NOVEL AND POTENT GASTRIN AND BRAIN CHOLECYSTOKININ ANTAGONISTS FROM *Streptomyces olivaceus*

TAXONOMY, FERMENTATION, ISOLATION, CHEMICAL CONVERSIONS, AND PHYSICO-CHEMICAL AND BIOCHEMICAL PROPERTIES

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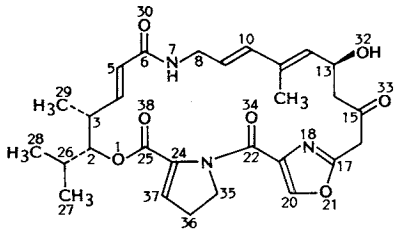
The discovery and physico-chemical characterization of three novel and minor virginiamycin M_1 analogs as potent gastrin antagonists from a culture of a strain of *Streptomyces olivaceus* are described. These analogs are L-156,586, L-156,587 and L-156,588. They are, respectively, 15-dihydro-13,14-anhydro-, 13,14-anhydro- and 13-desoxy-analogs of virginiamycin M_1. We also chemically converted virginiamycin M_1 (*via* L-156,587) to L-156,586 and its unnatural epimer, L-156,906. These analogs are competitive and selective antagonists of gastrin and brain cholecystokinin binding at nanomolar concentrations. These are the most potent gastrin/brain cholecystokinin antagonists from natural products. The same compounds showed poor Gram-positive antibiotic activity *versus* virginiamycin M_1. Structurally related Gram-positive antibiotics, griseoviridin and madumycin I, were inactive in gastrin and brain cholecystokinin binding at up to 100 μM.

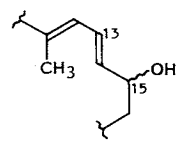
Gastrin and cholecystokinin (CCK) are peptide hormones found in gastrointestinal tissue and in the central nervous system. Antagonists to these regulators can be useful for treating diseases mediated by such hormones. Gastrin antagonists may be useful in the treatment and prevention of gastrin-related disorders of the gastrointestinal system such as ulcers, Zollinger-Ellison syndrome, gastrinoma and antral G-cell hyperplasia. Since gastrin and brain cholecystokinin share the same binding site, gastrin antagonists are also brain cholecystokinin antagonists. Brain cholecystokinin antagonists may be useful in potentiating and prolonging opiate-mediated analgesia as well as regulating appetite.

A screening for microbial metabolites as gastrin antagonists using guinea pig gastric mucosal glands led to the identification of virginiamycin M_1 (VM_1) (**1**) and three new analogs, L-156,586 (**2**), L-156,587 (**3**) and L-156,588 (**4**) (Fig. 1) from the aerobic fermentation of a strain of *Streptomyces olivaceus* (MA6287). These compounds represent a new class of gastrin/brain cholecystokinin antagonists. Preliminary biochemical evaluation suggested two of these minor analogs, **2** and **3**, were worthy of further investigation and larger quantities of these compounds were needed. To this end, a practical conversion of **1** to **2** *via* **3** was developed. In this report, we describe the taxonomy of the producing organism, the production, isolation and physico-chemical properties and chemical conversions of these compounds. In addition, we will describe some biochemical properties of these products and their comparison to other known members of VM_1 analogs, *viz* griseoviridin and madumycin I.

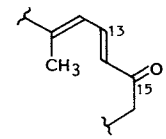
Fig. 1. Structures of VM_1 and analogs.



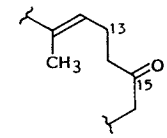
VM_1 (**1**)



L-156,586 (**2**),
L-156,906 (**5**), C-15 epimer



L-156,587 (**3**)



L-156,588 (**4**)

Taxonomy of Producer

The producing organism, strain MA6287, was isolated from a soil sample collected in Pimpri, India. It has been deposited in the American Type Culture Collection, Rockville, Maryland, U.S.A. and assigned ATCC No. 53527. Observations of growth, general cultural characteristics and carbon source utilization were made in accordance with the methods of SHIRLING and GOTTLIEB[1]. Chemical composition of the cells was determined using a modification of the methods of LECHEVALIER and LECHEVALIER[2]. Coloration of the culture was determined by comparison with color standards contained in the Inter-Society Color Council-National Bureau of Standards Centroid Color Charts[3].

Microscopic studies of MA6287 reveal that the aerial mycelium (0.57 μm) arises from a substrate mycelium and is branched and flexous. In mature cultures, the aerial mycelium commonly terminates in chains of spores ($10 \sim > 30$) arranged in short hooks, loops and extended spirals (*Spira*). No other special morphological features were observed.

The cultural characteristics of strain MA6287 on various media are given in Table 1. On most media, aerial mycelia were abundant and appeared within 7 days post inoculation at 27°C. Coloration of the aerial mass ranged from yellow-white to yellow-gray to dark gray. The reverse color ranged from pale yellow to light orange. On some media, colony edges were medium gray to dark gray. Starch is weakly hydrolyzed and hydrogen sulfide is produced. Soluble and melanoid pigments were not produced.

The utilization of carbon sources of MA6287 is presented in Table 2. L-Arabinose, cellobiose, D-fructose, inositol, α-D-lactose, β-D-lactose, D-maltose, D-mannitol, D-mannose, L-rhamnose, sucrose, and D-xylose were utilized for growth. No growth was observed on D-arabinose, D-raffinose, and L-xylose.

TLC of acid hydrolysates of MA6287 revealed that the cell wall contains L,L-diaminopimelic acid (Type I cell wall).

