MYCOPLANECINS, NOVEL ANTIMYCOBACTERIAL ANTIBIOTICS FROM *ACTINOPLANES AWAJINENSIS* SUBSP. *MYCOPLANECINUS* SUBSP. NOV.

III. STRUCTURAL DETERMINATION OF MYCOPLANECIN A

MUTSUO NAKAJIMA, AKIO TORIKATA, HIDETSUNE TAMAOKI, TATSUO HANEISHI, MAMORU ARAI

Fermentation Research Laboratories, Sankyo Co., Ltd.

TAKESHI KINOSHITA and HARUMITSU KUWANO

Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd.
2–58, 1-chome, Hiromachi, Shinagawa-ku, Tokyo 140, Japan

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The structure of mycoplanecin A was determined by the analysis of chemical degradation products and by mass and ^{1}H and ^{13}C NMR spectrometries.

Mycoplanecin A is a new cyclic peptide antibiotic composed of mol each of α-ketobutyric acid, glycine, L-leucine, L-proline, L-2-amino-5-methylhexanoic acid, *N*-methyl-D-leucine, *N*-methyl-L-threonine, methyl-L-proline and ethyl-L-proline and two mol of *N*-methyl-L-valine. Among these components, ethyl-L-proline is reported for the first time as a component of natural products. A newly developed mass analysis has been introduced for the differentiation of α-amino acid and its *N*-methyl derivative.

Mycoplanecin A is a lipophilic, neutral cyclic peptide antibiotic produced by *Actinoplanes awajinensis* subsp. *mycoplanecinus* subsp. nov.

The antibiotic showed specific activity against mycobacteria, such as *Mycobacterium tuberculosis* H37Rv and *M. intracellulare*, and other micoorganisms related to mycobacteria.

Taxonomy of the producing organism and fermentation, isolation and properties of mycoplanecin A were reported in the preceding papers[1,2]. This report deals with the structural elucidation of mycoplanecin A.

Structural Elucidation of Mycoplanecin A

Mycoplanecin A (**1**) with mp 161~167°C, $[\alpha]_D^{25}$ −66° (*c* 0.4, $CHCl_3$) was obtained as neutral, lipophilic and colorless powder. The molecular formula was determined to be $C_{61}H_{102}N_{10}O_{13}$ (mol. wt. 1,182) by field desorption mass spectrometry, elementary analysis and ^{13}C NMR spectrum. The IR spectrum of **1** in KBr pellet showed absorption at 1760, 1720 and 1670~1640 cm^{-1} corresponding to lactone, ester or ketone and amide groups, respectively. The ^{1}H NMR spectrum of **1** in $CDCl_3$ revealed the presence of four singlets due to *N*-methyl groups (2.89, 2.99, 3.15 and 3.32 ppm) and several broad signals in the lower field due to amide groups. In the ^{13}C NMR spectrum, all of the 61 carbons, including 12 carbonyl carbons and one carbon bearing on hydroxyl group (at 78 ppm), were observed. These characteristics of **1** suggested its structure to be a member of the cyclic peptide antibiotics. The structural studies of **1** were achieved by the following chemical degradation studies (Scheme 1).

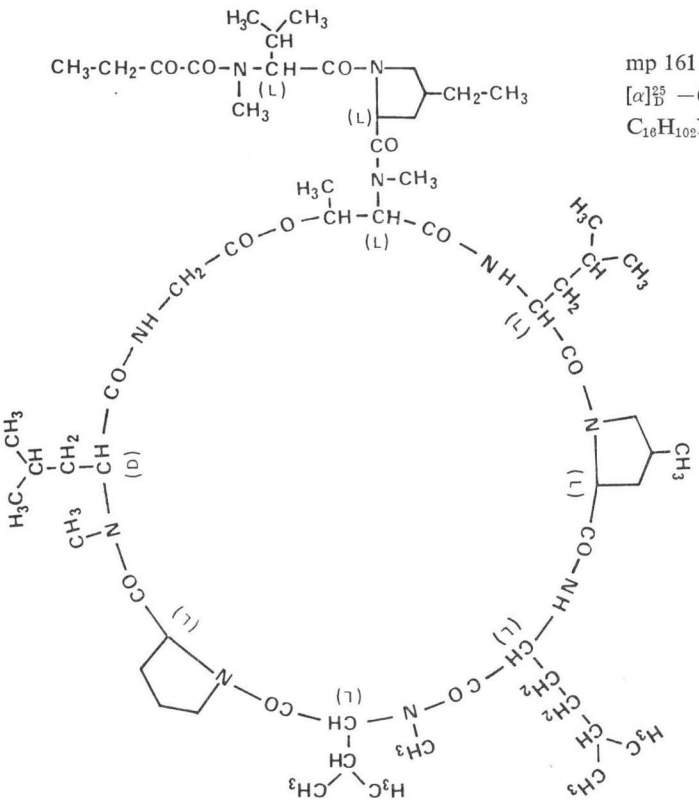
Acid hydrolysis (conc. HCl - AcOH, 1: 1, 105°C, 20 hours) of **1** followed by amino acid analysis indicated the presence of glycine, leucine and proline. However, other amino acids could not be identi-

