Biosynthesis of Lipstatin. Incorporation of Multiply Deuterium-Labeled (5*Z*,8*Z*)-Tetradeca-5,8-dienoic Acid and Octanoic Acid

Markus Goese,[†,‡] Wolfgang Eisenreich,[†] Ernst Kupfer,[§] Peter Stohler,[§] Wolfgang Weber,[§] Hans G. Leuenberger,[§] and Adelbert Bacher*[,†]

Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Federal Republic of Germany, and F. Hoffmann-LaRoche AG, Pharma Research Basel, Discovery Technologies, CH-4070 Basel, Switzerland

adelbert.bacher@ch.tum.de

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Fermentation experiments with *Streptomyces toxytricini* were performed using (5*Z*,8*Z*)-[10,11,12,12-^{2}H]tetradeca-5,8-dienoic acid or a mixture of [2,2-$^2H_2$]- and [8,8,8-$^2H_3$]octanoic acid as supplements. ^{2}H NMR and mass spectroscopy confirmed the incorporation of (5*Z*,8*Z*)-[10,11,12,12-^{2}H]tetradeca-5,8-dienoic acid into the C_{13} side chain as well as into the C_6 side chain of lipstatin. Moreover, deuterium was incorporated into the C_6 side chain of lipstatin from the 8-position but not from the 2-position of octanoate. The data establish that the β-lactone moiety of lipstatin is formed by condensation of a C_8 and a C_{14} fatty acid with a concomitant exchange of the H-2 atoms of the C_8 fatty acid.

Introduction

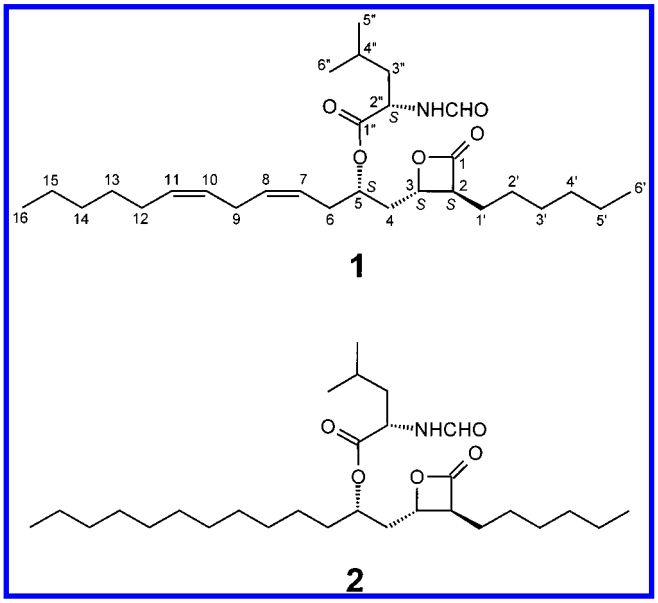
Lipstatin (**1**, Chart 1) is a lipophilic β-lactone produced by *Streptomyces toxytricini* that inhibits pancreatic lipase by formation of a covalent ester adduct.[1−3] The tetrahydro derivative **2** (Orlistat, Xenical; Chart 1) of lipstatin has been introduced recently for the treatment of severe obesity and hypercholesterolemia.

Tracer experiments using ^{13}C-labeled lipid mixtures or D_2O suggested the β-lactone moiety of lipstatin to be biosynthesized from two long-chain fatty acid moieties.[4,5] This paper describes incorporation studies designed to test specific fatty acids as committed precursors of lipstatin.

Results and Discussion

On the basis of well-established procedures of Pommier et al.[10] and Cohen et al.,[11] a modified method for the synthesis of multiply ^{2}H-labeled tetradeca-5,8-dienoic acid was developed. (5*Z*,8*Z*)-[10,11,12,12-^{2}H]Tetradeca-5,8-dienoic acid (**13**, Scheme 1) was prepared from 2-hexenal (**3**) as starting material. Catalytic deuteration of **3** afforded hexanal carrying deuterium atoms in positions 2−4 as shown by ^{2}H NMR and ^{1}H,^{2}H-decoupled ^{13}C NMR spectroscopy. Positional randomization of the deuterium label was observed in line with earlier results[6,7] and will be discussed in more detail below. Chain elongation of the ^{2}H-labeled hexanal (**4**) by two consecutive Wittig reactions afforded ^{2}H-labeled (5*Z*,8*Z*)-tetradeca-5,8-dienoic acid (**13**) as summarized in Scheme 1.

Chart 1



* To whom correspondence should be addressed. Tel: +49-89-289-13360. Fax: +49-89-289-13363.
† Technische Universität München.
‡ Present address: F. Hoffmann-La Roche Ltd, Vitamins Research Biotechnology, CH-4070 Basel, Switzerland.
§ F. Hoffmann-LaRoche AG.

