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Natural Products

Structural Revision of Natural Cyclic Depsipeptide MA026 Established by Total Synthesis and Biosynthetic Gene Cluster Analysis

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Abstract: *A revised structure of natural 14-mer cyclic depsipeptide MA026, isolated from Pseudomonas sp. RtlB026 in 2002 was established by physicochemical analysis with HPLC, MS/MS, and NMR and confirmed by total solid-phase synthesis. The revised structure differs from that previously reported in that two amino acid residues, assigned in error, have been replaced. Synthesized MA026 with the revised structure showed a tight junction (TJ) opening activity like that of the natural one in a cell-based TJ opening assay. Bioinformatic analysis of the putative MA026 biosynthetic gene cluster (BGC) of RtIB026 demonstrated that the stereochemistry of each amino acid residue in the revised structure can be reasonably explained. Phylogenetic analysis with xantholysin BGC indicates an exceptionally high homology (ca. 90 %) between xantholysin and MA026. The TJ opening activity of MA026 when binding to claudin-1 is a key to new avenues for transdermal administration of large hydrophilic biologics.*

It is known that the infectious hematopoietic necrosis virus (IHNV) causes infection and killing of salmon and trout, resulting in a significant influence on the revenue of the salmon farming industry.[1] By screening effective anti-IHNV compounds, MA026 was isolated in 2002 from *Pseudomonas* sp. RtlB026 which exists in the digestive tract of rainbow trout, and its structure was determined by amino acid, MS/MS and NMR analysis[2] to be a 14-mer cyclic depsipeptide (**1**) containing several D-amino acid residues and an acyl tail at the N-terminus (Figure 1). *Pseudomonas* spp. have also been reported to produce many other cyclic lipodepsipeptides with diverse biological activities (Supporting Information, Table S1).[3–7] Among them, a 14-mer cyclic depsipeptide, xantholysin A (Figure 1), which is an anti-microbial product

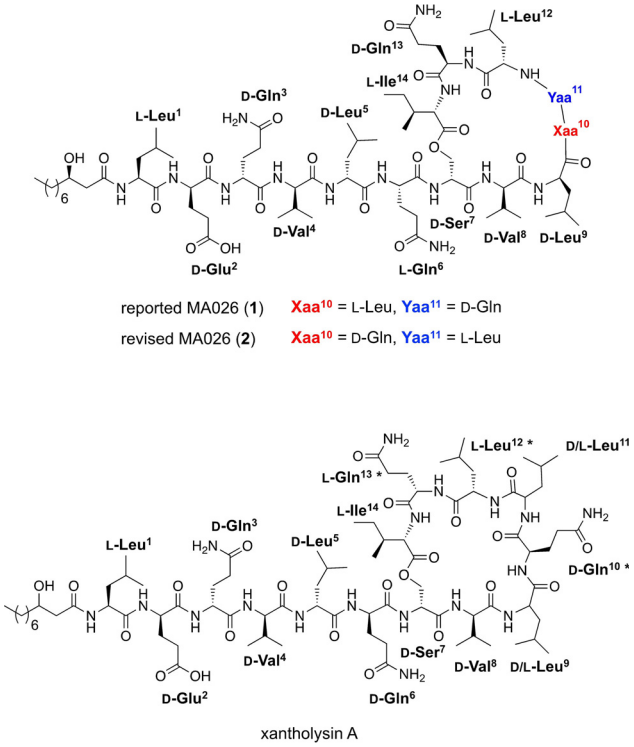

Figure 1. Reported (**1**)[2,9] and revised (**2**) structures of MA026 and the structure of xantholysin A.[3,4] *Stereochemistry of α-carbons is deduced from in silico experiments.[4]

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isolated in 2013 from *Pseudomonas putida* BW11M1 by Li et al.[3] and is known to show broad biological activities,[3,4,8] has a high similarity to MA026 in the amino acid sequence. A total solution-phase synthesis of MA026 and analysis of its anti-hepatitis C viral activity by binding to the TJ membrane protein claudin-1 were reported in 2013 by Shimura et al.[9] Kanda et al. reported in 2017 that natural MA026 has a tight junction (TJ) opening activity.[10]

The TJ is a continuous intercellular barrier between epithelial cells that separates the internal and external environments of cellular sheets, and controls invasion of foreign substances and a diffusion of solutes and water across the epithelium.[11] The TJ is composed of a multi-protein complex including several transmembrane proteins, such as claudins, occludin and several junctional adhesion molecules. Among these bio-molecules, claudins found in at least 23 kinds of proteins in human are key regulators of paracellular permeability.[11] Claudin-1 in particular, is highly expressed in skin and plays an essential role in the formation of an impermeable epidermal barrier.