Scheme 1.

Mycoplanecin A (**1**)

Acid hydrolysis	Acid hydrolysis	Reduction
conc. HCl - AcOH (1 : 1)	6 N HCl	$NaBH_4$ in MeOH
105°C, 20 hours	37°C, 72 hours	
Glycine (**2**)	Peptides	Dihydromycoplanecin A (**19**)
L-Leucine (**3**)	Compound **12**	Compound **20**
L-Proline (**4**)	Compound **13**	Me-ester of Compound **20** (**21**)
N-Me-L-threonine (**5**)	Compound **14**	Acid hydrolysis of **19**
N-Me-L-valine (**6**)	Compound **15**	conc. HCl - AcOH (1 : 1)
N-Me-D-leucine (**7**)	Compound **16**	105°C, 20 hours
L-2-Amino-5-Me-hexanoic acid (**8**)	Compound **17**	α-Hydroxybutyric acid (**22**)
Me-L-proline (**9**)	Compound **18**	
Et-L-proline (**10**)		
α-Ketobutyric acid (**11**)		

Chart 1. Structure of mycoplanecin A (**1**).



mp 161~167°C
$[\alpha]_D^{25}$ −66° (*c* 0.4, $CHCl_3$)
$C_{16}H_{102}N_{10}O_{13}$ (MW 1,182)

fied by conventional analysis. Amino acid-constituents were separately isolated from the acid hydrolysate by column chromatography on Sephadex G-10 equilibrated with the upper layer of a solvent mixture (BuOH - AcOH - H_2O, 4 : 1 : 5). Each amino acid was further purified by rechromatography on a

Sephadex G-10 column under the same conditions as described above.

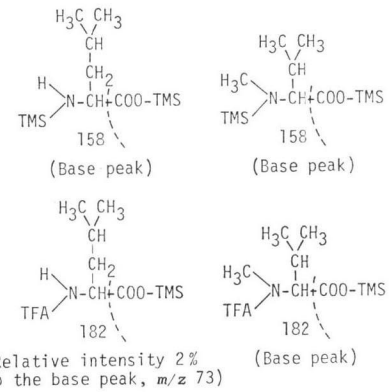
Identification of the *N*-methyl amino acids, *N*-methylvaline, *N*-methylleucine and *N*-methylthreonine, was carried out by mass spectral analysis of the derivatives obtained after trimethylsilylation[3,4] followed by treatment with *N*-methyl bistrifluoroacetamide.

The mass spectra of the derivatives of authentic leucine and *N*-methylvaline were recorded under the same conditions.

The ion intensities at *m/z* 158 of trimethylsilyl derivatives of leucine and *N*-methylvaline were very strong, while the ion intensity at *m/z* 182 of the *N*-trifluoroacetyl-*O*-trimethylsilyl derivative of *N*-methylvaline was much stronger than that of leucine (Scheme 2). A similar behavior in ion intensities was observed for the other *N*-methyl amino acids.

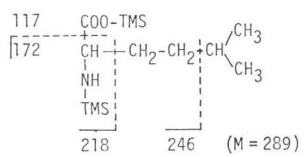
Such comparative studies by GC/MS spectral analysis and other spectral data were used for identification.

Scheme 2.



