HETEROCYCLES, Vol. 80, No. 1, 2010, pp. 281 - 288. © The Japan Institute of Heterocyclic Chemistry
Received, 17th February, 2009, Accepted, 11th March, 2009, Published online, 17th March, 2009
DOI: 10.3987/COM-09-S(S)11

M10709, A NEW CYCLIC PEPTIDE ANTIBIOTIC FROM CLINICALLY ISOLATED *STREPTOMYCES* SP.†

Takuya Kumamoto,[1] Hiroyuki Koshino,[2] Daisuke Watanabe,[1] Yuko Matsumoto,[3] Kazuki Aoyama,[3] Ken-ichi Harada,[4] Tsutomu Ishikawa,[1] and Yuzuru Mikami[3,*]

[1] Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan
[2] Advanced Science Institute, RIKEN, 2-1 Hirosawa, Wako 351-0198, Japan
[3] Medical Mycology Research Center (MMRC), Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan
[4] Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468-8503, Japan
e-mail: mikami@faculty.chiba-u.jp

Abstract – A new cyclic heptapeptide, M10709, was isolated from the mycelial cake of a clinically isolated *Streptomyces* sp. IFM 10709. The planar structure of M10709 was determined on the basis of IR, MS, NMR, and UV spectra. The absolute configuration of α-position of amino acid residues in the acid hydrolysate was determined by advanced Marfey's method to be L except that of tryptophan-derived amino acid. The compound showed antibacterial activity against *Micrococcus luteus* IFM 2066.

INTRODUCTION

New sources of bioactive metabolites such as marine microorganisms[1] and new microbes from different ecological niches have promoted recent advances in the discovery of new drugs.[2] However, such attention has never been paid to pathogenic microbes. We were interested in biologically active compounds produced by pathogenic microorganisms, especially from pathogenic actinomycetes[3] and fungi[4] because (i) pathogenic microorganisms should have unique competition mechanisms between

† This article is dedicated to Professor Akira Suzuki on the occasion of his 80th birthday.

pathogenic microbes and other microbes, plants, and animals, and (ii) pathogenic microbes should also have different metabolic pathways from those of non pathogenic microbes. During continuing studies on the search of biologically active compounds from clinical isolates of *Streptomyces* and *Nocardia* species,[5-7] one antimicrobial compound named M10709 (**1**) was isolated as a metabolite of the mycelial cake of a clinically isolated *Streptomyces* sp. IFM 10709.

RESULTS AND DISCUSSION

The physico-chemical properties of M10709 (**1**) are summarized in Table 1. It has molecular formula $C_{53}H_{78}N_8O_{10}$ confirmed by HRESIMS *m/z* 1009.5721 (calcd for $C_{53}H_{78}N_8NaO_{10}$: 1009.5739). In IR spectrum, absorption bands at 3600-3250 cm^{-1} and 1639 cm^{-1} suggested the presence of hydroxy, amide N-H and carbonyl groups, respectively. The existence of a hydroxy group was also supported from the result of FABMS; the characteristic fragment ion at 969 (MH^+-H_2O) was observed as a base peak. The ^{13}C NMR spectrum of **1** indicated the presence of thirteen methyls, four methylenes, twenty-two methines, and twelve quaternary carbons including seven amide carbonyl carbons (Table 2). Analysis of ^1H NMR spectrum (Table 2) by 2D DQFCOSY and TOCSY revealed the presence of five amide NH signals and two *N*-methyl signals. This evidence together with information of seven carbonyl carbons suggested that M10709 (**1**) is a heptapeptide. UV absorption at 221 and 283 nm suggested the existence of tryptophan or related indole ring system.[8] The physical properties and spectral data were compared with those of known cyclic peptide cyclomarin C (**2**) (Figure 1),[9-11] isolated as a metabolite of marine *Streptomyces* strain CNB-982 by Fenical and Clardy (Table 1, 2) and these compounds were found to have similar IR and UV spectra and NMR resonance except characteristic signals of 2-amino-3,5-dimethylhex-4-enoic acid (ADH) residue of cyclomarin C (**2**).

