Notes

L-156,602, A C5a ANTAGONIST WITH A NOVEL CYCLIC HEXADEPSIPEPTIDE STRUCTURE FROM *Streptomyces* sp. MA6348

FERMENTATION, ISOLATION AND STRUCTURE DETERMINATION

OTTO D. HENSENS, ROBERT P. BORRIS, LAWRENCE R. KOUPAL, CHARLES G. CALDWELL, SARA A. CURRIE, AKBER A. HAIDRI, CARL F. HOMNICK†, SUSAN S. HONEYCUTT, SUSAN M. LINDENMAYER, CHERYL D. SCHWARTZ, BARBARA A. WEISSBERGER, H. B. WOODRUFF††, DEBORAH L. ZINK, LAURETTA ZITANO, JILL M. FIELDHOUSE, THOMAS ROLLINS, MARTIN S. SPRINGER and JAMES P. SPRINGER

Merck Sharp & Dohme Research Laboratories, P. O. Box 2000, Rahway, N.J. 07065, and West Point†, P.A. 19486, U.S.A.
††Soil Microbiology Associates, Inc., Watchung, N.J. 07060, U.S.A.

(Received for publication July 3, 1990)

The recently discovered class of cyclic hexadepsipeptide antibiotics is exemplified by azinothricin[1] and A83586C[2]. In our search for novel substances with C5a antagonistic properties, a member of this class, L-156,602, was isolated from a newly isolated strain of streptomycete and found to be a competitive inhibitor of the binding of the anaphylatoxin C5a to its receptor on human PMNs[3]. Because of its broad range of biological properties, C5a has been implicated as a causative or aggravating agent in a variety of inflammatory and allergic diseases[4,5] and an inhibitor of such inflammatory actions would therefore be therapeutically beneficial in the treatment of such diseases. The producing organism, MA-6348, was isolated from a plant rhizosphere soil sample obtained from a Japanese garden. Comparison of spectral data suggested the compound to be identical to PD 124,966, but no structure has been published[6,7]. We report here primarily on the structure determination of L-156,602 including absolute stereochemistry, based on spectroscopic and X-ray diffraction analyses.

The culture was characterized by the International Streptomyces Project procedures described by SHIRLING and GOTTLIEB[8]. Pigment production, carbohydrate utilization and other diagnostic tests indicated that this culture was a unique strain of *Streptomyces*. A complete summary of culture characteristics is available upon request from the authors.

The seed culture was maintained as a frozen preparation using the following procedure. A transfer of hyphae containing spores was placed into a 250-ml baffled Erlenmeyer flask containing 54 ml of seed medium. This medium included glucose 1.0 g, soluble starch 10.0 g, Difco beef extract 3.0 g, Yeast autolysate (Ardamine) 5.0 g, NZ-Amine type E 5.0 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, Na_2HPO_4 0.37 g, $CaCO_3$ 0.5 g in 1 liter of distilled water. The pH was adjusted to 7.0~7.2 before autoclaving. The flask was incubated at 28°C on a rotary shaker set at 220 rpm for 1~2 days until good growth was observed. Aliquots of 2 ml each of the broth culture were each mixed with 1 ml of 20% glycerol and placed into sterile 4 ml screw cap vials. After mixing, the vials were frozen at −80°C until use.

