The biosynthesis of the pancreatic lipase inhibitor lipstatin was investigated by fermentation experiments using cultures of *Streptomyces toxytricini*, which were supplied with soybean oil and a crude mixture of U-13C-lipids obtained from algal biomass cultured with 13CO2. Lipstatin was analyzed by one- and two-dimensional NMR spectroscopy. 13C total correlation spectroscopy and INADEQUATE experiments show that two fatty acid fragments containing 14 and 8 carbon atoms, respectively, are incorporated en bloc into lipstatin. The 14-carbon fragment is preferentially derived from the unsaturated fatty acid fraction, as shown by an experiment with hydrogenated U-13C-lipid mixture, which is conducive to labeling of the 8-carbon moiety but not of the 14-carbon moiety. The data indicate that the lipstatin molecule can be assembled by Claisen condensation of octanoyl-CoA with 3-hydroxy-3,5,8,11-tetradecanoyl-CoA obtained by β oxidation of linoleic acid. The formation of lipstatin from acetate units by a polyketide-type pathway is ruled out conclusively by these data. The data show that surprisingly clear labeling patterns can be obtained in studies with crude, universally 13C-labeled precursor mixtures that are proffered together with a large excess of unlabeled material. One- and two-dimensional 13C total correlation spectroscopy analyses are suggested as elegant methods for the delineation of contiguously 13C-labeled biosynthetic blocks.

Lipstatin (Fig. 1, 1) and its tetrahydroderivative tetrahydro-lipstatin (Fig. 1, 2) are potent inhibitors of pancreatic lipase (1–4). Tetrahydrolipstatin is presently in clinical trials as an antiobesity agent. Tetrahydrolipstatin has been shown to form a covalent adduct with a serine moiety of human pancreatic lipase by transesterification (5).

Lipstatin is produced by *Streptomyces toxytricini* (1). The structure is characterized by a β lacton ring carrying two aliphatic residues with chain lengths of 6 and 13 carbon atoms (Ref. 6 and Fig. 1). One of the side chains contains two isolated double bonds and a hydroxy group esterified to N-formyl-leucine. Structurally, lipstatin is closely related to the esterase inhibitor esterastin, which contains a N-acetylasparagine side chain instead of N-formylleucine (7). Tetrahydrolipstatin can be obtained by catalytic hydrogenation of lipstatin (6). Total synthesis of tetrahydrolipstatin has been reported (8).

Nothing is known about the biosynthesis of lipstatin. Initial biosynthetic studies with isotope-labeled acetate were not conducive to the incorporation of label into lipstatin.  

The long alkyl side chains of lipstatin could be biosynthesized from acetate via a polyketide pathway or from preformed fatty acids. *S. toxytricini* can be grown on medium containing large amounts of lipids, and it appeared likely that these were actively metabolized by the microorganism. In that case, 13C-labeled lipids could be incorporated into lipstatin by partial degradation of preformed fatty acids or by total degradation via acetyl-CoA.

Triglycerides with appropriate 13C labeling were not commercially available. We therefore decided to use a mixture of universally 13C-labeled lipids, which was obtained by acetone extraction of algal biomass grown with 13CO2. This complex mixture was extensively diluted with soybean oil (natural 13C abundance) for the preparation of culture media. In conjunction with advanced NMR techniques, this approach was conducive to the elucidation of the building blocks of lipstatin. This method appears to be generally useful for biosynthetic studies on compounds with a putative origin from fatty acid precursors.

**MATERIALS AND METHODS**

*U-13C-Lipid—*The crude U-13C-lipid mixture used in this study was purchased from Dr. H. Oschkinat (European Molecular Biology Laboratory, Heidelberg, Germany). It was prepared by acetone extraction of algal biomass (*Scenedesmus obliquus*) grown on 13CO2. The acetone extract was evaporated to dryness under reduced pressure. The black, oily material was used as a supplement to the culture medium without purification.