On the basis of these observations, MA6287 was identified as a *Streptomyces* sp. These results were compared with published descriptions of validly named species of this genus[4~7]. MA6287 was most similar to *S. olivaceus* (Waksman) Waksman and Henrici[4]. A direct comparsion of MA6287 with the designated type strain, *S. olivaceus* ATCC 3335, was carried out to confirm these results. The data from these experiments are summarized in Tables 1 and 2. Although some subtle differences were observed in the coloration of the aerial and vegetative growth of these strains, MA6287 and ATCC 3335 were found to be essentially identical in micromorphology and carbon source utilization patterns. On the basis of these results, MA6287 is considered to be a strain of *S. olivaceus*.

Table 1. Cultural characteristics of strain MA6287 and the type strain of *Streptomyces olivaceus* ATCC 3335.

Medium	Amount of growth		Aerial mycelium		Soluble pigments		Substrate mycelium	
	MA6287	ATCC 3335	MA6287	ATCC 3335	MA6287	ATCC 3335	MA6287	ATCC 3335
Yeast extract-malt extract	Very good	Very good	Light gray (264 l. gy) with medium gray edges (265 med. Gy). Spores borne on loose coils	White (263 white) with some light gray (264 l. Gy) areas. Spores borne on loose and extended spirals	None	None	Light orange yellow (70 l. OY)	Deep yellow brown (75 d. yBr) with deep olive brown (96 d. olBr) edges
Glucose-asparagine	Good	Good	Yellow white (92 y, white). Spores borne on short spirals/hooks	White (263 white). Spores borne on loose and extended spirals	None	None	Pale yellow (89 pY)	Pale yellow (89 pY)
Inorganic-salts starch	Good, starch weakly hydro-lyzed	Moderate, starch weakly hydro-lyzed	Yellow gray (93 y. gray) with areas of yellow white (92 y. white) and medium gray (265 med. Gy). Spores borne in extended, loose spirals	White (263 white) with light gray (264 l. Gy) edges. Spores borne on loose and extended spirals	None	None	Pale yellow (89 pY) with medium gray edges (265 med Gy)	Pale yellow (89 pY)
Oatmeal	Moderate	Moderate	Yellow gray (93 y. gray) with white (263 white) and dark gray (266 d. Gy) edges. Spores borne in hooks, shoot extended spirals and loops	Dark olive brown (96 d. ol. Br) with white (263 white) areas. Sporophores are loose and extend spirals	None	None	Pale yellow (89 pY) with dark gray edges (266 med. Gy)	Dark gray brown (62 d.d Gy. Br) with brown black (65 br. black) edges
Water	Fair	Poor	Small tufts of loose and extended spirals	Yellow white (92 y. white) where present. Spores borne on loose and extended spirals	None	None	Sparse growth	Yellow white (92 y. white)
CZAPEK	Good	Good	Yellow white (92 y. white) with white (263 white) areas	White (263 white). Long flexous aerial mycelium, spores not present	None	None	Pale yellow (89 pY)	Yellow white (92 y. white)
Peptone iron	Good, H_2S produced	Good, H_2S produced			None	None		

Observation after incubation at 27°C for 21 days.

Color names and numbers (in brackets) from Inter-Society Color Council-National Bureau of Standards Centroid Color Charts, U.S. Dept. of Commerce National Bureau of Standards supplement to NBS Circular 553, 1985.

Table 2. Carbon utilization patterns of MA6287 and the type strain of *Streptomyces olivaceus* ATCC 3335.

Carbon source	MA6287	ATCC 3335	Carbon source	MA6287	ATCC 3335
D-Arabinose	0	0	D-Mannitol	2	2
L-Arabinose	2	2	D-Mannose	2	2
Cellobiose	2	2	D-Raffinose	0	0
D-Fructose	2	2	L-Rhamnose	2	2
Inositol	2	2	Sucrose	2	1
α-D-Lactose	2	2	D-Xylose	3	2
β-D-Lactose	2	2	L-Xylose	0	0
D-Maltose	2	2	α-D-Glucose (control)	2	2

Observation after incubation at 27°C for 21 days.
3: Good utilization, 2: moderate utilization, 1: poor utilization, 0: no utilization.

Fermentation

A lyophilized tube of MA6287 was added under aseptic conditions to 54 ml of a seed medium in a 250-ml baffled Erlenmyer flask. The seed medium consisted of glucose 1 g, soluble starch 10 g, beef extract 3 g, Ardamine PH 5 g, NZ amine 5 g, $MgSO_4 \cdot 7H_2O$ 0.08 g, KH_2PO_4 0.18 g, Na_2HPO_4 0.19 g and $CaCO_3$ 0.5 g in 1 liter water. The pH of the medium was between 7.0 and 7.2. The flask was maintained at 28°C on a 220-rpm rotary shaker for 48 hours. Ten ml of the 48-hour culture was aseptically transferred to 500 ml in a 2-liter baffled flask and incubated at 28°C on a 220-rpm rotary shaker for 24 hours. A 500-ml inoculum from the second seed culture was added to 10 liters of production medium in a 14-liter fermenter. The production medium contained corn gluten meal 5 g, Primatone HS 2.5 g, yeast extract (Fidco) 1 g, malt extract 10 g, sucrose 5 g, $CaCO_3$ 5 g and P-2000 2 ml in 1 liter water. The pH of the production medium was between 7.2 and 7.4. The inoculated production medium was incubated for 64 hours at 28°C, aerated at a flow of 3 liters per minute and agitated at 400 rpm.