Production of the antibiotic by fermentation was carried out in a 14-liter Microferm glass fermenter (New Brunswick Scientific Inc., N.J.) using a production medium (NPA5) which contained corn gluten 5.0 g, Primatone HS peptone (Sheffield Products, N.Y.) 2.5 g, Difco yeast extract 1.0 g, Difco malt extract 10.0 g, sucrose 5.0 g, and $CaCO_3$ 5.0 g per liter of distilled water. The pH of the medium was adjusted to 7.0~7.2 prior to sterilization at 121°C for 25 minutes. The contents of a frozen vial culture of MA6348 was used as the inoculum for a 54 ml volume of seed medium in a 250-ml baffled Erlenmeyer flask. The seed flask was incubated on a rotary shaker set at 220 rpm for 48 hours at 28°C. In the second stage seed culture, 8 ml of inoculum were transferred into each 2-liter baffled Erlenmeyer flask containing 500 ml of seed medium. These seed flasks were incubated at 28°C for 24~28 hours on a rotary shaker set at 200 rpm. Seed culture (0.5 liter) was transferred into each fermenter and the fermentation was carried out at 28°C with an air flow of 3 liters per minute and an impeller speed of 400 rpm. The antibiotic production was monitored by a paper disk agar diffusion method using *Staphylococcus aureus* (MB2865) as the test organism. Generally, the highest titers of 5.8~10.8 mg/liter were encountered after 70~94

hours of incubation.

After filtration through Celite, the culture solids were exhaustively extracted with methyl ethyl ketone. The residue afforded upon evaporation of the methyl ethyl ketone extract was defatted by partitioning between 95% MeOH and hexane. Initial purification was achieved by chromatography on a reverse-phase resin (LichroPrep-RP18, EM Science) using an acetonitrile - aq TFA step gradient, followed by gel filtration using Sephadex LH-20 in MeOH. Final chromatographic purification was achieved by repetitive preparative HPLC on a Dynamax-ODS column (Rainin Instruments) eluted with acetonitrile - 0.5% aq TFA (11 : 9). The column was operated at ambient temperature monitoring UV absorbance at 220 nm. The heart-cuts of the broad active zone of each separation were pooled, concentrated, and rechromatographed under the same conditions to afford 39 mg of the homogeneous active product, $[\alpha]_D$ +16.9° (*c* 1.00, MeOH). Repeated recrystallization of L-156,602 from warm acetonitrile yielded the antibiotic as colorless needles, mp 190~193°C.

Combustion analysis of the crystalline product after drying under vacuum at 25°C: *Anal* Calcd for $C_{38}H_{64}N_8O_{13}\cdot H_2O$: C 53.13, H 7.74, N 13.04. Found: C 53.11, H 7.81, N 13.11.

Cultures used for evaluation of biological activity included bacterial pathogens of human and animal origin, permeability mutants and selected laboratory strains carrying chromosomal or R-plasmid mediated β-lactamases. The MICs were determined using a modified version of a standard agar dilution method[9]. Briefly, L-156,602 was dissolved in 100% MeOH and diluted with sterile distilled water immediately prior to its addition to Trypticase Soy agar. The agar plates were inoculated with 10^4 cfu of each culture and were incubated at 35°C for 18~29 hours. Of the 21 Gram-positive and Gram-negative bacteria tested, L-156,602 demonstrated antibiotic activity only against Gram-positive organisms. L-156,602 competitively inhibited the binding of C5a to its receptor on human PMNs with an IC_{50} of 2 μM[3].

All NMR spectra were recorded on a Varian XL-400 NMR spectrometer. ^1H-^1H COSY were recorded at ambient temperature using the standard pulse sequence[10]. One-bond and long-range ^1H-^{13}C chemical shift correlation spectra (HETCOR) were recorded in C_5D_5N (45 mg in 0.45 ml) at 65°C using the standard pulse sequence[11] and optimized for a ^{13}C-^1H coupling constant of 130 and 7/10 Hz respectively. Despite slow decomposition, these conditions yielded optimal sensitivity, ^{13}C linewidths and ^1H NMR dispersion.

Mass spectral data were obtained on a Finnigan Mat 212 mass spectrometer at 90 eV. The exact mass measurements were made on the same instrument at HR by the peak matching method using perfluorokerosene (PFK) as internal standard. Samples were hydrolyzed in 6N HCl in tightly capped vials at 110°C for 18 hours and then evaporated to dryness under a stream of nitrogen. The hydrolysate residue was derivatized with a 1 : 1 mixture of BSTFA (or BSTFA-d_9): pyridine at 50°C for 30 minutes. GC-MS analyses were carried out using a J & W DB-5 Durabond capillary column (15 m × 0.3, 25 μm film). Components were identified by interpretation of their mass spectra and by comparison to library spectra.