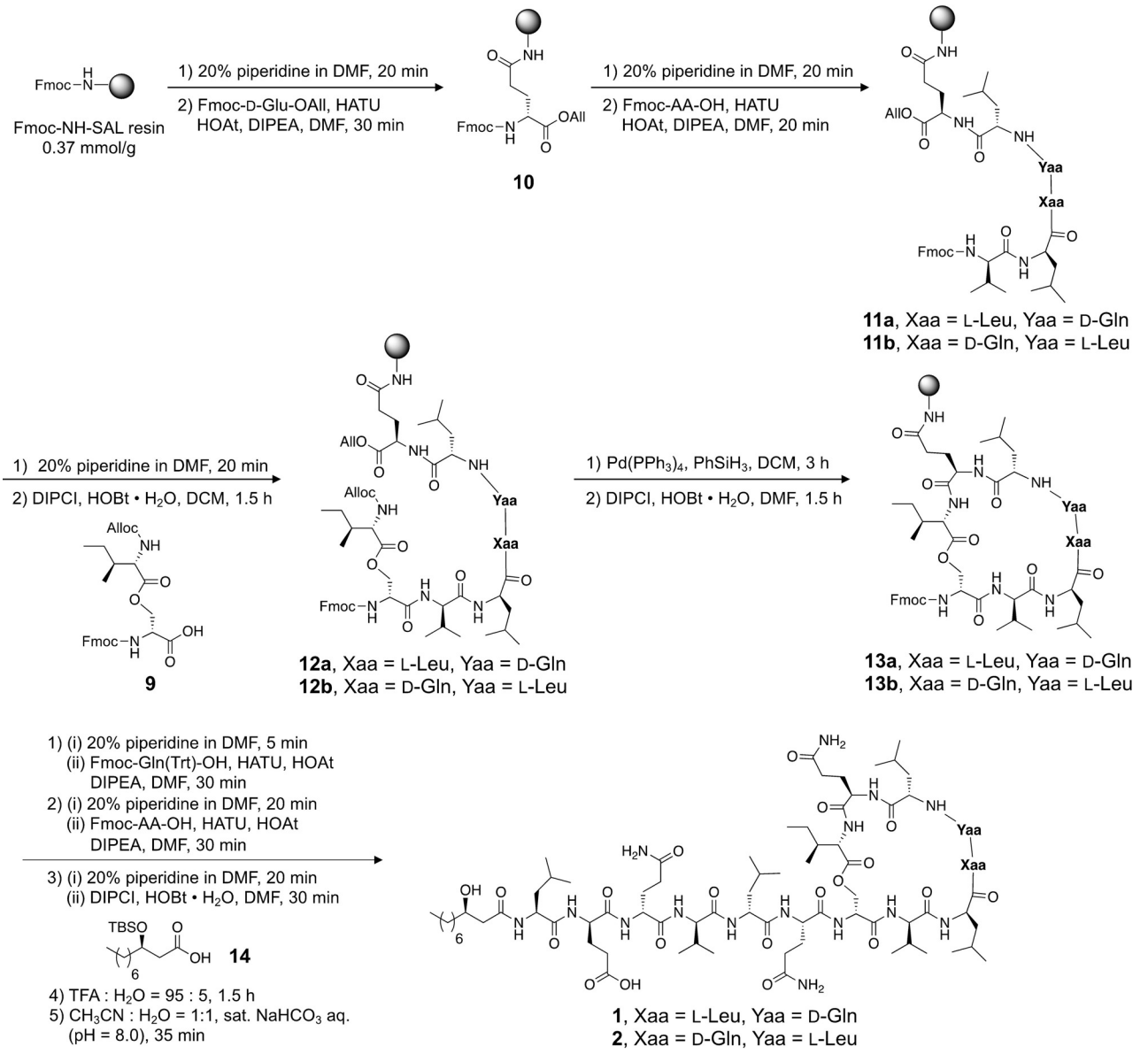
Oral administration of peptide/protein drugs encounters several problems.[12] With their high molecular weight and hydrophilicity, such drugs are unable to diffuse through the lipid bilayer of the cell membrane, and to solve this problem, paracellular delivery, particularly transdermal absorption, has received considerable attention.[13] Control of the TJ opening by attenuating the function of claudin-1 has proved to be attractive because it could allow safe and controllable transdermal administration of peptides or proteins and many other different hydrophilic macromolecular drugs.

