Biosynthetic Origin of a Branched Chain Analogue of the Lipase Inhibitor, Lipstatin

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The lipophilic β-lactone, lipstatin, inhibits pancreatic lipase and has been shown earlier to be biosynthesized by Claisen condensation of two fatty acid moieties. We present data from incorporation experiments with [U-$^{13}C_6$]leucine showing that a branched chain analogue of lipstatin is biosynthesized from leucine.

Introduction

The lipophilic β-lactone, lipstatin (**1**, Scheme 1), was isolated as an inhibitor of pancreatic lipase produced by *Streptomyces toxytricini*,[1,2] and its tetrahydro derivative **2**, Orlistat (Xenical), is used for the treatment of severe obesity (for review see ref 3). Chromatographic studies on a lipstatin-producing strain of *S. toxytricini* afforded evidence for minor fermentation products which were tentatively assigned as branched chain analogues of lipstatin on the basis of mass spectrometry data (unpublished). Structural analogues of lipstatin are potential lipase inhibitors, and the determination of their structures and biosynthetic origins are crucial for further developments.

Earlier, we have shown that the β-lactone moiety of lipstatin is biosynthesized by a Claisen condensation of the C_{14} carboxylic acid **5** or its thioester with octanoyl-CoA (**6**)[4−7] (Figure 1). On this basis, it appeared plausible that the biosynthesis of the branched chain lipstatin analogue **3** could involve a branched chain fatty acid or its thioester (more specifically, 7-methyloctanoyl-CoA) instead of octanoyl-CoA as precursor.

Branched chain fatty acids can be biosynthesized by methylation of linear precursors.[8,9] Alternatively, 7-methyloctanoyl-CoA could have been formed by a polyketide type pathway using the CoA-ester of a short branched chain fatty acid as starter unit which is elongated by a polyketide synthase. Thus, branched carboxylic acids which are biosynthetically incorporated into humulone in hops,[10] into hyperforin in *Hypericum perforatum*,[11] and into the antibiotics, avermectin[12] and IC101,[13] in *Streptomyces spp.* were shown to be obtained by oxidative decarboxylation of the amino acids, leucine[10,12,13] or valine.[11]

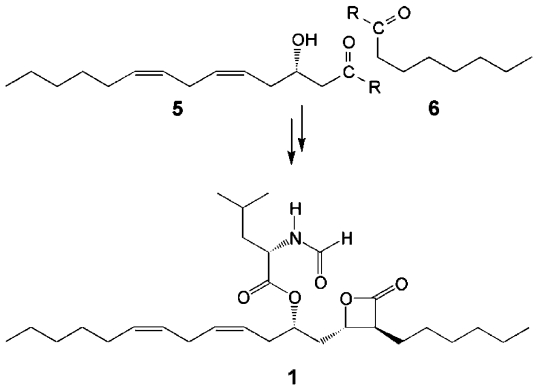
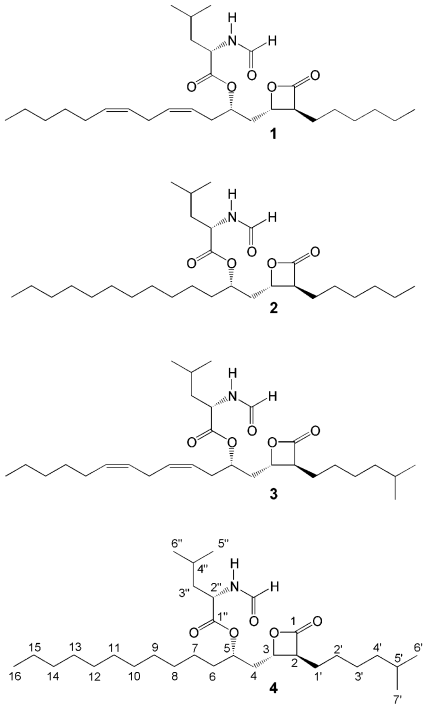


Figure 1. Biosynthesis of lipstatin.[4−7] R = OH or SCoA.

