FOUR DIASTEREOISOMERS OF CYCLO(-ASP-VAL-): INCONSISTENCY OF THEIR PROPERTIES WITH THE PROPOSED STRUCTURE OF CAIROMYCIN A

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The four diastereoisomers of cyclo(-Asp-Val-) were synthesized to compare with a proposed structure of cairomycin A. Their antimicrobial activities were determined against both Gram-positive and Gram-negative bacteria. The physico-chemical properties of the isomers were characterized by mp, ^{1}H NMR, IR, FAB-MS, and solubility in solvents, which were different from those reported for cairomycin A.

Cairomycins A (**1**), B and C were isolated from the culture broth of *Streptomyces* sp. strain AS-C-19.[1] Among them, **1** has been reported to be a potent peptide antibiotic against Gram-positive bacteria and to have a structure of cyclo(-L-Asp-L-Val-),††† 6-isopropyl-2,5-diketopiperazine-3-acetic acid.[2]

KUMAR *et al.* recently presented the conclusion that the diketopiperazine structure proposed by SHIMI *et al.* for **1** was correct by synthesizing cyclo(-L-Asp-L-Val-) and measuring antimicrobial activity against some organisms.[3]

Since an acidic antimicrobial peptide is of great interest, we synthesized several cyclic dipeptides including cyclo(-L-Asp-L-Val-) in order to investigate the structure-activity relationship of **1**. Unexpectedly, we noticed that the physico-chemical property of cyclo(-L-Asp-L-Val-) was not compatible with that of cairomycin A, suggesting that cairomycin A has a different structure from the proposed one.

In this paper, we report the syntheses of the four diastereoisomers of cyclo(-Asp-Val-) and the comparison of their physico-chemical and biological properties with those reported for cairomycin A (**1**).

Results

The synthetic strategy for cyclo(-L-Asp-L-Val-) (2LL) is shown in Fig. 1(A). The same procedure was applied to the other three diastereoisomers (2LD, 2DL and 2DD).†††† Z-L-Asp(OBzl)-OH (**3**) and H-L-Val-OMe (**4**) were synthesized according to the standard methods.[4,5] Mixed anhydride coupling reaction with isobutyl chloroformate[6] gave the protected dipeptide ester (**5**) in good yield. Catalytic hydrogenation (H_2 - Pd) removed the protecting groups on both the *N*-terminus and the side chain of the Asp residue simultaneously to give the desired deprotected compound in 75% yield. The cyclization reaction was carried out according to the method of SUZUKI *et al.*[7] in 86% yield.

The synthesis of the cyclic compound with a β-linkage between the Asp and Val residues (**6**) was

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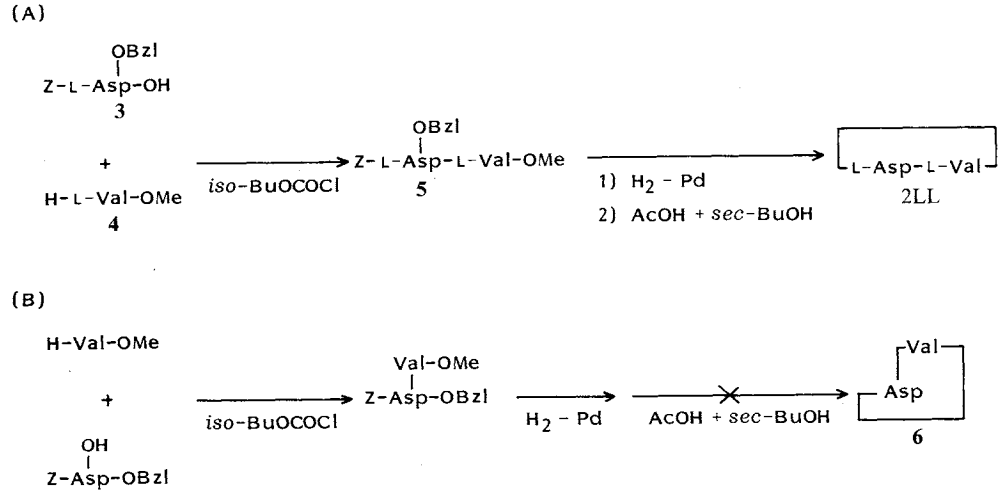
††† Abbreviations according to IUPAC-IUB Commission (Eur. J. Biochem. 138 : 9 ~ 37, 1984) are used throughout.

†††† Abbreviations for diastereoisomers of cyclo(-Asp-Val-) are as follows: cyclo(-L-Asp-L-Val-), 2LL; cyclo(-L-Asp-D-Val-), 2LD; cyclo(-D-Asp-L-Val-), 2DL; cyclo(-D-Asp-D-Val-), 2DD.

Fig. 1. Synthetic route for cyclic dipeptides.



Z: Benzyloxycarbonyl, Bzl: benzyl.

Table 1. Physico-chemical properties of cairomycin A, KUMAR's compound and compounds **2**.

