HIMASTATIN†, A NEW ANTITUMOR ANTIBIOTIC FROM *STREPTOMYCES HYGROSCOPICUS*

II. ISOLATION AND CHARACTERIZATION

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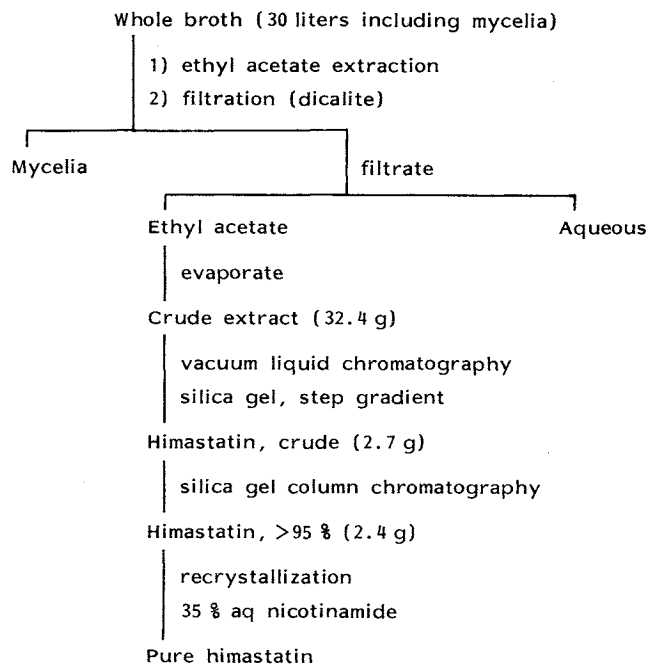
The novel antitumor antibiotic himastatin was isolated from cultured broth of *Streptomyces hygroscopicus* (ATCC 53653) and purified by vacuum liquid chromatography, column chromatography, and crystallization. Degradation and spectroscopic studies have shown that himastatin contains valine, leucine, threonine, α-hydroxyisovaleric acid, 5-hydroxypiperazic acid and a dimeric hexahydropyrroloindole system.

In the course of screening for new antitumor antibiotics from microorganisms, a new isolate of *Streptomyces hygroscopicus* (ATCC 53653) was selected for further evaluation[1]. This investigation led to the discovery of the novel antitumor antibiotic himastatin. This paper describes the purification and physico-chemical characterization of this compound. Details of the structure determination will be reported elsewhere.

Fig. 1. Procedure for isolation of himastatin.



† Himastatin was originally designated BMY-40800 in Eur. Pat. Appl. 329, 109, Aug. 23, 1989.

Isolation and Purification

The isolation of himastatin is summarized in Fig. 1. Fermentation broth (30 liters), prepared as described in the preceding paper[1], was extracted for 1 hour with ethyl acetate (20 liters). After filtration and phase separation, the organic layer upon evaporation yielded 32.4 g of crude solids.

Crude himastatin was obtained from this extract by vacuum liquid chromatography using 50 g of silica gel H (E. Merck, 10~40 μm) dry packed in a 150-ml, sintered glass funnel (medium porosity). After equilibrating the adsorbant with a solvent mixture of hexane-ethyl acetate (1:1), the extract in 200 ml of the same mixture was applied and pulled into the bed. The bed was sucked dry and then eluted in a stepwise fashion with 200 ml of each eluant in the following series: Ethyl acetate, chloroform, 1% methanol in chloroform, and 2% methanol in chloroform. The chromatography was monitored by silica gel TLC using short-wavelength UV light and ceric sulfate spray reagent for visualization. Himastatin was observed as the major component in the 1% and 2% methanol in chloroform fractions. These were pooled and evaporated to give 2.7 g of crude himastatin.

Further purification was accomplished by silica

Table 1. Physico-chemical properties of himastatin.