Isolation/Purification

Fractionation was guided by ^{125}I-gastrin binding activity on guinea pig gastric mucosal preparations. Initially, approximately 0.1 mg of VM_1 was isolated and identified (by EI-MS) from 75 ml of broth (without P-2000 as defoamer) with the observation that three additional more-potent components are present in smaller amounts. The following procedure is described in a practical scale. Forty liters of whole culture broth was extracted with 48 liters of methylethyl ketone. The organic layer containing gastrin binding activity was flash evaporated under reduced pressure at 40°C to give a residue. This residue was partitioned between hexane (1 liter) and MeOH (1 liter), with the hexane layer being discarded. The active methanolic layer was flash evaporated under reduced pressure at 40°C to yield a 50 g dry weight sample. Flash chromatography of this material in 5 runs on 500 g E. Merck Silica gel 60 (40 μm) using 50% acetone-hexane and then 75% acetone-hexane as the mobile phases yielded active fractions with a total dry weight of 3 g. Subsequent preparative reverse phase HPLC of this material in 3 runs on a DuPont Zorbax C-8 column (2.12 × 25 cm) using 40% acetonitrile-water as the isocratic mobile phase at room temperature and 15 ml/minute yielded, in order of elution, 92 mg of VM_1, 5.2 mg of L-156,586, 16.4 mg of L-156,587, and 25.9 mg of L-156,588. An analytical HPLC chromatogram of the reconstituted components is shown in Fig. 2.

Physico-chemical Properties

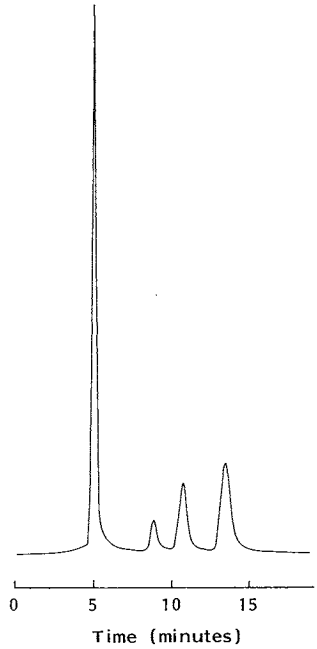
^{1}H and ^{13}C NMR spectra were recorded on a Varian SC-300 instrument. MS data were recorded on

a Finnigan-MAT MAT212 instrument at 90 eV in the EI mode. Exact mass measurements were recorded using perfluorokerosene as the internal standard and either the peak matching method or scanning HR and linked-scan metastable MS analyses utilizing both B^2/E and B/E methods. Trimethylsilyl derivatives were prepared with a 1 : 1 mixture of bistrimethylsilyltrifluoroacetamide and pyridine at 50°C for 1 hour.

VM_1 isolated from the present fermentation was identified by comparison with EI-MS (*m/z* 525 (M^+, $C_{28}H_{35}N_3O_7$), 507 $(M-H_2O)^+$), IR, ^1H and ^{13}C NMR data from the literature[8,9], and an authentic sample obtained by purifying a commercially available virginiamycin preparation. Both samples also showed identical chromatographic behavior in HPLC and TLC, UV spectra (λ_{max}^{MeOH} nm ($E_{1\,cm}^{1\%}$) 210.5 (535)), ^{125}I-gastrin and ^{125}I-cholecystokinin binding and antibiotic activities.

Fig. 2. HPLC chromatogram (in order of elution) of VM_1 (**1**), L-156,586 (**2**), L-156,587 (**3**) and L-156,588 (**4**).

Column: Zorbax ODS (150 × 4.6 mm, i.d.); mobile phase: acetonitrile-water (45 : 55); flow rate: 1.0 ml/minute; detector: UV 215 nm; temperature: 40°C.



Conversion of VM_1 to L-156,587

Methanesulfonyl chloride (0.2 ml) was added slowly to a solution of VM_1 (199.36 mg) in anhydrous pyridine (5 ml). The mesylation/dehydration reaction was allowed to proceed at room temperature for 20 minutes. Flash evaporation of the solvent, pyridine, under reduced pressure and at 40°C gave a residue. Partition of this residue in 1% NaCl-water (20 ml) and dichloromethane (2 × 25 ml) followed by drying the organic layers in anhydrous Na_2SO_4 and flash removal of solvent under reduced pressure yielded 245 mg of a crude product. Preparative reverse phase chromatography of the crude product on a DuPont Zorbax ODS column (2.12 × 25 cm) with 40% acetonitrile-water as the mobile phase at 15 ml/minute provided 88.15 mg of pure L-156,587.

Identification of L-156,587

The HREI-MS spectrum of L-156,587 revealed a molecular formula of $C_{28}H_{33}N_3O_6$ (Calcd: 507.2369, Found: 507.2369), *i.e.* 18 mass units less than **1**, with similarity to that of **1**, suggesting it to be an anhydro-virginiamycin M_1. A loss of one molecule of water from the sole hydroxyl on C-13 of **1** will result in a concomitant extension of the conjugated double bond and a red shift in the UV absorption. Its UV spectrum (λ_{max}^{MeOH} nm ($E_{1\,cm}^{1\%}$) 212 (545.5), 325 (417)) supported a conjugated trienone system. Its IR spectrum showed ν_{max} (CH_2Cl_2) cm^{-1} 3380, 1730, 1662, 1625. Its ^1H NMR spectrum (CD_2Cl_2, Fig. 4) revealed resonances at δ 5.89 (br d, J = 16 Hz) for 14-H, and δ 7.90 ppm (dd, J = 11.5 and 16 Hz) for 13-H, supporting a *trans* double bond between C-13 and C-14. A resonance at δ 5.88 (dd, J = 1 and 16.5 Hz) was observed for 5-H. Its broad band decoupled ^{13}C NMR spectrum (CD_2Cl_2) revealed resonances at 12.19, 18.78, 19.60, 21.37, 30.10, 30.22, 37.64, 40.65, 42.04, 52.33, 81.18, 125.38, 126.56, 126.79, 127.25, 129.46, 131.85,

