Structure of Cymbidine A, a Monomeric Peptidoglycan-Related Compound with Hypotensive and Diuretic Activities, Isolated from a Higher Plant, *Cymbidium goeringii* (Orchidaceae)

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The structure of a new monomeric peptidoglycan-related compound with hypotensive and diuretic activities, cymbidine A (1) isolated from the orchid *Cymbidium goeringii*, was elucidated mainly by spectroscopic analysis. The structure of 1 was shown to involve four amino acids (D-alanin, *meso*-diaminopimelic acid, D-gultamic acid, and L-valine) and two amino sugars (*N*-acetylglucosamine and 1,6-anhydro-*N*-acetylmuramic acid). The sequence of the amino acids and amino sugars was determined by the analysis of 2D NMR data. The absolute stereochemistries of the three amino acids (D-Ala, D-Glu and L-Val) were determined by the modified Marfey's method, and the (6*S*,10*R*) configurations of *meso*-diaminopimelic acid in 1 were indicated on the basis of the CD analysis. The absolute stereochemistry of 1,6-anhydro-*N*-acetylmuramic acid was also determined by CD data.

Key words monomeric peptidoglycan-related compound; cymbidine; *Cymbidium goeringii*; Orchidaceae

The research project for biologically active compounds from higher plants conducted by the former Tokyo Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. resulted in the isolation of cymbidines A and B from the orchid, *Cymbidium goeringii* in 1972.[1)] Cymbidines showed significant hypotensive and diuretic activities.[1)] The structural study of cymbidines was carried out as a joint-research by the Fujisawa group and professor Yamada's group of Tokyo College of Pharmacy (the former name of Tokyo University of Pharmacy and Life Sciences). Although several fragments of cymbidine A (valine, glutamic acid, alanin and glucosamine) were detected, the other fragments and the sequence of the fragments as well as the molecular formula were uncertain. Fortunately, the sample of cymbidine A was found to be stable in a refrigerator for over 30 years. Thus the structural study using modern analytical methods was restarted by the research group of Tokyo University of Pharmacy and Life Sciences in 2004 to determine the structure of cymbidine A. This paper describes the process for structural determination of cymbidine A.

The whole plants of *Cymbidium goeringii* (20 kg) were extracted with 80% aqueous ethanol. Successive separation of the extract by chromatography using active carbon, ion-exchange resin, and silica gel gave cymbidine A (**1**) as a pale-yellow amorphous substance (350 mg), which exhibited aqueous and hygroscopic properties. Biological activities of **1** are described in Experimental.

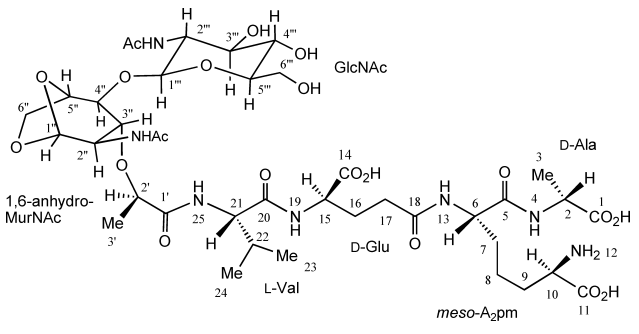

Fig. 1. Structure of Cymbidine A (**1**)

The molecular formula ($C_{39}H_{63}N_7O_{20}$) of cymbidine A (**1**) was obtained by the high-resolution electrospray ionization mass spectrum (HR-ESI-MS) and ^{13}C-NMR data. The ninhydrin color test of **1** showed a reddish-violet color. The IR spectrum of **1** showed absorptions at 3370 (OH, NH) and 1653 (CO) cm^{-1}. Compound **1** was hydrolyzed under an acidic condition, and the mixture of the products was analyzed by an amino acid analyzer. Four amino acids, alanine, valine, glutamic acid and an unidentified one, were detected.