*Hydrogenation of U-13C-Lipid—*A solution of 13C-labeled (crude algal extract, 277 mg) in 6 ml of ethanol was hydrogenated over 550 mg of Raney nickel (50 °C, 10-bar hydrogen, 3 h). The product still contained approximately 8% of C18:1 fatty acid according to GC analysis. The hydrogenation was therefore repeated using similar conditions, yielding 124 mg of fully hydrogenated material.

*Fatty Acid Composition of 13C-Algal Lipid—*An aliquot of the 13C-labeled lipid mixture (20 mg) and 1.25 mg of pentadecanoic acid were dissolved in 10 ml of 0.5 M sodium methyate in methanol. The mixture was heated to 60 °C in a screw cap glass for 30 min and was then acidified with 4 ml of 3.6% hydrochloric acid in methanol. An aliquot of 5 ml was diluted with 3 ml of water, and the mixture was extracted with 5 ml of n-hexane. The resulting fatty acid methyl esters were identified by GCMS on a capillary column (DBWAX, 20 m, 0.3-µm film; temperature gradient, 120–250 °C, 4 °C/min; splitless injection). Quantitative GC analysis of the methyl esters was performed on a capillary column (OV-225, 25 m, 0.25 µm; temperature gradient, 140–220 °C, 3 °C/min). Pentadecanoic acid methylester was used as internal standard for quantification, and dihomo-γ-linoleic acid ethyl ester (97.4% purity by weight) was used as a reference substance. The 13C content of fatty acid residues was determined by GCMS analysis (electron impact ioniza-

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1 W. Weber, E. Kupfer, and H. Leuenberger, unpublished data.

2 The abbreviations used are: GC, gas chromatography; MS, mass spectrometry; TOCSY, total correlation spectroscopy; COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; HMQC, heteronuclear multiple quantum correlation spectroscopy; HMQC, heteronuclear multiple quantum multiple bond correlation spectroscopy; MLEV, Malcom Levitt decoupling sequence.
For biosynthetic studies, the U-13C-labeled precursors had to be diluted extensively with unlabeled material. Per 35 ml of fermentation medium, 1.8 g of soybean oil and 0.1 g of the 13C-labeled lipid mixture or hydrogenated 13C-labeled lipid mixture were used. The lipids were emulsified in water using 0.5 g of soybean lecithin as an emulsifier. The medium also contained soybean flour (1.4 g) and glycerol (0.7 g). The pH was adjusted to 7.4 before sterilization (121°C, 20 min). After seeding with S. toxytricini, the culture was incubated for 7 days at 27°C and 70% relative humidity on a rotary shaker at 220 rpm and 5-cm throw.

Isolation of Lipstatin—Fermentation broth (58 ml) was extracted with 150 ml of aceton and 100 ml of hexane. After separation of the organic layer, the aqueous layer was extracted three times with 100 ml of a 1:1 mixture of acetone and hexane. The combined organic extracts were dried with sodium sulfate and concentrated to yield a green oil (3.1 g) containing 173 mg of lipstatin. The material was dissolved in hexane (40 ml) and placed on a column of silica gel (Bond Elut, 10 g). The column was developed with hexane (40 ml) and hexane/ethyl acetate at dilutions of 20:1 (v/v, 300 ml), 10:1 (200 ml), 20:3 (200 ml), and 5:1 (200 ml), yielding 220 mg of crude material after evaporation of solvent. Second chromatography on silica gel using the same procedure afforded 163 mg of semipure material. Further purification was done by reversed phase chromatography. Lipstatin was dissolved in 50% aqueous isopropanol (25 ml) and placed on a column of Bond Elut C-18 (10 g) which was developed with 50% isopropanol (225 ml) and 60% isopropanol (150 ml). Fractions containing lipstatin were concentrated by evaporation under reduced pressure, and the residue was extracted with ethyl acetate. The extract was dried with sodium sulfate and was concentrated to dryness, yielding 112 mg of lipstatin with 88% purity (high performance liquid chromatography, percentage of area) containing 9% of lipstatin analogs.