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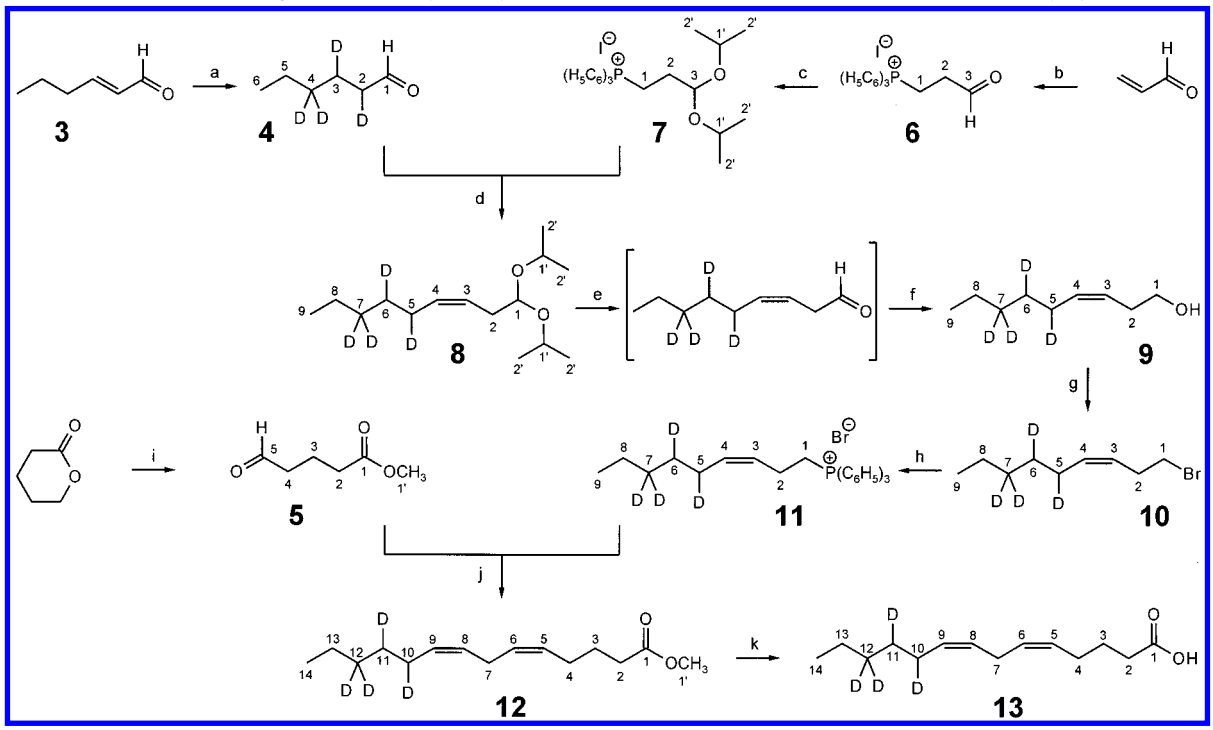
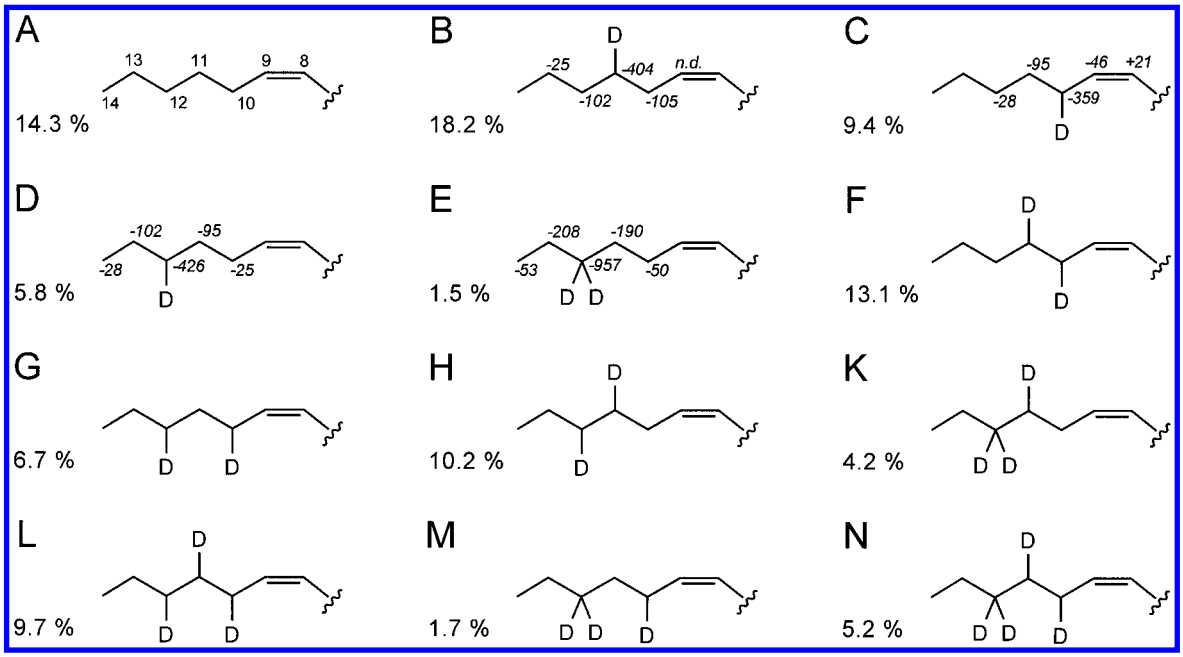
Scheme 1. Synthesis of (5*Z*,8*Z*)-[10,11,12,12-^{2}H]Tetradeca-5,8-dienoic Acid (13)[a]



[a] Reagents and conditions: (a) D_2, Pd/C, EtOAc, rt, 96 h; (b) $P(Ph)_3$, $(Ph)NH^+(Me)_2$ I^-, MeCN, 60 °C, 6 h; (c) $HC(OC_3H_7\text{-}i)_3$, MeCN/isoprop, 0 °C to rt, 2 h; (d) $NaN(SiMe_3)_2$, THF, −100 °C to rt, 24 h; (e) *p*-TsOH/H_2O, THF, 70 °C, 15 min; (f) (1) $LiAlH_4$, THF, −90 to −20 °C, (2) HCl, −20 °C to rt; (g) $(Ph)_3PBr_2$, MeCN/pyr, −7 °C to rt, 1 h; (h) $P(Ph)_3$, toluene, 90 °C, 48 h; (i) (1) H_2SO_4, MeOH, 70 °C, 5 h, (2) PCC, CH_2Cl_2, rt, 2 h; (j) $NaN(SiMe_3)_2$, THF, −100 °C to rt, 24 h; (k) (1) KOH, THF, 70 °C, 4 h, (2) HCl.