Careful interpretation of 2D NMR spectra including DQFCOSY, TOCSY, ^1H-^{13}C HSQC, ^1H-^{13}C HMBC,

Table 1. Physico-chemical properties of M10709 (**1**) and cyclomarin C (**2**)

Compound	M10709 (**1**)	cyclomarin C (**2**)[9]
Appearance	White powder	White solid
mp	158-163 °C	Not refered
[α]	(ORD) $[\alpha]_{589}^{24}$ -106.3, $[\alpha]_{510}^{24}$ -133.8, $[\alpha]_{436}^{24}$ -155.1 (c 1.9 x 10^{-2}, MeOH)	$[\alpha]_D^{20}$ -19.7 (*c* 1.0, $CHCl_3$)[10]
Molecular formula	$C_{53}H_{78}N_8O_{10}$	$C_{56}H_{82}N_8O_{10}$
HRMS (*m/z*, pos)	(HRESIMS)	(HRFABMS)
Found:	1009.5721	1049.6195
Calcd:	1009.5739 (for $C_{53}H_{78}N_8NaO_{10}$)	1049.6052 (for $C_{56}H_{82}N_8NaO_{10}$)
IR ν_{max} cm^{-1}	3600-3250, 1639	3400-3295, 1644
UV λ_{max} (ε) (MeOH)	221 (20,900), 283 (5,200)	220 (16,400)

Table 2. NMR data of M10709 (**1**) and cyclomarin C (**2**) (in $CDCl_3$)

		M10709 (**1**)				Cyclomarin C (**2**)[9]		
	no.	^{13}C	^{1}H	multi, *J* (Hz)	^{15}N	^{13}C	^{1}H	multi, *J* (Hz)
N-$Me_2C(CH{=}CH_2)$-	1	171.03				170.5		
β-OMe-Trp	2	53.98	4.70	dd, 5.1, 4.0		52.7	4.56	t, 4
	3	69.08	5.31	d, 5.1		68.4	5.30	d, 4.4
	4	123.22	7.27	s		123.0	7.30	s
	5	111.19				111.2		
	6	126.83				126.7		
	7	119.10	7.60	d, 7.6		118.6	7.49	d, 8
	8	119.58	7.05	dd, 7.6, 7.1		119.4	7.04	dd, 7, 7
	9	121.57	7.11	dd, 8.1, 7.1		121.4	7.18	dd, 8, 8
	10	114.36	7.49	d, 8.1		114.2	7.52	d, 8
	11	135.78				135.7		
	12	59.24				57.6		
	13	143.67	6.06	dd, 17.6, 11.1		143.6	6.06	dd, 17, 10
	14	113.82	5.22	d, 11.1		111.2	5.22	d, 10
			5.17	d, 17.6			5.17	d, 17
	15	27.81	1.69	s		24.9	1.56	s
	16	27.89	1.70	s		24.9	1.70	s
Val	17	172.38				172.5		
	18	58.99	4.05	dd, 9.5, 9.1		57.9	4.08	t, 5
	19	31.70	0.75	m		35.4	1.63	m
	20	18.49	0.64	d, 6.6		[a]	[a]	
	21	19.97	0.61	d, 6.6		18.4	0.64	d, 6.8
N-Me-Leu	22	168.45				168.3		
	23	58.83	4.78	br, d, 10.6		58.4	4.83	t, 10
	24	38.84	1.08	m		38.3	1.06	m
			2.27	ddd, 13.1, 10.6, 4.6			2.23	m
	25	25.10	1.47	m		25.6	1.42	m
	26	22.49	0.88	d, 6.6		23.3	0.82	d, 6.8
	27	23.51	0.91	d, 6.6		23.4	0.87	d, 6.3
Val	28	170.58				171.4		
	29	55.23	4.41	dd, 8.5, 8.5		55.2	4.36	t, 8.5
	30	30.79	2.21	m		30.7	2.20	m
	31	19.97	0.95	d, 6.6		18.6	1.05	d, 6.8
	32	19.27	1.06	d, 7.1		19.2	0.94	d, 6.5
β-OMe-Phe	33	169.73				169.5		
	34	55.83	4.86	dd, 5.5, 4.5		55.8	4.89	t, 5
	35	79.85	5.08	d, 5.5		79.8	5.07	d, 5
	36	134.97				135.0		
	37	127.99	7.18	m		[b]	[b]	
	38	128.11	7.24	m		[b]	[b]	
	39	128.63	7.24	m		[b]	[b]	
	40	57.77	3.36	s		57.6	3.36	s
Ala	41	171.52				171.5		
	42	50.81	4.78	dd, 4.3, 7.1		50.5	4.88	m
	43	20.83	1.22	d, 7.1		20.7	1.30	d, 7.3
N-Me-γ-OH-Leu	44	168.64				168.7		
	45	59.46	4.65	br, d, 9.6		59.2	4.78	m
	46	32.34	0.33	br, m		33.0	0.67	m
			2.18	br, m			2.28	m
	47	33.25	1.30	br, m		33.2	1.42	m
	48	66.18	3.12	br, dd, 10.5, 5.0		66.2	3.18	dd, 11, 5
			3.18	br, dd, 10.5, 3.0			3.23	dd, 11, 4
	49	17.68	0.63	br, d, 6.6		17.7	0.75	d, 6.8
	N-1				150.4			
	NH-2		7.24	d, 4.0	118.5		6.80	d, 3
	NH-3		8.17	d, 9.5	116.2		8.05	d, 10
	NH-5		8.03	br, d, 8.5	120.1		7.93	d, 8
	NH-6		7.10	br, d, 4.5	121.5		7.12	d, 5
	NH-7		8.48	d, 4.3	100.8		8.17	d, 10
	NMe-4	29.51	2.81	s	117.7	29.4	2.82	s
	NMe-8	29.28	2.66	br, s	120.2	29.2	2.71	s

