Biosynthetic Precursors of the Lipase Inhibitor Lipstatin

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Three putative intermediates in the biosynthesis of the lipase inhibitor lipstatin were synthesized in stable isotope-labeled form and were added to fermentation cultures of *Streptomyces toxytricini*. Biosynthetic lipstatin was isolated and analyzed by NMR spectroscopy. [3,10,11,12-^{2}H]-(3*S*,5*Z*,8*Z*)-3-hydroxytetradeca-5,8-dienoic acid (**9**) was shown to serve as a direct biosynthetic precursor of lipstatin. [7,8-$^2H_2$]Hexylmalonate (**11**) was also incorporated into lipstatin, albeit at a relatively low rate. The leucine moiety of [^{13}C-formyl,^{15}N]-*N*-formylleucine (**10**) was diverted to lipstatin under loss of the ^{13}C-labeled formyl residue.

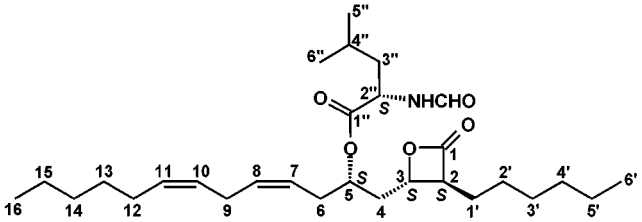
Introduction

The lipophilic β-lactone lipstatin is a product of *Streptomyces toxytricini*.[1,2] The compound irreversibly inhibits pancreatic lipase by covalent modification of a catalytic serine residue.[3] The tetrahydro derivative of lipstatin (Orlistat, Xenical) is used for the management of severe obesity (Chart 1).

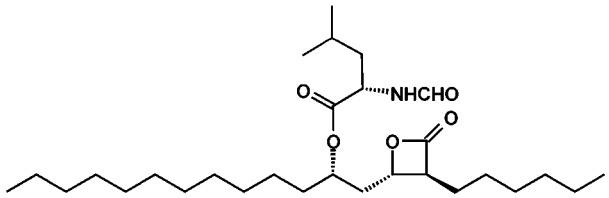
We have shown previously that the β-lactone moiety of lipstatin is biosynthesized by condensation of two fatty acid residues with chain lengths of 8 and 14 carbon atoms, respectively, which have been proposed to be obtained by incomplete β oxidation of long-chain fatty acids.[4,5] Former studies had also shown that [10,11,12-^{2}H]-(5*Z*,8*Z*)-tetradeca-5,8-dienoic acid is incorporated into lipstatin by growing cultures of *S. toxytricini*.[6] 3-Hydroxytetradeca-5,8-dienoic acid or a derivative thereof had been proposed as a further downstream intermediate in the biosynthetic pathway of lipstatin.[6]

To accurately determine the committed precursors, we synthesized isotope-labeled (3*S*,5*Z*,8*Z*)-3-hydroxytetradeca-5,8-dienoic acid (**9**), *N*-formylleucine (**10**), and hexyl malonate (**11**). The compounds were proffered to cultures of *S. toxytricini* in separate experiments. Labeling patterns of isolated lipstatin were determined by NMR spectroscopy.

Chart 1



Lipstatin



Orlistat

Results

[3,10,11,12-^{2}H]-(3*S*,5*Z*,8*Z*)-3-Hydroxytetradeca-5,8-dienoic acid (**9**) was prepared as described in the Experimental Section in an overall yield of 11%. Briefly, the dipropyl ester of 3-ketoglutarate was converted into [3-^{2}H]-3-hydroxyglutaric acid dipropyl ester (**2**) by catalytic deuteration. [3-^{2}H]-(3*S*)-3-Hydroxyglutaric acid monopropyl ester (**3**) was obtained by reaction of **2** with pig liver esterase. Compound **3** was converted into the 3-*tert*-butyldiphenylsiloxy derivative **4**. Subsequently, **4** was converted via **5** to [3-^{2}H]-(3*S*)-5-oxo-3-*tert*-butyldiphenylsiloxypentanoic acid propyl ester (**6**). Wittig reaction of **6** and [5,6,7-^{2}H]-(3*Z*)-triphenylnon-3-enylphosphonium bromide (**7**) afforded the *tert*-butyldiphenylsiloxy derivative **8**, which was finally deprotected to yield **9** (Scheme 1).

