LIPSTATIN, AN INHIBITOR OF PANCREATIC LIPASE, PRODUCED BY *STREPTOMYCES TOXYTRICINI*

II. CHEMISTRY AND STRUCTURE ELUCIDATION

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The structure of a new pancreatic lipase inhibitor, lipstatin, produced by *Streptomyces toxytricini* was determined as (2*S*,3*S*,5*S*,7*Z*,10*Z*)-5-[(*S*)-2-formamido-4-methylpentanoyloxy]-2-hexyl-3-hydroxy-7,10-hexadecadienoic lactone by spectroscopic and chemical methods. Structurally lipstatin is closely related to the known esterase inhibitor esterastin. It contains a *N*-formyl-L-leucine side chain instead of the *N*-acetyl-L-asparagine in esterastin.

Streptomyces toxytricini (NRRL 15443) was found to produce a specific inhibitor of pancreatic lipase which we have named lipstatin[1]. Lipstatin was found mainly in the mycelium and was extracted, after recovery of the biomass by centrifugation, with methanol. The organic extract was concentrated, the residue suspended in water and then extracted with hexane - ethyl acetate. This extract was chromatographed on silica gel. Further purification was done by preparative reverse phase chromatography on a Lobar Lichroprep RP-8 column, yielding pure lipstatin as a pale yellow oil[1].

Elemental analysis indicated the following composition:

Calcd for $C_{29}H_{49}NO_5$ (491.713): C 70.84, H 10.04, N 2.85.
Found: C 70.77, H 10.11, N 2.83.

The observation of peaks at *m/z* 509 $(M+NH_4)^+$ and 492 $(M+H)^+$ in the mass spectrum with chemical ionization (NH_3) and of 29 carbon signals in the ^{13}C NMR spectrum (see Table 1) supported the molecular formula $C_{29}H_{49}NO_5$. The IR spectrum of lipstatin shows a characteristic peak at 1823 cm^{-1}. This indicates the presence of a β-lactone moiety in the compound. In 1978 UMEZAWA *et al.*[2] described an esterase inhibitor of microbial origin, esterastin, containing a β-lactone moiety. A comparison of the ^1H NMR spectra of esterastin reported by UMEZAWA *et al.*[2] and of lipstatin (see Table 2) revealed some structural similarity of these two compounds. It was obvious, that the main difference is due to different amino acid side chains. Catalytic hydrogenation of lipstatin (**1**) gave a crystalline tetrahydrolipstatin (**2**). Chemical ionization mass spectra (CI-MS) (NH_3) *m/z* 513 $(M+NH_4)^+$, 496 $(M+H)^+$. Treatment of **1** with K_2CO_3 in methanol at 50°C gave the unsaturated δ-lactone **3** (MS *m/z* 332 (M)) and *N*-formyl-L-leucine methyl ester (**4**), which was identical with a reference sample prepared from L-leucine methyl ester. Alkaline hydrolysis of **1** at room temperature followed by catalytic *p*-toluenesulfonic acid treatment afforded the δ-lactone **5**, a degradation product of esterastin, found by UMEZAWA *et al.*[2] (^1H NMR, Table 2). Mild hydrolysis of **1** with $NaHCO_3$ in MeOH gave two methyl esters **6** and **7**. **6** could be identified as the δ-hydroxymycolic acid methyl ester† described by UMEZAWA *et al.*[2] (^1H NMR, Table 2).

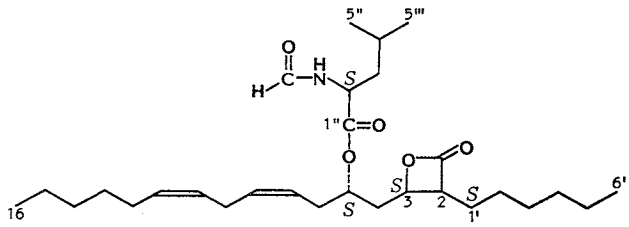
† Mycolic acid is the generic name of higher fatty acids possessing α-alkyl and β-hydroxy groups.

Table 1. Chemical shifts (ppm) of ^{13}C NMR spectra in $CDCl_3$ (assignments s, d, t and q show multiplicity on off-resonance experiments).