Chart 2. Isotopomers of (5*Z*,8*Z*)-[10,11,12,12-^{2}H]Tetradeca-5,8-dienoic Acid (13) Obtained by Catalytic Deuteration of *trans*-2-Hexenal[a]



[a] Key: ^{2}H-upfield shifts are indicated by values in italics. The relative abundance of each isotopomer (%) estimated from ^{1}H,^{2}H-decoupled ^{13}C NMR spectra is also shown.

The product was analyzed in detail by ^{1}H,^{2}H-decoupled ^{13}C NMR spectroscopy. Eight signals were found for C-11 and six signals were found for C-13 (Figure 1), reflecting the presence of 12 different isotopomers carrying up to four deuterium atoms at C-10, C-11, and/or C-12 (isotopomers A−N, Chart 2). ^{2}H isotope shifts are indicated in Figure 1. The chemical shift increments are additive in isotopomers with multiple deuterium substitution. The fraction of each respective isotopomer in the biosynthetic mixture could be determined from the signal integrals in the ^{1}H,^{2}H-decoupled ^{13}C NMR spectra (Chart 2). The average deuterium content per product molecule was 1.7 as estimated by NMR spectroscopy and 1.9 as estimated by mass spectrometry.

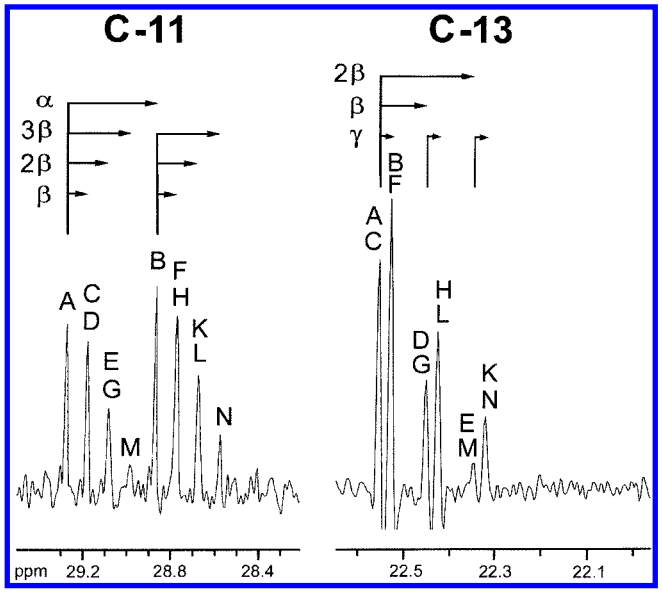


Figure 1. $^{1}H,^{2}H$-decoupled ^{13}C NMR signals of C-11 and C-13 of (5*Z*,8*Z*)-[10,11,12,12-^{2}H]tetradeca-5,8-dienoic acid. Capital letters denote isotopomers shown in Chart 2. α-, *β*-, and *γ*-^{2}H upfield shifts are indicated by arrows.

(5*Z*,8*Z*)-[10,11,12,12-^{2}H]Tetradeca-5,8-dienoic acid (**13**, Scheme 1) was proffered to a fed-batch fermentor culture of *S. toxytricini* together with an excess of unlabeled linoleic acid over a period of 1 h as described in the Experimental Section. The molar ratio of the labeled compound to unlabeled linoleic acid was 1.6%. Lipstatin was isolated and analyzed by mass spectrometry [5] and ^{2}H NMR spectroscopy. The deuterium abundance of lipstatin estimated by mass spectrometry was approximately 1% indicating that the proffered tetradecadienoic acid and linoleic acid were used as precursor of lipstatin with similar efficacy. The ^{2}H NMR spectrum of lipstatin showed two ^{2}H NMR signals at 2.0 and 1.25 ppm (Figure 2B).

Intact incorporation of (5*Z*,8*Z*)-[10,11,12,12-^{2}H]tetradeca-5,8-dienoic acid should result in deuterium enrichments at C-12, C-13, and C-14 of lipstatin (Scheme 2). On basis of the ^{1}H NMR signal assignments of lipstatin,[4] the signal at 2.0 ppm is caused by a deuterium atom at C-12, whereas the broad signal at 1.25 ppm could reflect deuterium atoms at positions C-13 and C-14 of lipstatin. The integral ratio of the C-12 signal and the C-13/C-14 signal was 1:5.2, whereas the corresponding ratio of the ^{2}H NMR signals for C-10 and C-11/C-12 of the precursor was 1:2.5 (Figure 2A). Since the ^{1}H NMR resonances of positions 2′, 3′, and 4′ of lipstatin are also found at chemical shift values of 1.3−1.2 ppm,[4] it appears likely that the ^{2}H NMR signal at 1.25 ppm reflects additional deuterium atoms at positions 2′−4′ of lipstatin. These results suggest that approximately 50% of the labeled lipstatin molecules arose from intact incorporation of the proffered (5*Z*,8*Z*)-[10,11,12,12-^{2}H]tetradeca-5,8-dienoic acid (**13**) and that 50% originated from indirect incorporation of **13** via breakdown to [4,5,6,6-^{2}H]−2-octenoyl-CoA by *β*-oxidation. Obviously, [4,5,6,6-^{2}H]−2-octenoyl-CoA could be reduced to [4,5,6,6-^{2}H]octanoyl-CoA. The latter could then serve as precursor conducive to the formation of [2′,3′,4′,4′-^{2}H]lipstatin (Scheme 2). It should be emphasized that the data clearly rule out total degradation of **13** to acetyl-CoA via *β*-oxidation with subsequent reincorporation via de novo synthesis of fatty acids.

