Himastatin, a New Antitumor Antibiotic from *Streptomyces hygroscopicus*

III. Structural Elucidation

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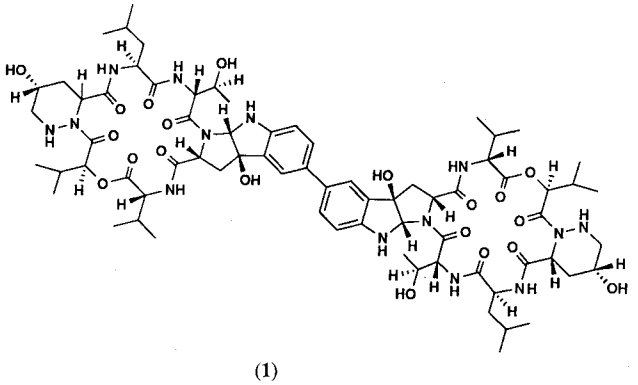
The structure of the antitumor antibiotic himastatin was determined using a combination of spectroscopic and chemical degradation techniques. Himastatin is a unique dimeric cyclohexadepsipeptide joined through a biphenyl linkage between two oxidized tryptophan units. The gross structure of the dimer was established through degradative ozonolysis. Himastatin consists of D-valine, D-threonine, L-leucine, L-α-hydroxyisovaleric acid, (3*R*,5*R*)-5-hydroxypiperazic acid, and (2*R*,3a*R*,8a*R*)-3a-hydroxyhexahydropyrrolo[2,3*b*]indole 2-carboxylic acid subunits.

Himastatin (**1**) is a novel depsipeptide antitumor antibiotic, produced in cultured broth of *Streptomyces hygroscopicus* (ATCC 53653). Details of the taxonomy of the producing strain, production, biological properties, isolation and physico-chemical properties of this substance have been previously reported[1,2]. In this paper we present details of the structure elucidation of himastatin using a combination of NMR techniques, mass spectrometry, and chemical degradation methods.

Results

Structural Elucidation

Himastatin (**1**) was isolated as a colorless microcrystalline solid from *Streptomyces hygroscopicus* strain ATCC 53653 as previously described[1,2]. The molecular formula of himastatin was determined to be $C_{72}H_{104}N_{14}O_{20}$ by high resolution fast atom bombardment mass spectrometry (HRFAB-MS) ($(M)^+$; *m/z* 1484.75897, calcd 1484.75507) and elemental analysis. The IR spectrum revealed ester carbonyl absorption at 1731 $^{-1}$ and amide carbonyl absorptions at 1675 and 1630 cm^{-1}. The UV spectrum (λ_{max} 286 nm (ε 27,900)) indicated a benzenoid aromatic system. Furthermore, the large extinction coefficient for the 286 nm absorption suggested extended conjugation. Acidic hydrolysis and amino acid analysis of himastatin revealed D-valine, L-leucine, D-threonine, and L-α-hydroxyisovaleric acid. The ^{13}C NMR spectrum revealed the presence of 36 carbon signals, which were attributed to seven methyl carbons, four methylene carbons, 12 methine carbons, three aromatic methine carbons, one quaternary oxycarbon, three aromatic quaternary carbons, and six carbonyls (amide, ester) from the gated coupled spectrum. Since the molecular formula indicated exactly twice this number of carbon atoms, it was concluded that himastatin is a symmetrical dimer. The structural arguments presented focus initially on the monomeric unit.



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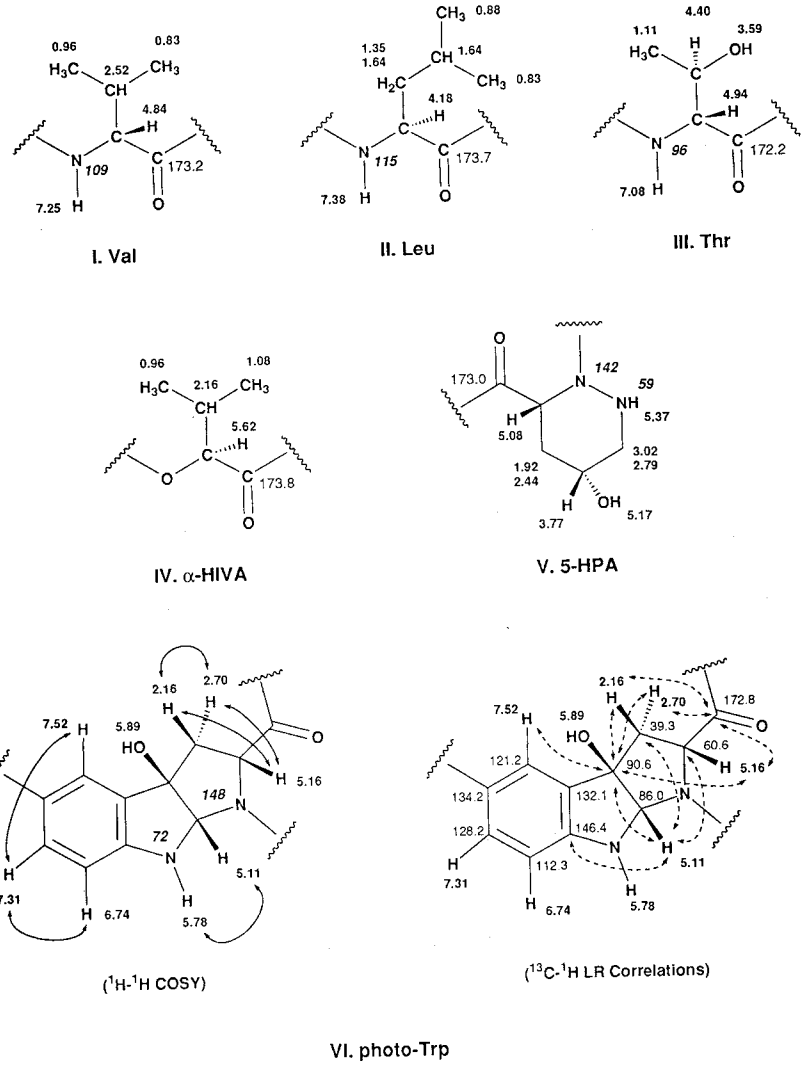
Analysis of 2D NMR correlation spectroscopy (COSY, long range COSY) spectra revealed the following structural fragments: I. (valine), II. (leucine), III. (threonine), IV. (α-hydroxyisovaleric acid), V. (5-hydroxypiperazic acid) and VI. (3a-hydroxypyrroloindole 2-carboxylic acid derivative) (Fig. 1). Assignment of the carbon signals was based on the ^1H-^{13}C shift correlated 2D-NMR (HETCOR) and inverse long range 2D correlation (HMBC) experiments (Table 1).

Fragment I (Valine): Strong splittings (J=6.8 Hz) were observed between the two γ methyl groups at δ 0.83, 0.96 and the β methine proton at δ 2.52, diagnostic of an

Fig. 1. Amino acid fragments in himastatin showing ^{1}H, ^{13}C, and ^{15}N chemical shifts in $CDCl_3$.



isopropyl group. A smaller coupling ($J=3.3$ Hz) was observed between the β C-H (δ 2.52) and α C-H (δ 4.84). A large coupling ($J=10.0$ Hz) between the α C-H (δ 4.84) and the N-H (δ 7.25) completed the spin system. The carbonyl carbon at δ 173.2 showed long range heteronuclear coupling with α C-H (δ 4.84).

Fragment II (Leucine): The typical isopropyl splitting pattern was observed between the two δ-methyl groups at δ 0.83, 0.88 and the γ C-H (δ 1.64) ($J=6.0$ Hz). The δ 1.64 resonance in the ^{1}H NMR spectrum integrated for two protons and showed correlations with carbon resonances at δ 25.1 (C-γ) and 40.8 (C-β) in the ^{1}H-^{13}C shift correlated 2D NMR experiment. It consists of two C-H protons, both belonging to leucine. The β methylene protons at δ 1.35, 1.64 showed significant couplings with α C-H (δ 4.18) which in turn is coupled to N-H (δ 7.38). The carbonyl carbon at δ 173.7 showed long range heteronuclear coupling with α C-H (δ 4.18) and β C-H (δ 1.35).

Fragment III (Threonine): In the COSY spectrum, a strong coupling ($J=6.6$ Hz) was observed between the γ CH_3 (δ 1.11) and β C-H (δ 4.40) groups and between the α C-H (δ 4.94) and N-H (δ 7.08) ($J=10.5$ Hz). The coupling between α C-H (δ 4.94) and β C-H (δ 4.40) was very weak (<1 Hz) in the COSY spectrum but was readily observed in the long range COSY experiment. The carbonyl (δ 172.2) displayed long range coupling with α C-H (δ 4.94) and β C-H (δ 4.40).