Scheme 1



Results and Discussion

To determine the biosynthetic origin of the lipstatin analogue, we cultured *S. toxytricini* with a supplement of [U-$^{13}C_6$]leucine. The mixture of lipstatin and its branched chain analogue was isolated by solvent extraction of the broth followed by chromatography on silica gel. To increase the stability of the biosynthetic β-lactones against oxygen and to facilitate their isolation, the mixture was subjected to catalytic hydrogenation over

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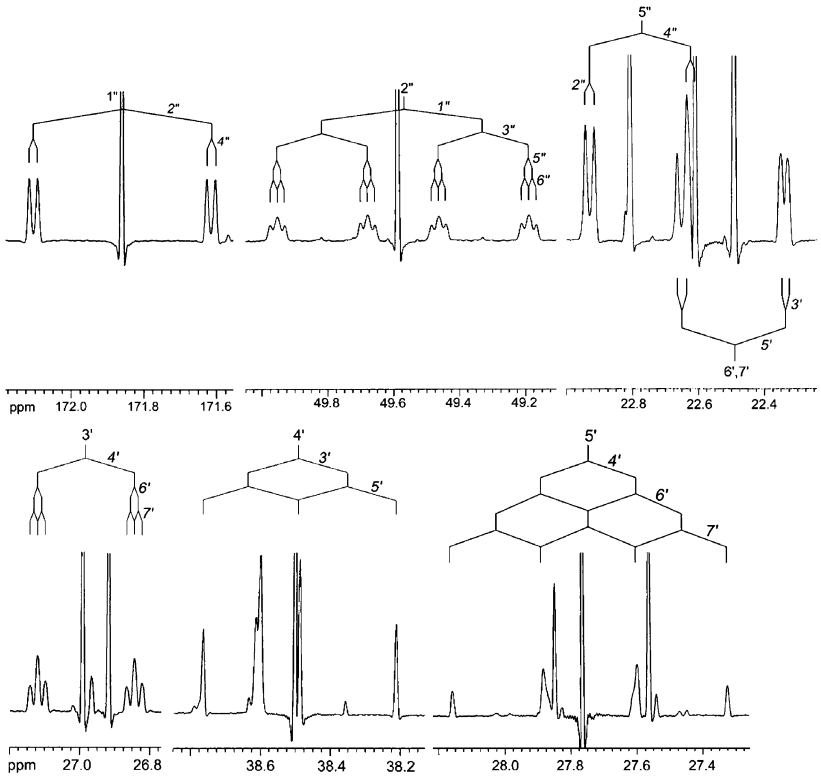

Figure 2. ^{13}C NMR signals of **4** from the experiment with [U-$^{13}C_6$]leucine. ^{13}C coupling patterns are indicated.

Table 1. NMR Data of 4

	chemical shifts, ppm[a]		coupling constants, Hz[b]	
position	$\delta^{13}C$	$\delta^{1}H$	J_{CC}	HMBC correlations
1″(C)	171.85		61.7(2″), 2.9(4″)	5, 2″, 3″
1(C)	170.76			3, 2, 1′
CHO(CH)	160.77	8.18		NH, 2″
NH		5.97		
3(CH)	74.71	4.26		5, 2, 4, 1′
5(CH)	72.62	5.00		3, 4, 6
2(CH)	56.91	3.19		4, 1′, 2′
2″(CH)	49.57	4.65	61.5(1″), 34.1(3″), 2.5(5″), 2.5(6″)	CHO, 3″
3″(CH_2)	41.33	1.63, 1.52	34.3(2″), 34.3(4″)	2″, 6″, 5″
4(CH_2)	38.59	2.14, 1.97		5, 2
4′(CH_2)	38.49	1.14	34.5(3′), 34.5(5′)	6′/7′, 5′, 3′
6(CH_2)	33.94	1.62, 1.56		5, 4
14(CH_2)	31.81	1.22		16, 15
8(CH_2)[c]	29.53	1.25		
9(CH_2)[c]	29.52	1.25		
10(CH_2)[c]	29.45	1.25		
11(CH_2)[c]	29.34	1.25		
12(CH_2)[c]	29.25	1.25		
13(CH_2)[c]	29.22	1.25		
5′(CH)	27.75	1.49	35.5(4′), 34.5(6′), 34.5(7′)	6′/7′, 4′
1′(CH_2)	27.55	1.77, 1.71		2, 3
3′(CH_2)	26.98	1.28	34.7(4′), 2.5(6′), 2.5(7′)	1′, 5′
2′(CH_2)	26.90	1.39, 1.28		2, 4′
7(CH_2)	25.00	1.27		5, 6
4″(CH_2)	24.78	1.65	34.7(3″), 34.7(6″), 34.7(5″), 2.7(1″)	5″, 6″, 2″, 3″
5″(CH_3)	22.79	0.94	34.9(4″), 3.3(2″)	4″, 6″
15(CH_2)	22.59	1.25		16, 14
6′/7′(CH_3)	22.48	0.84	34.9(5′), 2.5(3′)	6′/7′, 5′
6″(CH_3)	21.63	0.94	34.7(5″), 2.2(2″)	4″, 3″
16(CH_3)	14.04	0.85		15