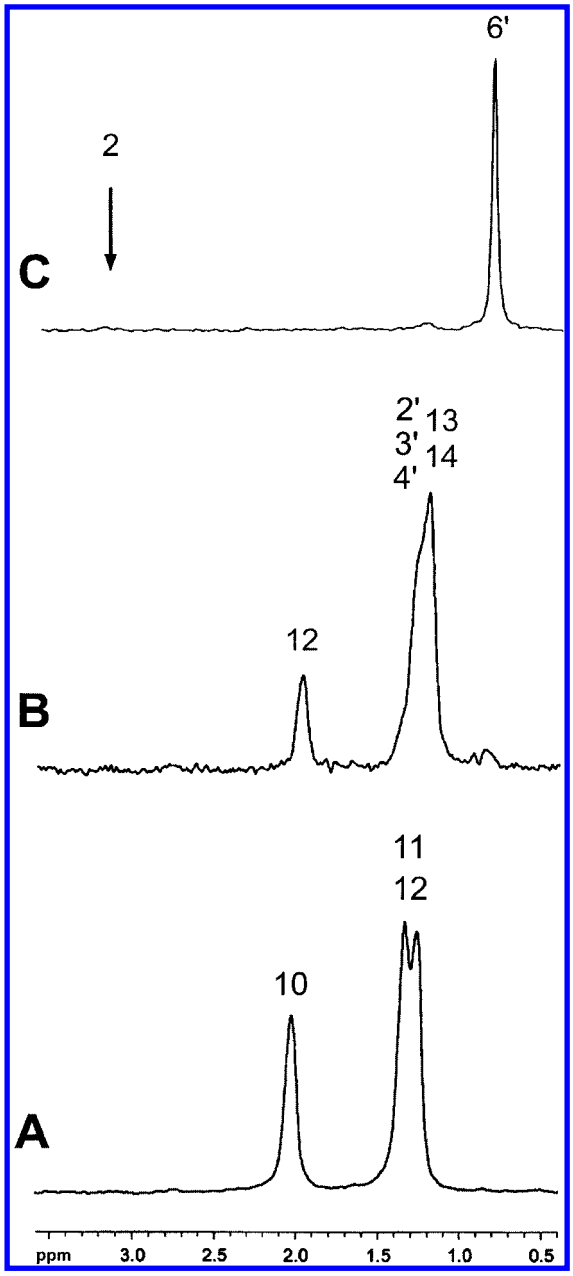
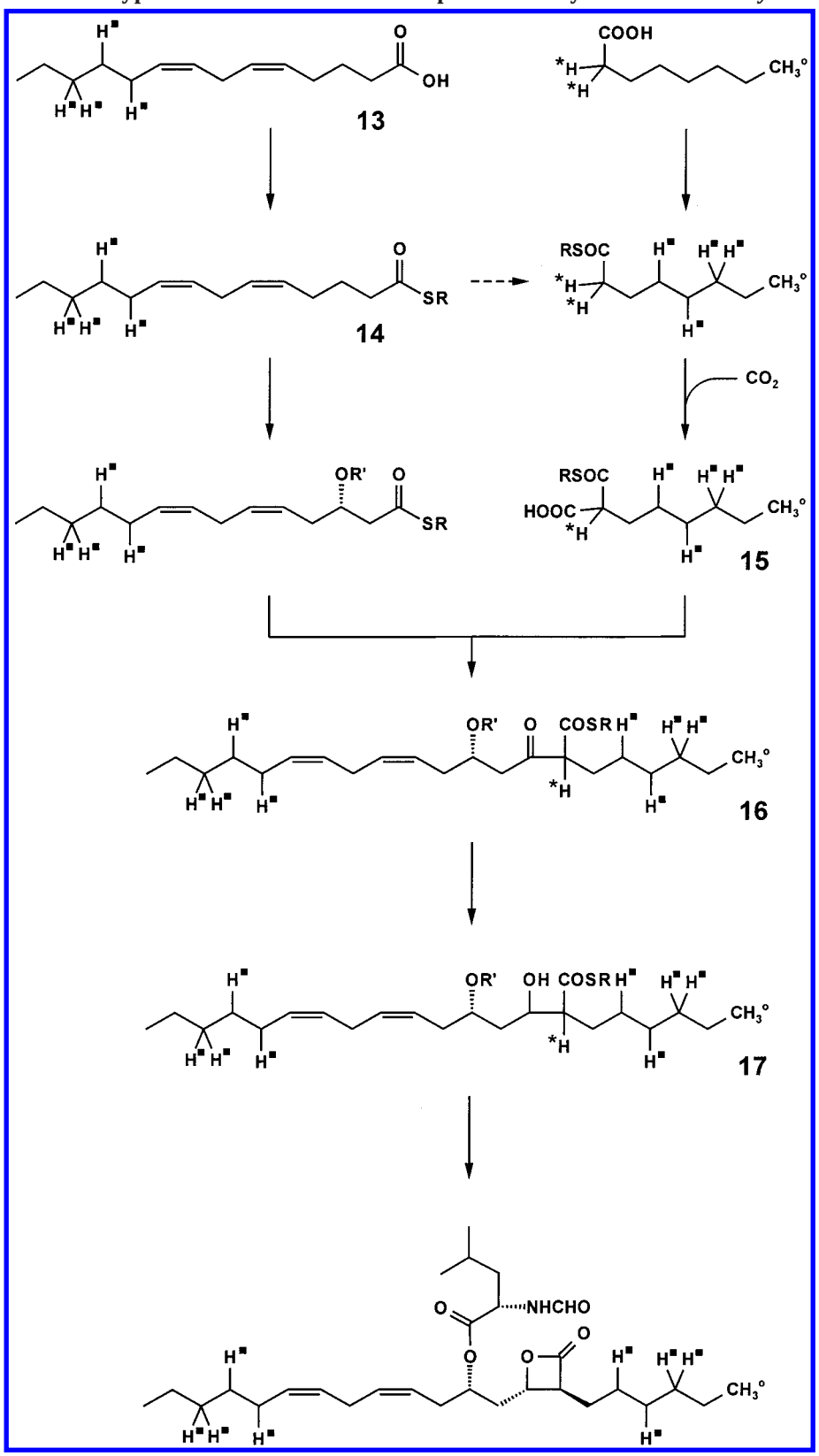


