The lipase inhibitor lipstatin is biosynthesized in Streptomyces toxytricini via condensation of a C14 precursor and a C8 precursor, which are both obtained from fatty acid catabolism. To study the mechanism of this reaction in more detail, S. toxytricini was grown in medium containing a mixture of U-13C-U-2H-lipids and unlabeled sunflower oil or in a medium containing 70% D2O. Lipstatin was isolated and analyzed by 1H,2H, and 13C NMR spectroscopy. Hydrogen atoms at C-2, C-3, and C-4 of lipstatin were found to be derived from solvent protons. The formation of the lipstatin precursor 3-hydroxy-Δ8,14-tetradecadienoyl-CoA by β oxidation of linoleic acid explains the incorporation of solvent hydrogen into the 4 position of lipstatin. The hydrogen in position 3 of lipstatin is most probably introduced from solvent by proton/deuterium exchange of a redox cofactor involved in the reduction of the keto group in the branched chain β keto acid arising by a decarboxylative condensation. The incorporation of solvent hydrogen at position 2 can be explained by epimerization of a chiral intermediate at C-2 and C-3. Epimerization may involve a dehydration-rehydration mechanism.

Lipstatin is an inhibitor of pancreatic lipase that is produced by Streptomyces toxytricini (1, 2). The tetrahydro derivative of lipstatin (Orlistat, Xenical®) is used for treatment of severe obesity. The lipophilic β-lactone irreversibly inactivates lipase by covalent modification of the serine residue of its catalytic triad (3).

Earlier in vivo incorporation studies using universally 13C-labeled lipids indicated that the β-lactone moiety of lipstatin is biosynthesized from a C14 and a C8 moiety, which are both obtained by partial catabolism of fatty acids (Fig. 1) (4). To analyze this biosynthetic transformation in more detail, we decided to study the fate of precursor hydrogen atoms by in vivo stable isotope incorporation experiments.

**EXPERIMENTAL PROCEDURES**

Materials—A U-13C-U-2H-lipid mixture was obtained by acetone extraction of algal biomass (Scenedesmus obliquus) grown with 13CO2 in D2O (H. Oschkinat, Freie Universität Berlin, Germany). The acetone solution was evaporated to dryness. The residue was used without purification.

Fermentation—Fermentation experiments with shaking cultures of S. toxytricini were conducted as described earlier (4). In the first experiment, the medium (50 ml) was supplemented with 2.7 g of sunflower oil and 0.5 g of U-13C,U-2H-lipid from S. obliquus. In the second experiment, the culture medium contained a mixture of D2O and H2O (7:3, v/v).

Lipstatin, isolated as described earlier (4), was 93% pure as judged by high performance liquid chromatography.

NMR Spectroscopy—NMR measurements were performed in CDCl3 at 17 °C using a four-channel Bruker DRX 500 spectrometer operating at 500.13 MHz for 1H experiments, 125.76 MHz for 13C experiments, and 76.77 MHz for 2H NMR experiments. The spectrometer was equipped with a lock switch unit for 2H decoupling experiments using the lock channel. One-dimensional 1H and 13C NMR experiments and two-dimensional 13C2H-correlated spectroscopy experiments (CHCOSY) were performed with standard Bruker software (XWINNMR). Simultaneous 1H and 2H decoupling of 13C was achieved with a WALTZ 16-pulse sequence during relaxation (1H) and acquisition (1H,2H). Prior to Fourier transformation, the free induction decay was multiplied with a Gaussian function. 1H and 13C signal assignments of lipstatin have been reported earlier (2, 4).

**RESULTS**

The β-lactone moiety of lipstatin is biosynthesized from two long-chain fatty acids (Fig. 1) (4). To map the origin of individual hydrogen atoms in the biosynthetic reaction sequence, S. toxytricini was grown in a medium containing U-13C-U-2H-lipids (obtained from an algal culture grown with 13CO2 in D2O) and sunflower oil at a ratio of 1:5.4 (w/w). A second, complementary fermentation experiment was performed with unlabeled sunflower oil in a medium containing 70% D2O.