Carbon	Lipstatin (1)	Tetrahydro-lipstatin (2)	Carbon	Lipstatin (1)	Tetrahydro-lipstatin (2)
1, 1″	171.75 s	171.93 s	4, 6, 9, 12 to 14, 1′ to 4′, 3″ (continued)	28.97 t	29.63 t
	170.61 s	170.73 s		27.64 t	29.57 t
NHCHO	160.55 d	160.83 d		27.28 t	29.46 t
7, 8, 10, 11	132.35 d	—		26.73 t	29.35 t
	130.97 d	—		25.77 t	29.35 t
	126.76 d	—	7*, 8*, 10*, 11*	—	28.99 t
	122.81 d	—		—	27.67 t
3, 5	74.77 d	74.74 d		—	26.73 t
	72.23 d	72.63 d		—	25.12 t
2, 2″	57.08 d	57.07 d	4″	24.89 d	24.93 d
	49.62 d	49.76 d	5″ or 5‴	22.87 q	22.87 q
4, 6, 9, 12 to 14, 1′ to 4′, 3″	41.55 t	41.44 t	15, 5′	22.58 t	22.69 t
	38.13 t	38.73 t		22.52 t	22.53 t
	31.89 t	34.07 t	5″ or 5‴	21.74 q	21.78 q
	31.52 t	31.93 t	16, 6′	14.08 q	14.10 q
	31.47 t	31.51 t		14.02 q	14.00 q
	29.26 t	29.63 t			

Table 2. Chemical shifts (ppm) of ^1H NMR spectra in $CDCl_3$.

Proton	Chemical shift (*J*, Hz)			
	Lipstatin (1)	δ-Lactone (5)	Methyl ester (7)	Methyl ester (6)
16,6′-H_3	0.89 t (7)	0.89 t (7)	0.89 t (6)	0.88 t (6)
	0.90 t (7)		0.90 t (6)	0.89 t (6)
5″,5‴-H_3	0.97 d (6)	—	0.97 m	—
13～15-H_2	1.2～1.85 m	1.2～2.16 m	1.16～1.9 m	1.16～1.74 m
1′～5′-H_2				
3″-H_2		—		—
4″-H		—		—
4-H_2	1.95～2.25 m	—	～1.6	～1.6 m
12-H_2		2.05 m	2.05 m	2.05 m
6-H_2	2.44 m	2.48 m	2.42 m	2.28 m
9-H_2	2.79 t (7)	2.79 t (7)	2.79 t (7)	2.80 t (7)
2-H	3.22 m (4, 7, 8)	2.29 m (3, 5, 8)	2.28 m	2.46 (5, 6, 9)
3-H	4.29 m (4, 5, 9)	4.3 br s	3.76 m	3.92 m
2″-H	4.69 m	—	4.7 m	—
5-H	5.03 m	4.78 m	5.1 m	3.92 m
7,8-H, 10,11-H	5.22～5.62 m	5.22～5.62 m	5.22～5.62 m	5.2～5.6 m
2″-NH	6.0 d (9)	—	6.1 d (8)	—
CHO	8.04 d (12)	—	8.04 d (12)	—
	8.22 s	—	8.22 s	—
4-H_{ax}	—	1.74 m (2, 12, 15)	—	—
4-H_{eq}	—	2.11 m (4, 4, 15)	—	—
3- or 5-OH	—	—	2.82 d (6)	3.17 d (2)
				3.58 d (6)
1-$COOCH_3$	—	—	3.72 s	3.72 s



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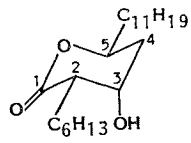

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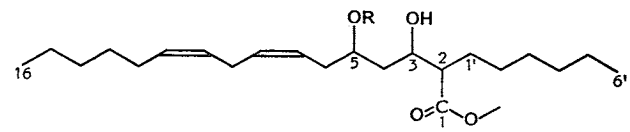
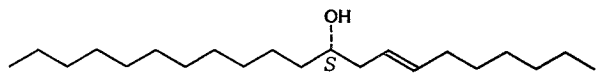


6 R = H

7 R = *N*-Formyl-(*S*)-leucine



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The IR spectrum of **7** was similar to that of lipstatin (**1**), only the characteristic peak at 1823 cm^{-1} (β-lactone) was missing. In the ^1H NMR the signals of the *N*-formyl-L-leucine side chain (Table 2) were present. In the MS peaks at *m/z* 505 ($M-H_2O$) and 492 ($M-OCH_3$) were observed.

Acetylation of **7** with acetic anhydride in pyridine gave a mono-*O*-acetyl derivative (CI-MS (NH_3) *m/z* 583 $(M+NH_4)^+$, 566 $(M+H)^+$).