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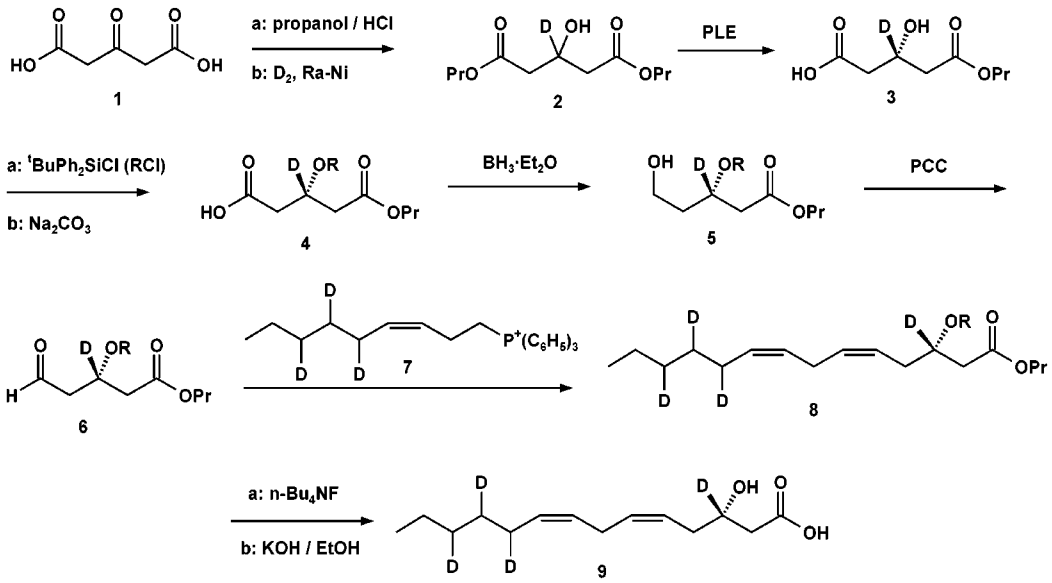
Scheme 1



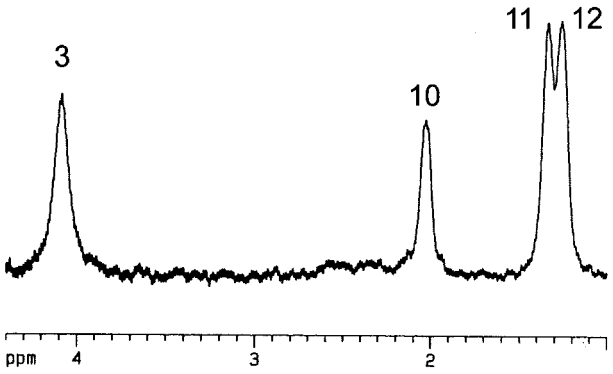


Figure 1. ^{2}H NMR spectrum of [3,10,11,12-^{2}H](3*S*,5*Z*,8*Z*)-3-hydroxytetradeca-5,8-dienoic acid (**9**).

Table 1. ^{2}H NMR Data of [3,10,11,12-^{2}H](3*S*,5*Z*,8*Z*)-3-Hydroxytetradeca-5,8-dienoic Acid (9) and Lipstatin from the Feeding Experiment with [3,10,11,12-^{2}H]-9

compd	position	δ ^{2}H (ppm)	integral
[3,10,11,12-^{2}H]-(3*S*,5*Z*,8*Z*)-3-hydroxytetradeca-5,8-dienoic acid (**9**)	3	4.1	1.0
	10	2.0	0.7
	11/12	1.3/1.2	1.8
lipstatin	5	5.0	1.0
	12	2.0	0.7
	13/14	1.3/1.2	3.2
	2′, 3′, 4′		

The ^{2}H NMR spectrum of **9** (Figure 1) showed four signals. Relative intensities of the ^{2}H signals are summarized in Table 1. On the basis of the ^{1}H NMR signal assignments of **9** (see the Experimental Section), the observed ^{2}H NMR signals were attributed to positions 3 and 10−12. The relative intensities of the signals of positions 10−12 reflected the isotopomer composition of the precursor **7**.[6]

[3,10,11,12-^{2}H]-**9** was dissolved in a 5-fold excess of linoleic acid and was proffered to a fermentation culture of *S. toxytricini* over a period of 30 min. Lipstatin was isolated and was analyzed by ^{1}H and ^{2}H NMR spectroscopy. ^{2}H NMR signals were observed at chemical shifts of 5.0, 2.0, 1.3, and 1.2 ppm (Figure 2B). On the basis of the known ^{1}H NMR signal assignments of lipstatin[4] (Figure 2A), the ^{2}H NMR signals at 5.0 and 2.0 ppm can be unequivocally attributed to H-5 and H-12, respectively. The approximate deuterium abundance of position 5 was 1.0% as determined by NMR spectroscopy using the signal of the unlabeled chloroform solvent as reference.

Intact incorporation of **9** into lipstatin should result in deuterium enrichments of positions 5, 12, and 13/14. The observed integral ratio of these signals was 1.0/0.7/3.2, whereas the corresponding ratio of the ^{2}H NMR signals of positions 3, 10, and 11/12 of compound **9** was 1.0/0.7/1.8 (Table 1). Therefore, it appears likely that the enhanced intensity of the ^{2}H signals of lipstatin at 1.2/1.3 ppm reflects not only ^{2}H label at positions 13/14 but additionally at positions 2′, 3′, and 4′.