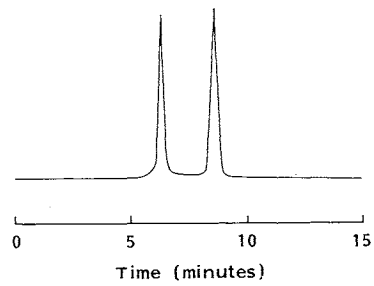
136.98, 142.91, 143.20, 143.32, 146.19, 160.28, 160.54, 167.12, and 192.78 ppm. The natural and semi-synthetic compounds showed identical chromatographic, spectroscopic and ^{125}I-gastrin and ^{125}I-cholecystokinin binding properties.

Conversion of L-156,587 to L-156,586 and Its "Unnatural" Epimer, L-156,906

Sodium borohydride (1.22 g) was added in portions over 5 minutes to a stirred solution of L-156,587 (15 g) in methanol (300 ml). The reaction was allowed to proceed at room temperature for 20 minutes and then quenched by addition of acetone (5 ml). After further stirring for 5 minutes, the reaction mixture was poured over saturated aqueous NaCl (750 ml) and extracted with dichloromethane (3 × 750 ml). The organic layers were washed once with saturated aqueous NaCl (500 ml), pooled, dried over anhydrous Na_2SO_4, and flash evaporated to dryness (dry weight 17.3 g). Purification on a ODS column (bed vol 2 liters, 40 μm) using 40% acetonitrile-water as the mobile phase at 250 ml/minute afforded in order of elution, 5.8 g of L-156,906, and 7.7 g of L-156,586. An analytical HPLC chromatogram of the products is shown in Fig. 3.

Fig. 3. HPLC chromatogram (in order of elution) of L-156,586 (**2**) and L-156,906 (**5**).

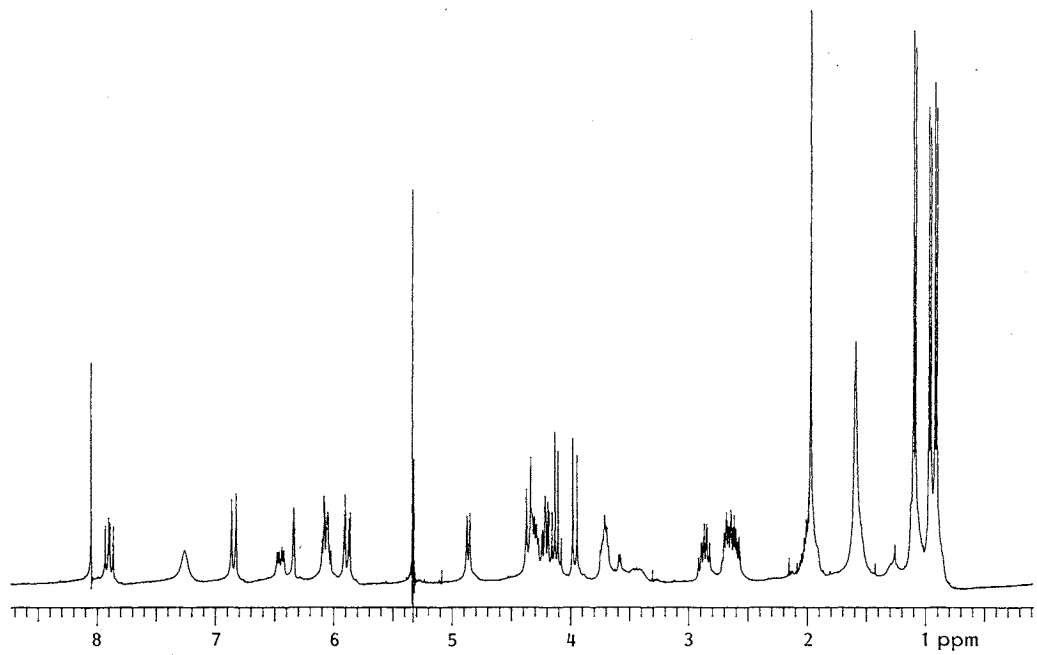
Column: Whatman Partisil 5 ODS-3 (100 × 4.6 mm, i.d.); mobile phase: acetonitrile-water (40:60); flow rate: 1.0 ml/minute; detection: UV 215 nm.



Formation of ^{3}H-L-156,586 and ^{3}H-L-156,906

By modification of the above procedure, using sodium borotritide (New England Nuclear) as the reducing agent, both [15-^{3}H]-L-156,586 and [15-^{3}H]-L-156,906 were obtained at 29.0 mCi/mg.

Fig. 4. ^{1}H NMR spectrum of L-156,587 (**3**) at 300 MHz in CD_2Cl_2.