[a] Resonaces on 124.7 ppm (for ^{13}C); 4.78 ppm (for ^{1}H, d, J = 10 Hz) for CH=, and 19.9, 27.7 ppm (for ^{13}C); 1.24, 1.27 ppm (for ^{1}H) for 2 x Me were observed. [b] Assigned as 127-128 ppm and 7.24-.26 ppm for ^{13}C and ^{1}H NMR, respectively.

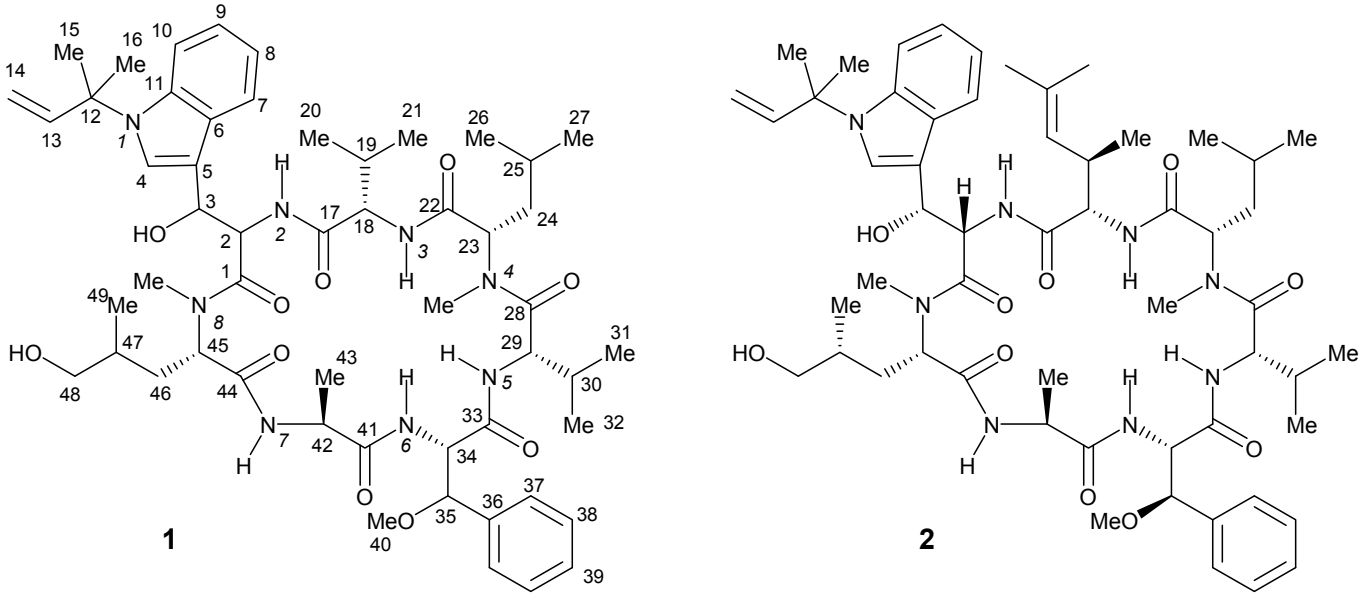

Figure 1. Structures of M10709 (**1**) and cyclomarin C (**2**)

and ^{1}H-^{15}N HSQC revealed that the seven amino acid residues are *N*-(1,1-dimethylallyl)tryptophan, *N*-methyl-γ-hydroxyleucine, alanine, β-methoxyphenylalanine, *N*-methylleucine, and two valines. Six amino acid residues except for one