From these observations the structure of **7** can be proposed to be the δ-hydroxymycolic acid methyl ester (**6**) esterified with *N*-formyl-L-leucine.

Tetrahydrolipstatin (**2**) was decarboxylated at 200°C followed by saponification to give the (*S*)-henicos-7*E*-en-10-ol (**8**) found by UMEZAWA *et al.*[2].

Periodate-permanganate oxidation of **8** gave (*S*)-3-hydroxymyristic acid (**9**), enanthic acid (**10**) also described by UMEZAWA *et al.*[2] and lauric acid (**11**).

From the fact that hydrolysis of esterastin and lipstatin afforded the identical δ-hydroxymycolic acid methyl ester (**6**) and δ-lactone **5**, it can be concluded that both inhibitors have the same δ-hydroxymycolic acid β-lactone moiety. This was further confirmed by the degradation products **8**, **9** and **10**. The hydrolysis products **4** and **7** indicate that in lipstatin δ-hydroxymycolic acid β-lactone is esterified with *N*-formyl-L-leucine. From the foregoing results, the structure of lipstatin can be proposed to be (2*S*, 3*S*, 5*S*, 7*Z*, 10*Z*)-5-[(*S*)-2-formamido-4-methylpentanoyloxy]-2-hexyl-3-hydroxy-7,10-hexadecadi-

enoic lactone. The structure of tetrahydrolipstatin (**2**) was recently confirmed by BARBIER and SCHNEIDER by total synthesis[3].

Experimental

Melting points were determined on a Tottoli capillary melting point apparatus and are uncorrected. IR spectra were determined on a Nicolet 7199 fourier transformation (FT)-IR instrument. NMR spectra were recorded in $CDCl_3$ on Bruker HX 270 and WM 400 spectrometers; chemical shifts in ppm downfield from internal TMS and coupling constants in Hz are given. The mass spectra were obtained on a MM 707F-DS 2050 mass spectrometer (Vacuum Generators, Altrincham, England), using direct introduction, vaporizing the samples from the tip of a glass rod close to the electron beam. Ionizing energy was 70 eV, ion-source temperature was 250°C.

Lipstatin (1)

Lipstatin was isolated as described previously[1].

$[\alpha]_D^{20}$ −19° (*c* 1, $CHCl_3$); UV λ_{max}^{EtOH} nm (ε) 270 (sh, 34); electron impact mass spectra (EI-MS) *m/z* (relative intensity, %) 332 (6), 163 (66), 150 (45), 114 (86), 93 (70), 69 (100), 55 (75), 43 (90); CI-MS (NH_3) *m/z* (relative intensity, %) 509 (17), 492 (14), 465 (2), 448 (6), 350 (2), 333 (15), 315 (5), 289 (74), 177 (100), 160 (34), 114 (6), 86 (16); IR (liquid film) cm^{-1} 3318, 3012, 2928, 2858, 2745, 1823, 1740, 1673, 1521, 1382, 1370, 1250, 1191.

Tetrahydrolipstatin (2)

138 mg **1** were stirred for 3 hours in 10 ml ethanol with 60 mg 5% Pd-C under a hydrogen atmosphere. The catalyst was removed by filtration through Celite. The filtrate was concd under reduced pressure and chromatographed on a silica gel column with $CHCl_3$. Upon standing, the oily product crystallized to yield 112 mg of crystalline tetrahydrolipstatin (**2**): MP 43°C; $[\alpha]_D^{20}$ −32.0° (*c* 1, $CHCl_3$); IR (liquid film) cm^{-1} 3332, 2956, 2921, 2853, 1838, 1731, 1709, 1665, 1524, 1383, 1249, 1200; ^1H NMR (270 MHz, $CDCl_3$) δ 0.89 (6H, t, *J*=7 Hz, 16-H_3 and 6′-H_3), 0.97 (6H, d, *J*=6 Hz, 5″-H_3 and 5‴-H_3), 1.15~1.85 (33H, m), 1.9~2.25 (2H, m, 4-H_2), 3.24 (1H, m, *J*=4, 7.5 and 7.5 Hz, 2-H), 4.32 (1H, m, *J*=4, 5 and 9 Hz, 3-H), 4.68 (1H, m, *J*=5, 9 and 9 Hz, 2″-H), 5.03 (1H, m, 5-H), 6.43 (1H, d, *J*=9 Hz, 2″-NH), 8.07 and 8.21 (1H, d, *J*=12 Hz and s, CHO); EI-MS *m/z* (relative intensity, %) 292 (30), 160 (17), 142 (20), 114 (100), 95 (36), 82 (34), 69 (56), 55 (40), 43 (48); CI-MS (NH_3) *m/z* (relative intensity, %) 513 (22, M+NH_4), 496 (29, M+H), 452 (12), 177 (100), 160 (46), 114 (27), 86 (52).