Appearance	White microcrystalline solid
MP	>200°C (dec)
Molecular formula	$C_{72}H_{104}N_{14}O_{20}$
Elemental analysis	
Calcd for:	$C_{72}H_{104}N_{14}O_{20}$
	C 58.25, H 7.06, N 13.21
Found:	C 58.16, H 6.94, N 12.84
FAB-MS (m/z)	
Calcd:	1,484.75507
Found:	1,484.75897 $(M)^+$
	1,507.5 $(M+Na)^+$
UV λ_{max}^{MeOH} nm (ε)	286 (27,900)
TLC[a] (Rf)	0.35
HPLC[b](Rt)	15 minutes

[a] Silica gel plates; $CHCl_3$-MeOH (95:5), ceric sulfate spray at room temperature; spot turns orange.

[b] Column: Whatman Partisil 10 ODS-3 (4.6 mm i.d. × 25 cm); eluant: acetonitrile-tetrahydrofuran-water (4:5:1); flow rate: 2.0 ml/minute; UV detection at 293 nm.

Fig. 2. UV spectrum of himastatin (MeOH).

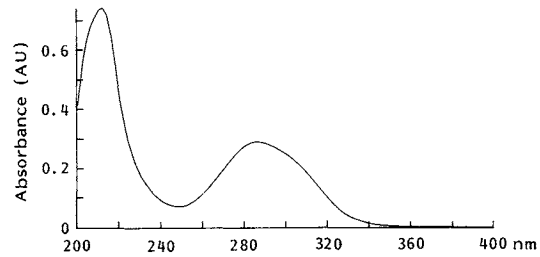
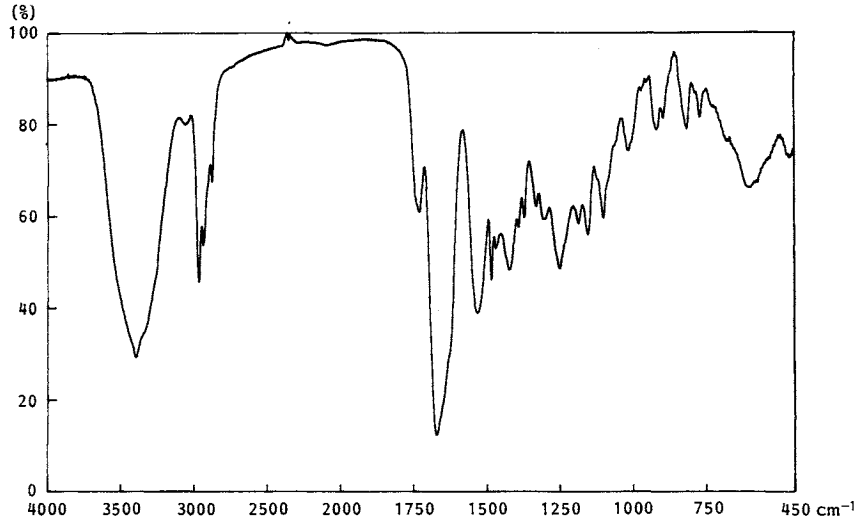


Fig. 3. IR (KBr) spectrum of himastatin.



gel column chromatography utilizing a stepwise gradient of chloroform to 2% methanol in chloroform. The chromatography was monitored and fractions were pooled and evaporated as before. Himastatin was obtained as a buff colored, amorphous solid (2.4 g, >95% by HPLC, ^{1}H NMR). The recovered solid was crystallized from 35% aqueous nicotinamide† to afford pure himastatin.

Fig. 4. ^{1}H NMR spectrum of himastatin (500.13 MHz, $CDCl_3$).