Fragment IV (α-Hydroxyisovaleric acid): The two γ-CH_3 groups at δ 0.96, 1.08 showed couplings ($J=$ 6.7 Hz) with the β C-H (δ 2.16) group. The β C-H (δ 2.16) group in turn is coupled with the α C-H (δ 5.62, d, $J=8.6$ Hz), thereby completing the spin system. The carbonyl carbon signal at δ 173.8 displayed long range

Table 1. Himastatin (**1**): ^{1}H and ^{13}C NMR Data ($CDCl_3$).

Position	^{13}C ppm (mult)	^{1}H ppm (mult, J (Hz))	^{1}H-^{1}H LR-COSY[a]	^{1}H-^{1}H ROESY[a]	long-range ^{13}C-^{1}H correlations (COLOC and HMBC combined)[a]
Leu					
NH	—	7.38 (d, 3.9)	α, 5-HPAα	α, β, γ	
CO	173.7 (s)	—			α, β1; ThrNH, α
Cα	54.1 (d)	4.18 (m)	NH, β, γ, δ, HPAα	NH, β, δ	NH, β
Cβ	40.8 (t)	1) 1.35 (dd, 10.5, 8.8)	α, γ, δ	NH, α, γ, δ2; HPAα	NH, α, γ, δ
		2) 1.64 (m)	α, γ, δ	NH, α, γ, δ; HPAα	
Cγ	25.1 (d)	1.64 (m)			α, β, δ
Cδ	1) 20.8 (q)	0.83 (d, 6.0)	α, γ	α, β2, γ	β, γ, δ2
	2) 22.8 (q)	0.88 (d, 6.0)	α, γ	α, β, γ	β, γ, δ1
Val					
NH	—	7.25 (d, 10.0)	α, pTrp2	α, γ1; pTrp2, 8a	
CO	173.2 (s)	—			α
Cα	57.0 (d)	4.84 (dd, 10.0, 3.3)	NH, β, γ, HIVAα	NH, β, γ	γ
Cβ	29.7 (d)	2.52 (dd, 6.8, 3.3)	α, γ	α, γ, HIVAγ	α, γ
Cγ	1) 16.3 (q)	0.83 (d, 6.8)	α, β	α, β, HIVAα	α, β, γ2
	2) 19.1 (q)	0.96 (d, 6.8)	β	α, β	α, β, γ1
Thr					
NH	—	7.08 (d, 10.5)	α, β	α, LeuNH, pTrp8a	
CO	172.2 (s)	—			α, β; pTrp2
Cα	53.6 (d)	4.94 (d, 10.5)	NH, OH, β; pTrp2, 8a	NH, γ, pTrp8a	NH, β, γ
Cβ	66.5 (d)	4.40 (q, 6.6)	NH, OH, α, γ	α, γ	γ
Cγ	17.2 (q)	1.11 (d, 6.6)	OH, β	α, β	OH, α, β
OH	—	3.59 (s)	α, β, γ		
α-HIVA					
CO	173.8 (s)	—			α; HPA-NH
Cα	77.1 (d)	5.62 (d, 8.6)	β, γ, Valα, HPA-NH	β, γ, Valγ	β, γ
Cβ	29.8 (d)	2.16 (sept., 6.7)	α, γ	α, γ	α, γ
Cγ	1) 18.1 (q)	0.96 (d, 6.7)	α, β	α, β	α, β, γ2
	2) 18.6 (q)	1.08 (d, 6.7)	α, β	α, β, Valβ	α, β, γ1
5-HPA					
NH	—	5.37 (d, 12.1)	α, δ, HIVAα	OH, α, δ, HIVAα	
CO	173.0 (s)	—			α, β, LeuNH
Cα	49.7 (d)	5.08 (d, 7.1)	NH, β, δ2; LeuNH, α	β; LeuNH, α, β	β1, γ
Cβ	28.4 (t)	1) 1.92 (ddd, 14.9, 7.1, 3.3)	OH, α, β2, γ	α, γ	α, δ2
		2) 2.44 (d, 14.9)	α, β1, γ, δ2	α, γ	
Cγ	58.5 (d)	3.77 (br s)	OH, β, δ	NH, β, δ	α, β2, δ
Cδ	52.4 (t)	1) 2.79 (t, 13.6)	NH, OH, δ2	NH, δ2, HIVAα	
		2) 3.02 (d, 12.6)	NH, α, β2, γ, δ1	NH, γ, δ1	
OH	—	5.17 (d, 4.5)	γ		
photo-Trp					
CO	172.8 (s)	—			2, 3; ValNH, α
C2	60.6 (d)	5.16 (d, 8.0)	3, ValNH, Thrα	3β, 3α (wk), 8a, ValNH	3, 8a
C3	39.3 (t)	α) 2.70 (d, 14.3)	OH, 2, 8a	2, 4	
		β) 2.16 (dd, 14.3, 8.0)	OH, 2, 8a	2, 4, 8a	OH, 2, 8a
C3a	90.6 (s)	—			OH, 2, 3, 4, 8a
OH	—	5.89 (s)	3, 4, 7, 8a	8a	
C3b	132.1 (s)	—			OH, 2, 7
C4	121.2 (d)	7.52 (d, 1.8)	NH, OH, 6, 7	6	6
C5	134.2 (s)	—			4, 6, 7
C6	128.2 (d)	7.31 (dd, 8.3, 1.8)	7	4, 7, 8a	4
C7	112.3 (d)	6.74 (d, 8.3)	NH, OH, 4, 6	6	
C7a	146.4 (s)	—			4, 6, 8a
NH	—	5.78 (d, 5.8)	4, 7, 8a		
C8a	86.0 (d)	5.11 (m)	NH, OH, 3, Thrα	OH, 2, 3β; ThrNH, α	2, 3α

[a] Proton correlations within the same amino acid listed unless specified otherwise.

heteronuclear coupling with α C-H (δ 5.62).

Fragment V (5-Hydroxypiperazic acid): In the COSY spectrum, the N-H group (δ 5.37) showed strong couplings with the geminal pair (δ 2.79, 3.02), δ CH_2. These protons showed connectivity with an oxygenated methine, γ C-H (δ 3.77). This methine proton showed

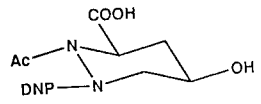
Table 2. Himastatin (**1**): ^{15}N NMR data.

δ ^{15}N	mult. (J, Hz)	^{1}H	^{15}N-^{1}H long range COSY	Amino acid
59	NH (75.7)	5.37	3.02, 3.77	5-HPA
72	NH (80.3)	5.78		photo-Trp
96	NH (92.5)	7.08	4.94, 4.40	Thr
109	NH (90.4)	7.25	4.84, 2.52	Val
115	NH (92.5)	7.38	4.18, 1.64, 0.83	Leu
142	N (10.1, *N-N*)		5.08, 3.02, 2.44	5-HPA
148	N (4.7, *N*-C-*N*)		5.16, 5.11, 2.70	photo-Trp

coupling with one O-H proton (δ 5.17) and a second methylene pair, β CH_2 (δ 1.92, 2.44). Finally, this gem pair coupled with a methine proton, α C-H (δ 5.08). The carbonyl carbon at δ 173.0 showed long range coupling with α C-H (δ 5.08) and β C-H (δ 1.92). Having thus far accounted for 3 of the 7 nitrogens (Val, Leu, Thr) of the empirical formula $C_{36}H_{52}N_7O_{10}$ (*e.g.* monomeric unit), we considered this spin system as part of an amino acid having more than one nitrogen. This led to our consideration of a piperazic acid system, a common component among known cyclodepsipeptides such as the monamycins[3]. Supporting evidence for this hypothesis was provided by ^{15}N NMR.

^{15}N-enriched himastatin was produced by fermentation using ^{15}N ammonium sulfate as the sole nitrogen source in the production medium. Work-up of the broth and subsequent purification provided a sample sufficient for ^{15}N NMR experiments in a practical time frame. Thus in the ^{15}N spectrum, 7 signals were observed of which 5 were NH's and 2 were tertiary N's (Table 2). ^{15}N-^{1}H correlation spectroscopy established the ^{15}N chemical shifts for each amino acid (Fig. 1). In addition, one of the tertiary Nitrogens (δ 142), showed N-N coupling (J=10.1 Hz) further supporting a hexahydropyridazine ring system.