valine are same components as those of cyclomarin C. Sequential assignments of seven amino acid residues of **1** were performed by analyses of ^{1}H-^{13}C and ^{1}H-^{15}N HMBC (Figure 2). Connectivity between *N*-(1,1-dimethylallyl)tryptophan and one valine residue was confirmed by NOE between H-7 of indole ring portion and Me-20 of the valine based on NOESY data (Figure 3). Other H-C connectivity was determined by ^{1}H-^{13}C long range correlations of HMBC

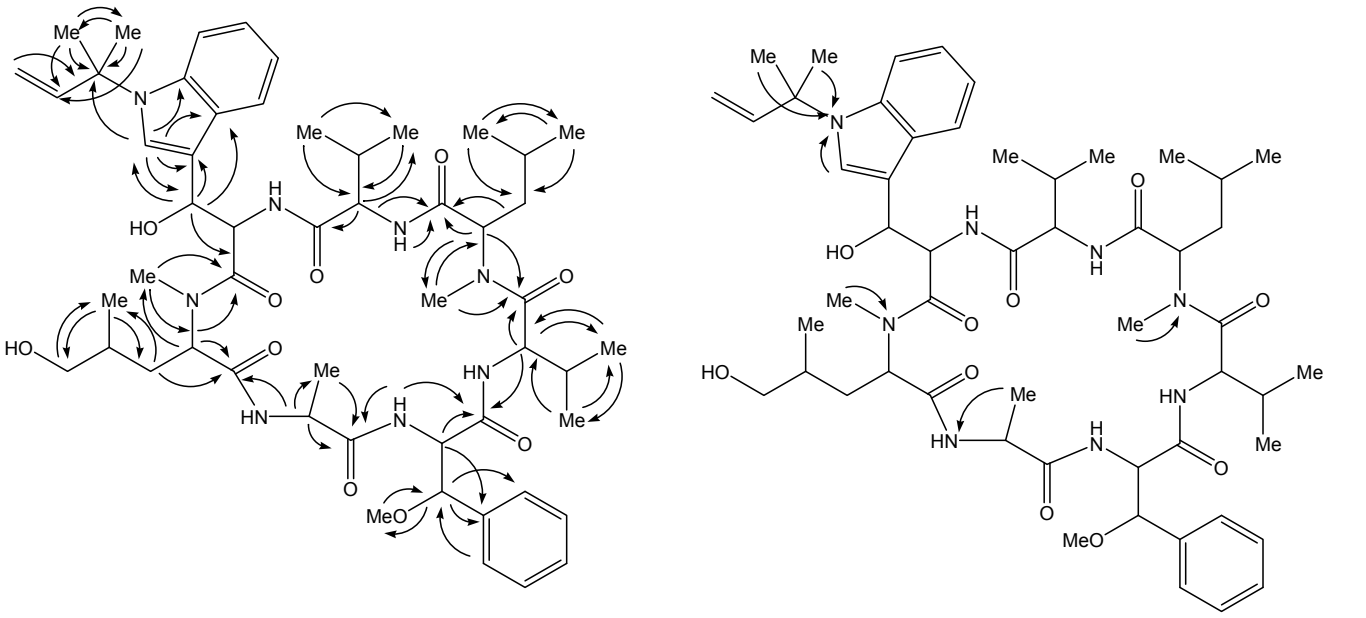

Figure 2. ^{1}H-^{13}C (left, selected) and ^{1}H-^{15}N (right) HMBC Correlations of M10709 (**1**)