X-Ray diffraction data to $2q \leq 114°$ were recorded at room temperature using an Enraf-Nonius CAD-4 diffractometer equipped with CuKα radiation ($\lambda = 1.5415$Å). Crystals formed from CH_3CN were sealed in thin walled capillaries for data collection.

To determine the absolute configuration, a sample of L-156,602 (0.71 mg, 0.84 nmol) was first hydrolyzed in 6N aq HCl at 110°C for 20 hours. After evaporation of the hydrolysate the residue was dissolved in 1 ml of a mixture of H_2O-$(C_2H_5)_3N$-CH_3CH_2OH (1 : 1 : 6). An aliquot (200 μl) of the solution was evaporated and the residue was redissolved in 200 μl of the basic alcohol solution. (*R*)-α-Methylbenzylisothiocyanate (10 μl, 70 nmol) was added and allowed to react for 30 minutes at room temperature. The reaction mixture was evaporated and the residue was dissolved in H_2O-CH_3CN (1 : 1, 1 ml). After extraction with butyl chloride (4 ml), 10~15 μl of the aqueous layer was injected onto a Hewlett-Packard 1090 HPLC system with a 15-cm column (Phenomenex) containing 3 μm Hypersil C18 packing and detection at 260 nm. The mobile phases consisted of ammonium acetate buffer (0.02 M, adjusted to pH 5.0 with acetic acid) and CH_3CN. The solvent mixture was initially 8% CH_3CN for 10 minutes, then a gradient with CH_3CN increasing to 50% was applied over 60 minutes at a flow rate of 0.7 ml/minute and an oven temperature of 50°C.

^{13}C NMR data of L-156,602 (^1H coupled/decoupled, APT) in CD_3CN suggested a carbon count of 38 and the following carbon types: 8 × CH_3, 8 × CH_2, 3 × CH, 3 × CH_2X, 7 × CHX, 1 × C–O, 1 × O–C–O and 7 × COX. ^1H NMR spectra in CD_3CN-CD_3OD and CD_3CN-DMSO-d_6 mixtures indicated the presence of eight active protons,

Table 1. ^{1}H and ^{13}C NMR data for L-156,602.