Molecular formula of compound **8**, one of the unknown amino acids, was determined to be $C_7H_{15}NO_2$ from high resolution GC/MS data of its trimethylsilyl derivative. The ^1H NMR spectrum of **8** indicated the presence of an isopropyl, two methylene and one methine groups. Therefore, compound **8** was logically identified as 2-amino-5-methyl hexanoic acid, which was deduced to be the L-isomer by the similarity of its specific rotation to that of L-leucine. The fragmentation pattern of the GC/MS spectrum is shown in Scheme 3.

Scheme 3.



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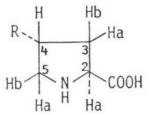
The structures of the unknown amino acids, compounds **9** and **10**, which were assumed to be proline analogues by their yellow color reaction with ninhydrin and their similar retention times to those of proline in amino acid analysis, were determined as follows: mass spectra of trimethylsilyl derivatives of compounds **9** and **10** suggested their molecular formulae to be $C_6H_{11}NO_2$ and $C_7H_{13}NO_2$, respectively. From analysis of ^1H NMR spectra compounds **9** and **10** can be identified as 4-methylproline and 4-ethylproline, respectively. Identification of compound **9** as *trans*-4-methyl-L-proline was achieved by comparative studies with *trans*-4-methyl-L-proline prepared from the acid hydrolysate of griselimycin isolated and purified from the culture broth of *Streptomyces caelicus* NRRL 2957[8].

^1H NMR and GC/MS spectra of compound **10** suggested its structure to be 4-ethylproline. As for the stereochemistry of compound **10**, it was revealed to be *trans*-4-ethyl-L-proline by ^1H NMR and CD spectra by comparison with those of compound **9** and by specific rotation data. The results of ^1H NMR studies of compounds **9** and **10** are summarized in Table 1.

The last constituent of **1**, compound **11**, $C_4H_6O_3$ (mol. wt. 102), was isolated from the acid hydrolysate of **1** on Dowex 50WX4 (H^+) column. From the analysis of ^1H NMR of compound **11** and GC/MS of its ditrimethylsilyl derivative, the structure was determined to be α-ketobutyric acid. The data on

Table 1. Comparative studies on proline analogues with ^1H NMR spectra.



Compound	Chemical shift (ppm)							Coupling constant (Hz)				
	2a	3a	3b	4-R	4	5a	5b	2a-3a	2a-3b	4-5a	4-5b	5a-5b
trans-4-Methyl-L-proline*	4.02	1.60~2.40	1.60~2.40	0.94 (CH_3)	1.60~2.40	2.69	3.40	4.4	8.6	8.3	6.7	11.0
cis-4-Methyl-D-proline*	4.00	1.47	2.10~2.60	0.94 (CH_3)	—	2.81	3.39	9.0	8.1	8.6	6.6	10.8
trans-4-Methyl-L-proline**	4.65	2.30~3.00	2.30~3.00	1.56	2.30~3.00	3.39	4.05	4.5	9.0	8.0	7.0	11.5
Compound **9***	4.66	2.30~2.80	2.30~2.80	1.55 (CH_3)	2.30~2.82	3.35	4.03	4.5	9.0	8.5	7.5	11.5
Compound **10***	4.61	2.30~2.80	2.30~2.80	1.38 (CH_3), 1.87 (CH_2)	2.30~2.80	3.35	4.03	4.0	9.0	8.0	6~7	12.0

* Cited from data of ABRAHAM[8] and measured in D_2O with dioxane as internal standard (3.57 ppm).
** Prepared from hydrolysate of griselimycin. Measured in D_2O with TMS as external standard.
*** Measured in D_2O with TMS as external standard.

compound **11** were identical with those of an authentic sample. The presence of α-ketobutyric acid moiety in **1** was supported by the following evidence: the ^{13}C NMR spectrum of **1** indicated a signal at 201 ppm due to a ketone group, which shifted to 70 ppm by reduction of **1** with $NaBH_4$ in accordance with the isolation of α-hydroxybutyric acid (**22**) from the acid hydrolysate of the reduced product of **1**, dihydromycoplanecin A (**19**).