spectrum. Presence of reverse *N*-prenylated tryptophan and two *N*-methylated amino acid residues were supported by ^{1}H-^{15}N HMBC data (Figure 2).

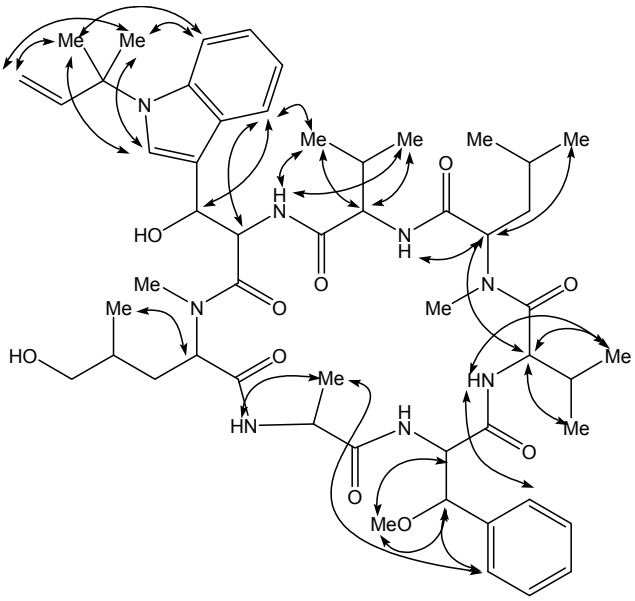

Figure 3. NOESY Correlation of M10709 (**1**)

For the confirmation of the stereochemistry on M10709 (**1**), advanced Marfey's method utilizing both enantiomers of 1-fluoro-2,4-dinitrophenyl-5-leucinamide (FDLA) was applied.[12,13] Acid hydrolysate of M10709 (**1**) was treated with D- and L-FDLA, respectively, and the each mixture of DLA derivatives of amino acids was subjected to LC/MS. Among the amino acids assigned by NMR analysis, five amino acids, *i.g.* alanine, β-methoxyphenylalanine, *N*-methyl-γ-hydroxyleucine, *N*-methylleucine, and valine, were detected as the corresponding DLA derivatives with the correct molecular weight. Tryptophan derivative was not detected provably due to the lability of tryptophan and/or 1,1-dimethylallyl parts in acidic condition. As to the determination of absolute configuration, the L-DLA derivatives of L-amino acids generally shows shorter retention time in HPLC analysis than corresponding D-DLA derivatives.[12] As a result, peaks derived from five detectable L-DLA derivatives were observed in shorter retention time than those of the corresponding D-derivatives. Thus, the absolute configurations of α-position of these amino acids were determined as L.[14] Even though some differences were observed on the chemical shifts on ^{1}H NMR [H-2, H-7, and H-15 on *N*-(1,1-dimethylallyl)tryptophan residue; H-45, H-46, H-47, and NMe-8 on *N*-methyl-γ-hydroxyleucine], they showed quite similar NMR data, implying that M10709 (**1**) could have the same stereochemistry as that of cyclomarin C (**2**).

In vitro antibacterial activities of M10709 (**1**) are shown in Table 3. The compound showed antibacterial activity only against *Micrococcus luteus* IFM 2066, and its MIC value was 5.0 μg/mL. The

compound did not show antibacterial activities against other gram-positive and negative bacteria such as *Staphylococcus aureus* and *Escherichia coli*. M10709 (**1**) did not show antifungal activities against *Candida albicans* and *Aspergillus niger*.