[a] Referenced to solvent signals. [b] Detected with the sample from the experiment with [U-$^{13}C_6$]leucine. [c] Assignments may be interchanged.

palladium on charcoal. The product was separated into two fractions by preparative HPLC. Fraction A coeluted with a reference sample of tetrahydrolipstatin. Analytical HPLC of fraction B revealed two major peaks (62% and 37% peak intensity, respectively).

This product mixture was further analyzed by NMR

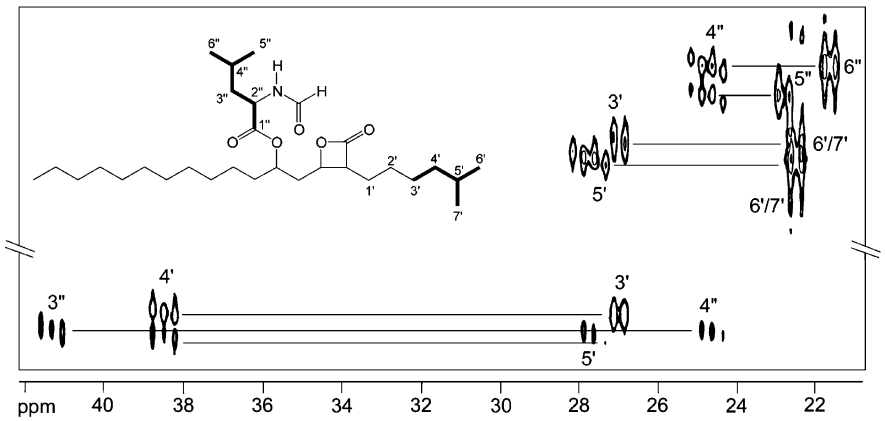

Figure 3. INADEQUATE spectrum of **4** from the incorporation experiment with [U-$^{13}C_6$]leucine. Pairs of signals at specific double quantum frequencies are connected by horizontal lines. Totally ^{13}C-labeled molecular fragments from leucine are indicated by bold lines in the structure of **4**.

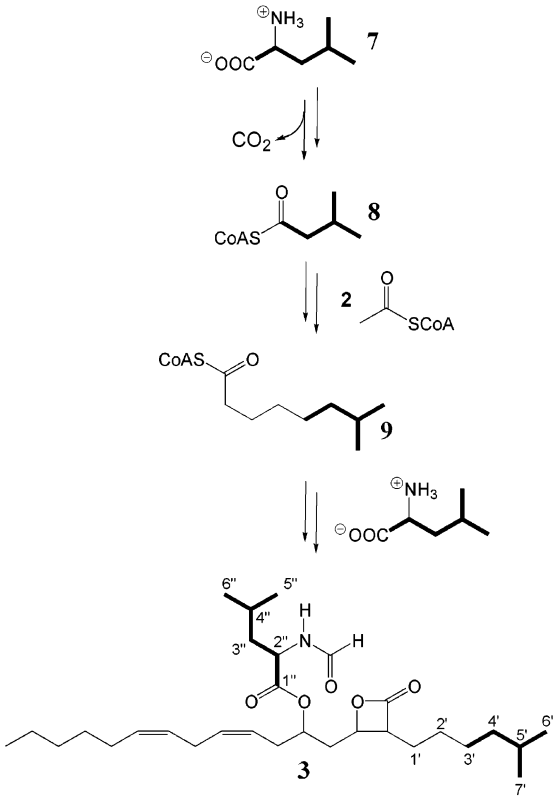

Figure 4. Hypothetical pathway for biosynthesis of **3**. The transfer of totally ^{13}C-labeled molecular fragments from leucine is indicated by bold lines.