In an effort to develop safe and effective paracellular permeability enhancers leading to improved drug bioavailability from skin, we studied the TJ opener MA026. Although we started a structure–activity relationship (SAR) study of MA026, physicochemical data of the material synthesized based on the reported structure were different from those of natural MA026 and failed to show any TJ opening activity. This led us to reconsider the structure of MA026 with further synthetic study and physicochemical analyses of **1** and its analogues, coupled with the latest phylogenetic analysis of nonribosomal peptide synthetase (NRPS) of microorganisms. In the new synthesis, the aim of which was the development of a medical tool from MA026, we developed an efficient solid-phase synthetic method, in which the complete cyclic peptide structure was built on the resin. In the phylogenetic analysis, virtual identification of natural cyclic peptides was used as a complementary method providing a rationale to the chemistry-based structure determination. This was also validated by a gene analysis of putative MA026 biosynthetic gene cluster (BGC) derived from the whole genome sequencing of RtIB026.

The first reported total synthesis of the published structure of MA026 was performed in the solution phase[2,9] and its solid-phase peptide synthesis (SPPS), which is an efficient method for the synthesis of various derivatives, was never reported. Generally, cyclization reactions in the solution phase synthesis must be carried out under high dilution conditions to prevent dimer formation.[14] In solid-phase synthesis, such concerns can be reduced because pseudo dilution conditions occur naturally on the solid support.[14] Our efficient solid-phase synthetic method features construction of the entire cyclic depsipeptide structure on the solid-support and subsequent one-shot deprotection. For the cyclization of the depsipeptide unit on the resin, an orthogonal protection strategy with allyl chemistry was adopted by use of a Pd^0-catalyzed deprotection method.[15] The planned SPPS of MA026 was expected to facilitate the efficient generation of a variety of derivatives to support an SAR study.

A retrosynthetic analysis of MA026 based on the standard Fmoc/*t*Bu-based SPPS was conducted (Supporting Information, Scheme S1). A key in this synthesis is construction of the cyclic depsipeptide unit, that is, a macrolide containing an ester bond, while a section containing a linear peptide chain (Leu1 to Glu6) with an N-terminal (*R*)-3-hydroxydecanoyl group can be constructed using conventional SPPS. Cyclization at the ester bond on the resin could reduce total yield and cause epimerization at Ile14. Because of this, the application of a dipeptide unit (Fmoc-D-Ser(Alloc-Ile)-OH, **9**), in which Ile14 is esterified with the β-hydroxy group of D-Ser7, was planned. In this analysis, the amide bond between Ile14 and D-Gln13 was disconnected and the amino group of Ile14 and the carboxy group of D-Gln13 were protected by an Alloc group and an allyl ester, respectively. This allows the loading of Fmoc-D-Glu-OAll onto the Fmoc-NH-SAL resin via the γ-amide bonding, leading to a conventional and sequential amide bond formation on the resin starting from D-Gln13.