Identification of L-156,586 and Its "Unnatural" Epimer, L-156,906

The HR mass spectrum of L-156,586 revealed a molecular formula of $C_{28}H_{35}N_3O_6$ (Calcd: 509.2526, Found: 509.2528). Since L-156,586 could be obtained from L-156,587 by sodium borohydride reduction, L-156,586 was a dihydro derivative of L-156,587. Its UV spectrum (λ_{max}^{MeOH} nm ($E_{1\,cm}^{1\%}$) 212 (557), 274.5 (764)) supported a conjugated triene system. Its IR spectrum showed ν_{max} (CH_2Cl_2) cm^{-1} 3595, 3375, 1730, 1670, 1620. Its ^1H NMR spectrum (CD_2Cl_2) is displayed in Fig. 5. Its broad band decoupled ^{13}C NMR spectrum (CD_2Cl_2) revealed resonances at 12.11, 18.82, 19.77, 20.34, 29.91, 30.13, 36.77, 38.87, 42.26, 51.08, 70.93, 82.46, 123.77, 123.85, 125.78, 126.46, 127.11, 127.72, 129.20, 132.85, 132.93, 135.20, 137.13, 142.29, 142.58, 161.37, 161.86, 167.13 ppm. The natural and semi-synthetic L-156,586 showed identical chromatographic, spectroscopic, and ^{125}I-gastrin and ^{125}I-cholecystokinin binding properties. Both L-156,586 and L-156,906 showed identical MS results and suggested they are epimers. The stereochemistry at the epimeric C-15 was not determined for either compound at this time. The ratio of L-156,586 and L-156,906, however, could be changed to 7:4 when isopropanol was used to predissolve the reducing agent. Also an examination of the 3D structure of VM_1[6] favored a β-attack of hydride. The ^1H NMR spectra (CD_2Cl_2) for L-156,906 is presented in Fig. 6. Its broad band decoupled ^{13}C NMR spectrum (CD_2Cl_2) revealed resonances at 12.03, 18.83, 19.67, 20.47, 30.07, 35.95, 39.10, 42.06, 51.04, 69.88, 81.75, 124.39, 124.47, 125.52, 126.98, 127.51, 127.57, 129.60, 129.62, 132.61, 135.19, 137.19, 143.22, 143.50, 161.23, 161.90, and 167.04 ppm. Its UV spectrum (λ_{max}^{MeOH} nm ($E_{1\,cm}^{1\%}$) 212 (522), 274.5 (701)) again supported a conjugated triene system. Its IR spectrum showed ν_{max} (CH_2Cl_2) cm^{-1} 3600, 3375, 1730, 1670, 1625.

Identification of L-156,588

The HR mass spectral data on the bistrimethylsilyl derivative of L-156,588 revealed a molecular

Fig. 5. ^1H NMR spectrum of L-156,586 (**2**) at 300 MHz in CD_2Cl_2.

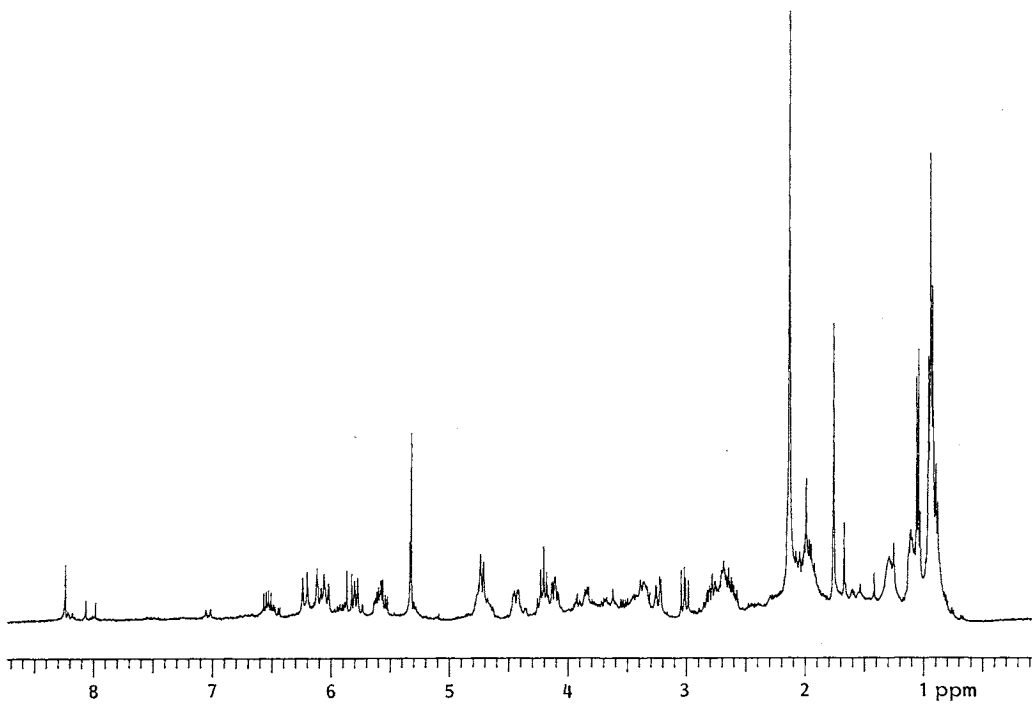


Fig. 6. ^1H NMR spectrum of L-156,906 (**5**) at 300 MHz in CD_2Cl_2.