NMR Spectroscopy—1H and 13C NMR spectra were recorded at 360 and 90.6 MHz, respectively, with a Bruker AM 360 spectrometer equipped with fast power switching of the transmitter output, an external pulse amplifier (BFX5, Bruker), and a selective excitation unit (Bruker). Chloroform-d was used as a solvent. 1H NMR spectra were measured as follows: 50° pulse (4 μs); repetition time, 2.5 s; spectral width, 5.3 kHz; data set, 16 kilowords; temperature, 25°C, and 0.1-Hz line broadening. 13C NMR spectra were measured as follows: 30° pulse (2 μs); repetition time, 2.6 s; spectral width, 25.0 kHz; data set, 32 kilowords zero filled to 64 kilowords; temperature, 25°C, and 1H composite pulse decoupling.

Two-dimensional double quantum-filtered COSY, DEPT, and INADEQUATE experiments were performed according to standard Bruker software (DISR87). Phase-sensitive two-dimensional TOCSY spectroscopy was done according to the method of Bax and Davis (9). 1H-Detected multiple quantum 1H-13C chemical shift correlation experiments (HMOC and HMBC) were performed according to the methods of Bax and Subramanian (10) and Bax and Summers (11). Samples were not rotated during two-dimensional experiments.

Data acquisition and processing parameters for two-dimensional experiments were: COSY, 32 scans/t1 increment, 2.0-s relaxation delay, 480 × 2048 raw data matrix size, zero filled to 2048 words in t1, and processed with 2-Hz Gaussian in the f2 dimension, and 90°-shifted sine bell filtering in the f2 dimension; TOCSY, 32 scans/t1 increment, 2.0-s relaxation delay, 62-ms MLEV-17 mixing period preceded and followed by 2.5-ms trim pulses, 90° pulse width, 44 μs, 512 × 2048 raw data matrix size, zero filled to 2048 words in t1 and processed with 2-Hz Gaussian in the f2 dimension, and 90°-shifted sinezine bell filtering in the f2 dimension; HMOC, 256 scans/t1 increment, start of coherence experiment 159 ms after a bilinear rotation decoupling pulse, 3.5-ms delay period for evolution of 1JCH corresponding to a coupling of 143 Hz, 13C decoupling during acquisition by globally optimized alternate phase rectangular pulse sequence 1, 500 × 1024 raw data matrix size, zero filled to 1024 words in t1, and processed with 10-Hz Gaussian in f2 and 90°-shifted sinezine bell filtering in f2; HMBC, 64 scans/t1 increment, 1.8-s relaxation delay, 3.5-ms delay for suppression of 1JCH, 60-ms delay period for evolution of long-range couplings (2JCH and 3JCH) corresponding to a coupling of 8 Hz, and 350 × 2048 raw data matrix size, zero filled to 1024 words in t1, and processed with 20-Hz Gaussian in f2 and in f3; and INADEQUATE, 128 scans/t1 increment, 1.5-s relaxation delay, Ernst-type phase cycle, 5.0-ms delay for evolution of 1JCC, and 350 × 2048 raw data matrix size, zero filled to 2048 words in t1, and processed with 90°-shifted sine bell filtering in f1 and f3.

Phase-sensitive two-dimensional 13C TOCSY experiments were performed with a MLEV-17-based mixing period (9). The 1H excitation pulse was generated in the transmitter high power output period (90° pulse, 5 μs). 13C pulses for mixing were generated in the transmitter low power output output period amplified with a BFX5 unit (90° pulse, 30 μs). The MLEV-17 mixing period was 45 ms and was preceded and followed by 2.5-ms trim pulses. The data were acquired in the phase-sensitive mode using time-proportional phase increments. Other data acquisition and processing parameters were: 48 scans per t1, increment, 2.0-s relaxation delay, and 400 × 2048 raw data matrix size, zero filled to 2048 in t1 and processed with 90°-shifted, squared sine bell functions in f2 and Gaussian broadening in f3.

One-dimensional 13C TOCSY experiments were performed with selective excitation using a Gaussian- or half-Gaussian-shaped pulse of 5 ms in length generated by the transmitter output of a selective excitation unit (Bruker). The transfer of magnetization between 13C atoms was achieved by a MLEV-17-based mixing period (45 ms) preceded and followed by trim pulses (2.5 ms). The pulses for mixing were generated in the low power output of the transmitter amplifier with a BFX5 unit (90° pulse, 30 μs).