All the 39 carbon signals were observed in the ^{13}C-NMR spectrum of **1** (Table 1); six methyls, seven sp^3 methylenes, seventeen sp^3 methines and nine quaternary carbons. The nine quaternary carbon signals between 170—185 ppm in the ^{13}C-NMR spectrum indicated the presence of carbonyl carbons due to amide, ester and caboxylic acid. No signal was detected in the olefinic region (110—160 ppm). Two methine signals at δ_C 102.7 and 103.6 ppm were attributed to the anomeric carbons in the sugar moieties. Seven oxymethine signals were observed in the region of 70—80 ppm, and two oxymethylene signals at δ_C 63.6 and 67.7 ppm were also observed. Seven methylene signals from 50 to 60 ppm indicated that these carbons bear a nitrogen atom. The ^{1}H-NMR spectrum (Table 1) demonstrated the four methyl doublets. Two of these at δ_H 0.93 (3H, d, J=6.8 Hz) and 0.95 (3H, d, J=6.8 Hz) ppm were assigned for an isopropyl group in valine and one of the remaining doublet methyls was assignable to that in alanine. Two methyl singlets at δ_H 2.06 (3H, s) and 2.08 (3H, s) ppm indicated the presence of two acetyl groups. Four signals due to two oxymethylens in sugars were observed between 3.7—4.3 ppm. The four methine signals between 4.2—4.3 ppm were assignable to the α-protons of the amino acids. The six doublets from 7.4 to 8.4 ppm indicated the presence of the six amide linkages.

Direct correlations between ^{13}C and ^{1}H signals were re-

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Table 1. ^{13}C- and ^{1}H-NMR Data[a] of Cymbidine A (**1**)

Unit	Position	δ_C	δ_H	Unit	Position	δ_C	δ_H
Ala	1 (CO_2H)	181.5		MurNAc	1′ (CO)	178.5	
	2 (CH)	53.2	4.22 (m)		2′ (CH)	78.7	4.26 (m)
	3 (CH_3)	20.0	1.34 (d, 7.2)		3′ (CH_3)	20.7	1.38 (d, 6.8)
	4 (NH)		8.10 (d, 6.9)		1″ (CH)	102.7	5.45 (br s)
A_2pm	5 (CO)	176.2			2″ (CH)	52.0	4.01 (d, 9.1)
	6 (CH)	56.8	4.28 (m)		3″ (CH)	79.8	3.65 (br s)
	7 (CH_2)	33.7	1.76 (m)		4″ (CH)	77.4	4.00 (br s)
			1.84 (m)		5″ (CH)	76.5	4.71 (br d, 6.0)
	8 (CH_2)	23.9	1.46 (quint, 7.7)		6″ (CH_2)	67.7	3.82 (dd, 6.0, 7.7)
	9 (CH_2)	33.0	1.84 (m)				4.28 (d, 7.7)
			1.93 (m)		NH		7.41 (d, 9.1)
	10 (CH)	57.6	3.74 (t, 7.0)		$\underline{CO}CH_3$	176.5	
	11 (CO_2H)	177.5			$CO\underline{CH_3}$	24.9	2.06 (s)
	13 (NH)		8.25 (d, 6.8)	GlcNAc	1‴ (CH)	103.6	4.68 (d, 8.4)
Glu	14 (CO_2H)	179.8			2‴ (CH)	58.5	3.78 (m)
	15 (CH)	56.9	4.24 (m)		3‴ (CH)	76.4	3.59 (dd, 8.1,10.3)
	16 (CH_2)	30.4	1.97 (m)		4‴ (CH)	72.8	3.48 (t, 10.3)
			2.14 (m)		5‴ (CH)	78.9	3.47 (dd, 8.2, 10.3)
	17 (CH_2)	34.7	2.36 (t, 7.7)		6‴ (CH_2)	63.6	3.77 (dd, 8.2, 11.5)
	18 (CO)	178.30[b]					3.90 (br d, 11.5)
	19 (NH)		8.22 (d, 7.6)		NH		8.33 (d, 9.5)
Val	20 (CO)	175.6			$\underline{CO}CH_3$	178.34[b]	
	21 (CH)	62.0	4.25 (m)		$CO\underline{CH_3}$	25.3	2.08 (s)
	22 (CH)	33.5	2.14 (m)				
	23, 24	20.4	0.93 (d, 6.8)				
	(each CH_3)	21.5	0.95 (d, 6.8)				
	25 (NH)		8.03 (d, 8.5)				