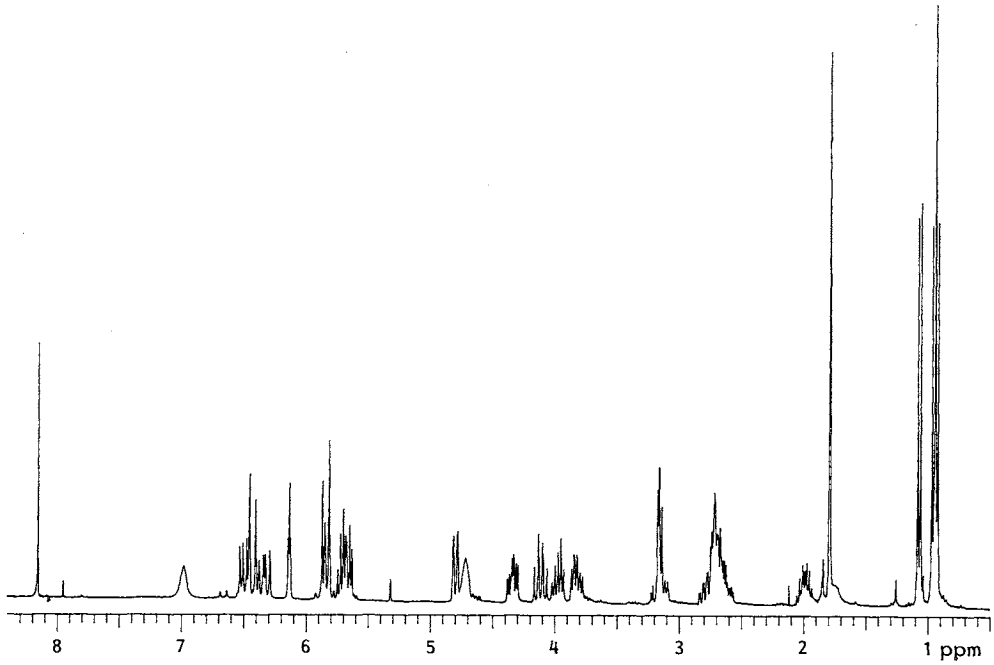
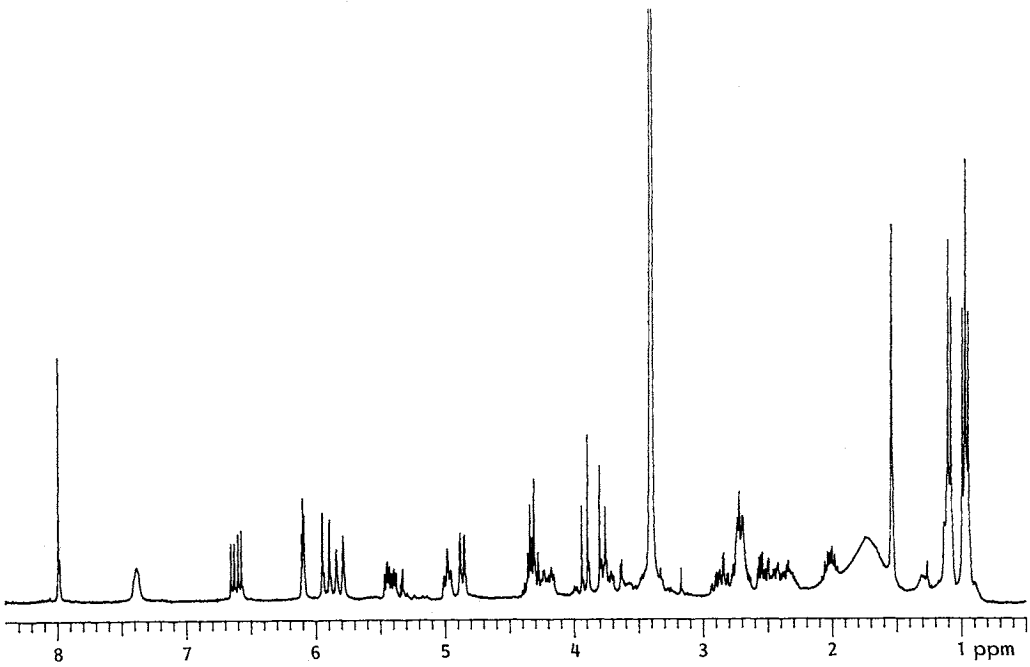


Fig. 7. ^1H NMR spectrum of L-156,588 (**4**) at 300 MHz in CD_2Cl_2.



formula of $C_{28}H_{35}N_3O_6+T_2$ (Calcd: 653.3316, Found: 653.3452 (T: trimethylsilyl)) and suggested it to be a desoxyvirginiamycin M_1. Its UV spectrum (λ_{max}^{MeOH} nm ($E_{1\,cm}^{1\%}$) 213 (542)) is identical to that of VM_1, suggesting a conservation of chromophores. A loss of the oxygen atom on C-13 will preserve the skeleton

and have little if any effect on any of the UV chromophores. Its ^1H NMR spectrum (CD_2Cl_2, Fig. 7) also supported the structure shown.

Biochemical Properties

Griseoviridin was generously provided by Parke Davis (Detroit, MI, U.S.A.).

Madumycin I was obtained by antibiotic activity directed reisolation from a fermentation of a known producer, *Nocardiopsis clava*, MA6340. The compound was characterized by comparing its spectroscopic data (UV, IR, ^1H NMR and MS) with those in the literature[10].