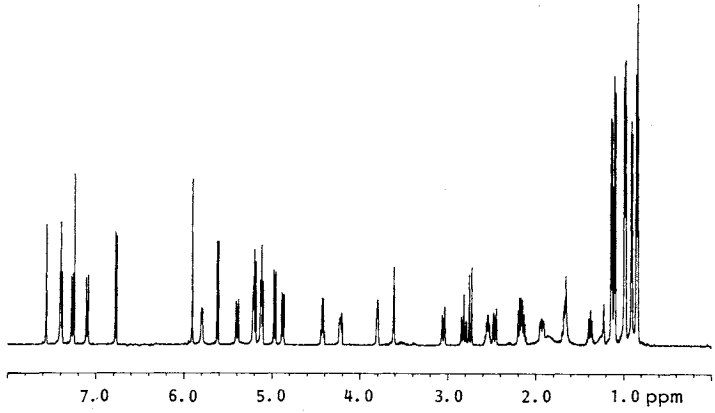


Table 2. ^{1}H and ^{13}C NMR data for himastatin (360.13/90.6 MHz, $CDCl_3$).

Assignment	^{1}H δ (ppm)	$^1J_{HH}$ (Hz)	^{13}C δ (ppm)
Leucine			
NH	7.38 d	(3.9)	—
C=O	—	—	173.67
αC–H	4.18 m	—	54.10
βC–H_2	1.35 dd	(8.8, 10.5)	40.77
	1.64 m	—	40.77
γC–H	1.64 m	—	25.09
δCH_3	0.83 d	(5.9)	20.81
	0.88 d	(6.1)	22.75
Valine			
NH	7.25 d	(10)	—
C=O	—	—	173.20
αC–H	4.84 dd	(3.2, 10)	57.02
βC–H	2.52 m	(3.3, 6.8)	29.66
γCH_3	0.83 d	(6.8)	16.27
	0.96 d	(6.8)	18.09
Threonine			
NH	7.08 d	(10.5)	—
C=O	—	—	172.17
αC–H	4.94 d	(10.5)	53.63
βC–H	4.40 q	(6.6)	66.47
γCH_3	1.11 d	(6.6)	17.17
OH	3.59 s	—	—
α-Hydroxy-isovaleric acid			
C=O	—	—	173.84
αC–H	5.62 d	(8.6)	77.08
βC–H	2.16 m	—	29.78
γCH_3	0.96 d	(6.8)	19.14
	1.08 d	(6.6)	18.64
5-Hydroxy-piperazic acid			
NH	5.37 d	(12.1)	—
C=O	—	—	173.02
αC–H	5.08 d	(7.1)	49.72
βC–H_2	1.92 ddd	(3.3, 7.1, 14.9)	28.44
	2.44 d	(14.9)	28.44
γC–H	3.77 br s	—	58.50
δC–H_2	2.79 t	(13.6)	52.44
	3.02 d	(12.6)	52.44
OH	5.17 d	(4.5)	—
Hexahydro-pyrroloindole			
2 C–H	5.16 d	(8)	60.62
2 C–*C*=O	—	—	172.79
3 C–H_2	2.16 m	—	39.30
	2.70 d	(14.3)	39.30
3a C	—	—	90.61
3a C–OH	5.89 s	—	—
3b C	—	—	132.08
4 C–H	7.52 d	(1.8)	121.18
5 C	—	—	134.21
6 C–H	7.31 dd	(1.8, 8.3)	128.21
7 C–H	6.74 d	(8.3)	112.30
7a C	—	—	146.41
8 NH	5.78 d	(5.8)	—
8a C	5.11 m	(5.4)	85.98

† A serendipitous observation. Aqueous nicotinamide is a routine solubilization vehicle used in compound preformulation studies. Developed by M. A. KAPLAN, Bristol-Myers Squibb Co., Syracuse, N.Y.

Physico-chemical Properties

Himastatin was isolated as a white microcrystalline compound. It was soluble in chloroform, dichloromethane, methanol, ethanol, butanol, ethyl acetate, pyridine, toluene, tetrahydrofuran and DMSO but insoluble in water, *n*-hexane and diethyl ether. The molecular formula of the compound was determined to be $C_{72}H_{104}N_{14}O_{20}$ by HRFAB-MS and elemental analysis. Additional physico-chemical and chromatographic properties are given in Table 1.

Acid hydrolysis and amino acid analysis revealed the presence of D-valine, L-leucine and D-threonine in equimolar amounts. In addition to these amino acids, the hydrolysate also yielded α-hydroxyisovaleric acid which was identified by spectroscopic analysis.