RESULTS

NMR Signal Assignment of Lipstatin

A 1H and 13C NMR analysis of lipstatin providing assignments for some of the carbon atoms has been reported (6). However, since the biosynthetic study depended crucially on unequivocal assignments for all 13C signals, a more detailed NMR analysis using double quantum-filtered COSY, 1H TOCSY, and inverse carbon-proton correlation experiments such as HMOC and HMBC was in order. Additional assignment information was afforded by 13C TOCSY and INADEQUATE analysis of multiple 13C-labeled lipstatin samples obtained in the labeling experiments described below. 1H and 13C NMR signal assignments of lipstatin are summarized in Table I.

Analysis of U-13C-Lipid Mixture

An acetone extract of totally 13C-labeled algal biomass obtained by growth of Scenedesmus obliquus on 99% enriched 13CO2 as a carbon source was obtained from Dr. H. Oeschkinat. After evaporation of the solvent, the viscous oil appeared almost black. The dark color was in part due to the presence of chlorophylls in considerable amount.

For assessment of the fatty acid content, the crude algal lipid mixture was subjected to methanolysis, and the resulting fatty acid methyl esters were analyzed by coupled gas chromatography/mass spectrometry (G. Oesterhelt, Hoffmann-La Roche

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**Fig. 1. Structures of lipstatin (1) and tetrahydrolipstatin (2).**
Biosynthesis of Lipstatin

13C NMR signals of lipstatin from fermentation with [U-13C] lipid. The data were multiplied with a Gaussian function prior to Fourier transformation. 13C coupled signals are indicated.

**Biological Studies**

**Fermentation with U-13C-Lipid**—Fermentation experiments were performed with *S. toxytricini* and the culture medium reported earlier (1). To detect the joint transfer of 13C-labeled atoms, it is important to dilute the U-13C-labeled precursors extensively with unlabeled material. We used the 13C-labeled lipid mixture and unlabeled soybean oil at a ratio of 1:17.4 (w/w) for the preparation of the fermentation medium. After cultivation for 7 days, lipstatin was isolated as described under “Materials and Methods.”

The analysis of the 13C satellites in the H NMR spectrum of lipstatin gave 13C enrichments of about 4%. This indicated that the 13C-labeled algal lipid had been metabolized by the organism at a similar rate as soybean oil and had served as a

**Analysis of fatty acids in lipid extract of Scenedesmus obliquus**

| Fatty acid | U-13C-lipid | Hydrogenated U-13C-lipid |
|------------|-------------|-------------------------|
| % (w/w)    | % (w/w)     |                         |
| 14:0       | 0.7         | 1.2                     |
| 16:0       | 6.1         | 12.1                    |
| 16:1       | 0.7         |                         |
| 16:1, ω-7  | 0.6         |                         |
| 16:2       | 0.5         |                         |
| 18:0       | 0.2         | 25.3                    |
| 18:1, ω-9  | 0.4         |                         |
| 18:1, ω-7  | 0.6         |                         |
| 18:2, ω-6  | 8.2         |                         |
| 18:3, ω-3  | 21.2        |                         |
| Sum        | 39.2        | 38.6                    |

AG). The results are shown in Table II. The combined fatty acid residues account for about 40% (w/w) of the total material. Eleven fatty acids with chain lengths of 14–18 carbon atoms were determined in widely different abundance. In the saturated fraction, palmitic acid was the dominant component. The combined unsaturated fatty acids accounted for 32% (w/w) of the crude material. The dominant components were linolenic acid (about 21%) and linoleic acid (about 8%). The 13C abundance was approximately 97%.

An aliquot of the U-13C-lipid mixture from algae was subjected to two consecutive cycles of catalytic hydrogenation over Raney nickel. Gas chromatographic/mass spectrometric analysis of fatty acid methyl esters obtained by methanolysis confirmed that virtually all double bonds had been removed by hydrogenation (Table II). The dominant fatty acid in the hydrogenated mixture was stearic acid (about 25%, w/w).
The $^{13}$C satellites of C-5 appeared at a distance of 78.2 Hz.