Earlier studies had shown that octanoate can serve as the C_8 building block in lipstatin biosynthesis.[4] In analogy with other biosynthetic Claisen condensations,[7] it appears possible that octanoate is activated by carboxylation to the assumed precursor hexyl malonate. To test this hypothesis, we prepared [7,8-$^2H_2$]hexyl malonate (**11**), which was applied to a culture of *S. toxytricini* with a large excess of linoleic acid.

The ^{2}H NMR spectrum of isolated lipstatin showed two signals with equal intensities at 1.2 and 0.8 ppm reflecting ^{2}H enrichments at positions 5′ and 6′ (Figure 2C). The approximate deuterium abundance of positions 5′ and 6′ was 0.2% as determined by NMR using the solvent signal as internal reference.

Finally, we studied the origin of the formyl leucine moiety since it was still unknown whether the formyl residue is attached to leucine before or after the aminoacylation of the lipophilic C_{22} precursor of lipstatin. [^{13}C-formyl,^{15}N]-*N*-formylleucine (**10**) was synthesized as described in the Experimental Section and was proffered to a fermentation culture of *S. toxytricini* together with unlabeled (*S*)-leucine at a ratio of about 1:9. Lipstatin was isolated and analyzed by ^{1}H NMR spectroscopy. The amide ^{1}H signal showed satellites which could be unequivocally attributed to ^{1}H^{15}N coupling by ^{15}N decoupling experiments (Figure 3). Their intensities correspond

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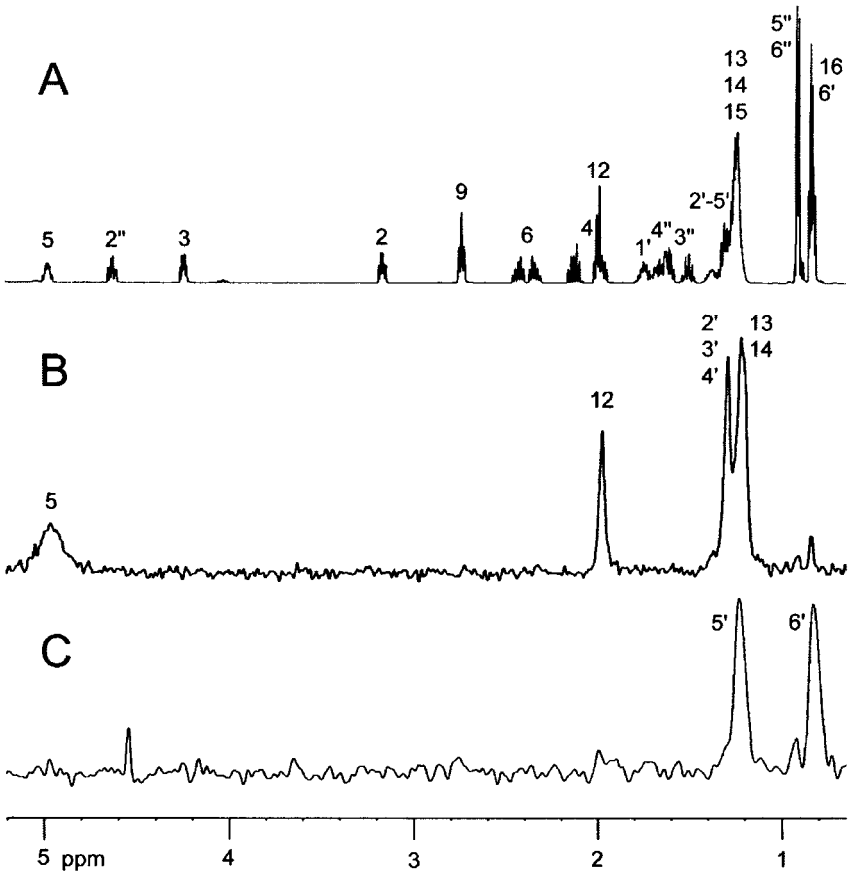

Figure 2. ^{1}H NMR spectrum of lipstatin isolated after feeding of **9** (A), the corresponding ^{2}H NMR spectrum (B), and ^{2}H NMR spectrum of lipstatin isolated after feeding of **11** (C).

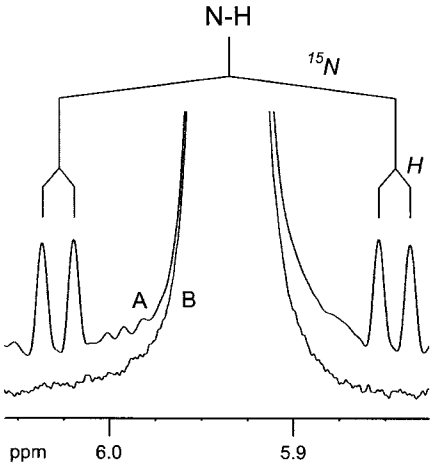

Figure 3. ^{1}H NMR signal of the amide proton of lipstatin isolated after feeding of **10**. Satellites in A are due to coupling with ^{15}N ($^{1}J_{HN}$ = 91 Hz). The integrals of the satellites correspond to 2.6% ^{15}N abundance, which is significantly above normal levels of 0.37%. The further splitting (J = 8.6 Hz), which can also be observed for the central signal if not truncated, is the result of ^{1}H^{1}H coupling. B was obtained under ^{1}H{^{15}N}-decoupling conditions.

to a ^{15}N abundance of 2.6%, which is significantly above the natural abundance level of 0.37%.