Figure 2. (A) ^{2}H NMR signals of (5*Z*,8*Z*)-[10,11,12,12-^{2}H]-tetradeca-5,8-dienoic acid (**13**). (B) ^{2}H NMR signals of lipstatin isolated from a fed-batch culture of *S. toxytricini* growing with (5*Z*,8*Z*)-[10,11,12,12-^{2}H]tetradeca-5,8-dienoic acid (**13**) as supplement. (C) ^{2}H NMR signals of lipstatin isolated from a fed-batch culture of *S. toxytricini* growing with an equimolar mixture of [2,2-$^{2}H_2$]- and [8,8,8-$^{2}H_3$]octanoic acid as supplement. The arrow points to the position of the absent deuterium signal at C-2 (for details see text).

In a second fermentation experiment with *S. toxytricini*, an equimolar mixture of [2,2-$^{2}H_2$]octanoate and [8,8,8-$^{2}H_3$]octanoate was proffered together with an excess of linoleic acid. The molar ratio of labeled octanoate to linoleic acid was 4.0%. Lipstatin was again isolated and analyzed by mass spectrometry and ^{2}H NMR spectroscopy (Figure 2C). Mass spectrometry established an incorporation rate of approximately 2%. The ^{2}H NMR signal at 0.83 ppm indicates that the position 6′ methyl

Scheme 2. Hypothetical Mechanism of Lipstatin Biosynthesis in *S. toxytricini*[a]



[a] Key: H■ denotes atoms that acquired deuterium labeling from (5*Z*,8*Z*)-[10,11,12,12-^{2}H]tetradeca-5,8-dienoic acid, H° denotes atoms that acquired deuterium labeling from [8,8,8-$^2H_3$]octanoic acid, and H* denotes atoms that were exchanged with the solvent in the course of biosynthetic transformation into lipstatin. R = CoA, R′ = H or formylleucine.

group of lipstatin had been labeled. A ^{2}H NMR signal at 3.16 ppm for the 2-position of lipstatin could not be detected. Accordingly, octanoic acid had been incorporated into lipstatin under complete exchange of the H-2 atoms with solvent water (Scheme 2), confirming earlier results obtained from fermentation experiments with ^2H_2O and ^{2}H-labeled lipid mixtures.[5]

The reported data support the biosynthetic formation

of lipstatin from an unsaturated C_{14} carboxylic acid (**13**) or its thio ester (**14**) and activated octanoate (**15**) as shown in Scheme 2. Decarboxylative condensation could afford a β-keto thioester (**16**). Reduction of **16** could yield the diol **17**. Lactonization of **17** could then yield the β-lactone ring, and formylleucine substitution at C-5 could afford lipstatin.

The absence of deuterium incorporation from the 2-position of octanoate puts stringent requirements on the mechanism of lipstatin biosynthesis. Specifically, one deuterium atom could be removed by carboxylation of octanoate leading to hexyl malonyl CoA (**15**). The observed removal of the second deuterium atom from [2,2-$^2H_2$]octanoate could proceed at the level of **15** during the condensation process or at the level of **17** by epimerization or a dehydration/rehydration process.[5] It should be noted that epimerization also appears to occur in the biosynthesis of the polyketide 6-deoxyerythronolide B.[13] A nonenzymatic loss of ^2H by enolization of **15**, **16**, or **17** does not appear plausible, since the rates of chemical exchange are slow in malonyl-CoA derivatives compared to the typical lifetimes of biochemical intermediates[14−16] (H. G. Floss, personal communication).

Experimental Section

Materials. [2,2-$^2H_2$]Octanoic acid (98% ^2H) and [8,8,8-$^2H_3$]-octanoic acid (98% ^2H) were purchased from Phychem (Düren, Germany). Deuterium (D_2, 99.6%) was from Cambridge Isotope Laboratories (Woburn, MA). All other chemicals were from Aldrich (Steinheim, Germany), Sigma (Deisenhofen, Germany), Fluka (Buchs, Switzerland), and Merck (Darmstadt, Germany). Solvents were redistilled and dried over molecular sieves (4 Å) or, in the case of THF and diethyl ether, sodium.

NMR Spectroscopy. NMR measurements were performed in $CDCl_3$ or $CHCl_3$ (^2H NMR experiments) at 17 °C using a Bruker DRX 500 spectrometer operating at 500.13 MHz for ^1H experiments, 125.76 MHz for ^{13}C experiments, and 76.77 MHz for ^2H NMR experiments, a Bruker AM 360 spectrometer operating at 360.13 MHz for ^1H experiments and 90.6 MHz for ^{13}C experiments, or a Bruker AC 250 spectrometer operating at 250.13 MHz for ^1H experiments, 62.9 MHz for ^{13}C experiments, and 101.3 MHz for ^{31}P experiments. The DRX 500 spectrometer was equipped with a lock-switch unit for ^2H-decoupling experiments using the lock channel. NMR experiments were performed with standard Bruker software (XWIN-NMR 1.3). Prior to Fourier transformation, the free induction decay was multiplied with a Gaussian function. NMR assignments were verified by two-dimensional NMR analysis ($^1H-^1H$-TOCSY, $^1H-^1H$-COSY, $^1H-^{13}C$-HMQC, $^1H-^{13}C$-HMBC) and referenced to the $CDCl_3$ solvent signal or external H_3PO_4 (^{31}P NMR). ^{13}C NMR data listed below were achieved by ^1H-decoupled measurements. ** in the spectroscopic data denotes NMR signals of atoms that are strongly weakened by ^2H substitution, * denotes NMR signals of atoms that are slightly weakened by ^2H substitution.