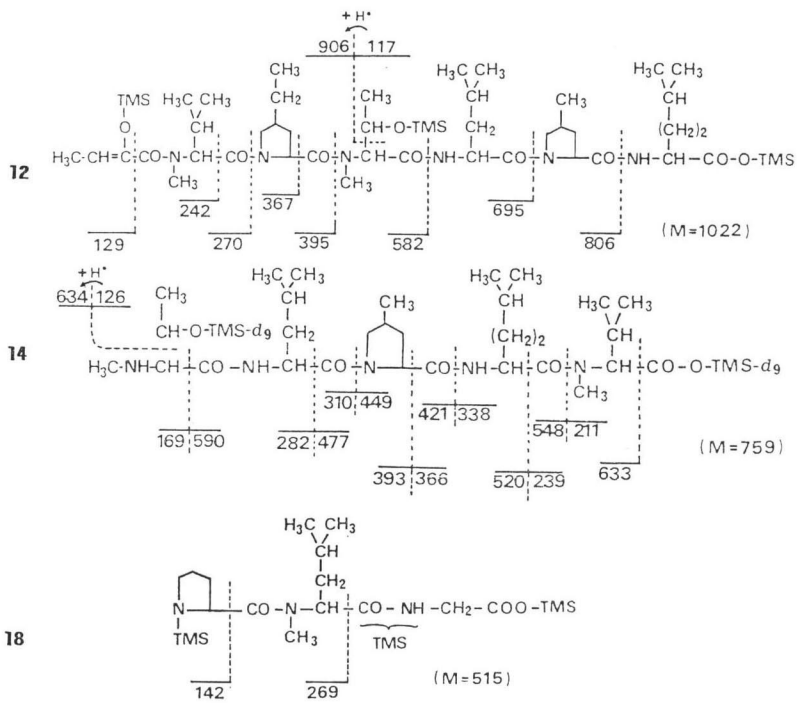
Thus, all of the constituents of **1** were isolated and characterized as follows: glycine, L-leucine, L-proline, *trans*-4-methyl-L-proline, *trans*-4-ethyl-L-proline, L-2-amino-5-methylhexanoic acid, *N*-methyl-L-threonine, *N*-methyl-L-valine, *N*-methyl-D-leucine and α-ketobutyric acid.

Four *N*-methyl signals and 11 carbonyl carbons in ^1H and ^{13}C NMR spectra of **1** suggested that one of the three *N*-methyl amino acids described above could exist in two molar equivalents.

The next step of structural elucidation was achieved by isolation of the oligopeptides from either mild acidic or alkaline hydrolysates followed by analysis of their amino acid composition and sequence.

Partial hydrolysis in 6 N HCl was carried out at 37°C for 72 hours. The hydrolysate was purified by column chromatography on Sephadex G-10 equilibrated with the upper layer of a solvent mixture (BuOH - AcOH - H_2O, 4: 1: 5) followed by preparative TLC on silica gel; seven peptides were separated. For determination of amino acid composition, each peptide was further hydrolyzed in conc. HCl - AcOH (1: 1) at 105°C for 20 hours; samples were submitted to amino acid analysis, GC/MS spectral analysis of their trimethylsilyl derivatives, and silica gel TLC (solvent system; BuOH - AcOH - H_2O, 4: 1: 2). The amino acid sequence of each peptide was determined by GC/MS, DIMS and FD/MS analyses. The fragmentation patterns of mass spectra of trimethylsilyl derivatives of peptides **12**, **14** and **18** are shown in Scheme 4.

Scheme 4.



The amino acid sequences of peptides are summarized in Table 2. Reduction of **1** with $NaBH_4$ gave a major product (**19**) and minor compound (**20**) which was obtained either by acid or mild alkaline hydrolysis of **19**. The IR spectrum of **20** exhibited disappearance of the band at 1760 cm^{-1} due to lactone or ester linkage, which was observed in the spectrum of **1** or **19**. Treatment of **20** with ethereal diazomethane afforded the reaction product (**21**). Mass spectral analysis indicated that **21** is the methyl ester of **20** obtained by anhydration at the threonine residue with cleavage of the lactone linkage in **19** under alkaline condition as shown in Scheme 5.