The intensity of ^{13}C satellites of the formyl ^{1}H signal was determined as 1.1%, reflecting the natural abundance of ^{13}C. In contrast, if **10** had been incorporated intact, one would expect an intensity of 3.3%. Additionally, a further splitting of the signal of the formamide hydrogen due to its coupling with the ^{13}C formyl carbon atom would have been expected ($J \approx 4$ Hz).

The CI mass spectrum signal of $[M - CO_2 + H^+]^+$ (m/z 448) showed an isotope peak at m/z 449 that was about 2 percentage points more intensive than the corresponding signal of an unlabeled reference, whereas the signal at m/z 450 was not enhanced (data not shown). These data show that the ^{15}N label but no ^{13}C label was transferred to lipstatin.

Discussion

The transfer of deuterium from the 3 position of proffered **9** shows that the β-carbon atom of **9** did not pass through an oxidation/reduction cycle, which would have resulted in the loss of the label. In conjunction with the data from our earlier incorporation studies,[4−6] we can rule out a structural modification of the hydroxydienyl side chain of **9** during the biochemical transformation into lipstatin.

The enhancement of the ^{2}H signal intensity at 1.2/1.3 ppm was also observed after feeding of the dehydroxy derivative of **9**.[6] It therefore appears likely that an incorporation of **9** into the hexyl side chain of lipstatin takes place, after its conversion to the thioester of octanoic acid and hexyl malonate.

From the data, it can be concluded that about one-third of the overall amount of precursor incorporated into lipstatin is incorporated via the hexyl side chain.

The relatively low incorporation of **11** leaves room for several different interpretations. Hexyl malonate could be the actual biosynthetic precursor but is less efficiently

converted to the required thioester form as compared to octanoate. Alternatively, the proffered hexyl malonate could be enzymatically decarboxylated prior to utilization for the biosynthesis of lipstatin. The incorporation data are not sufficient to conclusively prove the proposed hypothesis of α-carboxylation of octanoate.

We found that only ^{15}N label but no ^{13}C label is incorporated into lipstatin from compound **10**. Obviously, incorporation can only take place after hydrolysis of the formamide motif. The data also show that the assumed formate moiety liberated by hydrolysis of the proffered $^{15}N^{13}C$-labeled formyl leucine is not incorporated into lipstatin to a significant extent.

All reported data are consistent with the biosynthetic scheme shown in Figure 4. A fraction of the thioester of 3-hydroxytetradeca-5,8-dienoic acid is obviously converted to hexyl malonic thioester via an octanoic acid derivative. This hexyl malonic thioester undergoes a condensation in conjunction with decarboxylation affording a C_{22}-moiety with the main fraction of the hydroxyacyl thioester. Reduction of the keto group, ring closure to the *β*-lactone, and introduction of the leucine residue followed by its formylation afford lipstatin.

Experimental Section

Materials. Commercially available reagents were used without further purification. Pig liver esterase (EC 3.1.1.1) was purchased from Fluka (Buchs, Switzerland). The phosphonium salt **7** was synthesized as published and is a complex mixture of isotopomers.[6] The reactions were done in dry solvents at room temperature unless stated otherwise.

Synthesis of ^2H-Labeled Intermediates. (3*S*,5*Z*,8*Z*)3-Hydroxytetradeca-5,8-dienoic Acid (9). The synthesis of isotope labeled **9** was achieved by methods modified from ref 8.

[3-^2H]-3-Hydroxyglutaric Acid Dipropyl Ester (2). 3-Oxoglutaric acid (**1**) (21 g, 144 mmol) was dissolved in 50 mL of dry propanol (40 g, 0.66 mol) containing 8.75 g (0.24 mmol) of HCl. The mixture was incubated with shaking at 35 °C for 10 min and was then kept at room temperature overnight. The solution was extracted with diethyl ether. The organic phase was washed with aqueous sodium carbonate, dried over sodium sulfate, and evaporated to dryness under reduced pressure, yielding 30.9 g (134 mmol, 93%) of the liquid dipropyl ester of **1**.

Without further purification, the ester was dissolved in 160 mL of propanol containing 1% (v/v) of triethylamine. Catalytic deuteration was performed with D_2 at atmospheric pressure over Raney nickel (7.2 g) as catalyst. The catalyst was removed by filtration after 18 h, and the solution was evaporated under reduced pressure. Vacuum distillation of the residue (bp 100−112 °C, ∼0.1 mbar) afforded **2** (118 mmol, 27.5 g, 88%). The deuterium abundance at position 3 was about 75%, as the catalyst was produced in nondeuterated solvents.