The UV spectrum (Fig. 2) of himastatin exhibited a characteristic absorption maximum at 286 nm,

Fig. 5. ^{1}H-^{1}H COSY spectrum of himastatin (500.13 MHz, $CDCl_3$).

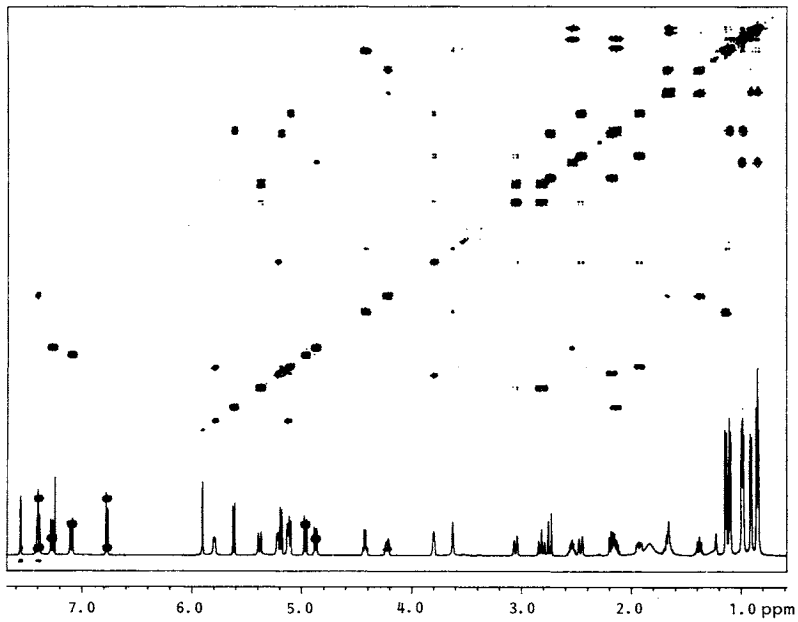
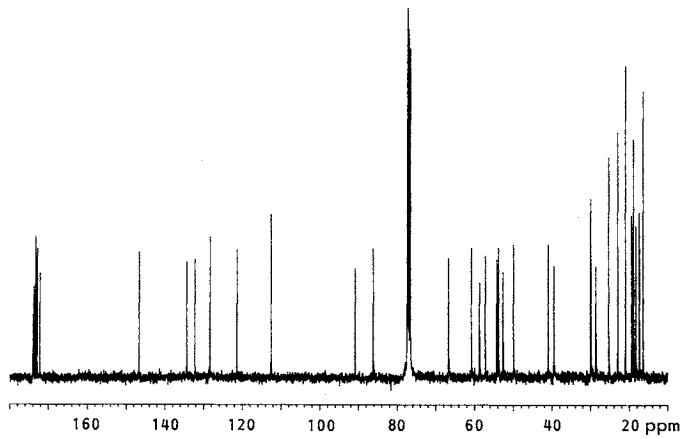
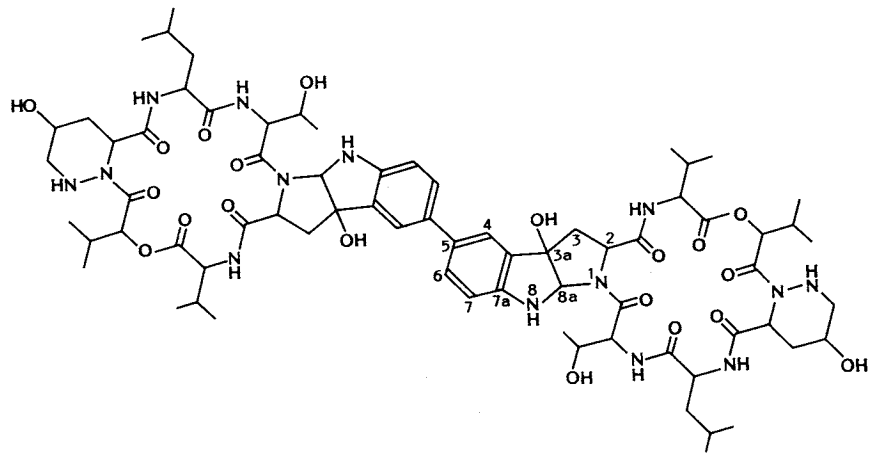