The mass spectral analysis of trimethylsilyl derivatives of peptides (**12**, **13**, **14** and **15**) always gave a very stable ion peak at *m/z* 117 due to a fragment, CH_3–CH=O^+–TMS, derived from the free hydroxyl group of the *N*-methylthreonine residue. However, the mass spectrum of the trimethylsilyl derivative of **1** did not show the fragment ion peak at *m/z* 117. Therefore, it was concluded that the hydroxyl group of *N*-methylthreonine residue in **1** was not in free form. Consequently, the structure of **1** was found to have a lactone linkage between the carboxyl group of glycine and the hydroxyl group of *N*-methylthreonine.

All evidences described above unequivocally led to the cyclic peptide structure (**1**) for mycoplanecin A (Chart 1.). The mass spectrum of **1** and the assignments of the main fragment ions are shown in Schemes 6 and 7, respectively.

All of the known peptide antibiotics with typical antimycobacterial activity, such as griselimycins[5,6,7], ilamycins[9], rufomycins[10], phegamycins[11] and triculamin[12] have been reported to be isolated from the culture broth of Streptomyces; mycoplanecin A is produced by Actinoplanes.

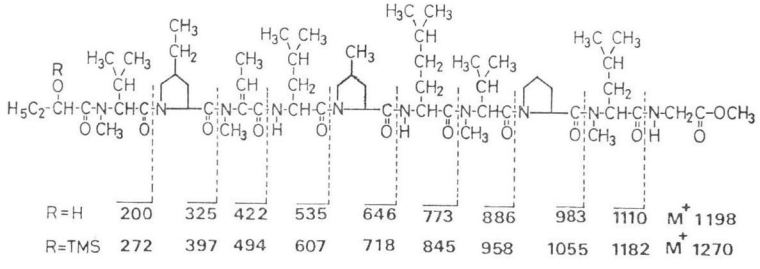
Table 2. Amino acid sequence of isolated peptides.

Compound	α-KBA	*N*-MeVal	EtPro	*N*-MeThr	Leu	MePro	AMHA	*N*-MeVal	Pro	*N*-MeLeu	Gly
12	α-KBA	—	—	—	—	—	AMHA				
13	α-KBA	—	—	*N*-MeThr							
14				*N*-MeThr	—	—	—	*N*-MeVal			
15				*N*-MeThr	—	—	AMHA				
16						MePro	AMHA				
17		*N*-MeVal	EtPro								
18									Pro	—	Gly
20	α-KBA	—	—	*N*-MeThr*	—	—	—	—	—	—	Gly

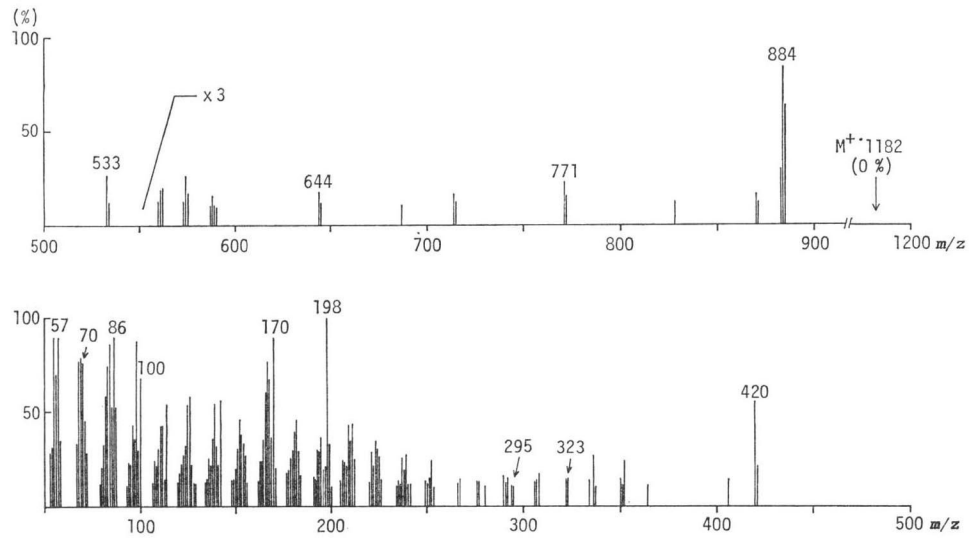
* *N*-Methylanhydrothreonine.