2: ^1H NMR *δ* (ppm) 0.9 (t, *J* = 8 Hz, 6H, 3′/3″), 1.6−1.7 (m, 4H, 2′/2″), 2.6 (s, 4H, 2/4), 3.5 (s, 1H, OH), 4.1 (t, *J* = 7 Hz, 4H, 1′/1″), 4.5 (m, 0.25H, 3); ^{13}C NMR *δ* (ppm) 10.2 (3′, 3″), 21.8 (2′, 2″), 40.6 (2, 4), 64.6 (3), 66.2 (1′, 1″), 171.7 (1, 5).

[3-^2H]-(3*S*)-3-Hydroxyglutaric Acid Monopropyl Ester (3). Pig liver esterase (3620 U) in 270 mL of 30 mM phosphate buffer (pH = 7) was added to 18 g (77.5 mmol) of **2**. The mixture was incubated with stirring at 4 °C. The pH was continually adjusted to 7.0 by the addition of 1 M NaOH. The reaction came to an end after the consumption of 1 equiv of the base (44 h). The solution was brought to pH 9 and extracted with diethyl ether. The aqueous phase was adjusted to a pH of 2 by the addition of 1 M hydrochloric acid and extracted with ethyl acetate. Evaporation of the solvent and distillation afforded 73 mmol of **3** (13.8 g, 94%, ee 87%, $[\alpha]^{25}_D$ +1.88° (*c* = 0.1 g/mL in acetone)).

3: ^1H NMR *δ* (ppm) 0.9 (t, *J* = 7 Hz, 3H, 3′), 1.5−1.6 (m, 2H, 2′), 2.5/2.6 (2s, 4H, 2/4), 4.0 (t, *J* = 7 Hz, 2H, 1′), 4.4 (m, 0.25H, 3); ^{13}C NMR *δ* (ppm) 10.2 (3′), 21.8 (2′), 40.4/40.5 (2/4), 64.6 (3), 66.5 (1′), 171.9 (1), 176.1 (5).

[3-^2H]-(3*S*)-3-*tert*-Butyldiphenylsiloxyglutaric Acid Monopropyl Ester (4). *tert*-Butyldiphenylsilyl chloride (17.3 g, 62.9 mmol) was added dropwise to a solution containing 5 g (26.3 mmol) of **3** and 6.44 g (94.6 mmol) of imidazole in 50 mL of dry CH_2Cl_2 at 3 °C under a nitrogen atmosphere. The solution was incubated at room temperature with shaking overnight. An aqueous solution of 5% sodium carbonate (160 mL) was added. After several days, CH_2Cl_2 was evaporated, and the remaining aqueous phase was extracted with diethyl ether. It was adjusted to pH 2 and extracted again. The organic phase was evaporated, affording a slightly yellow syrup (9.1 g, 21.3 mmol, 81%).

4: ^1H NMR *δ* (ppm) 0.9 (t, *J* = 7 Hz, 3H, 3′), 1.0 (s, 9H, ^{t}Bu-Me), 1.5−1.6 (m, 2H, 2′), 2.5−2.7 (br, 4H, 2/4), 3.9 (m, 2H, 1′), 4.5 (m, 0.25H, 3), 7.3−7.7 (m, 10H, Phe); ^{13}C NMR *δ* (ppm) 10.4 (3′), 19.2 (^{t}Bu-C), 21.8 (2′), 26.8 (^{t}Bu-Me), 41.4/41.6 (2/4), 66.1 (1′), 67.0 (3), 127.7/129.8/133.2/135.9 (Phe), 170.8 (1), 176.9 (5).

[3-^2H]-(3*S*)-5-Hydroxy-3-*tert*-butyldiphenylsiloxypentanoic Acid Propyl Ester (5). A solution of borane−THF complex in THF (11 mL, 1 M) was added to a solution of **4** (3.62 g, 8.4 mmol) in 13 mL of dry THF at −18 °C and under an inert atmosphere. The mixture was allowed to warm to 0 °C. After 7 h, one volume of ice−water and sodium carbonate solution were added slowly. The mixture was extracted with diethyl ether. The organic phase was dried over sodium sulfate. The solvent was evaporated under reduced pressure. The residue was applied to a column of silica gel 60 (48 cm × 3 cm) that was developed with hexane/ethyl acetate (2:1) yielding 3.1 g (7.4 mmol, 88%) of **5**.

5: ^1H NMR *δ* (ppm) 0.9 (t, *J* = 7 Hz, 3H, 3′), 1.0 (s, 9H, ^{t}Bu-Me), 1.5−1.6 (m, 2H, 2′), 1.7−1.8 (m, 2H, 4), 2.5 (s, 2H, 2), 3.6 (m, 2H, 5), 3.9 (t, *J* = 7 Hz, 2H, 1′), 4.3 (m, 0.25H, 3), 7.3−7.7 (m, 10H, Phe); ^{13}C NMR *δ* (ppm) 10.3 (3′), 19.2 (^{t}Bu-C), 21.7 (2′), 26.8 (^{t}Bu-Me), 38.9 (4), 41.9 (2), 59.1 (5), 66.0/68.6 (1′/3), 127.6/129.7/133.5/135.8 (Phe), 171.2 (1).