	^{13}C (ppm)[a]		^{1}H (ppm)[b]	$^{1}J_{HH}$ (Hz)
	CD_3CN	C_5D_5N		
β-OHLeu:				
NH			8.21 d	9.0
Cα	47.2 d (~140)	47.9 d (br)	6.57 dd	4.5, 9.0
Cβ	79.5 d (150)	79.8 d	5.31 dd	4.5, 9.4
Cγ	30.7 d (125)	30.6 d	2.31 m	
Cδ	18.5 q (126)	18.8 q	1.07 d	6.6
Cδ′	20.0 q (~126)	19.9 q	0.94 d	6.8
Gly:				
NH			7.54 br s	
Cα	42.6 t (144)	42.5 t	3.97 dd	3.8, 18.5
			3.81 v br d	~18.5
Piz:				
NH			5.44 br d	12.8
Cα	49.4 d (145)	50.1 d	6.01 br d	~6
Cβ	25.8 t	25.7 t	2.43 br d, 1.96 m	13.7
Cγ	22.3 t (130)	22.04 t	1.63 obsc, 1.47 m	
Cδ	47.7 t (~138)	47.8 t	3.07 br d, 2.81 m	12.2
Piz′:				
NH			5.00 v br d	~12
Cα	50.1 d (145)	51.0 d	5.76 dd	~3, ~7
Cβ	24.0 t	24.0 t	2.11 m, 1.82 m	
Cγ	21.1 t (130)	20.8 t	~1.60 obsc, 1.25 m	
Cδ	47.2 t (~139)	46.8 t	2.88 dt, 2.52 m	13.5, ~4.5
N-OHAla:				
Cα	53.3 d (140)	53.9 d	5.92 q	7.0
Cβ	12.6 q (129)	12.4 q	1.64 d	7.0
N-OHAla′:				
Cα	54.4 d (140)	54.4 d	5.54 q	7.0
Cβ	13.2 q (129)	13.8 q	1.62 d	7.0
THPP:				
C-2	72.3 d (145)	71.9 d	4.01 dq	9.8, 6.2
2-CH_3	20.1 q (126)	20.0 q	1.42 d	6.2
C-3	40.5 d (~128)	40.4 d	1.21 m	
C-4	24.6 t	24.9 t	1.77 m, 1.68 m	
C-5	28.1 t (125)	28.3 t	2.07 m, 1.93 m	
C-6	99.9 s	100.0 s		
C-7	77.2 s	77.3 s		
7-CH_3	22.0 q (130)	22.0 q	1.86 s	
C-1′	39.4 t (~128)	39.4 t	1.10 m, 1.05 m	
C-2′	31.8 d (~125)	31.6 d	1.37 m	
2′-CH_3	18.9 q (~126)	19.1 q	0.74 d	6.6
C-3′	31.7 t (125)	31.2 t	1.23 m, 1.13 m	
C-4′	11.9 q (~128)	11.6 q	0.83 t	7.4
Carbonyl carbons:				
COX	170.6 s	170.2 s		
	170.6 s	170.39 s		
	170.7 s	170.42 s		
	172.5 s	171.4 s		
	173.0 s	172.9 s		
	175.3 s	174.0 s		
	177.2 s	177.7 s		

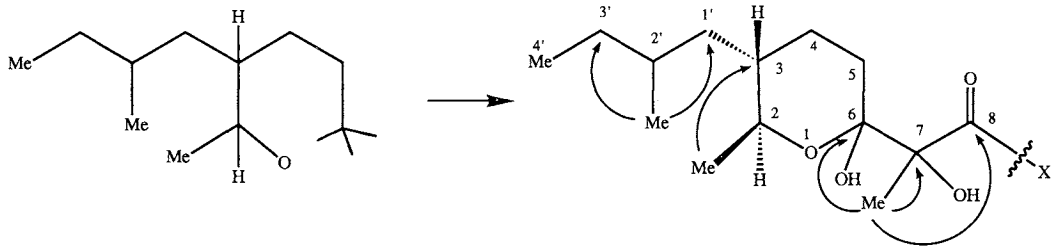
[a] ^{13}C Chemical shifts were recorded at 100 MHz in CD_3CN and C_5D_5N at ambient temperature at 65°C, respectively (referenced to solvent peaks at δ 1.3 and 123.5 downfield of TMS at 0 ppm, respectively). $^{1}J_{CH}$ are given in parentheses in Hz.

[b] ^{1}H Chemical shifts were recorded at 400 MHz at 65°C in C_5D_5N (referenced to solvent peak at δ 7.19 downfield of TMS).

Abbreviations: v, very; obsc, obscured.

Fig. 1. Partial structures of $C_{14}H_{25}O_4$ moiety.

Arrows indicate long-range ^1H-^{13}C correlations.



X Y

suggesting therefore a total proton count of 64. Negative-ion FAB measurements indicated a MW of 840 and, assuming a C/H/N/O compound, suggested a molecular formula of $C_{38}H_{64}N_8O_{13}$. This empirical formula was verified by HREI-MS under fast-heating conditions by peak matching on a weak $M^+ - H_2O$ peak (found m/z 822.4493; calcd for $C_{38}H_{62}N_8O_{12}$ m/z 822.4487) and elemental analysis. The formula would require eleven units of unsaturation, suggesting a depsipeptide structure having four rings in addition to the seven carbonyl groups.