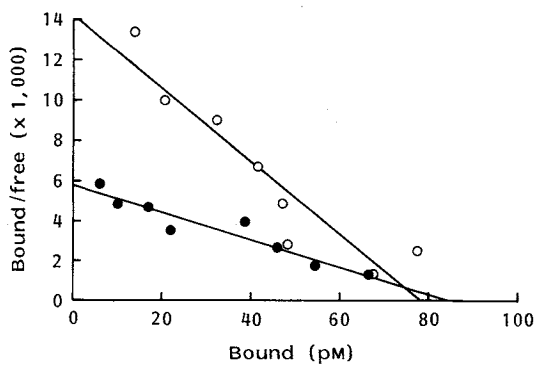
Gastrin Receptor Binding in Guinea Pig Gastric Glands

Guinea pig gastric mucosal glands were prepared by the procedure of PRAISSMAN *et al.*[11]. Stomachs from male Hartley guinea pigs (250~400 g body weight) were washed thoroughly and minced with fine scissors in HEPES buffer consisting of the following: NaCl 130 mM, $NaHCO_3$ 12 mM, NaH_2PO_4 3 mM, Na_2HPO_4 3 mM, K_2HPO_4 3 mM, $MgSO_4$ 2 mM, $CaCl_2$ 1 mM, glucose 5 mM and L-glutamine 4 mM, HEPES 25 mM at pH 7.4. The minced tissues were washed and incubated in 37°C shaker bath for 40 minutes, with HEPES buffer containing 0.1% collagenase and 0.1% bovine serum albumin, and bubbled with 95% O_2 and 5% CO_2. The tissues were passed twice through a 5 ml glass syringe to liberate the gastric glands, and then filtered through 200 mesh nylon. The filtered glands were centrifuged at $270 \times g$ for 5 minutes and washed twice by resuspension and centrifugation. The washed guinea pig gastric glands were resuspended in 25 ml of HEPES buffer containing 0.25 mg/ml of bacitracin. For binding studies, 14.2 μl of 50% MeOH-H_2O (for total binding) or gastrin (1 μM final concentration, for non-specific binding) or broth or test compound and ^{125}I-gastrin (New England Nuclear (NEN), 20,000 cpm, 20 μl) were added to 220 μl of gastric glands in triplicate tubes which were aerated with 95% O_2 and 5% CO_2 and capped. The reaction mixtures, after incubation at 25°C for 30 minutes in a shaking water bath, were filtered under reduced pressure on glass GF/B filters (Whatman) and immediately washed with 3 × 4 ml of HEPES buffer containing 0.1% BSA fraction V. The radioactivity on the filters was measured using a Beckman 5500 gamma-counter for ^{125}I-gastrin. For [^3H]pentagastrin binding, [^3H]pentagastrin was used in lieu of ^{125}I-gastrin in this procedure.

To determine the nature of the inhibition of gastrin receptor binding by VM_1 related compounds, Scatchard analyses of [^3H]pentagastrin binding in the presence and absence of these compounds were performed. All of them were competitive inhibitors. An example was depicted in Fig. 8: L-156,586 (22 nM) increased the dissociation constant (K_d) from 5.6 nM to 14.1 nM without significantly affecting maximal number of binding sites. The result was consistent with a competitive inhibition of gastrin receptors by L-156,586, with *Ki* value calculated to be 15 nM.

Fig. 8. Scatchard analysis of [^3H]pentagastrin binding in the presence and absence of L-156,586 (**2**) (22 nM).

● Presence, ○ absence.



Pancreatic Cholecystokinin Receptor Binding Assay

^{125}I-CCK-8 was purchased from New England Nuclear. Receptor binding was performed according

to INNIS and SNYDER[12], with the minor modification of adding phenylmethane sulfonyl flouride (PMSF) and *o*-phenanthroline. All assays were performed in triplicates. Pancreatic receptor membranes were prepared by homogenizing a gram of fresh rat pancreas tissue in 30 ml Tris-HCl (50 mM), pH 7.7 using a Brinkmann Polytron PT10. The homogenate was washed twice and collected by centrifugation at 48,000 × *g* at 4°C for 10 minutes. The membranes were resuspended in assay buffer consisting of dithiothreitol 5 mM, bacitracin 0.1 mM, $MgCl_2 \cdot H_2O$ 5 mM, heat-denatured bovine serum albumin 0.2%, PMSF 1.2 mM and *o*-phenanthroline 0.5 mM at a concentration of 100 ml per g of pancreas. To each incubation tube, in an ice-bath, containing 0.45 ml of membrane preparation, 25 μl of assay buffer (for total binding) and unlabeled CCK 26-33 (for non-specific binding) or broth or test compound for determining the inhibition of specific CCK binding, and 25 μl of ^{125}I-CCK were added. The reaction mixtures were mixed briefly and placed in a 37°C water bath with gentle shaking for 30 minutes. The reaction mixtures were then diluted with 4 ml of ice-cold Tris buffer, pH 7.7, containing 1 mg/ml bovine serum albumin and immediately filtered in Whatman GF/B filters. The filters were washed with 3 × 4 ml of the same buffer and the radioactivity associated with the filters was counted in a Beckman 5500 gamma-counter. Broths were prepared by mixing 1.4 volumes of methylethyl ketone to original broth. The organic layers were dried under a stream of nitrogen and then brought up in methanol-water (1 : 1) and ready for assay.

Brain Cholecystokinin Receptor Binding Assay

Brain CCK-8 binding was performed according to the description for the pancreatic method, with modifications by SAITO *et al.*[13]. Brains from male Hartley guinea pigs (300 ~ 500 g) were removed and placed in ice-cold 50 mM Tris-HCl plus 7.58 g/liter Trizma-7.4 (pH 7.4 at 25°C). The cerebral cortex was dissected and used as a receptor source. Each g of fresh guinea pig cerebral cortex was homogenized in 10 ml of Tris-Trizma buffer, pH 7.4, with a Brinkmann Polytron PT-10. The homogenates were centrifuged at 42,000 × *g* for 15 minutes, then the resulting pellets were resuspended in 200 volumes of binding assay buffer consisting of 10 mM HEPES, pH 7.7 at 25°C, $MgCl_2$, 1 mM EGTA, 0.4% BSA and 0.25 mg/ml bacitracin, pH 6.5. The remainder of the binding assay method was as described for the pancreatic method above, except that the reaction mixtures were incubated at 25°C for 2 hours before filtration and the filters were washed with HEPES buffer.