The stereochemistry of the 5-hydroxypiperazic acid fragment was addressed. The 2,4-dinitrophenyl (DNP) derivative (**2**) was prepared by treatment of the crude acid hydrolysate of himastatin with 2,4-dinitrofluorobenzene. The ^{1}H NMR data for the purified derivative was essentially identical with published data for the DNP derivative of (3*S*,5*S*)-5-hydroxypiperazic acid[4], indicating the same relative configuration. The specific rotation of himastatin derived material, however, ($[\alpha]_D$ +120° (*c* 0.04, acetone) was opposite in sign (lit.[4], 3*S*, 5*S* isomer: $[\alpha]_D$ −240°). Treatment of this derivative with acetic anhydride afforded the γ-lactone (**3**), (IR ν_{max} 1794 (γ-lactone C=O), 1696 cm^{-1} (*N*-acetyl C=O)),



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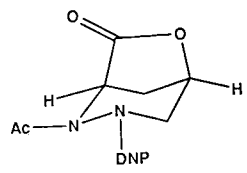


(3)

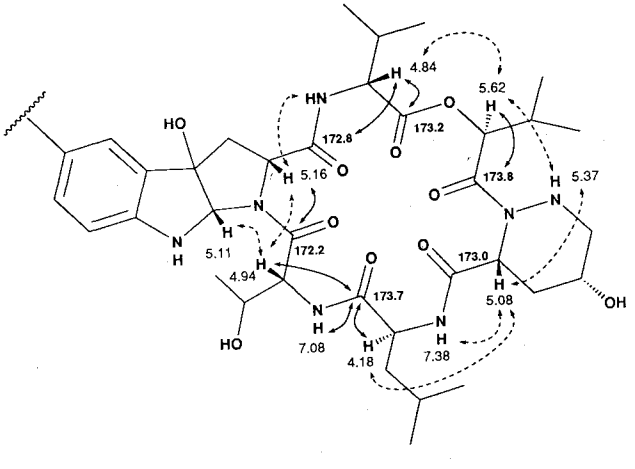
indicating a cis relationship between the C-5 hydroxyl and C-3 carboxylic acid groups. The ^{1}H NMR spectrum was again nearly identical with that reported for (3*S*,5*S*)-2-acetyl-1-(2,4-dinitrophenyl)-5-hydroxypiperazic acid lactone[4]. The specific rotation of our lactone ($[\alpha]_D$ −270° (*c* 0.05, dioxane)) was also opposite in sign to that of the 3*S*, 5*S* lactone (lit.[4] $[\alpha]_D$ +380°). The discrepancies in the $[\alpha]_D$ absolute values of **2** and **3** from those of the reference antipodes could possibly be due to impurities in the very limited quantities available. Based on the evidence presented, we have concluded that the 5-hydroxypiperazic unit in himastatin has the 3*R*, 5*R* configuration.

Fragment VI (3a-hydroxypyrroloindole derivative ("photo-Trp")): The hexahydropyrroloindole structure shown in Fig. 1 was proposed on the basis of COSY, ^{13}C-^{1}H long range heteronuclear correlation spectroscopy, and ^{15}N-^{1}H long range COSY data. Two 3-proton spin systems, including an aromatic ABX pattern (δ 7.52 (1H, d, J=1.8 Hz, H-4); 7.31 (1H, dd, J=8.3, 1.8 Hz, H-6); 6.74 (1H, d, J=8.3 Hz, H-7), and an aliphatic ABX system (δ 5.16 (1H, d, J=8.0 Hz, H-2; 2.70 (1H, d, J=14.3 Hz, H-3α; 2.16 (1H, dd, J=14.3, 8.0 Hz, H-3β) were evident from the COSY spectrum (Fig. 1). Other elements of fragment VI included a quaternary carbon (δ 90.6), an OH group (δ 5.89), a tertiary nitrogen (δ 148) and a carbonyl group (δ 172.8). Two additional sites of unsaturation (*e.g.* 2 rings) were necessary to satisfy the empirical formula. Additionally, the quaternary carbon (δ 90.6) bearing the hydroxyl group showed long range ^{13}C-^{1}H coupling with the aromatic proton (δ 7.52), two methines (δ 5.11, 5.16) and the geminal pair (δ 2.16, 2.70). The methine at (δ 5.11) showed long range coupling to the aromatic carbon at δ 146.4, the carbon (δ 60.6) bearing the δ 5.16 proton, and the carbon (δ 39.3) bearing the geminal pair (δ 2.16, 2.70). These same protons (δ 5.16, 2.16, 2.70) displayed long range coupling to the carbonyl group at δ 172.8. In the ^{15}N-^{1}H long range correlation spectrum, the tertiary nitrogen (δ 148) coupled with the two adjacent methine protons (δ 5.11, 5.16) and the δ 2.70 resonance of the gem pair. Fragment

VI is thus a 3a-hydroxyhexahydropyrrolo[2,3-*b*]indole 2-carboxylic acid, an oxidized cyclic tautomer of tryptophan not previously encountered among the depsipeptide antibiotics[5,6]. This ring system has been found in some fungal metabolites such as the sporidesmines and brevianamide E[6]. In addition, the recently reported antitumor cyclic heptapeptides, phakellistatin-3 and isophakellistatin-3 obtained from a marine sponge, also contain this moiety which was abbreviated by PETTIT *et al.* as photo-Trp[7]. The relative configuration of the photo-Trp unit in himastatin was deduced from a ROESY NMR experiment (Table 1). In this manner, correlations between between H-2 (δ 5.16) and H-8a (δ 5.11), and H-8a and C-3a-OH (δ 5.89) indicate that the three protons are on the same face of the hexahydropyrroloindole ring system.

The sequence of amino acids in himastatin was deduced from a combination of 2D NMR methods (Table 1). A crucial experiment optimized for the long range couplings between the carbonyl carbons and protons on the adjacent carbons provided valuable sequence information. The ^{1}H-^{1}H long range COSY experiment proved to be complementary. Thus, the carbonyl group (δ 172.8) of the 3a-hydroxypyrroloindole (*e.g.* photo-Trp) showed long range (3 bond) coupling with the α C-H (δ 4.84) of valine. In the ^{1}H-^{1}H long range COSY, coupling between the N-H (δ 7.25) of valine and the C2-H (δ 5.16) of the photo-Trp supported this connectivity. The α C-H (δ 4.84) of valine also showed long range coupling with the α C-H (δ 5.62) of α-hydroxyisovaleric acid. Long range ^{1}H-^{1}H coupling between this α C-H (δ 5.62) and the N-H (δ 5.37) of 5-hydroxypiperazic acid (5-HPA) indicated linkage between these two. The 5-hydroxypiperazic acid is in turn linked to leucine, as evidenced by long range couplings between the α C-H (δ 5.08) of 5-HPA to the N-H (δ 7.38) and α C-H (δ 4.18) groups of leucine. The carbonyl group of leucine (δ 173.7) displayed 3 bond heteronuclear coupling with the α C-H (δ 4.94) of threonine and 2 bond coupling with the NH (δ 7.08) group of threonine. Finally, the carbonyl group (δ 172.2) of threonine showed a 3 bond correlation with the C2-H (δ 5.16) proton of the photo-Trp unit. Long range ^{1}H-^{1}H coupling was seen between the α C-H of threonine (δ 4.94) and the two methines (δ 5.11, 5.16) of the photo-Trp as well. The cyclic peptide sequence of the monomeric unit of himastatin is thus as shown (Fig. 2).

Fig. 2. Himastatin: Sequence of amino acids by 2D NMR.
Solid lines: ^{13}C-^{1}H heteronuclear long range correlations.
Dotted lines: LR-COSY.



Derivatives, Selective Degradation Products

Additional derivatization and chemical degradation experiments were carried out in order to prove correct the proposed structure for himastatin (**1**). Methylation of himastatin was attempted in order to firm up several NMR chemical shift assignments. Permethylation of himastatin using methyl iodide was achieved using the method of JOHNSTONE and ROSE[8]. The major product was shown to consist of $C_{86}H_{132}N_{14}O_{20}$ by HRFAB-MS $(M+H)^+$ *m/z* 1681.9765, calcd 1681.9821), indicating incorporation of 14 methyl groups (*e.g.* 7 per monomer). Accordingly, seven new methyl carbon signals were observed in the ^{13}C NMR spectrum, of which 3 were OCH_3's and 4 were NCH_3's. The NH group of the 5-hydroxypiperazic acid unit was not methylated. To obtain possible amino acid sequence information from this reaction, the same permethylation procedure was repeated using ^{13}C-labeled methyl iodide. On a relatively small sample of ^{13}C-enriched permethylated derivative, a long range ^{1}H-^{13}C 2D correlation NMR experiment was now practical. This study firmed up the vicinal relationship between the N-H (δ 5.78) and 8a C-H (δ 5.11) in the photo-Trp unit of the parent himastatin. The methyl carbon chemical shifts and their respective correlations appear in Table 3.