1H and 2H NMR spectra of lipstatin isolated from these cultures are shown in Fig. 2. In the lipstatin sample obtained from the experiment with double-labeled U-13C-U-2H-lipid, no deuterium was observed by 2H NMR spectroscopy at position 2 of the β-lactone moiety, and little, if any deuterium was observed at position 3 (Fig. 2D). Of the two diastereotopic hydrogen atoms at C-4, the one resonating at lower field (H-4) was apparently devoid of deuterium label. Moreover, deuterium was absent at the α position of the leucine moiety (H-2”) and in the formyl moiety.

The distribution of deuterium observed in the experiment with unlabeled sunflower oil in D2O was complementary to that observed in the previous experiment. Specifically, deuterium was detected in positions 2, 3, and 4 (downfield shifted signal) of the β-lactone moiety (Fig. 2C). Deuterium was also detected in the α position of the leucine moiety (H-2”) and in the formyl moiety. The partial replacement of 1H by 2H in these positions is also reflected in the 1H NMR spectrum. A comparison between the 1H NMR spectra of unlabeled lipstatin (Fig. 2A) and the lipstatin sample from the experiment with 70% D2O (Fig. 2B) shows that the signal intensities of the hydrogen atoms 2, 3, and 4 (downfield shifted signal) are significantly reduced.

Additional information on the distribution of 1H and 2H in

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**Biosynthetic Origin of Hydrogen Atoms in the Lipase Inhibitor Lipstatin***

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**21192**
these lipstatin samples was gleaned from 1H,2H-decoupled 13C NMR spectra. Relevant signals from the experiment with unlabeled lipid in 70% D2O are shown in Fig. 3. The 13C signals of C-2 to C-6 are complex multiplets comprising up to eight lines. In a spectrum obtained with 1H decoupling but without 2H decoupling, the signal components shifted by more than 150 ppb were substantially broadened or absent (Fig. 3, bottom).

The signal multiplicity reflects the presence of multiple isotopomers carrying 0 to 3 deuterium atoms at C-2, C-3, and/or C-4. Heavy isotope shifts for substitution with one deuterium atom of carbon atoms in the respective α, β, and γ positions were found in the ranges of −426 to −198 ppb, −120 to −52 ppb, respectively, −47 to −18 ppb. The chemical shift increments are additive in isotopomers with multiple deuterium substitutions. Direct evidence on the origin of the signal multiplets in Fig. 3 was obtained by a 13C1H COSY experiment recorded under 2H decoupling with high resolution in the 13C dimension. As an example, the four 13C signals at high field in the complex signal pattern of C-4 correlated with one of the H-4 1H signals (H-4* at 1.97 ppm) (Fig. 4). The four 13C signals at lower field correlated with both signals of the diastereotopic H-4 atoms (H-4* and H-4). On the basis of these data, all 13C signals in Fig. 3 can be unequivocally attributed to individual isotopomers, designated A–H (Fig. 5).

The fraction of each respective isotopomer in the biosynthetic mixture can be determined from the signal integrals in the 1H,2H-decoupled 13C NMR spectra. However, it should be noted that the relaxation rates of 13C atoms are substantially modulated by directly bound 2H atoms. The respective signal components were therefore not included in the statistical evaluation of the label distribution.

An average deuterium content of 1.6 deuterium atoms per β-lactone moiety of lipstatin is obtained from the 13C-derived data in Fig. 5 and Table I. Mass spectrometric analysis of the derivatized β-lactone moiety (Fig. 6) indicates an average deuterium content of 1.8 deuterium atoms, in close agreement with the 13C NMR data. The average 2H enrichment at the 2H-labeled positions is therefore approximately 55%. This value is close to the D2O content of the culture medium.

The same type of analysis can be applied to the signals of the formyl-leucine moiety of the biosynthetic lipstatin. The pres-
ence of isotopomers K through N (Fig. 7) at relatively high abundance can be deduced from the $^{13}$C signals shown in Fig. 8. These data reflect the presence of 47% $^2$H at the $\alpha$-carbon of leucine and 48% $^2$H in the formyl group. Minor amounts of deuterium (13% relative $^2$H enrichment) were also detected by $^2$H NMR analysis in the 5$^9$9 and 6$^9$9 position of the leucine moiety.