a) ^{13}C-NMR: 125 MHz in H_2O containing 10% D_2O, ^{1}H-NMR: 500 MHz in H_2O containing 10% D_2O. TSP as an internal standard. *J* in Hz. Assignments of the ^{13}C and ^{1}H signals were made based on HMQC. *b*) Values with the same superscript are interchangeable.

vealed from the HMQC analysis as summarized in Table 1. The ^{1}H–^{1}H COSY and/or TOCSY correlations as depicted by the bold lines in Fig. 2 demonstrated the partial structures **a**—**g** in **1**. The ^{1}H–^{1}H COSY correlations (partial structure **f**) and the HMBC correlations as shown by the broken lines in Fig. 2 clarified that the unidentified amino acid detected by the amino acid analysis was 2,6-diaminopimelic acid (A_2pm). The presence of two *N*-acetyl amino sugars (*N*-acetyl hexose and *N*-acetyl 1,6-anhydrohexose) was also indicated by the ^{1}H–^{1}H COSY and HMBC correlations. The 1,6-anhydro structure of one of the amino sugars was elucidated by the HMBC correlations from H-1″ [δ_H 5.45 (1H, br s)] to C-5″ [δ_C 76.5 (CH)] and C-6″ [δ_C 67.7 (CH_2)], from H-5″ [δ_H 4.71 (1H, br d)] to C-1″ [δ_C 102.7 (CH)] and from H-6″ [δ_H 3.82 (1H, dd)] to C-1″. The glycosidic linkage between C-1‴ of the terminal *N*-acetyl hexose and C-4″ of the *N*-acetyl 1,6-anhydrohexose was indicated by the HMBC correlations from H-1‴ [δ_H 4.68 (1H, d)] to C-4″ [δ_C 77.4 (CH)] and from H-4″ [δ_H 4.00 (1H, br s)] to C-1‴ [δ_C 103.6 (CH)]. The presence of a lactic acid moiety (Lac) was indicated by the HMBC correlations from H-3′ [δ_H 1.38 (3H, d)] to C-1′ [δ_C 178.5 (C)] and from H-2′ [δ_H 4.26 (1H, m)] to C-1′. An ethereal linkage of the lactic acid moiety to the *N*-acetyl 1,6-anhydrohexose at C-3″ was demonstrated by the HMBC correlations from H-2′ to C-3″ [δ_C 79.8 (CH)] and from H-3″ [δ_H 3.65 (1H, br s)] to C-2′ [δ_C 78.7 (CH)]. The sequence of Lac-Val-Glu-A_2pm-Ala was clarified by the HMBC correlations shown in Fig. 2. From these spectroscopic findings, the gross structure of cymbidine A (**1**) was determined.

The absolute configurations of the three amino acids (Ala, Val and Glu) were established by the modified Marfey's

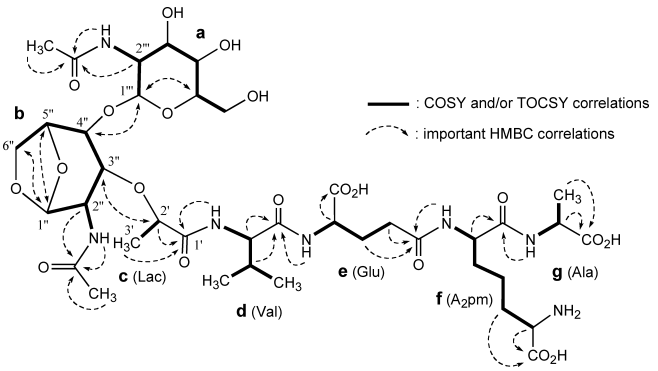

Fig. 2. ^{1}H–^{1}H COSY, TOCSY and HMBC Correlations of **1**

method.[2] The amino acid fraction of the acid-hydrolysate of **1** was treated with (1-fluoro-2,4-dinitrophenyl)-L-araninamide (FDAA) to give a mixture of FDAA derivatives of amino acids (FDAA-amino acids). The mixture was analyzed by LC-MS, and each retention time of FDAA-amino acid was compared with those of the standard FDAA-amino acids, revealing that Val was an L-form while both Ala and Glu had a D-type stereochemistry in **1**.