For example, the $^{13}$C signal of C-3 was characterized by a one-dimensional $^{13}$C NMR spectrum is summarized in Table III. These data show already that biosynthetic modules containing more than two carbon atoms were incorporated into building blocks can be addressed much better by two-dimensional $^{13}$C TOCSY experiment. The $^{13}$C-labeled precursors are diagnosed with high sensitivity. The 13C TOCSY experiment is the exchange of magnetization between directly coupled spins by a radiofrequency field (spin lock field). In the case of a two-dimensional experiment, the result of the mixing process is that a spin ($^1$H or $^{13}$C) shows a correlation cross-peak to each of the nuclei in a contiguous spin system. A transfer of magnetization under the influence of a spin lock field was introduced by Davis and Bax (12) in 1985. To improve the performance of the experiment, periodic phase alteration (MLEV-17) of the spin lock pulses was implemented (10). Traditionally, the TOCSY experiment is used to assign scalar coupled $^1$H spin systems. More recently, TOCSY pulse trains were also applied to transfer magnetization between $^{13}$C of labeled biopolymers (13). However, the $^{13}$C TOCSY experiment is evaluated infrequently in the evaluation of biosynthetic pathways.

The $^{13}$C NMR spectrum of lipstatin revealed the presence of extensive $^{13}$C-$^{13}$C coupling in the biolabeled molecule (Fig. 2). For example, the $^{13}$C signal of C-3 was characterized by a central signal at 74.9 ppm and by a doublet ($^3$J$_{CC}$, 39.2 Hz) resulting from coupling to one adjacent $^{13}$C atom (C-2 or C-4). The $^{13}$C satellites of C-5 appeared at a distance of 78.2 Hz. Coupling to either C-4 or C-6 should result in a coupling constant of about 40 Hz due to the aliphatic nature of C-4 and C-6. Therefore, the observed coupling signature must result from simultaneous coupling to two adjacent $^{13}$C atoms (i.e. C-4 and C-6) yielding a pseudo-triplet ($^3$J$_{CC}$, 39.1 Hz) where the central component overlaps with the uncoupled singlet (Fig. 2). It follows that the carbon atoms C-4–C-6 were derived en bloc from the uniformly $^{13}$C-labeled algal lipid. Similarly, the signal of C-2 was characterized by simultaneous $^{13}$C coupling to two $^{13}$C spins via one-bond coupling ($^3$J$_{CC}$, 42.4 Hz and 38.0 Hz, respectively) and to one $^{13}$C spin via two-bond coupling ($^5$J$_{CC}$, 3.6 Hz) (Fig. 2).

The analysis of $^{13}$C-$^{13}$C coupling in terms of multiplicities in the one-dimensional $^{13}$C NMR spectrum is summarized in Table III. These data show already that biosynthetic modules containing more than two carbon atoms were incorporated into the lipstatin molecule. However, the size of these respective building blocks can be addressed much better by two-dimensional NMR analysis as described below.

A section of a two-dimensional $^{13}$C INADEQUATE experiment is shown in Fig. 3. The double quantum-filtering technique monitors pairs of adjacent $^{13}$C atoms but not isolated $^{13}$C nuclei. Due to the low natural abundance of $^{13}$C, INADEQUATE is notorious for its low sensitivity. However, pairs of $^{13}$C atoms that were contiguously incorporated from multiple $^{13}$C-labeled precursors are diagnosed with high sensitivity. The