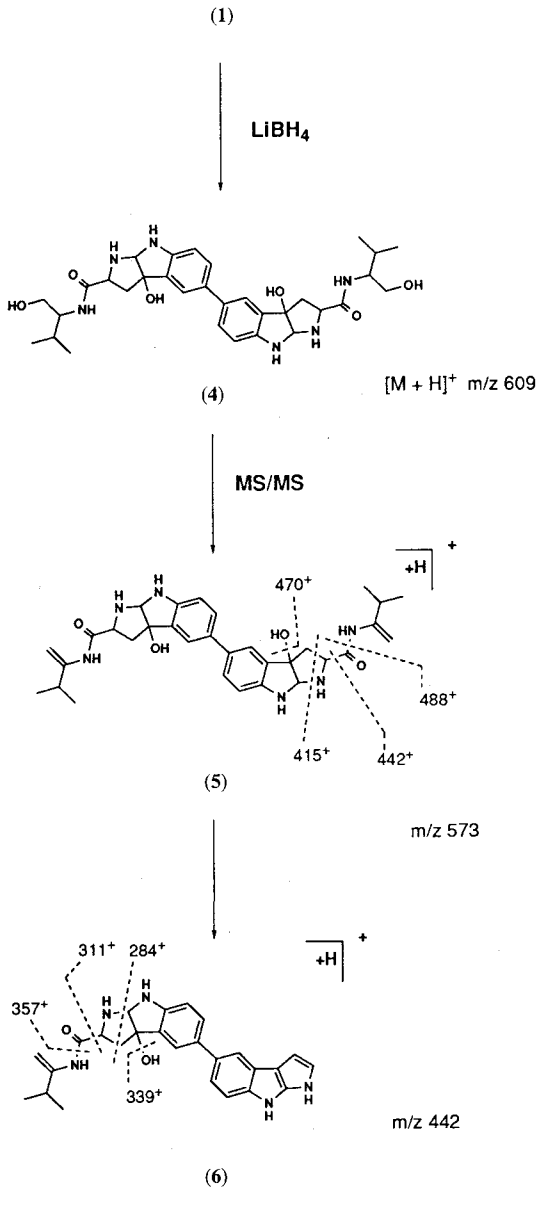
Table 3. Permethylated himastatin: NMR data.

δ ^{13}C	^{1}H	LR ^{1}H-^{13}C corr.	Assignment ^{13}C
28.9	2.98	4.18; α C-H Thr	N-CH_3
30.5	2.73	5.32; α C-H Leu	N-CH_3
30.8	3.08	5.00; α C-H Val	N-CH_3
37.6	3.10	5.83; 8a C-H photo-Trp	N-CH_3
51.5	2.92		photo-Trp O-CH_3
56.1	3.27		Thr O-CH_3*
56.2	3.19		5-HPA O-CH_3*

* Chemical shifts assignments may be interchangeable.

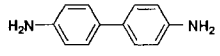
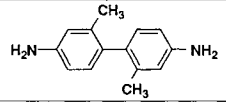
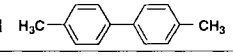
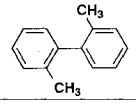
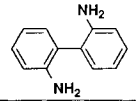
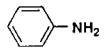
Himastatin (**1**) failed to undergo clean reduction with sodium borohydride, lithium aluminum hydride, and catalytic hydrogenation. Treatment with lithium borohydride yielded an aromatic fragment in low yield which we were able to characterize. The molecular formula of the $LiBH_4$ reduction product was determined to be $C_{32}H_{44}N_6O_6$ by HRFAB-MS ($(M)^+$ *m/z* 608.3292, calcd 608.3322). In the IR spectrum (KBr), amide carbonyl absorption at 1648 cm^{-1} was observed, but no ester carbonyl absorptions were present. The UV spectrum of this product (λ_{max} 292 nm (ε 21,500)) indicated essentially the same chromophore as in himastatin. The presence of only 16 resonances in the fully decoupled ^{13}C NMR spectrum again indicated a symmetrical dimer. Like the parent himastatin, the COSY spectrum revealed an aromatic ABX system, (CD_3OD: δ 7.46 (1H, d, J=1.7 Hz, H-4); 7.30 (1H, dd, J=8.2, 1.7 Hz, H-6); 6.64 (1H, d, J=8.2 Hz, H-7), a 3-proton aliphatic ABX pattern, (δ 3.68 (1H, m, H-2); 2.60 (1H, dd, J=12.0, 6.0 Hz, H-3β); 2.26 (1H, t, J=12.0 Hz, H-3α) and a spin system indicative of a reduced valine (*e.g.* valinol) moiety. The ^1H and ^{13}C NMR data are in agreement with structure (**4**) as shown (Fig. 3). Substructure analysis by MS/MS techniques revealed features consistent with the proposed structure, as shown in the fragmentation scheme (Fig. 3). Initial loss of two H_2O molecules from the valinol moieties results in the *m/z* 573 substructure (**5**). Formation of the *m/z* 488 substructure (loss of 85 amu) results from loss of the valinol moiety. Loss of H_2O from the pyrroloindole system results in the *m/z* 470 substructure. The *m/z* 442 and 415 substructures result from successive losses of CO and HCN, respectively. This fragmentation pattern was repeated as identical losses from the *m/z* 442 substructure (**6**) were observed *via* fragments at *m/z* 357, 339, 311, and 284. In summary, lithium borohydride reduction of himastatin resulted in cleavage of the ester linkage between the valine and α-hydroxyisovaleric acid subunits, and the amide linkage between the threonine and photo-Trp subunits, and provided additional confirmation for the linkage of the photo-Trp with valine.

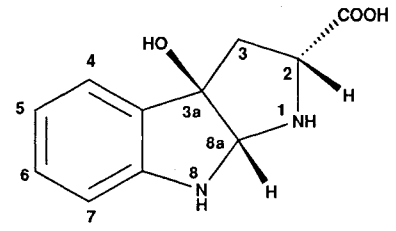
Fig. 3. Himastatin $LiBH_4$ reduction product (4): MS/MS data-possible fragmentation scheme.



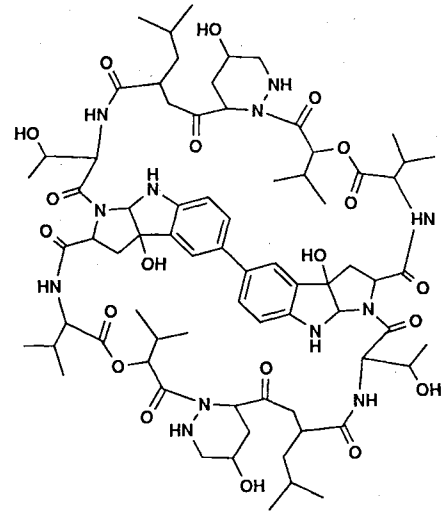
Since the aromatic lithium borohydride reduction product (**4**) is a symmetrical dimer, and the phenyl ring is trisubstituted, we reasoned that the two monomeric units of himastatin are joined together through a biphenyl linkage. The biphenyl hypothesis is supported by consideration of the UV data (Table 4)[9]. The relatively large extinction coefficients (ε) for the lithium borohydride product (**4**) and himastatin (**1**) suggest that both incorporate a coplanar biphenyl system. Biphenyls unsubstituted at the ortho position have a greater tendancy towards coplanarity and thus have relatively large UV extinction coefficients[10]. On the other hand, as illustrated in Table 4, biphenyls which have ortho substituents have markedly reduced extinction coefficients since their sterically favored conformations are not coplanar. The UV spectra of himastatin (**1**) and its $LiBH_4$ reduction product (**4**) also resemble that of benzidine (biphenyl, 4,4'-diamino). This supports the presence of a benzidine chromophore in himastatin. In addition, himastatin appears to be highly susceptable to oxidation. For example, when exposed to short wavelength UV light after development on a silica gel TLC plate, the normally colorless compound (*e.g.* spot) briefly turns deep yellow

Table 4. UV data: Himastatin and known biphenyls, benzene derivatives[9].