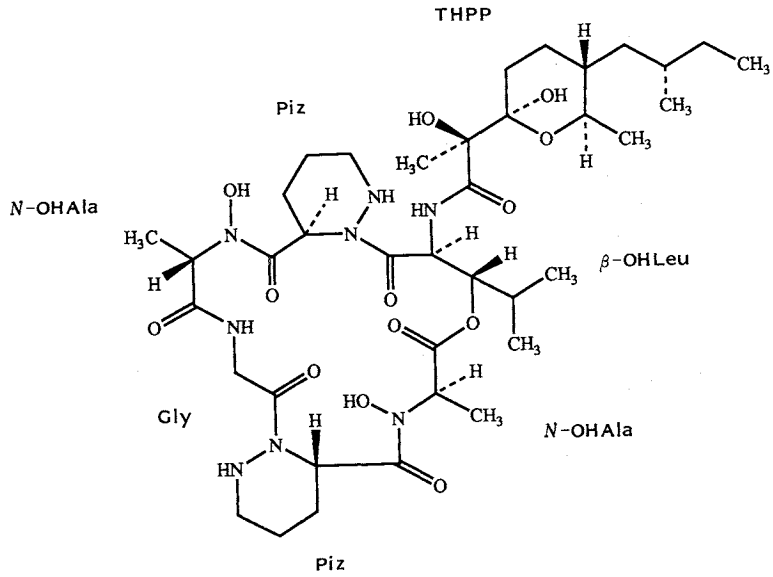
The NMR evidence indicated the presence of one equivalent each of glycine and β-hydroxyleucine (β-OHLeu) which was supported by GC-MS of the trimethylsilyl (TMS) derivatives of the total acid hydrolysate and two equivalents each of piperazic acid (Piz) and *N*-hydroxyalanine (*N*-OHAla). The low field chemical shift position of the β-*H* of the β-OHLeu residue suggested that the β-OH group was involved in an ester-type linkage (Table 1). These subunits account for $C_{24}H_{39}N_8O_9$ leaving $C_{14}H_{25}O_4$ which has one ring, assuming a cyclic depsipeptide molecule.

One-bond ^{13}C-^1H HETCOR experiments in pyridine-d_5 at 65°C allowed the methylene protons to be readily distinguished from methine protons in the severely overlapping δ 0.8 ~ 3.3 region of the ^1H NMR spectrum. Critically, the experiment led to assignment of all methylene proton pairs (Table 1) of the unknown C_{14} substructure which, with the aid of the COSY and ^1H decoupled NMR data, led to the partial structure **X** (Fig. 1). The long-range version of the HETCOR experiment further allowed elaboration of **X** to partial sequence **Y**, showing the relationship between the hitherto unaccounted for hemiketal, tertiary carbinol, methyl singlet and amide carbonyl functions comprising a novel tetrahydropyranylpropionic acid moiety (THPP) (Fig. 1). The large $J_{H2,H3}$ coupling of 9.8 Hz establishes a *trans*-diequatorial relationship of the substituents at C-2 and C-3 where the six membered ring is in a chair conformation. Completion of the structure in terms of sequence and absolute stereochemistry was accomplished by X-ray diffraction analysis. NMR assignments are listed in Table 1, although the data do not allow individual assignments of the two Piz and *N*-OHAla residues.