Unsaturated δ-Lactone 3 and *N*-Formyl-L-leucine Methyl Ester (4)

97 mg **1** dissolved in 10 ml MeOH were stirred with 30 mg K_2CO_3 for 1 hour at 50°C. The solvent was removed *in vacuo*, the residue was dissolved in $CHCl_3$ and washed with water. After evaporation of the solvent the residue was separated on a silica gel column with $CHCl_3$ - EtOAc (1 : 1) and $CHCl_3$ to afford 11 mg *N*-formyl-L-leucine methyl ester (**4**) and 27 mg of the unsaturated δ-lactone **3**.

Unsaturated δ-Lactone **3**: $[\alpha]_D^{20}$ +60.5° (*c* 1, MeOH); IR (liquid film) cm^{-1} 3011, 1721, 1651, 1383, 1246, 1218; ^1H NMR (270 MHz, $CDCl_3$) δ 0.87 (6H, t, *J*=7 Hz, 16-H_3 and 6′-H_3), 1.21~1.53 (14 H, m), 2.0~2.65 (8H, m, 4-H_2, 6-H_2, 12-H_2 and 1′-H_2), 2.79 (2H, t, *J*=7 Hz, 9-H_2), 4.40 (1H, m, 5-H), 5.24~5.62 (4H, m, 7-H, 8-H, 10-H and 11-H), 6.53 (1H, m, 3-H); EI-MS *m/z* (relative intensity, %) 332 (7, M), 261 (17), 181 (82), 163 (56), 136 (40), 93 (90), 79 (100), 87 (97), 55 (95), 41 (96).

N-Formyl-L-leucine Methyl Ester **4**: $[\alpha]_D^{20}$ −10.5° (*c* 1, MeOH); IR (liquid film) cm^{-1} 3292, 1746, 1530, 1360, 1254, 1205; EI-MS *m/z* (relative intensity, %) 117 (14), 114 (100), 85 (27), 72 (39), 69 (47), 46 (41), 43 (38); CI-MS (NH_3) *m/z* (relative intensity, %) 191 (62, M+NH_4), 174 (100, M+H).

A reference sample prepared from L-leucine methyl ester·HCl showed the identical IR and CI-MS; $[\alpha]_D^{20}$ −43.4° (*c* 1, MeOH).

δ-Lactone 5

172 mg **1** were dissolved in 0.7 ml 1 M KOH and 35 ml dioxane and stirred at room temp. After 120 minutes, 0.085 ml 1 M KOH and 15 ml dioxane were added and the reaction continued for 105

minutes. Then the solution was neutralized with 1 N HCl and concd *in vacuo*. The residue was taken up in diethyl ether, washed with satd NaCl soln and dried over Na_2SO_4. The ether was evaporated, the residue was dissolved in 50 ml CH_2Cl_2, a few mg of *p*-toluenesulfonic acid were added and the mixture was stirred for 90 minutes at room temp. The CH_2Cl_2 layer was evaporated and the residue was taken up in diethyl ether. The solution was washed with $NaHCO_3$ solution, satd NaCl soln, dried over Na_2SO_4 and concd *in vacuo*. The crude product was separated by column chromatography on silica gel with hexane - EtOAc (5:1) and (2:1) to give 51 mg δ-lactone **5**, 31 mg unsaturated δ-lactone **3** and 25 mg starting material.

δ-Lactone **5**: $[\alpha]_D^{24}$ +24.9° (*c* 1.02, $CHCl_3$) (literature[2]: $[\alpha]_D^{27}$ +21.5° (*c* 1, $CHCl_3$)); IR (liquid film) cm^{-1} 3444, 3011, 1708, 1237, 1180, 1068, 1040; EI-MS *m/z* (relative intensity, %) 350 (1, M), 332 (6), 189 (22), 155 (26), 136 (58), 79 (78), 55 (84), 45 (100), 43 (96).