The synthesis of the authentic structure of **1** and its analogues is depicted in Scheme 1. Briefly, in the conventional SPPS, Fmoc-deprotection was performed with 20% piperidine in DMF, and coupling of each Fmoc-protected amino acid was performed in DMF using the HATU/HOAt/DIPEA method.[16] The solid-supported D-Gln (**10**) was prepared by the coupling of Fmoc-D-Glu-OAll to the Fmoc-NH-SAL resin after Fmoc-deprotection (Scheme 1). After constructing the protected hexapeptide-resin (**11a**) and subsequent deprotection of its Fmoc group, the dipeptide unit Fmoc-D-Ser(Alloc-Ile)-OH (**9**; Supporting Information, Scheme S2) was coupled using the DIPCI/HOBt method[17] to obtain the resin-based depsipeptide (**12a**). The Alloc group and allyl ester of **12a** were removed in the presence of $Pd(PPh_3)_4$ and $PhSiH_3$[15] (Supporting Information, Figure S1), and on-resin cyclization was performed using the DIPCI/HOBt method to obtain the cyclic depsipeptide-resin (**13a**). HPLC and MS analysis (Supporting Information, Figure S2) revealed no significant level of dimerization. In the subsequent Fmoc deprotection of **13a** using 20% piperidine/DMF, an expected *O*-*N* intramolecular acyl migration[18] at D-Ser7 was effectively suppressed to 5.2% by reducing the piperidine-treatment time to 5 min (Supporting Information, Figure S3). Further elongation of the peptide chain from **13a** and addition of the carboxylic acid (**14**)[9] with the conventional SPPS gave a protected form of **1** on the resin. Final one-shot deprotection and cleavage of peptides from the resin with aqueous TFA (95%) afforded the crude peptide (**1**). Subsequent final HPLC purification gave **1** (RT = 26.8 min) in a yield of 15% with purity of > 99%. Peptide **2** was also synthesized in a yield of 11% from the resin (purity 98%)



Scheme 1. Solid-phase synthesis of MA026 and its analogues. HATU: *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt: 1-hydroxy-7-azatriazole, DIPEA: diisopropylethylamine, DIPCI: *N*,*N*′-diisopropylcarbodiimide, HOBt: 1-hydroxybenzotriazole.

with a protocol similar to that used for peptide **1**. These results suggest that the proposed on-resin synthetic route effectively tolerates diversity and could be applicable to other cyclic depsipeptides in the future. Details of the syntheses are described in the Supporting Information.

The HPLC retention time (RT) (26.8 min) of **1**, whose synthesis was based on the reported structure was different from that of natural MA026 (32.6 min; Figure 2A), although HR-MS analyses indicated that **1** and natural MA026 have the same molecular weight and a molecular formula of $C_{84}H_{146}N_{18}O_{23}$. In addition, the ^{1}H NMR data of **1** were also different from that of natural MA026 (Supporting Information, Figure S4). For example, natural MA026 showed peaks around 3.8 and 1.1 ppm which were absent from the NMR spectra of the synthetic **1**. Moreover, the synthetic **1** exhibited no TJ-opening activity in a transport assay (Supporting Information, Figure S5). These results suggest that the structure of MA026 is different from that of the reported peptide **1**. We synthesized a linear peptide (**15**) based on the reported structure **1**, but without cyclization. Hydrolysis of natural MA026 at the ester was also performed to produce a linear peptide (**16**). The comparison of HPLC RT of these linear peptides revealed that peptides **15** and **16** are not identical (Figure 2B). From these results, it was concluded that the amino acid sequence or stereochemistry of MA026 is different from that reported previously.

To confirm the amino acid sequence of natural MA026, we carried out an MS/MS analysis of the hydrolysate (**16**) derived from natural MA026 (Supporting Information, Figure S6). As a result, the detected y4 and b10 ions, corresponding to Leu11-Ile14 and Leu1-Gln10 ion fragments, respectively, indicated the existence of a Gln10-Leu11

