with water, dilute $NaHCO_3$, and brine, then dried (Na_2SO_4). Evaporation of the solvent *in vacuo* gave the intermediate sulfonate (**17**). This was dissolved in DMSO (20 mL) and mixed with K^{15}N (1.01 g, 15.2 mmol). After 45 min, methansulfonic acid (0.06 mL) was added and the mixture stirred at room temperature for 20 h and at 45 °C for 5 h. Half of the reaction mixture was treated with LiBr (60 mg) and heating continued for an additional 16 h. To the cooled reaction was added water (50 mL) and extracted with EtOAc (2 x 50 mL). The EtOAc layer was diluted with heptane (100 mL) and washed with water and brine, dried (Na_2SO_4) and evaporated *in vacuo* to give the crude cyanide. Recrystallization from hexane gave **18** (1.569 g, 5.8 mmol, 82% yield corrected for the split of the reaction mixture). IR identical to unlabeled reference sample except for the C^{15}N peak at 2220 cm^{-1} vs. CN at 2245 (CN). MS (CI+) *m/z*: 271, 255, 243, 215, 199, 185, 173, 157, 139, 115.

^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.35 (dd, J = 1.6, 2.3 Hz, 1H), 1.39 (s, 3H), 1.45 (s, 9H), 1.47 (s, 3H), 1.76 (d, J = 12.8 Hz, 1H), 2.40 (ddd, J = 6.6, 15.3, 35.6 Hz, 2H), 2.50-2.53 (m, 2H), 4.13 (m, 1H), 4.16 (m, 1H).

Metabolic studies. In a study of the metabolic fate of CI-981 in male and female Wistar rats (6 per gender), a mixture [d_5], [d_0],and [^{14}C]CI-981,[7a] at 4 μCi/mg, was administered as single oral suspension dose of 10 mg free acid equivalent/kg body weight. Biliary excretions were cannulated through 24 hours and examined by HPLC, and the identity of each of the major radioactive components were established by mass spectrometry.

All analyses were performed by using an Autospec Ultima-Q hybrid mass spectrometer of EBEqQ geometry. Ionization was by LSIMS[8b] using cesium ions with energies of 20 kV for the primary beam (anode potential, 20 kV; anode heater, 2.5 A). The liquid matrix was a mixture of 50:50:1:0.1 acetonitrile:water:glycerol:trifluoroacetic acid that was continuously introduced onto a modified LSIMS probe tip at a flow rate of 6 μL/min. Scan data were acquired under the control of the VG OPUS data system with 10-15 scans at a scan rate of 3 sec/decade[3] typically averaged (MS), and 20-30 scans at a scan rate of 3 secs/decade averaged (MS/MS). 1 μL of HPLC fraction was injected into the liquid matrix stream.

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REFERENCES and FOOTNOTES

1. (a) Synthesis of [^{14}C]Ring-Labeled Atorvastatin. Part I of the preceding papers. (b) Synthesis of [^{14}C]Side-Chain-Labeled Atorvastatin. Part II of the preceding papers.

2. Abbreviations used. APCI = atmospheric pressure chemical ionization. B/E = magnetic field/electrostatic field (type of scan used on double focusing mass spectrometer). Decade = 10 atomic mass units. DMSO = dimethyl sulfoxide. EI = electron impact ionization. ES = electrospray. LC = liquid chromatography. GC = gas chromatography. M = mass spectral molecular weight of the normal unlabeled isotope. See also 4(a) and 8(a).

3. (a) It is not feasible to do a bioequivalence study with radiolabeled atorvastatin. The plasma concentrations are very low (ca. <200 ng/mL) and would have required a very high dose of radiolabel to detect. (b) The metabolite mapping study also cannot not be achieved with ^{14}C, which allows detection but not identification in the presence of a lot of endogenous materials. In contrast, stable-label technique is useful in characterizing the structure of the molecule with MS/MS; the known position of the label aids the interpretation of the spectrum. The characteristic isotope doublet cluster helps identification of drug-derived material.

4. (a) On the other hand, traditional studies like mass-balance, bodily distribution and elimination require radiolabels like ^{14}C. The mass spectral technique that might serve as a replacement, chemical reaction interface ms (CRIMS), requires considerable further development because it is currently not quite as sensitive as radiodetection. (b) The use of stable label as a replacement to radioactivity is a topic of current interest, intensified by the lack of low-level radioactive waste disposal sites.[8]

5. (a) Leal, M., Hayes, M. J., and Powell, M. L.—Biopharm. Drug Dispos. 13: 617 (1992). (The Metabolism of CGS 15873 in Man Using Stable Isotope Pattern Recognition Techniques). (b) Baillie, T. A.—Int. J. Mass Spectrom. Ion Processes: 118, 289 (1992). (Advances in the Application of Mass Spectrometry to Studies of Drug Metabolism, Pharmacokinetics, and Toxicology).

6. (a) Brower, P. L., Butler, D. E., Deering, C. F., Le, T. V., Millar, A., Nanninga, T. N., and Roth, B. D.—Tetrahedron Lett. 33: 2279 (1992). (b) Baumann, K.L., Butler, D.E., Deering, C.F., Mennen, K.E., Millar, A., Nanninga, T.N., Palmer, C.W., and Roth, B.D.—*ibid.* 33: 2283 (1992); Butler, D. E., Deering, C. F., Millar, A., Nanningar, T. N., and Roth, B. D.—U.S. Patent 5,149,837, Sept. 22, 1992)

7. (a) The use of trace amount of ^{14}C label facilitated initial identification atorvastatin-related peaks in HPLC for the rat study. However, the present metabolite-mapping technique can be applied **without** the ^{14}C isotope and is thus particularly advantageous for use in human. (b) The *ortho*-hydroxy and *para*-hydroxy metabolites were differentiated by HPLC comparison with authentic reference samples obtained by independent syntheses (Woolf, Black, et al., manuscript in preparation).

8 (a) In the collisionally activated decomposition of an ion in mass spectrometry (also known as MS/MS or tandem mass spectrometry), an ion accelerated by about 8000 electron volts collides with a stream of helium. Kinetic energy is transformed to internal energy during the collision, resulting in decomposition of the molecule to smaller fragment. (b) LSIMS = liquid secondary ion mass spectrometry = FAB = fast atom bombardment. In LSIMS positive ion mass spectrum, the sample is ionized from an acidic matrix; in LSIMS negative ion mass spectrum, the sample is ionized from a basic matrix.

9. (a) Wacks, M. E.; Pasternak, A. D.; Rychlyk, T.; Meeden, A. R.—Proc. Int. Symp. Exper. Plann. Oper. Low Level Waste Disposal Facil. (1997), Meeting Date 1996, 153-164 Publisher: International Atomic Energy Agency, Vienna, Austria. (b) Gruber, W.—EI Digest; Environmental Information Ltd.: Minneapolis, MN. 2 (1992). (Michigan's Low-Level Waste Crisis).

10. Hurley, T. R., Colson, C. E., Clipper, S. A., Uhlendorf, S. E., and Reily, M. D.—Tetrahedron 49: 1979 (1993).

11. As a calcium salt, atorvastatin typically melts with decomposition over a wide temperature range.[1a]