Table 3. *In vitro* antimicrobial activity of M10709 (**1**)

Test organisms	MIC values (μg/mL)
Micrococcus luteus IFM 2066	5
Staphylococcus aureus 209P	> 80[b]
Bacillus subtilis PCI 219	> 80
Escherichia coli NIH JC-2	> 80
Candida albicans ATCC 90029	> 80
Aspergillus niger IFM 5368	> 80
Trichophyton mentagrophytes IFM 40092	> 80

[a] Partial inhibition was observed.

EXPERIMENTAL

Methods

Melting point was measured on a micro melting-point hot stage (Yanaco) and uncorrected. IR spectrum was recorded on a JASCO Infrared spectrophotometer FT/IR-300E. NMR spectra were recorded on a JEOL ECA-600 spectrometer at 600 and 150 MHz for ^{1}H and ^{13}C, respectively. UV spectra were recorded on a JASCO UV/VIS spectrophotometer V-530. ESIMS was recorded on a Shimadzu LCMS-2010EV mass spectrometer with ESI/APCI Dual source. FABMS was recorded on a JEOL JMS-SX102 mass spectrometer. Acid hydrolysis (reaction time: 9 h) and conversion of the hydrolysate to the corresponding DLA derivatives were carried out according to the reported procedure.[12,13] The separation of D- and L-DLA derivatives of amino acids was performed using an Agilent Model 1100 liquid chromatograph system. The separations were carried out on a YMC-Pack Pro C18 (100 × 4.6 mm i.d., 3 μm, YMC) column at 40 °C. Acetonitrile (MeCN)–water containing 5 mM of ammonium formate and 5 mM of formic acid was used as the mobile phase under a linear gradient elution mode (MeCN, 30 %-60 %, 20 min) at a flow rate of 1.0 mL/min, with UV detection at 340 nm and 210-600 nm by photodiode array detection. The ESIMS for LC/MS of Marfey's method were recorded on an Agilent LC/MSD mass spectrometer using a Model 1100 HPLC system. Peaks of each DLA derivatives were detected on the following retention time (D- and L-DLA derivatives, respectively, unit: min): *N*-methyl-γ-hydroxyleucine (7.5, 6.9), alanine (7.8, 5.4), β-methoxyphenylalanine (13.5, 8.8), *N*-methylleucine (15.6, 13.2), valine

(12.4, 7.6), *N*-(1,1-dimethylallyl)tryptophan (not detected). ORD was recorded on a JASCO J-720WI spectropolarimeter.

Taxonomic position of producing strain

Chemotaxonomic studies[15] on *Streptomyces* sp. IFM 10709 strain confirmed that it belongs to a genus *Streptomyces*. Sequence analysis studies of 16S rRNA gene showed that *Streptomyces* sp. IFM 10709 strain showed high similarity to *Streptomyces rectiverticillatus* NRBC 13709 with the similarity value of 99.85%.

Fermentation and isolation of active compound

The seed broth was prepared by inoculating mycelial elements of the producing strain (*Streptomyces* sp. IFM 10709, a clinical isolate from a stomach of a 85 years old patient with stomach cancer in Japan) grown on Brain heart infusion (BHI) agar (Difco, Detroit) in 10 mL of BHI broth with 2% glucose in 50-mL Erlenmeyer shake flask. The culture was incubated on a rotary shaker at 250 rpm for 96 h. Ten percent inoculum was transferred to a 500-mL Erlenmeyer flask containing 150-mL of the production medium composed of meat extract 0.5%, peptone 0.5%, glucose 1%, starch 1.0% and antifoam 0.005%. The pH was adjusted to 7.4, and the culture was incubated on a rotary shaker at 250 rpm for 6 days. After the incubation, the broth was filtered and mycelial cake was extracted with MeOH. The broth and the mycelial extract were combined and concentrated *in vacuo*. The crude (2.07 g) was dissolved in H_2O (300 mL) and was extracted with AcOEt (1 x 900 mL). The organic layer was evaporated and the residue was suspended in H_2O - MeCN, 1 : 1 (v/v). The mixture was centrifuged (3000 rpm, 10 min) and the supernatant was concentrated *in vacuo* and subjected to preparative HPLC [column: nacalaitesque, Cosmosil 5C_{18}-AR-II, 250 x 10 mm i.d., eluent: H_2O - MeCN, 40 : 60 (v/v), 4.0 mL/min] to give pale pink powder (1.4 mg), which was washed with hexane - AcOEt, 10 : 1 (v/v) to give M10709 (**1**) as colorless powder (1.2 mg).