[3-^2H]-(3*S*)-5-Oxo-3-*tert*-butyldiphenylsiloxypentanoic Acid Propyl Ester (6). A 3.65 g (8.8 mmol) portion of **5** was added to 3.75 g (17.4 mmol) of pyridinium chlorochromate in 30 mL of dry CH_2Cl_2. The solution was incubated for 3 h with shaking under a dry atmosphere. The mixture was extracted with ether. The organic phase was concentrated under reduced pressure and applied to a column of silica gel (43 cm × 3 cm), which was developed with hexane/ethyl acetate (2:1), yielding 2.34 g (5.7 mmol, 64%) of **6**.

6: ^1H NMR *δ* (ppm) 0.9 (t, *J* = 7 Hz, 3H, 3′), 1.0 (s, 9H, ^{t}Bu-Me), 1.5−1.7 (q, *J* = 7 Hz, 2H, 2′), 2.5−2.8 (br, 4H, 2/4), 3.9 (t, *J* = 7 Hz, 2H, 1′), 4.6 (m, 0.25H, 3), 7.3−7.8 (m, 10H, Phe), 9.6 (s, 1H, 5); ^{13}C NMR *δ* (ppm) 10.3 (3′), 19.1 (^{t}Bu-C), 21.8 (2′), 26.8 (^{t}Bu-Me), 41.9 (2), 50.3 (4), 65.9/66.1 (1′/3), 127.7/129.9/133.2/135.7 (Phe), 170.5 (1), 200.7 (5)

[3,10,11,12-^2H]-(3*S*,5*Z*,8*Z*)-3-*tert*-Butyldiphenylsiloxytetradeca-5,8-dienoic Acid Propyl Ester (8). The phosphonium salt of labeled bromononene (**7**) (4.31 g, 9.2 mmol) was added to 9.0 mL of a 1 M solution of sodium bis-silyl amide at −17 °C in a dry THF/toluene solution (5:1) under nitrogen. The orange-colored solution was allowed to warm to room temperature and was then cooled to −90 °C. A 3.1 g portion of **6** (7.5 mmol) in 5 mL of THF was added dropwise, and the mixture was shaken overnight with warming to room temperature. A saturated aqueous solution of ammonium chloride was added, and the mixture was extracted with diethyl ether. The extract was concentrated under reduced pressure. The residue was applied to a column of silica gel (37 cm × 2.5 cm), which was developed with pentane/diethyl ether (97:3), yielding pure **8** (2.42 g, 4.6 mmol, 62%).

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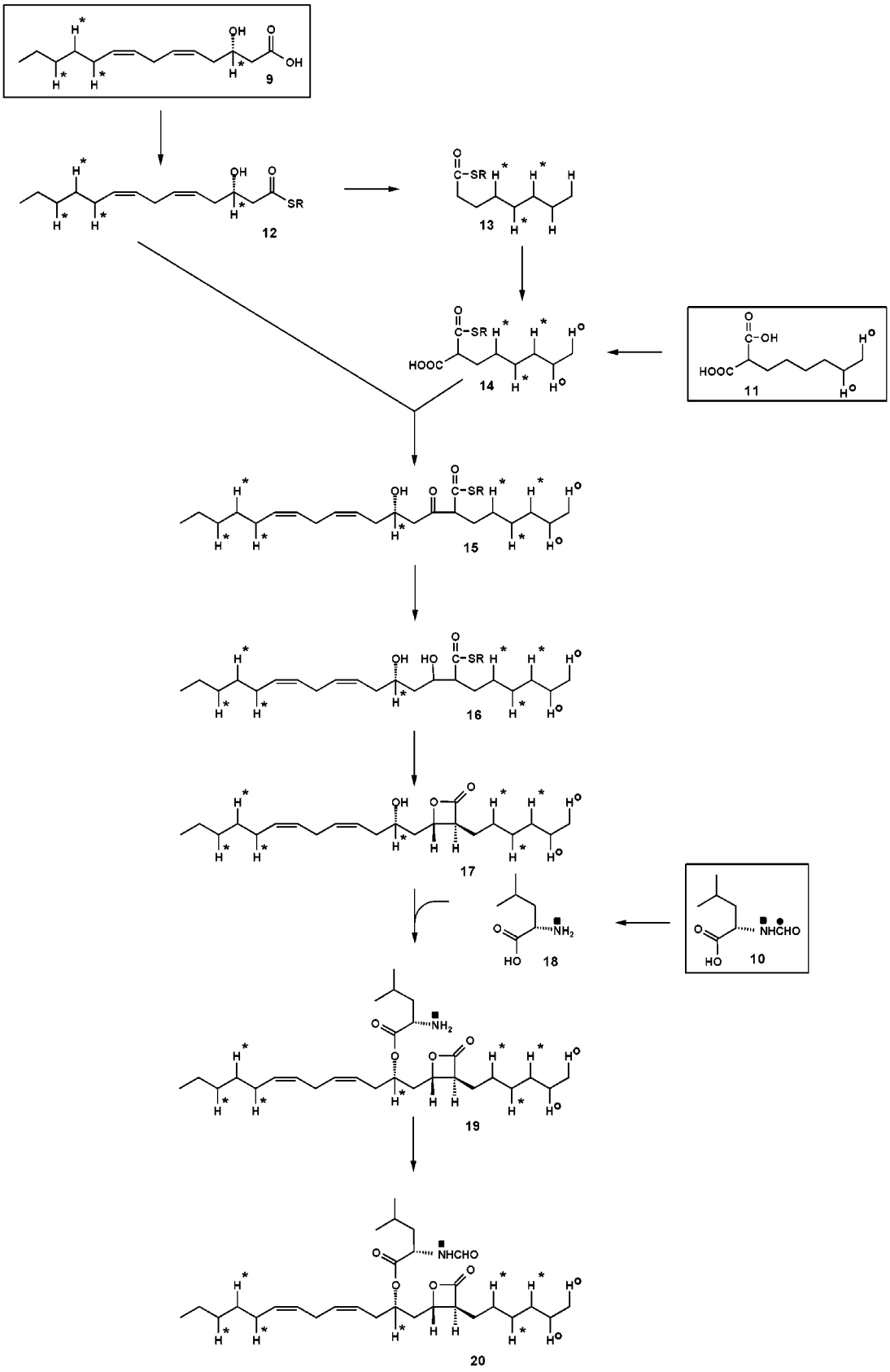