Preparation of Labeled Compounds. Methyl 5-oxopentanoate (5) was prepared as described:[8] ^1H NMR (250 MHz) δ ppm 9.78 (t, ^3J = 1.3 Hz, 1 H-5), 3.68 (s, 3 H-1′), 2.55 (dt, ^3J = 7.1 Hz, 1.3 Hz, 2 H-4), 2.39 (t, ^3J = 7.1 Hz, 2 H-2), 1.96 (quint, ^3J = 7.1 Hz, 2 H-3); ^{13}C NMR (62.9 MHz) δ ppm 201.4 (C-5), 173.2 (C-1), 51.5 (C-1′), 42.8 (C-4), 32.8 (C-2), 17.2 (C-3).

(3-Oxopropyl)triphenylphosphonium iodide (6) was prepared as described:[9] ^1H NMR (360 MHz) δ ppm 9.60 (t, ^3J = 1.9 Hz, 1 H-3), 7.75−7.56 (m, 15 $H_{arom.}$), 3.75 (dt, $^2J_{HP}$ = 12.9 Hz, ^3J = 7.1 Hz, 2 H-1), 3.07 (dt, $^3J_{HP}$ = 13.5 Hz, ^3J = 7.1 Hz, 2 H-2); ^{13}C NMR (90.6 MHz) δ ppm 197.0 (C-3, $^3J_{CP}$ = 11.3 Hz), 135.5 (3 $C_{arom.}$, $^4J_{CP}$ = 3.3 Hz), 133.7 (6 $C_{arom.}$, $^2J_{CP}$ = 10.0 Hz), 130.6 (6 $C_{arom.}$, $^3J_{CP}$ = 12.6 Hz), 117.0 (3 $C_{arom.}$, $^1J_{CP}$ = 86.9 Hz), 35.7 (C-2, $^2J_{CP}$ = 3.1 Hz), 15.7 (C-1, $^1J_{CP}$ = 55.9 Hz); ^{31}P NMR (101.3 MHz, $H_3PO_{4\ ext.}$) δ ppm +25.5.

(3,3-Diisopropoxypropyl)triphenylphosphonium iodide (7). A 92.30 g portion of **6** (0.207 mol) was dissolved in 450 mL of a mixture of dichloromethane and 2-propanol (5:4, v/v) and cooled to 0 °C in an ice bath. A 116.9 mL portion of triisopropyl orthoformate (0.414 mol) and 1.5 mL of HCl concentrated were added. The solution was stirred for 2 h and allowed to warm to room temperature. Triethylamine was added until an alkaline pH was reached, and approximately half of the solvent was removed under reduced pressure. A 500 mL portion of a mixture of dichloromethane, diethyl ether, and pentane (1:2:2, v/v/v) was added to induce crystallization, and the mixture was cooled at − 80 °C overnight. The resulting white crystals were removed by filtration, washed with cold pentane, and dried in vacuo: yield 116.80 g (97%); ^1H NMR (360 MHz) δ ppm 7.76−7.64 (m, 15 $H_{arom.}$), 5.10 (t, ^3J = 4.7 Hz, 1 H-3), 3.86 (sept, ^3J = 6.1 Hz, 2 H-1′), 3.62 (m, 2 H-1), 1.85 (m, 2 H-2), 1.15 (d, ^3J = 6.1 Hz, 6 H-2′), 1.11 (d, ^3J = 6.1 Hz, 6 H-2′); ^{13}C NMR (90.6 MHz) δ ppm 135.2 (3 $C_{arom.}$, $^4J_{CP}$ = 3.1 Hz), 133.5 (6 $C_{arom.}$, $^2J_{CP}$ = 10.0 Hz), 130.5 (6 $C_{arom.}$, $^3J_{CP}$ = 12.6 Hz), 118.0 (3 $C_{arom.}$, $^1J_{CP}$ = 86.3 Hz), 98.3 (C-3, $^3J_{CP}$ = 17.7 Hz), 69.7 (2 C-1′), 29.0 (C-2, $^2J_{CP}$ = 3.8 Hz), 23.2 (2 C-2′), 22.9 (2 C-2′), 18.2 (C-1, $^1J_{CP}$ = 53.5 Hz); ^{31}P NMR (101.3 MHz, $H_3PO_{4\ ext.}$) δ ppm +25.5.

[2,3,4,4-^2H]Hexanal (4). A 17.64 g (0.180 mol) portion of *trans*-2-hexenal (**3**) was added to a mixture of 1.7 g of palladium (5%) on charcoal and 100 mL of ethyl acetate. The mixture was flushed with nitrogen, evacuated, and then incubated in a D_2-atmosphere for 96 h under room temperature and normal pressure. After consumption of 4.1 L of deuterium (0.183 mol), the reaction was stopped and the mixture filtered through Celite. The filter cake was washed twice with diethyl ether. The solvent was removed under reduced pressure at room temperature. The resulting aldehyde was distilled immediately prior to the subsequent Wittig reaction (bp 135 °C/ 1013 mbar): yield 11.00 g (60%); ^1H NMR (360 MHz) δ ppm 9.70 (t, ^3J = 1.8 Hz, 1 H-1), 2.37 (dt-like, H-2**), 1.56 (quint-like, H-3**), 1.26 (m, H-4** + H-5), 0.84 (t, ^3J = 7.7 Hz, 3 H-6); ^{13}C NMR (90.6 MHz) δ ppm 202.6 (C-1), 43.6 (C-2**), 31.3 (C-4**), 22.4 (C-5**), 21.7 (C-3**), 13.7 (C-6*); ^2H NMR (76.7 MHz) δ ppm 2.36 (br s, D-2), 1.58 (br s, D-3), 1.24 (br s, D-4).