A 75-mg sample of L-156,602 was recrystallized three times from CH_3CN (1.5 ml) to give 31 mg of colorless needles, mp 190 ~ 193°C. Chemical analysis of crystals isolated with minimal drying, showed the presence of two molecules of CH_3CN for each molecule of L-156,602. These crystals had space group symmetry $P2_12_12_1$ with $a=16.568(4)$ Å, $b=30.141(5)$ Å and $c=10.616(3)$ Å for $Z=4.4005$ potential diffraction peaks were measured of which 2,151 were observed ($I \geq 3\sigma I$). Application of a multi-solution tangent formula approach to phase solution gave an initial model for the structure† which was subsequently refined with least squares and Fourier methods. Anisotropic temperature parameters were refined for the nonhydrogen atoms. Hydrogens were placed in calculated positions, isotropic temperature factors were applied and the hydrogens were refined as riding on their parent atoms. The function $\Sigma\omega(|F_o|-|F_c|)^2$ with $\omega = 1/(\sigma F_o)^2$ was minimized with full matrix least squares to give an unweighted residual of 0.080. The final fractional coordinates, temperature parameters, bond distances and bond angles have been deposited in the Cambridge Crystallographic Data Centre. Some of the bond distances and angles differ slightly from their generally accepted values presumably because of the low data/parameter ratio.

Reaction of (*R*)-α-methylbenzyl isothiocyanate[15] with (2*S*,3*S*)-3-hydroxyleucine and (2*R*,3*R*)-3-

† The following library of crystallographic programs was used: See refs 12 to 14.



L-156,602

hydroxyleucine[16] produced derivatives having Rt's of 30.8 and 31.8 minutes, respectively, using the chromatographic system described above. HPLC analysis of the material obtained by hydrolysis and derivatization of L-156,602 showed only the presence of the peak at 30.8 minutes thereby establishing the absolute configuration of the 3-hydroxyleucine of L-156,602 as (2*S*,3*S*).

L-156,602 is analogous to azinothricin and A83586C but has significant differences. All three compounds have 19-membered rings composed of six amino acids and novel side chains. The position and stereochemistry of 3-hydroxy-L-leucine, D-piperazic acid and L-piperazic acid are strictly conserved throughout all three compounds and the position of *N*-hydroxy-L-alanine in L-156,602 is the same as in A83586C. However, the D-threonine and *N*-methyl-L-alanine residues of azinothricin and A83586C are replaced by *N*-hydroxy-L-alanine and glycine, respectively. In addition, L-156,602 has a C-14 moiety in contrast to the C-20 and C-21 carbocyclic chains of the other two compounds. Despite these differences, corresponding asymmetric centers among all three compounds have identical absolute stereochemistries. Subsequent to the recent disclosure of a total synthesis of L-156,602[17,18], Dr. JAMES C. FRENCH has informed us of an unpublished X-ray structure determination on PD 124,966 done in collaboration with Dr. J. CLARDY. This study, as well as side by side physical comparison with an authentic sample of PD 124,966 kindly supplied by Dr. FRENCH, confirms that the two compounds are indeed identical.

Acknowledgments

We wish to thank Mr. JACK SMITH for FAB-MS measurements and Dr. JERROLD M. LIESCH for helpful discussions.

Addendum in Proof

The structures excluding absolute stereochemistry of variapeptin[19] and citropeptin[19,20], two other members of this hexadepsipeptide family of antibiotics, have very recently been reported. Variapeptin is characterized by the same C-14 tetrahydropyranylpropionic acid side-chain moiety as in L-156,602.

References

1) MAEHR, H.; C.-M. LIU, N. J. PALLERONI, J. SMALLHEER, L. TODARO, T. H. WILLIAMS & J. F. BLOUNT: Microbial products. VIII. Azinothricin, a novel hexadepsipeptide antibiotic. J. Antibiotics 39: 17~25, 1986
2) SMITKA, T. A.; J. B. DEETER, A. H. HUNT, F. P. MERTZ, R. M. ELLIS, L. D. BOECK & R. C. YAO: A83586C, a new depsipeptide antibiotic. J. Antibiotics 41: 726~733, 1988
3) DURETTE, P. L.; F. BAKER, S. S. BONDY, C. G. CALDWELL, J. L. GOULET, M. L. HAMMOND, J. F. KINNEARY, I. E. KOPKA, T. J. LANZA, R. A. ZAMBIAS, M. S. SPRINGER, D. N. CIANCIARULO, J. M.