There are three stereoisomers [*RR*, *SS* and *meso* (*RS* or *SR*)] for A_2pm. Commercial A_2pm gave three peaks in the HPLC analysis using a chiral column. Each peak was separated and assigned by the CD spectrum[3] as an *RR* for the first peak, a *meso* for the second one, and an *SS* for the last one. The retention time of A_2pm of **1** in the HPLC using a chiral column of the acid-hydrolysate of **1** coincided with that of *meso*-A_2pm. The next question in relation to the stereochimistry of A_2pm was which chiral center (*R* or *S*) bearing

amino and carboxylic groups in A_2pm constituted the amino acid skeletal chain, and the question was solved by the CD analysis of its 2,4-dinitrophenylhydrazone (DNP). Cymbidine A (**1**) was treated with 2,4-dinitrophenylhydrazine followed by acid hydrolysis. The mono-DNP of A_2pm was separated from the acid-hydrolysate. Comparison of the CD data of the mono-DNP of A_2pm from **1** with those in the literature[4] concluded that the chiral center with the *S* configuration constituted the peptide skeletal chain.

The relative stereochemistries of the two amino sugars **a** and **b** were determinened by analysis of NOE data and consideration of ^{1}H coupling constants. The large coupling constants between H-1‴ and H-2‴ (8.4 Hz), H-2‴ and H-3‴ (8.1 Hz), H-3‴ and H-4‴ (10.3 Hz), and H-4‴ and H-5‴ (10.3 Hz) as shown by the solid lines in Fig. 3 indicated each axial configuration for these protons in amino sugar **a**. The NOE correlations as shown by the broken lines also support this stereochemistry. These findings indicated that the amino sugar **a** was a pyranoside of *β*-*N*-acetylglucosamine.

On the other hand, very small coupling constants of nearly zero Hz between H-1″ and H-2″, H-2″ and H-3″, H-3″ and H-4″, and H-4″ and H-5″ indicated that the dihedral angles between these protons were nearly 90°, thus demonstrating the relative stereochemistry from H-1″ to H-5″ to be that depicted in Fig. 3. These findings revealed that the amino sugar **b** was 1,6-anhydro-*N*-acetylglucosamine. The relative stereochemistry between two amino sugars **a** and **b** was indicated by the NOE correlation between H-4″ and H-1‴. Furthermore, the above-mentioned HMBC correlations in Fig. 2 indicated the connection of lactic acid to the C-3″ position of amino sugar **b**. This connection indicated the presence of the 1,6-anhydro-*N*-acetylmuramic acid moiety in **1**. The absolute stereochemistry of 1,6-anhydro-*N*-acetylmuramic acid (1,6-anhydroMurNAc) was determined by CD measurement. The acid-hydrolysate of cymbidine A (**1**) was subjected to HPLC analysis to show a peak with a retention time of 18.5 min which was almost identical with that for authentic muramic acid consisted of D-glucosamine and (*R*)-lactic acid. The CD spectrum of the purified compound showed two negative Cotton effects at λ_{ext} 206 and 271 nm, and a positive one at λ_{ext} 243 nm which were almost identical with those of authentic muramic acid consisted of D-glucosamine and (*R*)-lactic acid.[5] Thus the 1,6-anhydroMurNAc moiety in **1** was found to consist of D-1,6-anhydro-*N*-acetylglucosamine and (*R*)-lactic acid. The absolute stereochemistry of amino sugar **a** (*N*-acetylglucosamine) was tentatively assigned as a D-series.