Because of extensive $^{13}$C-$^{13}$C coupling, the $^{13}$C NMR spectrum of the lipstatin sample from the experiment with U-$^{13}$C,U-$^2$H-lipid is rather complex. Nevertheless, these data can be interpreted using the heavy isotope shift values described above and the $^{13}$C-$^{13}$C coupling constants reported earlier (4). The signal of C-2 shows $^{13}$C-$^{13}$C coupling to C-1, C-1', and C-2' (Fig. 9). By comparison with the singlet representing the lipstatin fraction derived from the unlabeled sunflower oil, the multiplet is shifted to higher field by 223 ppb. This shift is due to the combined heavy isotope shifts of multiple $^{13}$C and $^2$H neighbor atoms (two $\beta$- and two $\gamma$-$^2$H upfield shifts). Signal components shifted to even higher field by 365 ppb ($\alpha$-$^2$H shift) would have resulted if deuterium had been carried over to position 2 from the double-labeled lipid precursor. The absence of these hypothetical signal components (indicated by arrows below the spectrum in Fig. 9) confirms that the lipstatin fraction biosynthesized from double-labeled lipid is devoid of deuterium in position 2.

The $^{13}$C signal of C-4 of lipstatin from the experiment with the double-labeled lipid is a pseudotriplet arising from the coupling of C-4 to two adjacent $^{13}$C atoms in position 3 and 5. The pseudotriplet is shifted to higher field by 561 ppb (comprising $^{13}$C isotope shifts and one $\alpha$-, one $\beta$-, and two $\gamma$-$^2$H shifts). The presence of a fractional amount of deuterium at C-3 or of a second deuterium atom at C-4 in this sample would have been conducive to additional signal components shifted to higher field by 130 ($\beta$-$^2$H shift) or 360 ppb ($\alpha$-$^2$H shift), respectively. Again, the absence of these hypothetical signal components (Fig. 9) documents that no detectable amount of deuterium was carried over into position 3 and one of the H-4 positions of lipstatin via the C-4 moiety derived from the double-labeled lipid precursor.

**Fig. 5.** Isotopomers of the $\beta$-lactone moiety of lipstatin isolated from *S. toxycriniti* grown in medium containing 70% $^{2}$H$_2$O. The relative abundance of each isotopomer estimated from $^1$H,$^2$H-decoupled $^{13}$C NMR spectra is displayed. $^2$H upfield shifts are indicated by negative values in italics.

<table>
<thead>
<tr>
<th>Position</th>
<th>$^2$H enrichment from:</th>
<th>$^1$H</th>
<th>$^{13}$C</th>
<th>$^2$H</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td></td>
<td>48.4</td>
<td>46.6</td>
<td>36.9</td>
</tr>
<tr>
<td>2'</td>
<td></td>
<td>54.9</td>
<td>46.5</td>
<td>45.4</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>42.4</td>
<td>39.7</td>
<td>39.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>64.3</td>
<td>63.2</td>
<td>63.8</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>60.9</td>
<td>59.9</td>
<td>56.7</td>
</tr>
</tbody>
</table>

$a$ Absolute enrichment.  
$b$ Relative enrichment (calculated from the average of all relevant integrals).  
$c$ Reference value from the $^1$H, $^2$H-decoupled $^{13}$C NMR spectra.
DISCUSSION

An analysis of lipstatin derived from a fermentation experiment with deuterated water shows that the hydrogen atoms at positions C-2 and C-3 and one hydrogen atom at C-4 of lipstatin have been biosynthetically introduced from solvent water. The exchange with the solvent goes to apparent equilibrium as shown by the experiment with U-13C,U-2H-lipid as precursor.

3-Hydroxy-Δ5,8-tetradecadienoyl-CoA (compound 2, Fig. 10) obtained by β oxidation of linolyl-CoA (compound 4) has been proposed to serve as the committed precursor for the formation of the β-lactone moiety of lipstatin by Claisen condensation with an octanoyl derivative (4). The proposed hydration of the putative intermediate 5 is conducive to the introduction of solvent hydrogen into position 2 of the molecule.