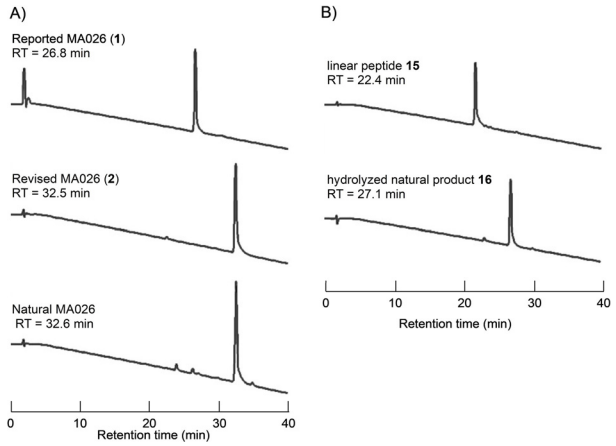
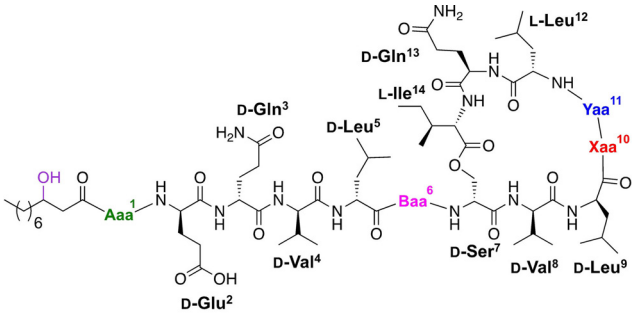


Figure 2. HPLC charts of A) reported MA026 (**1**), revised MA026 (**2**) and natural MA026, and B) synthetic linear peptide **15** and hydrolyzed natural product **16**. HPLC conditions: a linear gradient starting from 30 to 70% CH_3CN in aqueous TFA over 40 min at flow rate of 1.0 mL min^{-1}, UV: 220 nm.



	peptide	3-OH	Aaa^1	Baa^6	Xaa^{10}	Yaa^{11}
reported structure	**1**	*R*	L-Leu	L-Gln	L-Leu	D-Gln
	1'	*S*	L-Leu	L-Gln	L-Leu	D-Gln
revised structure	**2**	*R*	L-Leu	L-Gln	D-Gln	L-Leu
	2'	*S*	L-Leu	L-Gln	D-Gln	L-Leu
	3	*R*	L-Leu	L-Gln	L-Gln	L-Leu
	4	*R*	L-Leu	L-Gln	L-Gln	D-Leu
	5	*R*	L-Leu	L-Gln	D-Gln	D-Leu
	6	*R*	D-Leu	L-Gln	D-Gln	L-Leu
	7	*R*	L-Leu	D-Gln	D-Gln	L-Leu
	8	*R*	D-Leu	D-Gln	D-Gln	L-Leu

Figure 3. Reported (**1**)[2,9] and revised (**2**) structures of MA026 and the structures of MA026 derivatives (**1'**, **2'** and **3**–**8**).

sequence in **16**, which differs from that reported (L-Leu10-D-Gln11). These results reveal that the amino acid sequence is reversed at the positions 10 and 11 of MA026.

Therefore, a cyclic depsipeptide (**2**) with a D-Gln10-L-Leu11 sequence (Figure 1) was synthesized using the method described above (Scheme 1). We found that the HPLC RT of **2** (32.5 min) is the same as that of natural MA026 (Figure 2 A; Supporting Information, Figure S8A). Furthermore, ^{1}H and ^{13}C NMR data of **2** showed good agreement with those of natural MA026 (Supporting Information, Figure S7, Tables S2 and S3). Furthermore, we synthesized three stereoisomers (**3**–**5**) with sequences L-Gln10-L-Leu11, L-Gln10-D-Leu11 and D-Gln10-D-Leu11, respectively (Figure 3) using the same route that was used for peptide **1**. In HPLC analysis, these three peptides showed RTs different from that of natural MA026 (Supporting Information, Figure S8B), and it was concluded that the structure of **2** is a correct structure for natural MA026.