FIELDHOUSE, S. KOBAYASHI, T. ROLLINS & S. SICILIANO: The C5a antagonistic properties of the novel cyclic hexadepsipeptide L-156,602 and analogs. Abstracts of the 200th ACS National Meeting, Div. of Med. Chem., No. 177, Washington, Aug. 26~31, 1990

4) HUGLI, T. E.: The structural basis for anaphylatoxins and chemotactic functions of C3a, C4a, and C5a. Crit. Rev. Immunol. 1: 321~366, 1981 and refs therein

5) WEIGLE, W. O.; M. G. GOODMAN, E. L. MORGAN & T. E. HUGLI: Regulation of immune response by components of the complement cascade and their activated fragments. Springer Semin. Immunology 6: 173~194, 1983

6) HURLEY, T. R.; R. H. BUNGE, N. E. WILLMER, G. C. HOKANSON & J. C. FRENCH: PD 124,895 and PD 124,966, two new antitumor antibiotics. J. Antibiotics 39: 1651~1656, 1986

7) BUNGE, R. H.; J. C. FRENCH, T. R. HURLEY & N. E. WILMER: CL-1957D Antibiotic compound and its production. U.S. 4,696,794, 1987 [C.A. 108: 9308z, 1988]

8) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966

9) NCCLS Approved Standard. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, M7-A. Vol. 5. No. 22, 1985

10) BAX, A.; R. FREEMAN & G. A. MORRIS: Correlation of proton chemical shifts by two-dimensional Fourier transform NMR. J. Mag. Reson. 42: 164~168, 1981

11) BAX, A. & G. A. MORRIS: An improved method for heteronuclear chemical shift correlation by two-dimensional NMR. J. Mag. Reson. 42: 501~505, 1981

12) SHELDRICK, G. M.: SHELXS-86. Univ. of Göttingen, Göttingen, West Germany, 1986

13) MOTHERWELL, W. D. S. & W. CLEGG: PLUTO. University of Cambridge, England, 1978

14) OKAYA, Y. & B. A. FRENZ and associates: SDP Plus VI. 1, College Station, Texas, 1984

15) GAL, J. & A. J. SEDMAN: (*R*)-α-Methylbenzyl isothiocyanate, a new and convenient chiral derivatizing agent for the separation of enantiomeric amino compounds by high-performance liquid chromatography. J. Chromatogr. 314: 275~281, 1984

16) CALDWELL, C. G. & S. S. BONDY: A convenient synthesis of (2*S*,3*S*)- or (2*R*,3*R*)-3-hydroxyleucine. Synthesis 1990: 34~36, 1990

17) DURETTE, P. L.; F. BAKER, P. L. BARKER, J. BOGER, S. S. BONDY, M. L. HAMMOND, T. J. LANZA, A. A. PESSOLANO & C. G. CALDWELL: Total synthesis of L-156,602, a novel cyclic hexadepsipeptide antibiotic. Tetrahedron Lett. 31: 1237~1240, 1990

18) CALDWELL, C. G.; K. M. RUPRECHT, S. S. BONDY & A. A. DAVIS: Synthesis of the lipophilic side chain of the cyclic hexadepsipeptide antibiotic L-156,602. J. Org. Chem. 55: 2355~2361, 1990

19) NAKAGAWA, M.; Y. HAYAKAWA, K. FURIHATA & H. SETO: Structural studies on new depsipeptide antibiotics, variapeptin and citropeptin. J. Antibiotics 43: 477~484, 1990

20) HAYAKAWA, Y.; M. NAKAGAWA, Y. TODA & H. SETO: A new depsipeptide antibiotic, citropeptin. Agric. Biol. Chem. 54: 1007~1011, 1990