α-KBA; α-Ketobutyric acid, *N*-MeVal; *N*-methylvaline, EtPro; Ethylproline, *N*-MeThr; *N*-methylthreonine, Leu: Leucine, MePro; Methylproline, AMHA; 2-amino-5-methylhexanoic acid, Pro; Proline, *N*-MeLeu; *N*-methylleucine, Gly; Glycine.

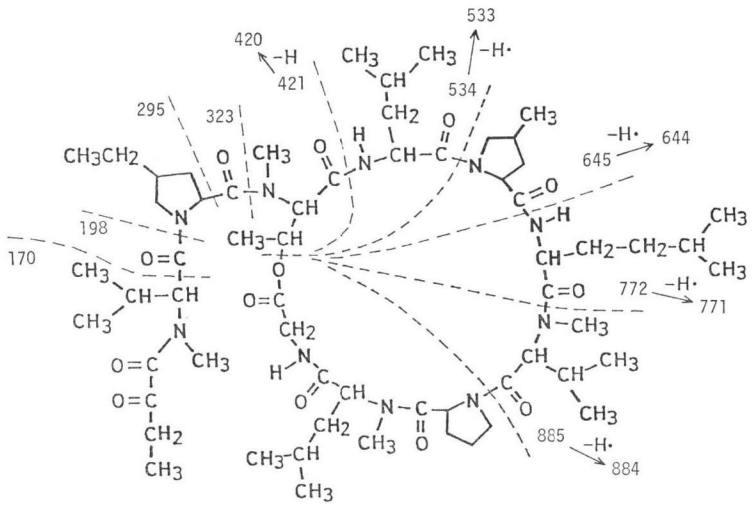
Scheme 5.



Scheme 6.



Scheme 7.



Mycoplanecin A is closely related to griselimycin, but clearly differs by its amino acid and fatty acid components. It appears that the difference between these two antibiotics lies in the presence in mycoplanecin A of 2-amino-5-methylhexanoic acid, ethylproline and α-ketobutyric acid which are replaced by leucine, methylproline and acetic acid in griselimycin.

Experimental

^{1}H and ^{13}C NMR spectra were taken with a Varian HA-100 (100 MHz) and a Varian XL-100 (25.2 MHz) spectometer, respectively. Electron impact mass spectra were measured on a JEOL-01SG mass spectrometer using the direct-inlet probe and field desorption mass spectra on a double focusing JEOL-01SG instrument equipped with a combined f. d. f. i. e. i. ion source. GC/MS spectra were combined with a JEOL JMS D-100. Amino acid analysis was carried out on a Hitachi KLA-5 automatic amino acid analyzer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter.

Acid Hydrolysis of **1**

A 1 g sample of **1** was hydrolyzed in 20 ml of conc. HCl - AcOH (1: 1) in a sealed tube for 20 hours at 105°C. The hydrolysate was concentrated several times from water under reduced pressure to remove excess acids. For separation of each amino acid the residue was subjected to a cellulose column (Avicel, Funakoshi Yakuhin Co., Ltd., Japan) packed and developed with 90% aqueous isopropanol followed by Sephadex G-10 column equilibrated with the upper layer of BuOH - AcOH - H_2O (4: 1: 5). Each amino acid was further purified by preparative TLC on silica gel with BuOH - AcOH - H_2O (4: 1: 2). Finally each amino acid was adsorbed on Dowex 50WX4 (H^+) and eluted with 0.5 N NH_4OH. Recovery and optical rotation of each amino acid was as follows: glycine (54 mg), leucine (30 mg) $[\alpha]_D^{25}$ +6.8° (*c* 0.3, 1 N HCl), proline (70 mg) $[\alpha]_D^{25}$ −38.96° (*c* 0.58, 1 N HCl), *N*-methylleucine (15 mg) $[\alpha]_D^{25}$ −19.2° (*c* 0.59, 1 N HCl), *N*-methylthreonine (20 mg) $[\alpha]_D^{25}$ −10.53° (*c* 0.20, 1 N HCl), *N*-methylvaline (85 mg) $[\alpha]_D^{25}$ +19.7° (*c* 0.39, 1 N HCl), compound **8** (87 mg) $[\alpha]_D^{25}$ +9.77° (*c* 0.45, 1 N HCl), compound **9** (80 mg) $[\alpha]_D^{25}$ −19.1° (*c* 0.46, 1 N HCl) and compound **10** (60 mg) $[\alpha]_D^{25}$ −30.5° (*c* 0.25, 1 N HCl).