(3*Z*)-[5,6,7,7-^2H]Non-3-enal Diisopropyl Acetal (8). An 88.76 g portion of dry **7** (0.162 mol) was suspended in 1200 mL of THF, and 162 mL of sodium bis-trimethyl silyl amide (1 M in THF, 0.162 mol) was added slowly under nitrogen. The orange solution was stirred for 1 h at room temperature and then cooled to −100 °C using liquid nitrogen in ethanol. An 11.00 g portion of compound **4** (0.108 mol) was added dropwise using a syringe, and the solution was allowed to warm to room temperature overnight. An aqueous saturated solution of ammonium chloride (250 mL) and 500 mL of diethyl ether were added. The organic phase was separated, and the aqueous phase was washed three times with 250 mL of diethyl ether. The combined organic solutions were washed repeatedly with aqueous saturated sodium chloride and then dried over magnesium sulfate. The solvent was removed under reduced pressure, and the crude product was purified by distillation in vacuo (bp 72 °C/ 0.5 mbar): yield 17.08 g (65%); ^1H NMR (500 MHz) δ ppm 5.45 (m, 1 H-4), 5.39 (m, 1 H-3), 4.54 (t, ^3J = 5.6 Hz, 1 H-1), 3.87 (sept, ^3J = 6.2 Hz, 2 H-1′), 2.34 (t, ^3J = 6.2 Hz, 2 H-2), 2.03 (q-like, H-5**), 1.28 (m, H-6** + H-7** + H-8), 1.19 (d, ^3J = 6.2 Hz, 6 H-2′), 1.14 (d, ^3J = 6.2 Hz, 6 H-2′), 0.88 (t, ^3J = 6.6 Hz, 3 H-9); ^{13}C NMR (125.8 MHz) δ ppm 132.1 (C-4*), 124.3 (C-3), 100.1 (C-1), 67.8 (C-1′), 33.8 (C-2), 31.6 (C-7**), 29.3 (C-6**), 27.5 (C-5**), 23.4 (2 C-2′), 22.6 (2 C-2′), 22.5

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(C-8**), 14.1 (C-9*); ^{2}H NMR (76.7 MHz) δ ppm 2.01 (br s, D-5), 1.29 (br s, D-6 + D-7).

(3*Z*)-[5,6,7,7-^{2}H]Non-3-en-1-ol (9) was prepared using the procedure of Pommier et al.[10] (3*Z*)-Non-3-enal diisopropyl acetal was replaced by **8**: ^{1}H NMR (500 MHz) δ ppm 5.51 (m, 1 H-4), 5.32 (m, 1 H-3), 3.60 (t, ^3J = 6.6 Hz, 2 H-1), 2.30 (q, ^3J = 6.6 Hz, 2 H-2), 2.01 (t-like, H-5**), 1.78 (br s, OH), 1.26 (m, H-6** + H-7** + H-8), 0.84 (t, ^3J = 7.1 Hz, 3 H-9); ^{13}C NMR (125.8 MHz) δ ppm 133.5 (C-4*), 124.7 (C-3), 62.2 (C-1), 31.4 (C-7**), 30.8 (C-2), 29.3 (C-6**), 27.3 (C-5**), 22.5 (C-8**), 14.0 (C-9*); ^{2}H NMR (76.7 MHz) δ ppm 2.02 (br s, D-5), 1.27 (br s, D-6 + D-7).

(3*Z*)-[5,6,7,7-^{2}H]1-Bromonon-3-en (10) was prepared using the procedure of Pommier et al.[10] (3*Z*)-Non-3-en-1-ol was replaced by **9**: ^{1}H NMR (500 MHz) δ ppm 5.52 (m, 1 H-4), 5.35 (m, 1 H-3), 3.36 (t, ^3J = 7.2 Hz, 2 H-1), 2.61 (q, ^3J = 7.1 Hz, 2 H-2), 2.04 (t-like, H-5**), 1.29 (m, H-6** + H-7** + H-8), 0.89 (t, ^3J = 6.6 Hz, 3 H-9); ^{13}C NMR (125.8 MHz) δ ppm 133.2 (C-4*), 125.7 (C-3), 32.3 (C-1), 31.4 (C-7**), 30.9 (C-2), 29.2 (C-6**), 27.3 (C-5**), 22.5 (C-8**), 14.0 (C-9*); ^{2}H NMR (76.7 MHz) δ ppm 2.01 (br s, D-5), 1.29 (br s, D-6 + D-7).

(3*Z*)-[5,6,7,7-^{2}H]Triphenylnon-3-enylphosphonium Bromide (11). The compound was prepared by the procedure of Cohen et al.[11] (3*Z*)-1-Iodonon-3-ene was replaced by **10**: ^{1}H NMR (500 MHz) δ ppm 7.86−7.73 (m, 15 $H_{arom.}$), 5.55 (m, 1 H-3), 5.38 (m, 1 H-4), 3.80 (m, 2 H-1), 2.44 (m, 2 H-2), 1.76 (t-like, H-5**), 1.20 (m, H-6** + H-7** + H-8), 0.83 (t, ^3J = 7.3 Hz, 3 H-9); ^{13}C NMR (90.6 MHz) δ ppm 135.0 (3 $C_{arom.}$, $^4J_{CP}$ = 2.7 Hz), 133.5 (6 $C_{arom.}$, $^2J_{CP}$ = 10.3 Hz), 132.3 (C-4), 130.3 (6 $C_{arom.}$, $^3J_{CP}$ = 12.6 Hz), 125.6 (C-3, $^3J_{CP}$ = 14.1 Hz), 117.6 (3 $C_{arom.}$, $^1J_{CP}$ = 85.8 Hz), 31.0 (C-7**), 28.6 (C-6**), 26.8 (C-5**), 22.7 (C-1, $^1J_{CP}$ = 48.8 Hz), 22.1 (C-8**), 20.0 (C-2, $^2J_{CP}$ = 3.4 Hz), 13.8 (C-9); ^{2}H NMR (76.7 MHz) δ ppm 1.75 (br s, D-5), 1.18 (br s, D-6 + D-7); ^{31}P NMR (101.3 MHz, $H_3PO_{4\ ext}$) δ ppm + 24.5.