Figure 4. Hypothetical mechanism of lipstatin biosynthesis. Isotope label transferred from [3,10,11,12-^{2}H](3S,5Z,8Z)-3-hydroxytetradeca-5,8-dienoic acid (**9**) is indicated by *. Isotope label transferred from [7,8-$^2H_2$]hexyl malonate (**11**) is indicated by ○, and isotope labeled transferred from [^{13}C-formyl, ^{15}N]-*N*-formylleucine (**10**) is indicated by ■.

8: ^{1}H NMR δ (ppm) 0.9 (t, J = 7 Hz, 6H, 14/3′), 1.0 (s, 9H, ^{t}Bu-Me), 1.2−1.4 (br, 4.5H, 11−13), 1.5−1.7 (br, 2H, 2′), 2.0 (br, 1.5H, 10), 2.2 (d, J = 7 Hz, 2H, 4), 2.5 (s, 2H, 2), 2.6 (br, 2H, 7), 3.8−4.0 (m, 2H, 1′), 4.2−4.3 (m, 0.25H, 3), 5.2−5.4 (m, 4H, 5/6/8/9), 7.3−7.8 (m, 10H, Phe); ^{13}C NMR δ (ppm) 10.3 (3′), 14.0 (14), 19.2 (^{t}Bu-C), 21.8 (2′), 22.5/29.2/31.4 (11/12/13), 25.6 (7), 26.9 (^{t}Bu-Me), 27.1 (10), 34.9 (4), 41.5 (2), 65.9 (1′), 70.3 (3), 124.5/127.3/127.5/129.6/130.4/130.8/133.8/135.9 (5/6/

8/9/Phe), 171.5 (1); GC t_{ret} = 28.4 min; MS main peaks for unlabeled **8** were m/z = 463 ($[M - {}^tBu]^+$) and m/z = 199 ($[Ph_2SiOH]^+$). ^3J coupling constants of the olefinic protons were 10.8 Hz. This is in the typical range for the (*Z*)-configuration and very similar to the value observed in lipstatin (11.0 Hz).

[3,10,11,12-^{2}H]-(3*S*,5*Z*,8*Z*)-3-Hydroxytetradeca-5,8-dienoic Acid (9). A solution containing 1.60 g (3.05 mmol) of **8** in 15 mL of dry THF was added to a tetrabutylammonium fluoride solution in THF (1 M, 10.7 mL) at 0 °C. The mixture was incubated with shaking for 2 days at room temperature and was then added to the same volume of water. The mixture was extracted with diethyl ether, and the solvent was evaporated. The residue was applied to a column of silica gel (20 cm × 2.5 cm), which was developed with pentane/diethyl ether (18:5), yielding 0.48 g (1.68 mmol, 55%) of the propyl ester of **9**.

The propyl ester was dissolved in 5 mL of 10 M KOH/ethanol (1:4) and refluxed at 95 °C. A 15 mL portion of water was added, and the solution was extracted with diethyl ether. HCl (2 N) was added to adjust the pH to 2. The aqueous solution was again extracted with diethyl ether. The organic phase was concentrated under reduced pressure and afforded **9** (0.36 g, 1.48 mmol, 88%) as a clear oil.