The structure of cymbidine A (**1**) is closely related to those of the monomeric portion of peptidoglycans which form bacterial cell-walls. The peptidoglycan-derived tracheal cytotoxin (TCT)[6] which was isolated from a Gram-negative bacterium *Bordetella pertussis* responsible for causing whooping cough (pertussis), a respiratory illness in humans, has a structure of *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramylalanyl-*γ*-glutamyldiaminopimelylalanine. The compound with the same structure was first found in *Neisseria gonorrhoeae*[7] and was also isolated from human urine as a promoter of slow-wave sleep substance.[8,9] Although the difference of the structures between the monomeric peptidoglycan-related compounds exemplified by TCT and cymbidine A lies only in the part of muramylamino acid (muramylalanine in TCT and muramylvaline in cycmbidine A), TCT was not reported to exhibit hypotensive and diuretic activities which were characteristic for cymbidine A.

Cymbidine A (**1**) is the first example of a monomeric peptidoglycan-related compound from higher plants.

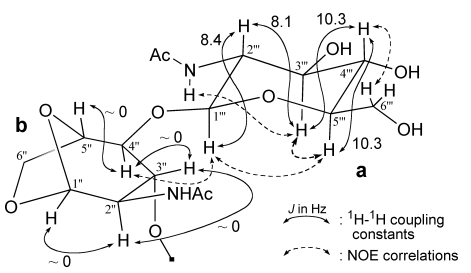

Fig. 3. ^{1}H–^{1}H Coupling Constants and NOE Correlations of Aminosugar Moiety of **1**

Experimental

General Procedures Optical rotation was measured on a JASCO DIP-360 digital polarimeter, and circular dichroism (CD) spectra on a DIP J-720 spectrometer. IR spectra were recorded on a Perkin Elmer PARAGON 1000 FT-IR spectrometer. ^{1}H, ^{13}C and all 2D NMR (^{1}H–^{1}H COSY, TOCSY, HMQC, HMBC and NOESY) spectra were taken with a Bruker DRX-500 spectrometer (^{1}H; 500 MHz, ^{13}C; 125 MHz) in H_2O–D_2O (90 : 10). Chemical shifts are given on a δ (ppm) scale with 3-(trimethylsilyl)propionic acid-D_4 sodium (TSP) as an internal standard. High-resolution ESI mass spectrum (HR-ESI-MS) was measured on a Micromass LCT spectrometer.

Isolation and Purification of Cymbidine A Whole plants (20 kg) of *Cymbidium goeringii* were immersed in 80% EtOH (EtOH–H_2O=80 : 20, 60 l) for 3 d. The solution was concentrated under reduced pressure. The extract (300 g) was dissolved in H_2O (3 l) and then active charcoal (3 l) was added to the solution. The active charcoal was washed with H_2O and eluted by a mixed solvent (acetone–pyridine–H_2O=2 : 1 : 1, 4 l). The eluent was concentrated and then dissolved in H_2O (1.5 l). The aqueous solution was washed with 1-butanol twice in a separatory funnel and concentrated to dryness. The residue was dissolved in H_2O and absorbed on a cation-exchange resin column (Dowex 2×8, OAc form). Elution with 2 M AcOH in pyridine (4 l) followed by concentration gave a residue, which was dissolved in H_2O and then absorbed on an anion-exchange resin column (Dowex 50×2, H^+ form). Elution with 5% pyridine in H_2O (1 l) followed by concentration gave a residue (0.85 g), which was chromatographed on a silica gel column. Elution with 1-butanol–AcOH–H_2O (4 : 1 : 1) gave ninhydrin-test positive fractions. Finally, preparative TLC of the combined ninhydrin-test positive fractions by development with 1-butanol–AcOH–H_2O (4 : 1 : 2) gave cymbidine A (**1**) (350 mg) with an *Rf* value of 0.3.