spectroscopy. In the low-field region, the ^{1}H NMR spectrum was virtually identical to the ^{1}H NMR spectrum of tetrahydrolipstatin. More specifically, signals reflecting the formyl moiety (8.2 ppm), the β lactone ring (4.3 ppm, H-3; 3.2 ppm, H-2), and the *O*-leucyl ester (5.0 ppm, H-5; 4.6 ppm, H-2″) were observed (Table 1). Broad intense signals were observed in the high-field region of the spectrum (0.8−2 ppm).

HMQC and HMBC experiments afforded information about the ^{13}C NMR chemical shifts (Table 1). Again, all ^{13}C signals characteristic of the lactone ring and the leucyl side chain of tetrahydrolipstatin were detected. It was therefore concluded that fraction B contained structural analogues of tetrahydrolipstatin with modified C_{14} or C_6 side chains. For one component of the mixture, the structure of the side chain was determined by analysis of the ^{13}C spin network which resulted from the incorporation of [U-$^{13}C_6$]leucine. More specifically, 10 ^{13}C NMR signals showed ^{13}C−^{13}C coupling satellites of relatively high intensity (cf. Figure 2). From the well resolved multiplet structures of the satellite signals ^{13}C−^{13}C coupling constant could be determined (Table 1). The sizes of the coupling constants indicated scalar couplings between ^{13}C atoms via one to three bonds. The other signals of the sample did not show ^{13}C−^{13}C coupling satellites above the natural abundance level. Information about the carbon connectivities was obtained by carbon−carbon correlation experiments (INADEQUATE) which featured two spin systems comprising five and six contiguous ^{13}C atoms, respectively (Figure 3). This double-quantum filtered experiment reveals scalar couplings between adjacent ^{13}C atoms; hence, only molecular species with adjacent ^{13}C atoms in the same molecule are observed. The spin system comprising six ^{13}C atoms was attributed to a leucyl moiety.

The second ^{13}C spin system comprises two methyl groups (both resonating at 22.5 ppm) and three aliphatic carbon atoms (resonating at 38.5, 27.7, and 27.0 ppm). The complex multiplets are again characterized by satellites due to ^{13}C−^{13}C coupling via one and/or three bonds (cf. Figure 2 and Table 1). As an example, the multiplet structure of the signal at 27.7 ppm (C-5′) indicated simultaneous one-bond couplings to three ^{13}C atoms which could be assigned as C-4′, C-6′, and C-7′ by the two-dimensional INADEQUATE spectrum. On the basis of the NMR data described above, the second ^{13}C spin system was attributed to carbon atoms 3′ through 7′ of the iso-C_7-analogue of tetrahydrolipstatin (**4**).

The data also show that a block of five contiguous ^{13}C-labeled carbon atoms was incorporated from [U-$^{13}C_6$]-leucine into the β-lactone side chain providing carbon atoms 3′ through 7′. This is well in line with formation of isovaleryl-CoA (**8**) from leucine (**7**) by the consecutive action of leucine transaminase and α-ketoisocapryl decarboxylase (Figure 4). Stepwise elongation of isovaleryl-CoA by two acetyl moieties could afford 7-methyloctanoyl-CoA (**9**) which could serve as a precursor of **3** via the lipstatin biosynthetic pathway described earlier.[7]

Notably, the leucine moiety of **4** can serve as internal standard of ^{13}C enrichment. The ^{13}C abundances of the

Table 2. ^{13}C Enrichments of **4** from the Experiment with [U-$^{13}C_6$]leucine

position	%^{13}C[a]	%$^{13}C-^{13}C$[b]
1″	2.72	59.5
2″	2.76	60.2
3″	2.69	59.1
4″	2.70	59.3
5″	2.95	62.8
6″	2.74	59.8
3′	2.38	53.7
4′	2.67	58.8
5′	2.56	56.1
6′/7′	2.44	54.9

[a] ^{13}C abundances. [b] Fraction of ^{13}C-coupled satellite signals in the global ^{13}C NMR signal of a given atom.