| Carbon atom$^a$ | U-$^{13}$C-lipid | Hydrogenated U-$^{13}$C-lipid |
|----------------|------------------|-------------------------------|
| 1$^b$          | d                | d                             |
| 2$^b$          | 2               | 2                             |
| CHO            | d                | d                             |
| 8              | dd               | dd                            |
| 11             | dd               | dd                            |
| 10             | dd               | dd                            |
| 7              | dd               | dd                            |
| 3              | d                | 4,6                           |
| 5              | 4,6              | 4,6                           |
| 2              | 1,1              | 1,1                           |
| 3$^c$          | t                | t                             |
| 4              | 3,5              | 3,5                           |
| 6              | nd               | t                             |
| 14             | nd               | t                             |
| 13             | nd               | t                             |
| 3$^e$          | nd               | t                             |
| 1              | 2,4              | 2,4                           |
| 12             | nd               | t                             |
| 2$^f$          | 3,1              | t                             |
| 9              | t                | t                             |
| 4$^g$          | d                | d                             |
| 5              | 4,4              | 4,4                           |
| 5$^h$          | 6,4              | 6,4                           |
| 15             | 6,4              | 6,4                           |
| 6$^i$          | d                | 5,4                           |
|               | 5,4             | 5,4                           |
|               | 2,1             | 2,1                           |
|               | 5,4             | 5,4                           |
|               | 2,1             | 2,1                           |

$^a$ Carbon atoms are listed according to Table II.

$^b$ Multiplicity of $^{13}$C-coupled satellite signals (d, doublet; dd, double doublet; t, pseudo-triplet).

$^c$ Two-dimensional $^{13}$C TOCSY experiment encompassed the aliphatic spectral region (80–10 ppm). Observed cross-peaks are indicated.

$^d$ nd, not determined due to signal overlapping.

TABLE III
$^{13}$C-$^{13}$C-coupling pattern of lipstatin biosynthesized from a mixture of U-$^{13}$C-lipid and soybean oil or from a mixture of hydrogenated U-$^{13}$C-lipid and soybean oil

| Mult$^b$ | INADEQUATE | 2D-$^{13}$C TOCSY | 1D-$^{13}$C TOCSY | Mult$^b$ | INADEQUATE | 2D-$^{13}$C TOCSY | 1D-$^{13}$C TOCSY |
|----------|------------|-------------------|-------------------|----------|------------|-------------------|-------------------|
| 1         | d          | 2                 | d                 | d        | 2          | 2,1,2,3,4,5,6   | 1–6              |
| 1         | d          | 2                 | d                 | d        | 2          | 2,1,2,3,4,5,6   | 1–6              |
and therefore requires some technical comments.

The most important difference between $^1H$ and $^{13}C$ TOCSY methods is the larger spectral width of the $^{13}C$ chemical shift range. Efficient transfer of magnetization by the spin lock pulse requires a sufficiently high power of spin lock field $B_1$. Typically, $gB_1$ should exceed the spectral width. However, the maximum strength of the $B_1$ field is limited by the thermal and electronic stability of the probe head during the relatively long mixing period (typically 10–60 ms).

To optimize the experimental parameters, we performed a series of two-dimensional $^{13}C$ TOCSY experiments with [U-13C]lysine. A spin lock field of 8 kHz applied for 45 ms enabled the transfer of magnetization over a relatively wide frequency range (15 kHz) between all of the six carbon atoms of lysine. However, it should be noted that the cross-peak intensity between the carboxylic carbon and the side chain carbon atoms decreased approximately by a factor 10 relative to the other cross-peaks, which had similar intensities.

To improve the limited digital resolution of this two-dimensional experiment (e.g. for extraction of coupling constants), we performed a selective excitation of a single $^{13}C$ frequency followed by a $^{13}C$ TOCSY mixing process. The selective excitation was achieved by a 5-ms Gauss or semi-Gauss pulse. In model experiments with [U-13C]lysine, we were able to transfer magnetization from the α-carbon to C-3–C-6 with similar efficiency and to C-1 with decreased intensity. Obviously, the spin lock field (8 kHz) was too weak to achieve efficient magnetization transfer between C-1 and C-2 of lysine, which are separated by approximately 12 kHz from each other. Since the intensities of C-3–C-6 in the one-dimensional $^{13}C$ TOCSY spectrum were similar, the applied spin lock field was optimal for a frequency range of 3–4 kHz.