9: ^{1}H NMR δ (ppm) 0.9 (t, J = 7 Hz, 3H, 14), 1.2−1.4 (m, 4.5H, 11−13), 2.0 (t, J = 7 Hz, 1.5H, 10), 2.2−2.4 (m, 2H, 4), 2.4−2.6 (m, 2H, 2), 2.8 (t, J = 7 Hz, 2H, 7), 4.1 (m, 0.25H, 3), 5.3−5.6 (m, 4H, 5/6/8/9); ^{13}C NMR δ (ppm) 14.1 (14), 22.6/29.3/31.5 (11/12/13), 25.8 (7), 27.3 (10), 34.3 (4), 40.5 (2), 67.9 (3), 124.2/127.1/130.8/131.9 (5/6/8/9), 177.8 (1); ^{2}H NMR δ (ppm) 1.25/1.32 (2s, 1.40H, 11/12), 2.0 (s, 0.47H, 10), 4.1 (s, 0.75H, 3) (Figure 1); $[\alpha]^{25}_D$ = +1.9° (c = 0.084 g/mL, acetone).

Synthesis of [^{13}C-Formyl,^{15}N]-*N*-Formylleucine (10). Racemic [^{15}N]leucine (2.0 g, 15.1 mmol) was added to 20 g (0.43 mol, 15.1 mL) of [^{13}C]formic acid. The mixture was cooled with ice, and 5.3 mL of acetic anhydride was added dropwise. After incubation overnight, 7 mL of water was added dropwise. The solution was concentrated under reduced pressure. The precipitate was purified by incubation in water with shaking overnight affording 1.83 g (11.4 mmol, 75%) of pure **10**.

10: ^{1}H{^{13}C,^{15}N} NMR δ (ppm) 1.0 (d, J = 6 Hz, 6H, 5/5′), 1.6 (m, 1H, 4), 1.8 (m, 2H, 3), 4.8 (m, 1H, 2), 5.9 (d, J = 8 Hz, 1H, NH), 8.2 (s, 1H, CHO); ^{13}C{^{15}N} NMR δ (ppm) 20.6 (5′), 22.3 (5), 24.4 (4), 40.6 (3), 52.6 (2), 163.6 (formyl), 179.5 (1)

Synthesis of [7,8-$^2H_2$]-Hexylmalonate (11). 6-Bromohex-1-ene (37.4 mmol, 5 mL) was added to a solution of diethyl malonate (37.4 mmol, 5.68 mL) and 37.4 mmol of NaOEt in 20 mL of dry ethanol. The mixture was refluxed for 16 h and was then extracted with diethyl ether. The solvent was evaporated to yield crude 7-hexenylmalonic acid diethyl ester (8.4 g, 34.7 mmol), which was deuterated under atmospheric pressure with 0.6 g of Rh/C as catalyst in methanol within 3 h. KOH (14 M)/ethanol (1:2) was added, and the mixture was refluxed for 2 h. The mixture was brought to a pH of 1 under cooling and was then extracted with diethyl ether. The solution was evaporated under reduced pressure. The resulting solid was recrystallized from ethyl acetate affording **11** as white crystals (5.1 g, 26.5 mmol) with an overall yield of 85%.

11: ^{1}H NMR δ (ppm) 0.9 (m, 2H, 8), 1.2−1.3 (m, 7H, 4−7), 1.8 (m, 2H, 3), 3.2 (t, J = 7 Hz, 1H, 2); ^{13}C NMR δ (ppm) 14.2 (8), 23.5, 28.4, 30.0, 32.7, 53.1 (2), 173.3 (1, 1′); ^{2}H NMR δ (ppm) 0.9 (s, 1H, 8), 1.3 (s, 1H, 7).

Fermentation and Feeding Experiments. *S. toxytricini* was grown in 8 L of medium in a 14 L Chemap fermentor (Männedorf, Switzerland) according to published procedures.[9]

In experiment A, 0.2 g of **9** diluted in 1.1 g linoleic acid was added in seven aliquots at intervals of 5 min.

In experiment B, 1.1 g of **10** in 20 mL of water was added continuously within 20 h.

In experiment C, 1.62 g of **11** in 100 mL of NaOH (2%) was added continuously within 10 h.

Fermentation was stopped by heating to 70 °C for 10 min.

Isolation and Purification of Lipstatin. Lipstatin was isolated and purified as described previously.[4]

Analytical Methods. NMR spectra were obtained using Bruker AVANCE DRX 500 or AM 360 spectrometers. ^{1}H NMR spectra were measured without decoupling or with ^{15}N-decoupling, ^{13}C NMR spectra with ^{1}H-decoupling, and ^{2}H NMR spectra with ^{1}H-decoupling. Chemical shifts were referenced to internal tetramethylsilane.

Optical rotation was measured using a Perkin-Elmer 241 MC polarimeter.

A GC/MS system consisting of a GC 17A with a MS QP 5000 from Shimadzu was used to characterize synthetic products. A DB5 column (length: 30 m) was used with a helium flow of 0.8 mL/min. Samples were injected at a temperature of 180 °C. After 3 min, the column was heated to 290 °C at a rate of 5 °C/min.

Chemical ionization mass spectra were measured with ammonia as reactand gas and a ionizing energy of 230 eV.

TLC was performed on precoated silica gel 60 F_{254} sheets from Merck. Column chromatography was done with silica gel 60 from Fluka.

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