Compound	λ max	ε
Himastatin (1)	286 nm	27900
$LiBH_4$ Reduction Product (4)	292	21500
Benzidine 	287	25120
m-Tolidine 	290	3980
Biphenyl, 4,4'-dimethyl 	274	10000
Biphenyl, 2,2'-dimethyl 	267	680
Biphenyl, 2,2'-diamino 	295	5750
Aniline 	285	1700
Indoline 	291	2000



(7)



(8)

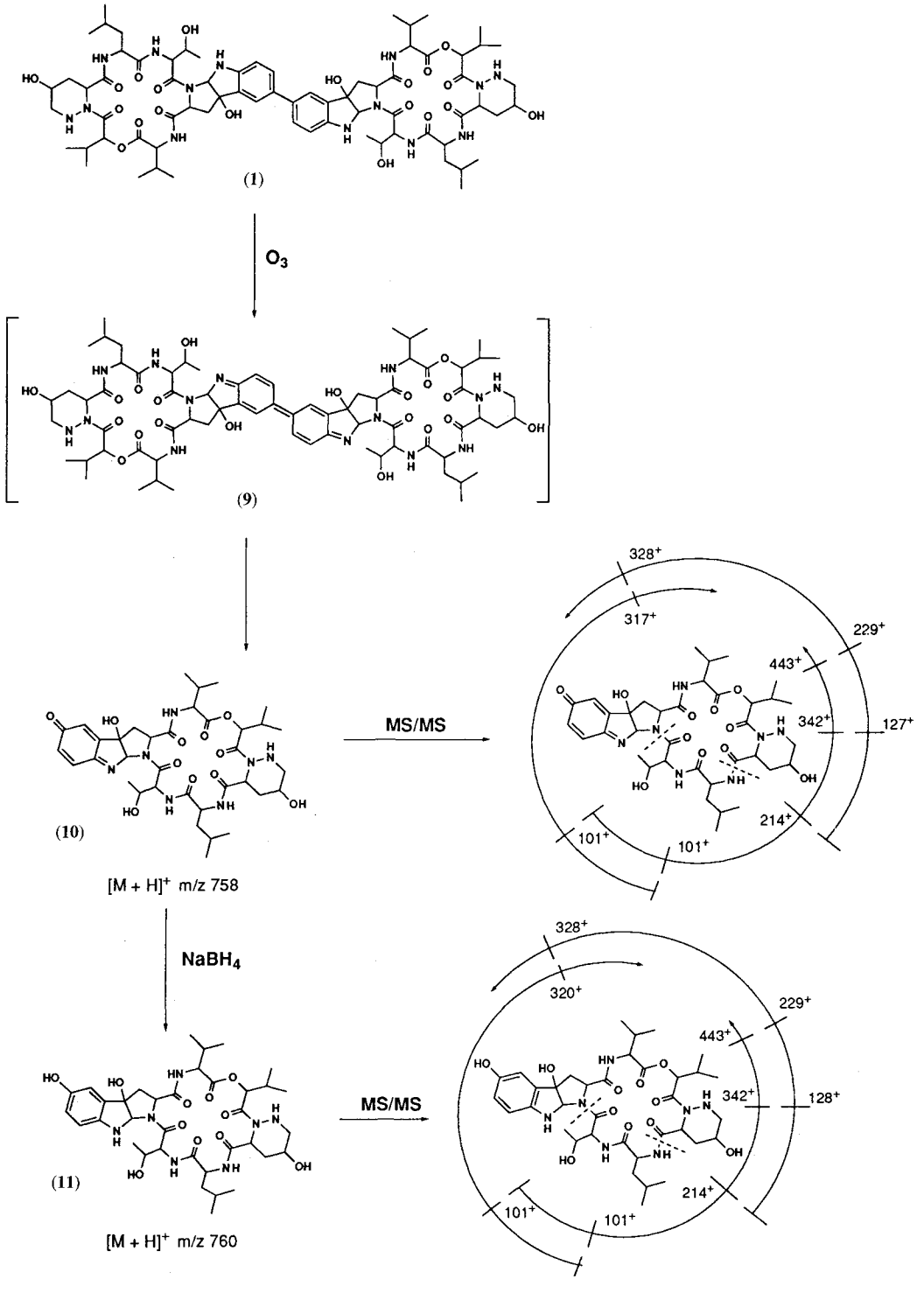
in color, and then fades back to colorless. When himastatin is exposed to activated charcoal, or treated with mild oxidizing agents such as manganese dioxide, a rapid transformation to a deep yellow compound results, with a large bathochromic shift as detected by HPLC-UV, λ_{max} 286, 298, 432 nm. This process also appears to be reversible. When exposed to methanol, THF, or DMSO the yellow solution over several minutes reverts back to colorless. In comparison, benzidine itself is readily oxidized to a reddish-yellow product having UV absorption maxima λ_{nm} (EtOH) 285, 413, 426, 446, 662 nm[11].

The absolute stereochemistry of the photo-Trp unit was deduced from circular dichroism (CD) measurements on the $LiBH_4$ reduction product (**4**). Based on ROESY NMR data for **1**, photo-Trp C-3a-OH, H-2 and H-8a are all syn to each other. In the CD spectrum of **4**, negative Cotton effects at 210 and 292 nm were observed (UV λ_{max} 214, 292 (ε 34,000, 21,500). The shape and sign of the CD curve is analogous to that reported for 1,2,3,3a,8,8a-hexahydro-3a(*R*)-hydroxy-8a(*R*)-pyrrolo-[2,3-*b*]indole-2(*R*)-carboxylic acid (**7**), an oxidized D-tryptophan derivative[5]. On this basis, we have proposed the stereochemical assignments for the photo-Trp unit in himastatin to be 2(*R*), 3a(*R*), and 8a(*R*).

With the structural fragments and their sequence in himastatin now established, it was necessary to consider the gross structure of this unusual dimeric cyclodepsipeptide. In our earlier communication[2], we proposed two possible structures for this symmetrical dimer: "dumbbell" structure (**1**) and "globular" structure (**8**), since at that time we were unable to distinguish between the two by spectroscopic or chemical means. The UV (ε) data and ease of oxidation of the benzidine chromophore in himastatin seemed to favor structure (**1**) over structure (**8**), since the two macrocyclic rings bridging the chromophore in **8** would introduce conformational constraints and thus could prevent coplanarity of the biphenyl system. The question was finally settled by degradation through ozonolysis (Fig. 4). Treatment of a chilled (−78°C) solution of himastatin with an ozone stream resulted in rapid loss of starting material. Following reductive workup, initial profiling of the crude product by LC/MS analysis revealed numerous components. The majority of these were in the 600~800 MW range, with a major component having MW 757, nearly half the mass of starting material. This result clearly favors structure (**1**) for the dimer, since under similar

conditions one would expect analogous products derived from structure (**8**) to be in the 1200~1600 MW range. The major, UV absorbing component (**10**) was isolated from the crude mixture, and its molecular formula of $C_{36}H_{51}N_7O_{11}$ was established by HRFAB-MS ($(M+H)^+$ *m/z* 758.3742, calcd 758.3725). Its NMR spectrum was similar to that of starting material. A quinoid moiety was evident from the large proton-proton coupling constants for the ortho protons: δ 7.31 (1H, d, $J=10.0$ Hz, H-7); 6.62 (1H, br d, $J=10.0$ Hz, H-6). The UV spectrum, λ_{max} 266 nm (ε 12,800), was also indicative of a *p*-quinone-imine chromophore[12~14]. Substructure analysis of this monomeric derivative (**10**) by MS/MS techniques revealed the following amino/hydroxy acid sequences (clockwise): α-HIVA–5-HPA–Leu–Thr (*m/z* 443, 342, 214, 101), Val–α-HIVA–5-HPA (*m/z* 328, 229,

Fig. 4. Himastatin ozonolysis: Summary of MS/MS data.



127), and Thr–photo-Trp-quinone-imine (*m/z* 317), and confirmed their proposed cyclic arrangement in himastatin (Fig. 4). Thus, under ozonolysis conditions, the benzidine chromophore in himastatin was presumably oxidized to a diphenoquinone-diimonium intermediate (**9**). Cleavage of the linking carbon-carbon double bond of this intermediate yielded the monomeric quinone-imine (**10**) as shown (Fig. 4), thus confirming the "dumbbell" structure (**1**) for himastatin.

The ozonolysis product (**10**) was reduced with sodium borohydride. The phenol (**11**) was formed (FAB-MS *m/z* 760 ($(M+H)^+$)), as evidenced by a significant shift in the UV spectrum (λ_{max} 310 nm (ε 3770)[14]). As with the ozonolysis product, analysis of **11** by MS/MS techniques revealed the following sequences (clockwise): α-HIVA–5-HPA–Leu–Thr (*m/z* 443, 342, 214, 101), Val–α-HIVA–5-HPA (*m/z* 328, 229, 128), 5-hydroxy-photo-Trp–Val (*m/z* 318), and Thr–5-hydroxy-photo-Trp (*m/z* 320), again confirming the cyclic sequence of amino acid fragments in himastatin (Fig. 4).

Discussion

The structure of himastatin (**1**) was solved using a combination of spectroscopic and chemical degradation techniques. Himastatin is a unique dimeric cyclohexadepsipeptide joined through a biphenyl linkage between two oxidized tryptophan units. The stereochemistry of the photo-Trp unit was determined by ROESY NMR and CD experiments. The chiralities of the amino acids valine, leucine, threonine, and the hydroxy acid α-hydroxyisovaleric acid were established by chiral GC methods. The stereochemistry of the 5-hydroxypiperazic acid unit was deduced from NMR and optical data for two 2,4-dinitrophenyl derivatives. The gross structure (**1**) was finally established through degradative ozonolysis of the dimer. Himastatin showed antimicrobial activity against gram-positive bacteria, and had *in vitro* cytotoxicity in tumor cell lines (HCT-116 IC_{50} 9.7 μg/ml; B16-F10 IC_{50} 9.7 μg/ml; Moser Human Colon Cells IC_{50} 15.6 μg/ml). It also had modest *in vivo* activity in murine tumor models (IP dosing/IP implant): P388 T/C 140 at 0.8 mg/kg/dose; B16 melanoma T/C 136 at 1.2 mg/kg/dose[1]. The compound, however, was inactive in IV models. Studies by MAMBER *et al.* suggest that micelle formation and the interaction between himastatin and cell membrane lipids may explain the lack of distal site activity against murine tumors *in vivo*[15]. The importance of the photo-Trp unit and/or benzidine chromophore in himastatin to the antitumor activity is unknown. Further studies with himastatin analogs will be necessary to address these issues.