These results served as a basis for the $^{13}C$ TOCSY experiments with lipstatin. Fig. 4 shows a part of a phase-sensitive two-dimensional $^{13}C$ TOCSY experiment with lipstatin from the fermentation with U-13C-lipid encompassing the aliphatic spectral region (80–10 ppm). The transfer of magnetization is highlighted in Fig. 4 among C-3–C-6 and among C-2 and C-1–C-6. Again, no transfer of magnetization was observed between C-2 and C-3. Additionally, a series of selective one-dimensional $^{13}C$-TOCSY experiments was performed (Table III). Fig. 5 shows spectra obtained by selective excitation of C-3 and C-2, respectively, and subsequent isotropic mixing. Due to the physical basis of the experiment, only signals were observed that result from magnetization transfer from the excited carbon. Consequently, the highly crowded $^{13}C$-coupled one-dimensional $^{13}C$ spectrum can be edited by this spectroscopic technique (Fig. 5). It should be noted that the $^{13}C$ spin lock field was not strong enough to achieve magnetization transfer from the aliphatic C-6 to the alkene carbon atoms. However, the signal of C-6 in the one-dimensional $^{13}C$ TOCSY experiment is a pseudo-triplet (Fig. 5B), which clearly indicates contiguous $^{13}C$ coupling to the unsaturated C-7 of lipstatin. In combination with signal multiplicities in the one-dimensional $^{13}C$ NMR spectrum (i.e. double doublets of the inner chain carbon atoms, indicating contiguous coupling) and the INADEQUATE results, this proves the presence of isotopomers with contiguous $^{13}C$ labeling extending from C-16 to C-3, and from C-1 to C-6 (Fig. 6A). We conclude that the lipstatin molecule is assembled from a 14-carbon (C-16–C-3) and an 8-carbon (C-1–C-6) moiety, which can both be derived en bloc from the U-13C-lipid mixture supplied as a precursor.

Two pairs of labeled carbon atoms (i.e. C-1°/C-2°, and C-4°/C-5°) were incorporated into the leucine side chain from the $^{13}C$-labeled lipid mixtures. This signifies the diversion of multiple $^{13}C$-labeled components of the lipid mixture to the biosynthesis of the amino acid.

**Fermentation with Hydrogenated U-13C-Lipid**—The presence of two isolated double bonds in the 14-carbon moiety suggested that it might be derived preferentially or exclusively from the unsaturated fatty acid fraction of the $^{13}C$-labeled precursor mixture (i.e. more specifically, from linoleic acid). To check this hypothesis, we used a totally hydrogenated U-13C-lipid mixture for an incorporation experiment.

The hydrogenated U-13C-lipid was mixed with soybean oil at a ratio of 1:17.4 (w/w), and the mixture was proffered to a growing culture of *S. toxytricini*. Lipstatin was isolated and analyzed by NMR spectroscopy as described above. The $^{13}C$ NMR signals of the 8-carbon moiety were again characterized by the presence of $^{13}C$-coupled satellites, whereas the carbon...
atoms of the 14-carbon moiety showed no $^{13}\text{C}^{13}\text{C}$ coupling in the one-dimensional NMR experiment (Table III). Analysis by two-dimensional INADEQUATE and $^{13}\text{C}$ TOCSY spectroscopy is summarized in Table III and confirmed that the 8-carbon moiety but not the 14-carbon moiety was consecutively labeled (Fig. 6B).

It follows that the unsaturated 14-carbon moiety was not biolabeled in the experiment with the hydrogenated $\text{U}^{13}\text{C}$-
Apparentl,y this part of lipstatin was exclusively derived from the unsaturated fraction of the unlabeled soybean oil supplement in this experiment. The labeling pattern of the leucine residue was the same as in the experiment described above.

**DISCUSSION**

We have demonstrated that the lipstatin backbone is assembled from two moieties consisting of 8 and 14 carbon atoms, respectively, which were both contiguously labeled with $^{13}$C from a U-$^{13}$C-lipid mixture. Since the uniformly $^{13}$C-labeled lipids were proffered together with a large excess of unlabeled lipid material, this result shows that both building blocks were derived from precursor lipids by partial catabolism of fatty acid residues. The *de novo* synthesis of the building blocks from smaller units such as acetate could not possibly yield lipstatin molecules with uninterrupted $^{13}$C labeling of long alkyl chains, since labeled and unlabeled precursor molecules would be interspersed at random, thus conducing to noncontiguous $^{13}$C labeling, as shown in the formylleucine moiety of lipstatin. In this case, the TOCSY transfer of magnetization along the alkyl chain would be interrupted.