From this structural revision of MA026, it became apparent that xantholysin A has the same amino acid sequence as revised MA026. Recently, its D/L configuration of each amino acid residue has been partially reported,[4] but some differences to our revised structure remain. To reveal whether or not MA026 and xantholysin A are the same compound, we determined the whole genome sequence of *Pseudomonas* sp. RtlB026. The putative BGC of MA026 encodes the nonribosomal peptide synthetase (NRPS) which has 14 modules, and terminal tandem thioesterase (TE) domains that are frequently observed in BGCs of other cyclic lipodepsipeptides[19] and has been suggested to be involved in productivity enhancement[20,21] (Figure 4). All modules except for modules 1, 12 and 13 contained condensation/epimerization (C/E), adenylation (A), and thiolation (T) domains. The modules 1, 12, and 13 have condensation (C) domains instead of a C/E domain. The predicted substrate specificity of the A domains by antiSMASH techniques[22] showed that the amino acid sequence of MA026 is Glu10-Leu11, identical with that in the xantholysins.[3,4] Furthermore, the NRPSs encoded in the BGC of MA026 have more than 90% sequence identity with those encoded in the BGC of xantholysin (Supporting Information, Table S4). Phylogenetic analysis of NRPS domains revealed the corresponding A, C, C/E, and TE domains of MA026 and xantholysin NRPSs are clustered into same clades (Supporting Information, Figures S9–S11). These results strongly suggest that MA026 produced by *Pseudomonas* sp. RtI026 is identical to xantholysin A. Molina-Santiago et al. however, reported that xantholysin A contains D-Gln6 and L-Gln13,[4] while revised MA026 (**2**) should have L-Gln6 and D-Gln13 from our analysis. Therefore, it is likely that the two natural products are identical but impossible to prove at this moment.

From the position of the C/E domains of NRPSs, there were two residues whose predicted stereochemistries were not consistent with the structure of MA026 (**2**); the stereochemistries of Leu1 and Gln6 were predicted to be D while our synthetic MA026 (**2**) has L. Therefore, we further synthesized 3 diastereomers of MA026; D-Leu1/L-Gln6 (**6**), L-Leu1/D-Gln6 (**7**), and D-Leu1/D-Gln6 (**8**) (Figure 3) and compared with natural MA026. However, the RTs of these compounds were different from that of natural MA026 (Supporting Information, Figure S12). Taken together with absolute configuration of Leu and Gln/Glu from hydrolysate of natural MA026 (Supporting Information, Figure S13), we concluded that MA026 contains L-Leu1/L-Gln6. When we analyzed HHI/LxxxxGD motif, which is conserved in C/E domains but not in C domains,[23,24] it is not precisely conserved in modules 2 and 7 (Supporting Information, Table S5). This observation indicated that epimerase activities are probably eliminated in these domains and suggests

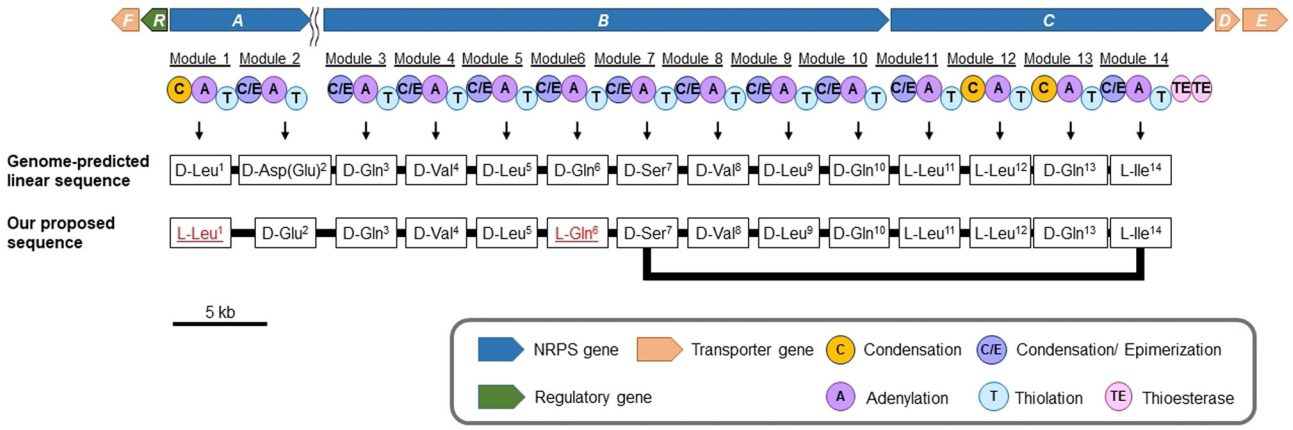


Figure 4. MA026 biosynthetic gene cluster of *Pseudomonas* sp. RtIB026. The organization of the genomic region with the MA026 synthetase genes (*A*, *B*, and *C*), the associated regulatory gene (*R*) and putative transporter genes (*D*, *E*, and *F*) is shown (GenBank accession numbers: AP023348).