Antibiotic Activity

Antibiotic activity was determined by a method described by MATSEN and BARRY[14]. The MICs were determined by applying serial dilutions of the compounds to standard filter paper discs. The concentration of the test compounds ranged from 0.05 μg to 3.0 μg per disc in 20 μl of diluent. The test organism, *Micrococcus luteus*, strain M1101, ATCC No. 9341 was inoculated on the surface of an agar based nutrient medium in petri plates and the discs placed on the surface of the medium. The nutrient media consisted of nutrient broth supplemented with 0.2% yeast extract and 1.5% agar. The innoculated plates were incubated for 15 hours at 28°C and the zones of inhibition determined.

The effects of the present VM_1 analogs on ^{125}I-gastrin and ^{125}I-cholecystokinin binding and their inhibition on *M. luteus* growth are summarized in Table 3. L-156,586 is the most potent and selective natural gastrin/brain cholecystokinin antagonist reported to date, with IC_{50}'s at 12 and 7.8 nM, respectively. Structurally related Gram-positive antibiotics, griseoviridin and madumycin I, are inactive at up to 100 μM

Table 3. Effects of VM_1 analogs on ^{125}I-gastrin and ^{125}I-CCK binding, and *Micrococcus luteus* growth.

Compounds	Receptor binding, IC_{50} (nM)			MIC (μM)
	^{125}I-Gastrin	^{125}I-CCK-8		
	Gastric gland	Brain	Pancreas	*M. luteus*
VM_1	710	571.0	>100,000	1.5
L-156,586	12	7.8	>10,000	90.0
L-156,906	13	6.1	>10,000	NT
L-156,587	90	13.3	>10,000	250.0
L-156,588	360	270	NT	15.0
Griseoviridin	>100,000	>100,000	NT	NT
Madumycin I	>100,000	>100,000	NT	NT

NT: Not tested.

in ^{125}I-gastrin and ^{125}I-brain cholecystokinin binding. Hence a distinct structural activity relationship was observed.

Incubation of ^{3}H-L-156,906 with a Rat Liver Homogenate

^{3}H-L-156,906 (at 100 mM and 10 mCi/ml final concentration) was incubated with a rat liver homogenate (12.2 g wet weight of liver and 48.8 ml buffer consisting of 1.15% KCl and 20 mM Tris, pH 7.4) at 37°C for 0, 15 and 45 minutes in triplicates. The incubation volume was 2 ml. The reaction was worked up by extraction with methylethyl ketone. The organic layer was analyzed by HPLC (column: Whatman Partisil 5 ODS-3 0.46 × 10 cm; mobile phase: 35% acetonitrile - 0.1% TFA (aq) at 1 ml/minute) using Knauer UV (215 nm) and Berthold radioactivity detectors in series.

Table 4. Time course of incubating rat liver homogenate with ^{3}H-L-156,906 at 37°C.

Incubation time (minutes)	^{3}H-L-156,906 recovered (%)
0	100
15	52
45	32

Recovery of ^{3}H-L-156,906 was determined by HPLC analyses using both UV and radioactivity detection.

Results of incubating ^{3}H-L-156,906 in a rat liver homogenate are summarized in Table 4. At 0 minute incubation, 100%, 15 minutes incubation, 52%, and 45 minutes incubation, 32%, of L-156,906 remained intact, as quantitated by both radioactivity and UV detection in HPLC. In addition, ^{3}H-L-156,906 was the only radioactive lipophilic species detected in this experiment, and may penetrate the blood brain barrier as such in *in vivo* experiments.

Discussion

The virginiamycin class of antibiotics have been known for some time and excellent reviews have appeared[15,16]. So far most VM_1 analogs in the literature, including virginiamycin M_2[17] and semisynthetics[18~20] may be considered as adducts to the dehydroproline moiety of VM_1. Others include VM_1-monoacetate[21], VM_1-carboxymethyl oxime[22] and dihydro-VM_1[23]. The new compounds described in this paper represent the first members of a new series of VM_1 analogs. In view of recent advances in cholecystokinin antagonism and its potential in therapy, further investigation in these potent VM_1 analogs is in progress. Since our initial discovery[24,25], GOTTSCHALL *et al.*[26] incubated virginiamycin with cattle rumen fluid and found trace conversion products which may be identical to the present metabolites. In addition, we have examined a) regrowths of some known virginiamycin/related antibiotic producers

(*Streptomyces graminofaciens* MA317, *S. virginiae* MA6341, *S. mitakaensis* MA6342, and *Nocardiopsis flava* MA6340) in the present fermentation conditions and b) Stafac† by analytical HPLC not to contain these new components. Also a preparative fractionation of a commercial preparation of virginiamycin as guided by ^{125}I-gastrin binding activity gave VM_1 as the only active ingredient. Hence, strain MA6287 may be a unique producer of L-156,586, L-156,587 and L-156,588.

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† Stafac is a commercial preparation of virginiamycin antibiotic complex by SmithKline Beecham as an animal feed additive (ref The Merck Index. Eleventh Edition. No. 9910, p. 1574, 1989).

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