Antimicrobial activities

Antimicrobial activities of the compound were determined by microbroth dilution method using BHI broth for bacteria and Sabouraud dextrose broth (Difco, Detroit) for fungi, respectively.

ACKNOWLEDGEMENT

Ms. Yuriko Nozawa, Taisho Pharmaceutical Co. Ltd., was acknowledged for performing the advanced Marfey's method.

REFERENCES AND NOTES

1. G. M. Konig and A. D. Wright, "Trends in marine biotechnology", in 'Drug discovery from nature', ed. by G. Grabley and R. Thiericke, Springer, Berlin, 1999, pp. 180-185.

2. U. Graefe and Y. Mikami, "Novel antibacterial drugs from microorganisms", in 'Drug discovery from nature', ed. by G. Grabley and R. Thiericke, Springer, Berlin, 1999, pp. 281-296.
3. Y. Mikami, *Actinomycetologica*, 2007, **21**, 46.
4. S. Nakadate, K. Nozawa, H. Horie, Y. Fujii, M. Nagai, T. Hosoe, K. Kawai, T. Yaguchi, and K. Fukushima, *J. Nat. Prod.*, 2007, **70**, 1510.
5. H. Shigemori, H. Komaki, K. Yazawa, Y. Mikami, A. Nemoto, Y. Tanaka, T. Sasaki, Y. In, T. Ishida, and J. Kobayashi, *J. Org. Chem.*, 1998, **63**, 6900.
6. A. Nemoto, Y. Hoshino, K. Yazawa, A. Ando, Y. Mikami, H. Komaki, Y. Tanaka, and U. Grafe, *J. Antibiot*., 2002, **55**, 593.
7. A. Nemoto, Y. Tanaka, Y. Karasaki, H. Komaki, K. Yazawa, Y. Mikami, T. Tojo, K. Kadowaki, M. Tsuda, and J. Kobayashi, *J. Antibiot.*, 1997, **50**, 18.
8. E. Pretsch, W. Simon, J. Sebl, and T. Clerc 'Tables of spectral data for structure determination of organic compounds', Springer, Berlin, 1989.
9. M. K. Renner, Y.-C. Shen, X.-C. Cheng, P. R. Jensen, W. Frankmoelle, C. A. Kauffman, W. Fenical, E. Lobkovsky, and J. Clardy, *J. Am. Chem. Soc.*, 1999, **121**, 11273.
10. For the total synthesis of cyclomarin C: a) S.-J. Wen, T.-S. Hu, and Z.-J. Yao, *Tetrahedron*, 2005, **61**, 4931; b) S.-J. Wen and Z.-J. Yao, *Org. Lett.*, 2004, **6**, 2721. The optical rotation of the synthetic samples ($[\alpha]_D$ -72.8) was reported to be higher than that of the natural material.
11. For the biosynthesis of cyclomarins and isolation of cyclomarin D from the marine bacterium *Salinispora arenicola* CNS-205: A. W. Schultz, D.-C. Oh, J. R. Carney, R. T. Williamson, D. W. Udwary, P. R. Jensen, S. J. Gould, W. Fenical, and B. S. Moore, *J. Am. Chem. Soc.*, 2008, **130**, 4507.
12. K. Fujii, Y. Ikai, T. Mayumi, H. Oka, M. Suzuki, and K.-i. Harada, *Anal. Chem.*, 1997, **69**, 3346.
13. K. Fujii and K.-i. Harada, *Anal. Chem.*, 1997, **69**, 5146.
14. Partial epimerization of *N*-methylleucine (L : D = *ca.* 6 : 4) during the acid hydrolysis was observed, even in the shorter period for hydrolysis (30 min).
15. S. Miyadoh, "Identification procedure at the genus level", in 'Identification manual for actinomycetes', eds. by S. Miyadoh *et al.*, Business Center for Academic Societies, Tokyo, 2001, pp. 9-19.