that the amino acid changes in consensus sequence, His to Leu of first amino acid residue, His to Tyr of second amino acid residue, or deletion, lead the loss of epimerase activity. Interestingly, xantholysin biosynthetic gene clusters still retain epimerase activity although the consensus sequence of the C/E domain of module 7 is not conserved (Supporting Information, Table S5).

Finally, we evaluated the TJ opening activity of revised MA026 (**2**) with transport assay using FD4 (FITC-dextran 4 kDa).[10,13] In this assay, the fluorescence intensity from FITC is measured in the transmembrane part and recognized as the TJ opening activity. As shown in Figure 5, revised MA026 (**2**) efficiently transports FD4 similarly to natural MA026, but the reported MA026 (**1**) and its epimer at C3 position of N-terminal acyl tail (**1′** in the Supporting Information, Figures S5 and S14) failed to do so. The revised MA026 (**2**) induced the time-dependent increase of cumulative fluorescence intensity and the activity was equivalent to that of the natural product (Supporting Information, Figures S14 and S15). Furthermore, an epimer (**2′**) of the revised

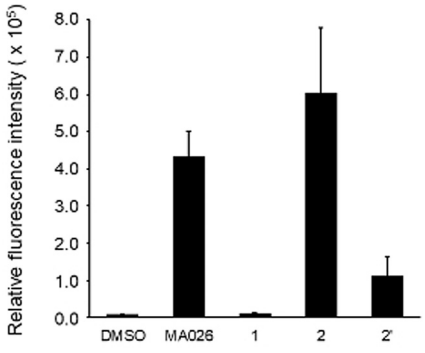


Figure 5. Transport assay of revised MA026 (**2**) and its epimer at C3 position of N-terminal acyl tail (**2′**), reported MA026 (**1**), and natural product (MA026) (each 3 μM). Vehicle: DMSO, the MDCK II monolayer was treated with each compound for 30 min, and fluorescence intensity of transported FD4 was measured. Triplicate. Values were mean ± standard deviation ($n=3$).

MA026 in the N-terminal acyl section had drastically decreased transport activity, suggesting the importance of the stereochemistry of the hydroxyl group at the N-terminal acyl group. On the other hand, two epimers of MA026; D-Leu1/L-Gln6 (**6**) and L-Leu1/D-Gln6 (**7**) showed more potent transport activity, but the transport activity of D-Leu1/D-Gln6 (**8**) resembled that of **2** (Supporting Information, Figure S16). The epimers **6** and **7** could be seed compounds to obtain a peptide that is more potent than natural MA026.

In conclusion, we successfully achieved an Fmoc-based SPPS of MA026 in which the whole structure of MA026 was constructed on the resin. This synthesis allowed the efficient preparation of various MA026 derivatives. With a synthetic study, we determined the revised structure (**2**) of MA026 based on the evidence from HPLC, NMR and MS(/MS) analyses and a TJ opening activity assay. BGC's genomic analysis, which serves to identify the structure of this natural cyclic peptide, worked very well and agreed with the physicochemical analyses. The revised structure contains D-Gln10-L-Leu11 which is different from the reported structure (**1**). **1** has no transport activity, but diastereomers of **2** (**2′** and **6**–**8**) showed transport activity (Figures 5 and S16). These results suggest that the stereochemistry of each amino acid residue, at least those in side chains, is not important for transport activity compared to amino acid sequence. Detailed SAR study of MA026 is in progress and SAR information will contribute to the development of efficient drug administration methods using MA026 analogues.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: bioinformatics · MA026 · natural products · structure elucidation · xantholysin A

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