Experimental

General Procedures

Solvents used for chromatography and chemical modifications were ACS grade and were not redistilled. TLC analyses were carried out using Uniplate Silica Gel GHLF precoated (scored 10 × 20 cm, 0.25 mm) plates. The thin layer chromatograms were detected with short wavelength UV light, ceric sulfate and/or iodoplatinate spray reagents. Preparative TLC separations were performed with E. Merck precoated Silica Gel (Kieselgel 60 F_{254} 20 × 20 cm, 0.5 mm) plates. Vacuum liquid chromatography (VLC) was performed with in-house vacuum, using E. Merck LiChroprep Silica gel 60, 25 ~ 40 μm particle size, or Baker C-18, 40 μm particle size. HPLC analyses were performed using a Rainin C-18 reverse phase column ("Short-One" 4.6 mm i.d. × 10 cm l. 3 μ particle size, 100 Å pore size; a Waters Associates, Model 590 solvent delivery system, and a Hewlett Packard HP-1040A diode array detector. The mobile phase used was a potassium phosphate buffer (0.01 M, pH 3.5)—acetonitrile gradient according to the method of D. J. HOOK *et al.*[16]. Preparative HPLC separations were accomplished using the Beckman System Gold workstation (model 126 pump module, model 166 detector module).

All ^1H, ^{13}C, and ^{15}N NMR spectra were recorded using either a Bruker WM-360 instrument operating at 360.13, 90.56, 36.49 mHz, respectively, or a Bruker AM-500 operating at 500.13, 125.76, 50.66 mHz, respectively, each using a 5 mm broadbanded probe. Chemical shifts are reported in ppm relative to solvent ($CDCl_3$: δ_H 7.24, δ_C 77.0; DMSO-d_6: δ_H 2.49, δ_C 39.6; MeOH-d_4 δ_H 3.34, δ_C 49.8). ^{15}N shifts are relative to external anhydrous liquid ammonia (δ_N 0.0). Spectra were recorded at ambient temperature. UV absorption spectra were determined using a Hewlett Packard 8452A diode array spectrophotometer. IR spectra were obtained on KBr discs using a Perkin Elmer 1800 fourier transform spectrometer. CD spectra were recorded with a Jasco J-720 spectropolarimeter. Specific rotations were recorded with a Perkin-Elmer 241 polarimeter. Low resolution mass spectrometric analyses were performed with a Finnigan MAT TSQ70 tandem quadrupole mass spectrometer in the positive ion FAB mode, using a m-nitrobenzyl alcohol matrix and argon as the primary particle source. DCI MS measurements were performed with a Finnigan 4500 quadrupole instrument. Electrospray MS analyses were conducted with a Finnigan TSQ7000 triple sector quadrupole instrument, using 0.1% sodium hydroxide in acetonitrile - water 1 : 1 as the mobile phase. Accurate mass measurements were obtained by peak matching either on a V.G. Instruments, 70SE double focusing mass spectrometer with either substance P or nitrobenzyl alcohol as a reference, or the Kratos MS50 with a cesium iodide saturated glycerol solution as the reference. MS/MS substructure analyses were performed with a Finnigan MAT TSQ70

instrument, using argon for collisionally activated dissociation (CAD) with an indicated collision gas pressure of 1.0 mTorr and collision energies of 40 ~ 60 eV. Full scan mass spectra were acquired with a 1 second scan rate while MS/MS spectra were acquired with a 1 ~ 4 second scan rate. LC/MS analyses were obtained using a Sciex API III mass spectrometer equipped with a Perkin Elmer Series 410 HPLC pump and a Perkin Elmer LC 90 UV spectrophotometer; Chromatographic conditions used: Zorbax C-18, 4.6 mm × 25 cm HPLC column, mobile phase acetonitrile - ammonium acetate buffer pH 5.0, 2 mM, gradient, 1 : 9 to 9 : 1 over 30 minutes, held for an additional 10 minutes, flowrate 1.0 ml/minute, UV detection at 254 nm.

Himastatin (**1**): Physical Data

Himastatin (**1**) was isolated from *Streptomyces hygroscopicus* fermentations as previously described[2]. $C_{72}H_{104}N_{14}O_{20}$; TLC Rf 0.60 ($CHCl_3$ - MeOH 9 : 1); $[\alpha]_D$ −34° (*c* 0.35, MeOH); MP >200°C (dec.); HRFAB-MS *m/z* 1484.75897 ($(M)^+$; calcd 1484.75507); IR ν_{max} (KBr) 3393, 3335, 2965, 2932, 2876, 1731, 1675, 1630 sh, 1532, 1484, 1469, 1454, 1422, 1392, 1330, 1308, 1250, 1187, 1154, 1101, 1019, 918, 898, 817 cm^{-1}; UV λ_{max} (MeOH) 286 nm (ε 27,900); CD λ nm ($\Delta\varepsilon$) (MeOH) 293 (−4.7), 262 (0), 236 (+23.3); ^1H and ^{13}C NMR data: see Table 1; ^{15}N NMR data: see Table 2.

Amino Acid Analysis

Himastatin (**1**) (30 mg) was hydrolyzed in 6 N HCl by refluxing for 18 hours. The acidic hydrolysate was neutralized with Amberlite IRA-400 (OH form) anion exchange resin. The hydrolysate was analyzed with a Beckman System Gold Amino Acid Analysis HPLC System, using a 3 × 250 mm Spherogel AA Na^+ cation exchange column, and ninhydrin detection. Retention times of amino acids in the hydrolysate were compared against those of reference amino acids. In this manner, the common amino acids present in the sample were confirmed as valine, leucine, and threonine. The determination of chirality was by GC using a Perkin-Elmer Sigma 2000 Capillary Chromatograph with an Alltech Chiralsil-Val III 25 m × 0.25 mm fused silica capillary column; injector at 250°C, detector at 300°C. Prior to GC analysis, the amino acids in the hydrolysate were converted to their respective pentafluoropropyl isopropyl esters using the Alltech PFP-IPA Amino Acid Derivatization Kit (Cat No. 18093), as described in the product bulletin. In this manner, the major components shown to be present were D-valine, D-threonine, and L-leucine.

Chirality Determination for α-Hydroxyisovaleric Acid

Himastatin (**1**) (100 mg) was hydrolyzed in 6 N HCl by heating in a sealed tube at 100°C for 20 hours. Silica Gel TLC using isopropanol - ammonium hydroxide 3 : 1 followed by detection with iodoplatinate spray reagent revealed a bluish-white spot which matched in terms of Rf with authentic α-hydroxyisovaleric acid. Preparative TLC of the crude hydrolysate using the same conditions provided an enriched sample of α-hydroxyisovaleric acid for chiral GC analysis. Prior to analysis, the sample was derivatized to the 2-trimethylsiloxycarboxamide and analysed using a Perkin-Elmer Sigma 2000 Capillary Chromatograph equipped with a Chiralsil-Val III 25 m × 0.25 mm fused silica capillary column; injector at 250°C, detector at 300°C[17]. Upon comparison with standards of DL- and D-α-hydroxyisovaleric acid using this procedure, the major enantiomer present in this sample was shown to be L-α-hydroxyisovaleric acid.