The mechanistic and stereochemical details of the proposed condensation are still unknown. A hypothetical mechanism among several possibilities is shown in Fig. 10. By analogy with the stereochemistry of erythromycin polyketide synthase (5, 6), it appears plausible that the C8 precursor could be carboxylated yielding a S-hexylmalonyl intermediate (compound 6). A decarboxylative condensation could then proceed by inversion of this chiral center yielding intermediate 7 with a 2R configuration. As in fatty acid biosynthesis (7), the reduction of the position 3 keto group of intermediate 7 could yield intermediate 8 with a 2R,3R configuration. It would then be necessary to invert the chiral centers 2 and 3 of intermediate 8 to obtain the 2S,3S configuration of lipstatin. In accordance with the biochemical pathways of unsaturated fatty acids, these epimerizations could proceed by a sequence of dehydration and rehydration steps, yielding compound 10 (8, 9). The rehydration of

FIG. 8. 13C NMR signals of lipstatin (formyl-leucine moiety) isolated from S. toxytricini grown in medium containing 70% D2O. Top, 1H,2H-decoupled signals; bottom, 1H-decoupled signals. K, L, M, and N indicate the isotopomers shown in Fig. 7.

FIG. 9. Labeling pattern of lipstatin from the experiment with U-13C,U-2H-lipid (top) and 1H, 2H-decoupled 13C NMR signals of C-2 and C-4. Multiple 13C labeling is indicated by bold lines. Isotope shift values (the sum of 1H and 13C shifts) are indicated by arrows, respectively, shown in italic letters for selected positions. 13C-13C couplings are indicated, and coupling partners are given in italic letters. *, indicates 13C NMR signals of lipstatin isotopomers with natural 13C abundance. Arrows below the spectra indicate the absent signals of hypothetical lipstatin species (see text).

FIG. 10. Hypothetical mechanism of lipstatin biosynthesis in S. toxytricini. Protons introduced from the solvent during fermentation are indicated by circles.
compound 9 would be conducive to the incorporation of solvent hydrogen at C-2 of lipstatin, which is not easily explained otherwise.

The presence of solvent hydrogen at position 3 of lipstatin suggests that a reducing agent involved in the generation of reducing equivalents for the reduction of the position 3 keto group of intermediate 7 in Fig. 10 is subject to hydrogen exchange with solvent water. Such an exchange process would be similar to solvent hydrogen exchange of NADPH observed in the context of fatty acid biosynthesis in Escherichia coli (10) and could proceed at the level of a reduced flavocoenzyme. In line with this hypothesis, we have found that S. toxytricini grown in D₂O-enriched culture medium forms [5,5-²H₂]proline by reduction of glutamate, apparently via a deuterated reducing agent (data not shown).

We have also found that the α-hydrogen of the leucine moiety of lipstatin is derived from solvent hydrogen. On the other hand, only a relatively small amount of solvent hydrogen is found in the methyl groups of the leucine moiety. The biosynthesis of leucine from two molecules of pyruvate and one molecule of acetyl-CoA involves the introduction of one solvent hydrogen atom into each of the leucine methyl groups because of the transformation of phosphoenol pyruvate to pyruvate (11–13). Additional solvent hydrogen can be introduced into pyruvate by spontaneous proton exchange because of the relatively high acidity of the pyruvate methyl group. Nevertheless, the ²H enrichments of the formyl-leucine methyl groups in lipstatin from the labeling experiments reported in this paper were low, thus suggesting that only a minor fraction of leucine incorporated into lipstatin has been biosynthesized de novo, whereas the bulk of lipstatin has been derived from unlabeled leucine present in the complex culture medium. This proposition is well in line with earlier incorporation experiments with ¹³C-labeled lipids (4). Moreover, the labeling pattern of the leucine moiety in lipstatin suggests that the preformed leucine in the culture medium is subject to extensive transamination involving proton exchange of the pyridoxal phosphate intermediate with the solvent.

The hydrogen atom of the formyl group in lipstatin has also been subject to equilibration with solvent hydrogen. Hydrogen exchange could have occurred at the level of methenyl tetrahydrofolate (14).¹

The hypothetical biosynthetic pathway in Fig. 10 is in line with all of the observed labeling patterns. The stereochemical features of lipstatin biosynthesis require additional studies.

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