Methyl Esters **6** and **7**

110 mg **1** were dissolved in 11 ml MeOH, 19 mg $NaHCO_3$ added and the mixture stirred for 20 hours at room temp. Then the reaction mixture was filtered, the filtrate concd under reduced pressure and the residue chromatographed on silica gel. Hexane - EtOAc (1:1) eluted 13 mg **6** as an oil: IR (liquid film) cm^{-1} 3420, 3010, 1740, 1200, 1170; EI-MS *m/z* 382 (M), 364 (M$-H_2O$), 346 (M$-2H_2O$) and 43 mg **7** as an oil: IR (liquid film) cm^{-1} 3340, 3010, 1740, 1670, 1200, 1170; EI-MS *m/z* (relative intensity, %) 505 (1, M$-H_2O$), 492 (1), 372 (8), 364 (5), 346 (28), 332 (10), 187 (70), 181 (53), 160 (57), 155 (42), 142 (47), 114 (100), 69 (76), 55 (78), 41 (82). 20 mg **7** were acetylated in 0.1 ml pyridine with 0.1 ml acetic anhydride. After chromatography on silica gel in hexane - EtOAc (1:1) 20 mg of an oil were obtained: EI-MS *m/z* (relative intensity, %) 505 (1, M$-$AcOH), 406 (4), 346 (35), 287 (10), 189 (71), 188 (72), 114 (90), 69 (71), 46 (32), 43 (100); CI-MS (NH_3) *m/z* (relative intensity, %) 583 (4, M+NH_4), 566 (8, M+H), 506 (15), 407 (35), 347 (63), 333 (60), 142 (80), 114 (75), 86 (100).

(*S*)-Henicos-7*E*-en-10-ol (**8**)

243 mg tetrahydrolipstatin (**2**) were heated for 40 minutes at 200°C *in vacuo*. The reaction product was hydrolyzed in 15 ml 0.1 M NaOH (22 hours at room temp). The solution was diluted with water and extracted with diethyl ether. The extract was washed with satd NaCl soln and dried over Na_2SO_4. The ether was evaporated *in vacuo* and the residue chromatographed on a silica gel column with hexane - EtOAc (19:1) to give 113 mg henicos-7*E*-en-10-ol (**8**) as white solid: MP 41~42°C (literature[2]: MP 40.5~41°C); IR (KBr) cm^{-1} 3337, 3250, 1065, 958; gas chromatography (GC)-EI-MS of the TMS-derivative $C_{24}H_{50}SiO$, *m/z* 382 (M), 367 (M$-CH_3$), 257 (M$-C_9H_{17}$); ^1H NMR δ 0.9 (6H, br t, 1-H_3 and 21-H_3), 1.09~1.59 (16H, br), 1.61 (s, 10-OH), 1.81~2.41 (6H, m, 6-H_2, 9-H_2 and 11-H_2), 3.57 (10-H, m), 5.35 and 5.58 (2H, m, *J*=16 Hz, 7-H and 8-H).

Periodate-permanganate Oxidation of (*S*)-Henicos-7*E*-en-10-ol (**8**)

60 mg **8** dissolved in 45 ml *tert*-BuOH were added to a solution of 624 mg $NaIO_4$ and 12 mg $KMnO_4$ in 130 ml of water adjusted to pH 8.5 with Na_2CO_3. The mixture was stirred for 15 hours at room temp and then filtered. The filtrate was adjusted to pH 2 and extracted with $CHCl_3$. The organic layer was washed with satd NaCl soln and the solvent was evaporated *in vacuo*. The residue was purified by TLC on silica gel with $CHCl_3$ - MeOH (19:1). 15 mg (*S*)-3-hydroxymyristic acid (**9**) and 20 mg of an oil, containing enanthic acid (**10**) and lauric acid (**11**) were obtained.

(*S*)-3-Hydroxymyristic Acid **9**: MP 71~72°C; $[\alpha]_D^{23}$ +12.5° (*c* 0.8, $CHCl_3$) (literature[2]: MP 72.5~73°C, $[\alpha]_D^{27}$ +13° (*c* 1, $CHCl_3$)); GC-EI-MS of the TMS-methylester derivative $C_{18}H_{38}SiO_3$, *m/z* 330 (M), 315 (M$-CH_3$), 257 (M$-CH_2COOCH_3$), 175 (M$-C_{11}H_{23}$).

Enanthic Acid (**10**): GC-EI-MS of the TMS-derivative $C_{10}H_{22}SiO_2$, *m/z* 202 (M), 187 (M$-CH_3$), 117 (M$-C_6H_{13}$).

Lauric Acid (**11**): GC-EI-MS of the TMS-derivative $C_{15}H_{32}SiO_2$, *m/z* 272 (M), 257 (M$-CH_3$), 117 (M$-C_{11}H_{23}$).

Acknowledgment

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