(3*R*,5*R*)-1-(2,4-dinitrophenyl)-5-Hydroxypiperazic Acid (**2**)

Himastatin (**1**) (200 mg) was hydrolysed in 6 N HCl (3 ml) by heating in a sealed tube at 100°C for 4 hours. The hydrolysate was evaporated to dryness, redissolved in 4 ml sat. sodium bicarbonate - ethanol 3 : 1, and mixed with 2 ml ethanol solution containing 195 μl of 2,4-dinitrofluorobenzene (~12 eqs., excess). The reaction mixture turned deep yellow immediately. Upon standing at room temperature for 3 hours, the reaction mixture was acidified with 6 N HCl, and extracted with diethyl ether. The yellow, ether extract was absorbed onto diatomaceous earth (dicalite, 2 g) and applied to a 2.5 × 7.5 cm column dry packed with 9 g dicalite. Elution using house vacuum was with hexane (100 ml) followed by toluene, and diethyl ether. DNP-5-HPA was detected in the diethyl ether fraction by ^1H NMR and by TLC ($CHCl_3$ - MeOH - AcOH 90 : 10 : 1). This fraction was further developed by C18 VLC (Baker, 40 μm, 2.5 × 10 cm column). A step gradient was begun with H_2O - CH_3CN 9 : 1, followed by 8 : 2, 7 : 3, 6 : 4, and 5 : 5 H_2O - CH_3CN (100 ml each). Final purification of the H_2O - CH_3CN 8 : 2 fraction (8 mg) was achieved by silica gel preparative TLC ($CHCl_3$ - MeOH - H_2O - AcOH 80 : 20 : 2 : 0.5, and then $CHCl_3$ - MeOH - H_2O 65 : 35 : 5), yielding 6 mg 2,4-dinitrophenyl-5-hydroxypiperazic acid (**2**): $[\alpha]_D$ +120° (*c* 0.04, acetone); ESI-MS *m/z* 311 $(M-H)^-$; UV λ_{max} (MeOH) 225, 372 nm (ε 9,450, 11,400); CD λ nm ($\Delta\varepsilon$) (MeOH) 442 (−0.1), 385 (−1.1), 328 (+0.7), 298 (0), 277 (+0.5), 256 (+0.7), 223 (−1.4); ^1H NMR (DMSO-d_6): δ 8.27 (1H, d, J=2.3 Hz, H-3′), 8.13 (1H, dd, J=9.5, 2.3 Hz, H-5′), 7.17 (1H, d, J=9.5 Hz, H-6′), 5.33 (1H, m, OH), 4.32 (1H, d, J=11.6 Hz, NH), 3.92 (1H, dd, J=12.1, 4.1 Hz, H-6eq), 3.58 (1H, br sept., H-5), 2.90 (1H, dt, J=11.6, 2.9 Hz, H-3), 2.73 (1H, t, J=11.1 Hz, H-6ax), 2.07 (1H, br d, J=12.2 Hz, H-4eq), 1.17 (1H, q, J=11.6 Hz, H-4ax).

(3*R*,5*R*)-2-Acetyl-1-(2,4-dinitrophenyl)-5-Hydroxypiperazic Acid Lactone (**3**)

A 3 mg sample of (3*R*,5*R*)-1-(2,4-dinitrophenyl)-5-hydroxypiperazic acid (**2**) was dissolved in 0.2 ml acetic anhydride and allowed to stand for 48 hours at room temperature. Following evaporation of solvent, purification by silica gel TLC (ethyl acetate - hexane 6 : 4) yielded

1.7 mg of the lactone (**3**): $[\alpha]_D$ −270° (*c* 0.05, dioxane); DCI-MS *m/z* 337 $(M+H)^+$; IR ν_{max} (thin film) 1794, 1696, 1604, 1522, 1340, 1270, 1192, 1122, 1062, 1000, 968 cm^{-1}; ^1H NMR (pyridine-d_5): δ 8.26 (DNP H-5), 7.67 (DNP H-6), 5.51 (1H, br s, H-3), 5.21 (1H, br s, H-5), 4.16 (1H, d, J=12.8 Hz, H-6), 3.59 (1H, br d, J=12.8 Hz, H-6), 2.50 (2H, m, H-4), 2.19 (3H, s, Me).

Fermentation and Isolation of ^{15}N Enriched Himastatin

The himastatin producing culture, ATCC 53653, was identified as a strain of *Streptomyces hygroscopicus*[1]. To prepare an inoculum for the shake flask culture, surface growth from a slant culture of ATCC 53653 was transferred to a 500 ml Erlenmeyer flask containing 100 ml of the vegetative medium consisting of 2% glycerol, 0.1% $MgSO_4$, 0.05% KH_2PO_4, 0.05% NaCl, 0.2% $CaCO_3$ and 0.2% $(^{15}NH_4)_2SO_4$. This vegetative culture was incubated at 28°C on a Gyrotary shaker (Model G53, New Brunswick Scientific) set at 250 rpm. After 5 days, 5 ml aliquots were transferred to ten 500 ml Erlenmeyer flasks containing 100 ml of the production medium with the same composition as the vegetative medium. The production culture was incubated at 28°C, and 250 rpm on the same shaker for 7 days. ^{15}N enriched himastatin was isolated from the above culture (1 liter) by extraction of the whole broth with an equal volume of ethyl acetate. The crude extract was chromatographed by silica gel VLC (2.5×10 cm column). Elution was begun with (100 ml each) ethyl acetate, followed by chloroform, 1%, 2%, and 3% methanol in chloroform. The chromatogram was followed by TLC ($CHCl_3$-MeOH 9:1). The 1~2% methanol fractions were further purified by silica gel preparative TLC using $CHCl_3$-MeOH 95:5 as the developing solvent to afford 29 mg ^{15}N-himastatin. The relevant ^{15}N-^1H NMR data appear in Table 2.

Permethylation of Himastatin

Himastatin (**1**) was permethylated according to method of JOHNSTONE and ROSE[8]. A 50 mg quantity of powdered KOH was added with stirring to a vial containing 1 ml dry DMSO. After 5 minutes, himastatin, 25 mg, was added directly to the slurry, resulting in a red-brown solution. Excess methyl iodide, 0.5 ml, was added and the contents allowed to stir at room temperature for 2 hours. The reaction mixture was diluted with 5 ml water and extracted with CH_2Cl_2. The organic layer was washed with water to remove trace DMSO. Purification of the crude organic extract by silica gel preparative TLC ($CHCl_3$-MeOH 95:5) afforded 4 mg permethylated himastatin: $C_{86}H_{132}N_{14}O_{20}$; TLC Rf 0.73 ($CHCl_3$-MeOH 9:1); HRFAB-MS, *m/z* 1681.9765 $((M+H)^+$; calcd 1681.9821); ^1H NMR ($CDCl_3$) photo-Trp: δ 7.43 (1H, d, J=8.0 Hz, H-6), 7.29 (1H, s, H-4), 6.54 (1H, d, J=8.4 Hz, H-7), 5.83 (1H, s, H-8a), 5.62 (1H, d, J=8.1 Hz, H-2), 2.92 (3H, s, OMe), 2.70 (1H, m, H-3), 2.41 (1H, m, H-3), Val: δ 5.00 (1H, d, J=11.3 Hz, H-α), 3.08 (3H, s, NMe), 2.30 (1H, m, H-β), 0.92 (3H, d, J=6.7 Hz, Me), 0.88 (3H, d, J=6.1 Hz, Me), α-HIVA: δ 6.12 (1H, d, J=10.3 Hz, H-α), 2.30 (1H, m, H-β), 0.97 (3H, d, J=6.3 Hz, Me), 0.88 (3H, d, J=6.1 Hz, Me), 5-HPA: δ 5.34 (1H, m, NH), 5.20 (1H, m, H-α), 3.20 (2H, m, H-γ, δ), 3.19 or 3.27 (3H, s, OMe), 2.67 (1H, m, H-δ), 2.30 (1H, m, H-β), 2.02 (1H, m, H-β), Leu: δ 5.32 (1H, m, H-α), 2.73 (3H, s, NMe), 1.58 (3H, m, H-β, γ), 0.80 (6H, J=6.9 Hz, 2×Me), Thr: δ 4.18 (1H, d, J=9.4 Hz, H-α), 3.73 (1H, m, H-β), 3.19 or 3.27 (3H, s, OMe), 2.98 (3H, s, NMe), 0.88 (3H, d, J=6.1 Hz, Me). Using the same procedure, the ^{13}C-permethyl derivative was prepared using ^{13}C-labeled methyl iodide. The key ^{13}C-^1H 2D NMR long range couplings appear in Table 3.