leucine moiety (1″ through 6″) and the β-lactone side chain atoms (3′ through 7′) in isotope-labeled **4** are 2.76 ± 0.10 and 2.51 ± 0.13%, respectively (Table 2). These values correspond to a ^{13}C excess of 1.66 ± 0.1% and 1.41 ± 0.13%, respectively. Since the proffered [U-$^{13}C_6$]-leucine had been diluted with unlabeled leucine at a ratio of 1:39 (w/w), the molar fraction of ^{13}C-labeled leucine was approximately 2.4%. On this basis, the specific incorporation of leucine into the leucine moiety and the β-lactone side chain atoms (3′ through 7′) of **4** was 69% and 59%, respectively. It can be inferred that carbons 3′ through 7′ in **3** are derived predominantly from the proffered leucine. Contributions by alternative pathways (e.g., via fatty acid methylation) are of minor relevance, if any.

Experimental Section

[U-$^{13}C_6$]Leucine was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). Fermentation of *S. toxytricini* during the growth phase was conducted as described earlier.[6] At the beginning of the lipstatin production phase (at ca. 30 h), a solution (360 mL) containing 0.74 g of [U-$^{13}C_6$]leucine and 28.8 g of unlabeled S-leucine was added continuously at a rate of 3.75 mL per hour to an 8 L culture during a period of 96 h. The extraction and enrichment of lipstatin from the fermentation broth was performed as described earlier.[4] The lipstatin fraction containing product **3** was hydrogenated in ethyl acetate with 20% Pd/C (5%) at 10 bar hydrogen and room temperature. The mixture was separated by preparative HPLC using a column of Supelcosil ABZ$^+$Plus (12 μm, 8 × 27 cm; Supelco) which was developed with 80% aqueous acetonitrile. The concentrated peak fractions containing approximately 23% of **4** and 26% of tetrahydrolipstatin were extracted with ethyl acetate and rechromatographed on Supelcosil ABZ$^+$Plus (12 μm, 2 × 25 cm; Supelco) with 85% aqueous acetonitrile. The product, free of tetrahydrolipstatin and lipid impurities, was finally chromatographed on 10 g of silica gel (Mega Bond Elut Cartridge; Varian) with a stepwise gradient of hexane/ethyl acetate (100−0 to 50−50 (v/v)) and crystallized from hexane to give a mixture of **4**, another analogue of tetrahydrolipstatin, and an unknown impurity (62:37:1; HPLC peak area; HPLC method: two columns of Hypersil Elite C18, 5 μm, 250 × 4.6 mm (Hypersil); 870 mL acetonitrile/130 mL water/0.00025% concentrated phosphoric acid; 30 °C; 1.0 mL/min; 195 nm; duration: 75 min).

An aliquot (30 mg) of the mixture containing **4** and another analogue of tetrahydrolipstatin was dissolved in 0.5 mL of $CDCl_3$. ^{13}C NMR spectra were recorded at 125.6 MHz using an AVANCE 500 MHz NMR spectrometer from Bruker Instruments, Karlsruhe, Germany. The transmitter pulse length was 5 μs (30°). ^1H decoupling was achieved by composite pulse decoupling during the relaxation and acquisition period. The relaxation delay (10 s) was adjusted in order to avoid differential relaxation effects by ^{13}C couplings. Each satellite in the ^{13}C NMR spectra was integrated separately. The relative fractions of each respective satellite pair (corresponding to a certain coupling pattern, cf. Table 1) in the total signal integral of a given carbon atom were calculated (% $^{13}C-^{13}C$ in Table 2). Assuming that the central singlet signal reflected the natural abundance contribution (i.e., 1.1% ^{13}C) for **4**, absolute ^{13}C abundances for each carbon atom were then calculated (cf. Table 2).

Two-dimensional COSY, HMQC, and HMBC experiments were measured with standard Bruker parameters (XWINMMR 3.0). Two-dimensional INADEQUATE experiments were performed with the Bruker pulse program inad using a 90° read pulse (8.0 μs). Further parameters were as follows: td2, 2k; ns, 64; aq, 0.163 s; d1, 2s, d4, 6ms; td1, 800; sw2, 50 ppm; sw1, 25 ppm; aq-mode, qsim; mc2, qf; wdw2, gm; lb2, −0.6; gm2, 0.02; wdw1, qsine; ssb1, 2.

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