(5*Z*,8*Z*)-Methyl [10,11,12,12-^{2}H]Tetradeca-5,8-dienoate (12). A 5.83 g portion of dry **11** (0.0125 mol) was suspended in 100 mL of THF and cooled to 0 °C. A 12.5 mL portion of sodium bis-trimethyl silyl amide (1 M in THF, 0.0125 mol) was added slowly under nitrogen. The orange solution was stirred for 1 h at room temperature and then cooled to −100 °C. A 0.71 g (0.0084 mol) portion of compound **5** was added with a syringe, and the solution was allowed to warm to room temperature overnight. A 20 mL portion of aqueous saturated ammonium chloride was added followed by 50 mL of diethyl ether. The organic layer was separated from the aqueous layer ,and the latter washed three times with 25 mL of diethyl ether. The combined organic layers were washed with aqueous saturated sodium chloride repeatedly and then dried over magnesium sulfate. The solvent was removed under reduced pressure and the crude product purified by distillation in vacuo (bp 92 °C/ 0.4 mbar): yield 1.59 g (65%); ^{1}H NMR (500 MHz) δ ppm 5.41−5.28 (m, 1 H-5 + 1 H-6 + 1 H-8 + 1 H-9), 3.67 (s, 3 H-1′), 2.76 (t, ^3J = 6.9 Hz, 2 H-7), 2.32 (t, ^3J = 7.6 Hz, 2 H-2), 2.11 (q, ^3J = 7.1 Hz, 2 H-4), 2.04 (q-like, H-10**), 1.70 (quint, ^3J = 7.4 Hz, 2 H-3), 1.30 (m, H-11** + H-12** + H-13), 0.89 (t, ^3J = 7.1 Hz, 3 H-14); ^{13}C NMR (125.8 MHz) δ ppm 174.1 (C-1), 130.3 (C-9*), 129.2 (C-6), 128.6 (C-5), 127.5 (C-8*), 51.5 (C-1′), 33.4 (C-2), 31.5 (C-12**), 29.3 (C-11**), 27.1 (C-10**), 26.5 (C-4), 25.5 (C-7), 24.7 (C-3), 22.5 (C-13**), 14.1 (C-14*); ^{2}H NMR (76.7 MHz) δ ppm 2.02 (br s, D-10), 1.28 (br s, D-11 + D-12); IR (CCl_4) ν cm^{-1} 3009, 2926, 2856, 1742, 1436, 1244, 1206, 1156, 722.

(5*Z*,8*Z*)-[10,11,12,12-^{2}H]Tetradeca-5,8-dienoic Acid (13). A 1.00 g portion of **12** (0.00417 mol) was added to a mixture of 30 mL of THF and 10 mL of 2 M KOH. The solution was stirred for 4 h under gentle reflux and then acidified using 2 M HCl. The product was extracted repeatedly with diethyl ether. The combined organic layers were washed with aqueous saturated sodium chloride and water and dried over magnesium sulfate, and the solvent was removed under reduced pressure: yield 0.63 g (67%); ^{1}H NMR (500 MHz) δ ppm 5.37−5.28 (m, 1 H-5 + 1 H-6 + 1 H-8 + 1 H-9), 2.75 (t, ^3J = 7.0 Hz, 2 H-7), 2.35 (t, ^3J = 7.5 Hz, 2 H-2), 2.11 (q, ^3J = 7.2 Hz, 2 H-4), 2.02 (q-like, H-10**), 1.69 (quint, ^3J = 7.5 Hz, 2 H-3), 1.28 (m, H-11** + H-12** + H-13), 0.86 (t, ^3J = 7.2 Hz, 3 H-14); ^{13}C NMR (125.8 MHz) δ ppm 180.3 (C-1), 130.4 (C-9*), 129.4 (C-6), 128.2 (C-5), 127.5 (C-8*), 33.4 (C-2), 31.5 (C-12**), 29.3 (C-11**), 27.2 (C-10**), 26.4 (C-4), 25.6 (C-7), 24.4 (C-3), 22.6 (C-13**), 14.1 (C-14*); ^{2}H NMR (76.7 MHz) δ ppm 2.01 (br s, D-10), 1.28 (br s, D-11 + D-12); IR (CCl_4) ν cm^{-1} 3009, 2926, 2857, 1709, 1456, 1413, 1240, 935, 723.

Fermentation. Fed-batch fermentation experiments with *S. toxytricini* were performed using a 14 L Chemap fermentor (Männedorf/Zürich, Switzerland) with a working volume of 8 L according to published procedures.[12] Two hours after feed-start, a mixture of 0.198 g of carboxylic acid **13** and 0.92 g of unlabeled linoleic acid (Fluka, Switzerland) was applied at a rate of 37 mg min^{-1}. In a second experiment, a mixture of 0.484 g of [2,2-$^2H_2$]octanoic acid, 0.479 g of [8,8,8-$^2H_3$]octanoic acid, and 4.84 g of unlabeled linoleic acid was inoculated at a rate of 45 mg min^{-1}. The molar ratio of the labeled compound to the total added fatty acids was 1.6% in the first experiment and 4.0% in the second experiment. After an incubation time of 1 h, both experiments were stopped and the fermentation broth was heated to 70 °C for 10 min.

Isolation of Lipstatin. Lipstatin was isolated essentially as described [4] and was 94.7% pure (first experiment) and 98.8% pure (second experiment) as judged by high-performance liquid chromatography (HPLC).

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