Lithium Borohydride Reduction Product (**4**)

Himastatin (**1**) (100 mg) was dissolved in 3 ml dry THF, and added slowly to a suspension of lithium borohydride (~10 mg, 6 eqs.) in 20 ml THF. The contents were heated to reflux, with stirring for 30 minutes. Mild foaming was observed. The mixture was cooled and excess reagent quenched with 2 ml acetone, adjusted to pH 7 with dropwise addition of 1 N HCl, and filtered. The filtrate was purified by silica gel preparative TLC using chloroform - methanol - water - ammonium hydroxide 120:45:8:0.5 as the developing solvent, affording 5 mg reduction product (**4**): $C_{32}H_{44}N_6O_6$; TLC Rf 0.48 ($CHCl_3$ - MeOH - H_2O - NH_4OH 120:45:8:0.5); $[\alpha]_D$ −167° (*c* 0.15, MeOH); HRFAB-MS, *m/z* 608.3292 $((M)^+$; calcd 608.3322); FAB MS/MS *m/z* 609, 573, 488, 470, 442, 415, 357, 339, 311, 284; IR ν_{max} (KBr) 3394, 2960, 2932, 2874, 1648, 1534, 1482, 1416, 1388, 1338, 1258, 1190, 1116, 1074, 816 cm^{-1}; UV λ_{max} (MeOH) 292 (ε 21,500); CD λ ($\Delta\varepsilon$) (MeOH) 292 (−8.2), 252 (−1.3), 230 sh (−2.8), 213 (−10.6); ^1H NMR (CD_3OD) photo-Trp: δ 7.46 (1H, d, J=1.7 Hz, H-4), 7.30 (1H, dd, J=8.2, 1.7 Hz, H-6), 6.64 (1H, d, J=8.2 Hz, H-7), 5.00 (1H, s, H-8a), 3.68 (1H, m, H-2), 2.60 (1H, dd, J=12.0, 6.0 Hz, H-3β), 2.26 (1H, t, J=12.0 Hz, H-3α), Valinol: δ 3.68 (1H, m, H-α), 3.55 (2H, m, H-β'), 1.87 (1H, sept, J=6.7 Hz, H-β), 0.94 (3H, d, J=6.7 Hz, H-γ), 0.89 (3H, d, J=6.7 Hz, H-γ); ^1H NMR (DMSO-d_6): photo-Trp: δ 7.33 (1H, s, H-4), 7.18 (1H, br d, J=8.2 Hz, H-6), 6.47 (1H, dd, J=8.2, 1.4 Hz, H-7), 5.97 (1H, s, exch., 8-NH), 5.57 (1H, s, exch., NH or OH), 5.56 (1H, s, exch., NH or OH), 4.82 (1H, s, H-8a), 3.42 (1H, dd, J=11.7, 5.6 Hz, H-1), 2.37 (1H, dd, J=11.7, 5.6 Hz, H-3β), 2.00 (1H, t, J=11.7 Hz, H-3α), Valinol: 7.49 (1H, d, J=9.5 Hz, exch., NH), 4.54 (1H, m, exch, OH), 3.54 (1H, m, H-1), 1.78 (1H, sept, J=6.7 Hz, H-2), 0.78 (3H, d, J=6.7 Hz, H-3), 0.73 (3H, d, J=6.7 Hz, H-4); ^{13}C NMR (CD_3OD) photo-Trp: δ 176.2 (C=O), 62.4 (C-2), 48.1 (C-3), 91.3 (C-3a), 133.9 (C-3b), 123.8 (C-4), 135.0 (C-5), 128.7 (C-6), 111.8 (C-7), 151.4 (C-7a), 87.3 (C-8a), Valinol: 58.6 (C-1), 30.8 (C-2), 20.8 (C-3), 19.5 (C-4), 63.9 (C-5).

Manganese Dioxide Oxidation of Himastatin

Himastatin (**1**), 2 mg, was dissolved in dichloromethane (1 ml) and to the solution was added excess manganese dioxide (20 mg). The solution turned deep yellow in color, was sonicated for 15 minutes, and filtered. TLC ($CHCl_3$-MeOH 9:1) revealed one component (yellow): λ_{max} (CH_2Cl_2) 292, 302, 438 nm (ε 13,380, 13,220, 76,880).

Ozonolysis of Himastatin

Himastatin (**1**), 500 mg, was dissolved in 20 ml ethyl acetate and chilled to −78°C (dry ice-acetone). Ozone, generated from oxygen with a Welsbach ozone generator (110 V), was bubbled into the solution at a flowrate of 0.3 LPM. The reaction was followed by TLC, ($CHCl_3$-MeOH 9:1). After 2.5 minutes, all starting material was consumed. Following a nitrogen sweep of residual ozone, methyl sulfide (1 ml) was added (*e.g.* reductive workup) to the mixture and allowed to stand at room temperature for 3 hours. The crude product was chromatographed by silica gel VLC (2.5 × 15 cm column). Elution was begun with hexane-ethyl acetate 1:1, followed by ethyl acetate, chloroform, 1%, 2% (5×), and 5% methanol in chloroform (100 ml each). The chromatogram was followed by TLC ($CHCl_3$-MeOH 9:1). The first 2% methanol in chloroform was highly enriched in a UV quenching zone; this material was recovered by silica gel preparative TLC using $CHCl_3$-MeOH 9:1 as the developing solvent to afford 7 mg ozonolysis product (**10**): $C_{36}H_{51}N_7O_{11}$; TLC Rf 0.36 ($CHCl_3$-MeOH 9:1); $[\alpha]_D$ −175° (*c* 0.2, MeOH); HRFAB-MS *m/z* 758.3742 ($(M+H)^+$; calcd 758.3725); FAB MS/MS *m/z* 759, 758, 741, 724, 640, 443, 399, 342, 328, 317, 300, 229, 214, 211, 201, 191, 127, 101, 100, 72; IR ν_{max} (thin film) 3388, 3320, 3254, 2964, 2933, 2876, 1728, 1670, 1643, 1528, 1414, 1299, 1262, 1193, 1148, 1100, 994, 923, 898 cm^{-1}; UV λ_{max} (MeOH) 206, 266 nm (ε 25,730, 12,880); CD λ ($\Delta\varepsilon$) (MeOH) 364 (−2.9), 302 (0), 272 (+2.5), 255 (+0.9), 233 (+7.8), 210 (−6.8); ^1H NMR (DMSO-d_6) photo-Trp: δ 7.31 (1H, d, J=10.0 Hz, H-7), 6.62 (1H, br d, J=10.0 Hz, H-6), 6.45 (1H, br s, H-4), 6.03 (1H, s, OH), 5.67 (1H, s, H-8a), 5.16 (1H, d, J=8.1 Hz, H-2), 2.55 (1H, d, J=14.7 Hz, H-3), 1.97 (1H, m, H-3); Val: δ 7.10 (1H, d, J=10.0 Hz, NH), 4.85 (1H, dd, J=10.0, 2.8 Hz, H-α), 2.60 (1H, m, H-β), 0.97 (3H, d, J=7.0 Hz, H-γ), 0.87 (3H, d, J=7.0 Hz, H-γ); α-HIVA: δ 5.63 (1H, d, J=8.7 Hz, H-α), 2.15 (1H, m, H-β), 1.10 (3H, d, J=6.8 Hz, H-γ), 0.97 (3H, d, J=6.8 Hz, H-γ); 5-HPA: δ 5.37 (1H, m, NH), 5.12 (1H, d, J=6.8 Hz, H-α), 3.79 (1H, br s H-γ), 3.04 (1H, d, J=13.8 Hz, H-δ), 2.82 (1H, t, J=12.7 Hz, H-δ), 2.48 (1H, d, J=14.6 Hz, H-β), 1.92 (1H, m, H-β); Leu: δ 7.36 (1H, d, J=4.5 Hz, NH), 4.15 (1H, m, H-α), 1.62 (2H, m, H-β, γ), 1.33 (1H, m, H-β), 0.91 (3H, d, J=5.7 Hz, H-δ), 0.84 (3H, d, J=5.7 Hz, H-δ); Thr: δ 7.19 (1H, d, J=10.3 Hz, NH), 5.32 (1H, d, J=10.3 Hz, H-α), 4.38 (1H, q, J=6.4 Hz, H-β), 3.79 (1H, s, OH), 1.16 (3H, d, J=6.4 Hz, H-γ); ^{13}C NMR (DMSO-d_6) δ 186.9, 173.9, 173.8, 173.0, 172.7, 172.4, 172.1, 164.6, 152.7, 136.7, 133.3, 122.9, 93.8, 86.3, 77.3, 66.8, 62.0. 58.7, 57.3, 54.4, 53.5, 52.6, 49.8, 40.7, 36.9, 29.8, 29.4, 28.4, 25.1, 22.9, 21.0, 19.2, 18.8, 18.1, 17.5, 16.3.

$NaBH_4$ Reduction of Ozonolysis Product (**10**)

The ozonolysis product (**10**) (2 mg) was dissolved in 1 ml MeOH and to the solution added a pinch (1 mg) of $NaBH_4$. After 5 minutes, TLC ($CHCl_3$-MeOH 9:1) revealed completion of reaction; HPLC-UV revealed essentially one product. The mixture was evaporated to dryness and partitioned between chloroform and water. The chloroform extract was purified by preparative C18 HPLC using an acetonitrile-water gradient to afford 0.2 mg reduction product (**11**): FAB MS/MS *m/z* 760 ($(M+H)^+$), 742, 724, 643, 612, 443, 399, 342, 328, 320, 300, 229, 214, 211, 201, 191, 128, 101, 100, 72; UV λ_{max} (MeOH) 208, 232, 310 nm (ε 32,190, 12,170, 3,770); CD λ ($\Delta\varepsilon$) 311 (−2.0), 260 (0), 234 (+14.1).

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