	Cairomycin A (**1**)[2]	KUMAR's compound[3]	Cyclo(-L-Asp-L-Val-) (2LL)
MP (°C)	110~112	238~240	230~235
$[\alpha]_D$		−56.7° (*c* 0.84, AcOH)	−91.0° (*c* 1.0, DMF)
Solubility			
Soluble:	Chloroform, Me_2CO, EtOAc		DMF, DMSO
Insoluble:	Petroleum ether, water		Chloroform, benzene, EtOH, MeOH, Me_2CO
Rf[a]	0.59	0.54	0.00
Anal Found:	C 50.51, H 6.51, N 12.93	C 50.62, H 6.59, N 12.89	C 49.92, H 6.37, N 13.02
Calcd for $C_9H_{14}N_2O_4$:	C 50.46, H 6.59, N 13.08		
IR (cm^{-1})	1735, 1625, 1030	3200, 1740, 1455, 1370, 1260, 830	3189, 1728, 1660, 1454, 1349, 1263, 848
^1H NMR	(60 MHz, chloroform-d_3) 1.02 (6H, d), 1.56 (1H, h), 4.25 (1H, d), 5.51 (1H, t), 3.04 (2H, d), 9.05 (2H, br)	(90 MHz, chloroform-d_3 +a drop of TFA) 1.0, 1.12 (6H, Valγ), 2.25~2.6 (1H, Valβ), 2.95~3.2 (2H, Aspβ)	(90 MHz, DMSO-d_6) 0.87, 0.94 (6H, Valγ), 2.21 (1H, Valβ), 2.64 (2H, Aspβ), 3.78 (1H, Valα), 4.23 (1H, Aspα), 8.02 (2H, br, amide)
MS (*m/z*)	214 (M^+)		215 ($(M+1)^+$)

	2LD	2DL	2DD
MP (°C)	234~240	235~238	232~236
$[\alpha]_D$	+18.4° (*c* 1.0, DMF)	−20.0° (*c* 1.0, DMF)	+94.3° (*c* 1.0, DMF)
Solubility			
Soluble:	DMF, DMSO	DMF, DMSO	DMF, DMSO
Insoluble:	Chloroform, benzene, EtOH, MeOH, Me_2CO	Chloroform, benzene, EtOH, MeOH, Me_2CO	Chloroform, benzene, EtOH, MeOH, Me_2CO
Rf[a]	0.00	0.00	0.00
Anal Found:	C 50.30, H 6.57, N 12.97	C 50.47, H 6.63, N 12.87	C 50.29, H 6.48, N 13.08
Calcd for $C_9H_{14}N_2O_4$:	C 50.46, H 6.59, N 13.08		
IR (cm^{-1})	3215, 1725, 1674, 1472, 1348, 1261, 834	3214, 1725, 1678, 1463, 1348, 1262, 834	3188, 1728, 1661, 1454, 1349, 1263, 849
MS (*m/z*)	215 ($(M+1)^+$)	215 ($(M+1)^+$)	215 ($(M+1)^+$)

[a] Measured with chloroform-MeOH (9:1).

Table 2. Antimicrobial activity of compounds **2**.[a]

Organism	MIC (μg/ml)			
	2LL	2LD	2DL	2DD
Staphylococcus aureus FDA 209P	>100	>100	>100	>100
S. aureus 308A-1	>100	>100	>100	>100
S. epidermidis ATCC 12228	>100	>100	>100	>100
Streptococcus pneumoniae Type-1	>100	>100	>100	>100
Bacillus subtilis PCI 219	>100	>100	>100	>100
Escherichia coli NIHJ JC-2	>100	>100	>100	>100
E. coli O-111	>100	>100	>100	>100
Klebsiella pneumoniae DT	>100	>100	>100	>100
Proteus mirabilis IFO 3849	>100	>100	>100	>100
Pseudomonas aeruginosa U-31	>100	>100	>100	>100

[a] Medium: Mueller-Hinton agar (Difco), 10% horse blood added. Inoculum size: 10^6 cell/ml.

unsuccessful (Fig. 1B). The cyclization of the deprotected dipeptide ester by the same procedure as in the case of compounds **2** gave a brown gummy solid, on which no characterization was made.

Compounds **2** synthesized in this study gave a single spot on TLC (silica gel 60 G), which was ninhydrin-negative and sulfuric acid-positive. Characterization of compounds **2** by ^{1}H NMR, IR, elemental analysis, and FAB-MS showed that the compounds have the desired structure, cyclo(-Asp-Val-). Some physico-chemical properties of compounds **2** are listed in Table 1 together with those of cairomycin A (**1**) and KUMAR's compound.

Antimicrobial activity was tested against over 25 kinds of organisms including Gram-negative and Gram-positive bacteria, some results being shown in Table 2.