Fig. 6. ^{13}C NMR spectrum of himastatin (90.6 MHz, $CDCl_3$).



suggesting a benzenoid aromatic system. Its IR spectrum (Fig. 3) showed an ester carbonyl absorption at 1731 cm^{-1} and amide carbonyl absorptions at 1675 and 1630 cm^{-1}. The ^1H NMR spectrum of himastatin (Fig. 4) displayed 52 proton signals. Of these, eight signals were exchanged with D_2O. Between δ 0.83~1.11, there were seven signals for methyl groups, six belonging to isopropyl groups. The spectra also had nearly first-order spin patterns for methylene and methine protons evenly distributed between δ 1.3~6.0. A three proton ABX pattern in the δ 6.74~7.52 region was attributed to a 1,2,4-trisubstituted phenyl ring (Table 2). Readily assignable by 2D ^1H-^1H correlation spectroscopy (COSY, Fig. 5) were signals belonging to valine, leucine, threonine and α-hydroxyisovaleric acid subunits. Heteronuclear NMR experiments allowed for identification and assignment of the two remaining spin systems as those belonging to 5-hydroxypiperazic acid and a hexahydropyrroloindole system. Several additional homo- and

Fig. 7. Himastatin proposed structures.



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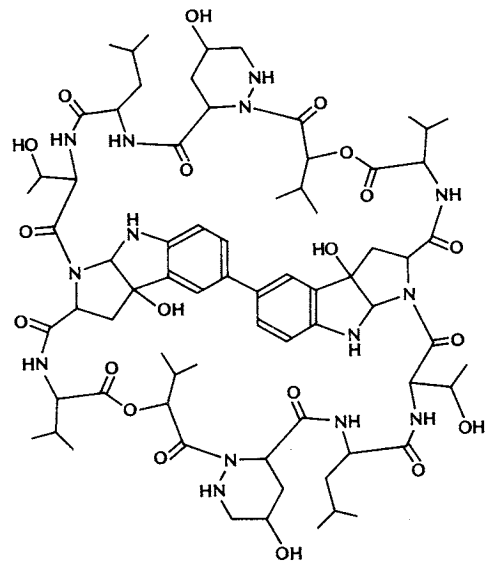

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heteronuclear NMR experiments were necessary to determine the sequence of the amino acid subunits and attachment to the aromatic core. The ^{13}C NMR spectrum of himastatin (Fig. 6) displayed 36 carbon resonances. Since the molecular formula indicated exactly twice this number of carbon atoms, the compound was inferred to be a symmetrical dimer. The ^{1}H NMR resonances are listed in Table 2 together with the corresponding ^{13}C chemical shifts. The proton-carbon correlations were determined by ^{1}H-^{13}C shift correlated 2D NMR analysis.

Discussion

Himastatin is a novel antitumor antibiotic produced by a strain of *S. hygroscopicus* sp. It is a crystalline compound exhibiting UV absorption at 286 nm. Chemical degradation and spectroscopic studies have established that himastatin is a new dimeric cyclohexadepsipeptide containing piperazic acid and a unique central aromatic core. This latter feature readily distinguished it from known piperazic acid containing cyclodepsipeptides such as azinothricin[2], A83586C[3], luzopeptin E_1, X and F (unpublished results), and monamycins[4]. Based in part upon the data presented here, two possible structures have been proposed for himastatin; "dumbbell" structure **1** and "globular" structure **2** (Fig. 7). Experiments to distinguish between these two possibilities are in progress. A detailed description of the structure determination will be presented in a separate paper.

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