Catalytic hydrogenation destroys the potential of the $^{13}$C-labeled lipid mixture to serve as a precursor for the 14-carbon moiety. However, the hydrogenated lipid mixture is still used efficiently as a precursor of the 8-carbon moiety and of leucine. It follows that the 14-carbon moiety is specifically derived from an unsaturated component of the U-$^{13}$C-lipid mixture. Linoleic acid has the same pattern of double bonds as the unsaturated side chain of lipstatin and could be converted to an appropriate precursor by $\beta$ oxidation (Fig. 7). Alternative pathways for the partial degradation of linoleic acid, such as the pathway described recently in rat liver (14, 15), cannot be ruled out on basis of the available data.

On the basis of the observed labeling patterns, we propose the following pathway for the biosynthesis of lipstatin (Fig. 8).
The 14-carbon moiety is derived from linoleic acid by partial β oxidation, resulting in the release of 2 acetate units. The CoA ester of 3-hydroxy-Δ5,8-tetradecanoic acid (Fig. 8, 3) could serve directly as a component for Claisen condensation. Similarly, the 8-carbon component could be obtained by partial β oxidation of long chain fatty acids. The resulting octanoyl-CoA (Fig. 8, 4) could then undergo the proposed Claisen condensation with the CoA ester of 3-hydroxy-Δ5,8-tetradecanoic acid, resulting in the formation of compound 5. This sequence of reactions explains the formation of the carbon skeleton of lipstatin. The 14- and 8-carbon modules can both be derived from U-13C-lipid or from unlabeled lipid. The Claisen condensation will use labeled and unlabeled fragments at random. Consequently, 13C-13C coupling is not observed between C-2 and C-3 of lipstatin.

A possible scenario for the subsequent reaction steps is immediately obvious, but the details require further study. The hydroxy group of the 14-carbon module could be aminoacylated prior to or after the Claisen condensation. Reduction of the carbonyl group generated by the Claisen condensation should yield a hydroxy group, and the β-lactone ring of compound 7 could be formed with the CoA moiety as a leaving group.

It should be noted that this biosynthetic pathway was observed in a medium containing large amounts of saturated and unsaturated fatty acids. Under these conditions, the 14-carbon moiety is entirely derived from the unsaturated fatty acid pool, as shown by the experiments with the hydrogenated U-13C-lipid mixture. Apparently, desaturation of fatty acid does not play a significant role under these culture conditions.

The data also show that degradation products of the proffered lipid are diverted to the biosynthesis of leucine. The incorporation of two 13C pairs from the precursor mixture is well in line with the biosynthetic pathway of the amino acid, as summarized in Fig. 9. The biosynthesis of leucine involves the condensation of pyruvate and acetyl-CoA molecules. A mixture of 13C3-pyruvate and 13C3-acetyl-CoA with the respective unlabeled precursors should yield the observed labeling pattern, as shown in Fig. 9. Whereas the details have not been investigated, 13C3-pyruvate could be formed during the fermentation from the glycerol part of the U-13C-lipid mixture. This should be conducive to the observed isotope distribution.

The use of a crude mixture of 13C-labeled precursors for biosynthetic studies is an unusual approach. As shown with the present example, it can yield results of optimum clarity under appropriate conditions. The crucial part of the present experiments is the use of a mixture of totally 13C-labeled lipids with a large excess of unlabeled lipids. In conjunction with modern one- and two-dimensional NMR technology, this approach can define the length of the biosynthetic building blocks with a minimum of experimental effort. This strategy is not limited to mixtures of lipids. Indeed, we have shown independently that it can also be used successfully using crude mixtures of 13C-labeled carbohydrates or amino acids.

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