Discussion

Data in Table 1 show that there are many physico-chemical differences not only between cairomycin A (**1**) and cyclo(-L-Asp-L-Val-) (2LL) but also between **1** and other compounds **2**. Compound **1** has a relatively low mp as pointed out by KUMAR *et al.*[3] This low mp could be explained by the reason that the compound was not completely pure. Compound **1** and compounds **2** showed quite different solubility. Compounds **2** were soluble only in DMF and DMSO, but hardly soluble in solvents such as chloroform, EtOAc, Me_2SO and benzene, in which **1** was reported to be dissolved easily. Furthermore, they showed Rf values quite different from those reported by SHIMI *et al.*[2]

KUMAR's compound and 2LL are similar in many aspects such as mp, elemental analysis, and IR spectra. KUMAR *et al.*, however, did not describe the result of MS and solubility sufficiently. Therefore, we could not conclude that they were totally the same compound.

None of compounds **2** showed any activity against the bacteria tested. This result was inconsistent with those of SHIMI *et al.*,[2] and even with those of KUMAR *et al.*[3] that cairomycin A was active against *Staphylococcus aureus* and *Escherichia coli*.

The physico-chemical and biological properties of cyclo(-L-Asp-L-Val-) obtained in this study differed from those of cairomycin A reported by SHIMI *et al.* and KUMAR *et al.* This suggests that cairomycin A has another structure which is different from the one proposed by SHIMI *et al.* The correct structure still remains unknown.

Experimental

All melting points are uncorrected. Rf values were determined on TLC plates (precoated with Merck silica gel 60 G) with the developing solvents such as Rf_1, *n*-BuOH - EtOH - H_2O (3 : 1 : 1): Rf_2,

chloroform - MeOH (9 : 1). Sulfuric acid and ninhydrin were used as visualizing reagents. Specific rotations were measured with a Union High Sensitivity Polarimeter PM-71. IR spectra were obtained with a Jasco FT/IR-7300 using KBr disk method. ^{1}H NMR spectrum was obtained with a Jeol FX-90Q spectrometer at 29°C, TMS being used as an internal standard. FAB-MS were obtained with a Jeol JMS DX-300 mass spectrometer.

H-D-Asp(OBzl)-OH,[4] Z-L-Asp(OBzl)-OH (**3**),[4] and H-D-Val-OMe[5] were prepared according to the standard methods, respectively, and characterized by their melting points and specific rotations. Their physico-chemical properties were in good agreement with those in the literature. Other reagents and solvents were commercial products of special grade.

The following experimental procedures are for the synthesis of 2LL, and the same procedure were applied to other three compounds **2**.

Z-Asp(OBzl)-Val-OMe (**5**)

This compound was synthesized according to the standard mixed-anhydride method by VAUGHAN *et al.*[6] A solution of **3** (1.79 g, 5 mmol) in THF (10 ml) and triethylamine (0.7 ml) was stirred vigorously at −15°C, and isobutyl chloroformate (0.65 ml) was added. Four minutes later a solution of **4** hydrochloride (0.84 g, 5 mmol) and triethylamine (0.7 ml) in dichloromethane (10 ml) was further added under vigorous stirring condition. The final solution was stirred for 1 hour below 0°C and overnight at room temperature. After evaporation, the residue was taken up in ethyl acetate (71.4 ml) and water (28.6 ml), washed with 2% HCl, 4% $NaHCO_3$ and water, successively, and dried over Na_2SO_4. After filtration and evaporation the residue was solidified from ether - petroleum ether. Yield 65%. mp 102~108°C (mp 110.5~112°C[8]). $[\alpha]_D$ +5.4° (*c* 1.0, chloroform).

H-Asp-Val-OMe

5 (0.47 g, 1 mmol) was dissolved in MeOH (6 ml) and hydrogen was passed through the solution in the presence of Pd-black with stirring. Pd-black was filtered off and the residue was solidified from ether - petroleum ether. Yield 75%. mp 135~137°C (mp 214~218°C[8]). $[\alpha]_D$ −7.0° (*c* 1.0, H_2O).

Cyclo(-Asp-Val-) (2LL)

Synthesis was carried out in a mixture of AcOH and *sec*-BuOH according to the method of SUZUKI *et al.*[7] H-Asp-Val-OMe (0.123 g, 0.5 mmol) was dissoved in 0.1 M AcOH - *sec*-BuOH (7.5 ml) and the solution was refluxed. After evaporation the residue was crystallized with ether - petroleum ether. Recrystallization was carried out from hot water. Yield 86%. Rf_1 0.36. Rf_2 0.00. mp 230~235°C. $[\alpha]_D$ −91.0° (*c* 1.0, DMF).

Antibacterial Activity Assay

The antibacterial activity assay was carried out by a dilution method using Mueller-Hinton agar (Difco) medium according to the literature.[9] About 5 μl of bacterial suspension containing 10^6 cells/ml was inoculated with a multiple inoculator onto agar plates containing 2-fold serial dilution of each peptide. The plates were incubated for 18 hours at 37°C, and the minimum concentration of the peptide that inhibited growth was noted.

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