Mild Acid Hydrolysis of **1**

A 400 mg sample of **1** was hydrolyzed in 12 ml of 6 N HCl at 37°C. The hydrolysate was concentrated several times from water under vacuum to remove excess HCl. The residue was applied to a Sephadex G-10 column equilibrated and developed with the upper layer of BuOH - AcOH - H_2O (4: 1: 5). The crude oligopeptides thus separated were purified on Sephadex G-10 column using the lower layer of BuOH - AcOH - H_2O - $CHCl_3$ (4: 1: 5: 2). Each peptide isolated on Sephadex G-10 column was subjected to preparative TLC on silica gel with BuOH - AcOH - H_2O (4: 1: 2) and finally purified by adsorption on a small column of Dowex 50WX4 (H^+) followed by elution with 0.5 N NH_4OH. Isolated peptides were: compound **12**, 43 mg; compound **13**, 33.9 mg; compound **14**, 30 mg; compound **15**, 22 mg; compound **16**, 8 mg; compound **17**, 33 mg and compound **18**, 44 mg.

Compound **8**: mp 210°C (dec.), $[\alpha]_D^{25}$ +9.77° (*c* 0.45, 1 N HCl), $C_7H_{15}NO_2$ (mol. wt. 145), ^1H NMR ($\delta_{ppm}^{D_2O}$) 0.91 (3H×2, d, J=6 Hz), 1.6 (1H, m), 1.35 (2H, m), 2.0 (2H, m) and 4.15 (1H, t, J=6 Hz).

Compound **9**: $[\alpha]_D^{25}$ −19.1° (*c* 0.46, 1 N HCl), $C_6H_{11}NO_2$ (mol. wt. 129). See text for ^1H NMR spectral data.

trans-4-Methyl-L-proline

trans-4-Methyl-L-proline (60 mg) was obtained from the acid hydrolysate of griselimycin (300 mg) prepared from the culture broth of *Streptomyces caelicus* NRRL 2957. $[\alpha]_D^{25}$ −21.5° (*c* 0.6, 1 N HCl); $C_6H_{11}NO_2$ (mol. wt. 129).

Compound **10**: $[\alpha]_D^{25}$ −30.5° (*c* 0.25, 1 N HCl); $C_7H_{13}NO_2$ (mol. wt. 143).

Reduction of **1**

To a solution of **1** (2 g) dissolved in 20 ml of methanol was added 150 mg of $NaBH_4$ upon stirring at 0°C for one hour. The reaction mixture was concentrated to dryness and the residue extracted with 50 ml of ethyl acetate after adjustment of the pH to 4.0. The extract was washed twice with 50 ml of saturated aqueous sodium chloride and evaporated to afford 2 g of the reaction product, which was treated with 10 ml of cold acetonitrile to give 1 g of dihydromycoplanecin A (**19**) as colorless needles.

The mother liquor was subjected to preparative TLC ($CHCl_3$ - MeOH, 4: 1) to afford 700 mg of the acidic substance as another product **20**, which was treated with ethereal diazomethane to yield monomethyl ester (**21**). Compound **20** could also be obtained by treatment of **19** with 0.1 N NaOH at room temperature for one hour.

Preparation of **22**

A 310 mg of **19** was hydrolyzed in 20 ml of conc. HCl - AcOH (1: 1) at 105°C for 20 hours. The hydrolysate was concentrated several times from water under reduced pressure to remove excess HCl and were applied on a column of Dowex 50WX4 (H^+) (50 ml). The effluent and washings were combined and extracted twice with 50 ml of ethyl acetate. After washing with saturated aqueous sodium chloride the extract was dried over Na_2SO_4 and concentrated to give 13.4 mg of **22**. $C_4H_8O_3$ (mol. wt. 104), ^1H NMR $\delta_{ppm}^{CDCl_3}$ 0.85 (3H, t), 1.65 (2H, m) and 4.2 (1H, m).

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