Cymbidine A (**1**): Pale yellow amorphous substance; $[\alpha]_D^{22}$ −28.4° (c=0.69, H_2O); IR (KBr) ν_{max} 3370, 1653, 1419, 1112, 802 cm^{-1}; ^{1}H- and ^{13}C-NMR, see Table 1; HR-ESI-MS *m*/*z* 950.4061 $[M+H]^+$ (Calcd for $C_{39}H_{64}N_7O_{20}$ 950.4206).

Biological Activities of 1[1] Cymbidine A (**1**) exhibited hypotensive activity in DCA hypertensive rats at a dose of 0.03 mg/rat (i.p.), and also exhibited diuretic activity in Donryu rats at a dose of 0.15 mg/kg (i.p.). The LD_{50} of **1** was 10 mg/kg (i.p.) in Donryu rats.

Acid Hydrolysis of 1 and Amino Acid Analysis A solution of cymbidine A (**1**) (2.0 mg) in 6 M HCl (1 ml) was heated for 24 h in a sealed tube. The solution was neutralized with aqueous NaOH solution and concentrated under reduced pressure. About one-tenth of the acid hydrolysates were dissolved in H_2O (10 μl), and amino acids were analyzed by an Applied Biosystem Model 421 amino acid analyzer.

Absolute Configuration of Amino Acids (Modified Marfey's Method)[2] The above-mentioned acid hydrolysates were desalted by absorbing on a cation exchange resin (Amberlite CG-120) followed by washing with H_2O. The acid hydrolysates were desorbed from the resin by elution with 10% aqueous ammonia and concentrated under reduced pressure. The residue (0.7 mg) was dissolved in H_2O (50 μl) and 1 M $NaHCO_3$ solution (20 μl) was added. To the solution of 1-fluoro-2,4-dinitrophenyl-5-alaninamide (FDAA, Marfey's reagent, 1 mg) in acetone (100 μl) was added, and the mixture was stood at 37 °C for 60 min. After an addition of 1 M HCl (20 μl), the solution was diluted with acetonitrile (810 μl), and 1 μl of the

acetonitrile solution was analyzed by LC-MS; Agilent Technologies Agilent 1100 liquid chromatography (LC) with a ThermoQuest Finnigan LCQ Duo spectrometer (MS), column TSKgel ODS-120T, flow rate 0.2 ml/min, detection 340 nm, solvent 0.1% trifluoroacetic acid solution–acetonitrile (90 : 10 to 55 : 45). Retention times (min) of the amino acid derivatives were as follows: D-Glu (24.74), D-Ala (28.44), and L-Val (31.95), which were identical with those derived from authentic amino acids [L-Glu (23.19), D-Glu (24.54), L-Ala (25.06), D-Ala (28.41), L-Val (31.96), and D-Val (36.88)].

Identification of 2,6-Diaminopimelic Acid (A_2pm) Commercially available 2,6-diaminopimelic acid (Wako Pure Chemical Industries) was analyzed by using a chiral column (CROWNPAK CR(+), Daicel Chemical Industries). Elution with perchloric acid (pH 1.5) gave three peaks. CD measurement of each peak exhibited that the first peak with a retention time of 4.93 min was DD-A_2pm (*RR*), the second peak with a retention time of 21.00 min was *meso*-A_2pm, and the third peak with a retention time of 27.81 min was LL-A_2pm (*SS*). Similar analysis of the acid hydrolysates of **1** using the chiral column showed the peaks for amino acids; D-Ala (3.41 min), D-Glu (3.93 min), L-Val (5.28 min), and A_2pm (21.65 min). The retention time of A_2pm from **1** was found to be almost identical with that (21.00 min) of the authentic *meso*-A_2pm.

The Position of the Amino Acid Moiety of *meso*-2,6-Diaminopimelic Acid in the Peptide Skeletal Chain To a solution of **1** (0.5 mg) in 1% $K_2B_4O_7$ solution (100 μl) was added a solution of 0.01 M 1-fluoro-2,4-dinitrobenzene (50 μl), and the mixture was stood at 60 °C for 30 min. After addition of concentrated HCl (150 μl), the mixture was heated at 115 °C for 15 h for hydrolysis. The mixture was diluted with H_2O (600 μl) and was washed with ether. The aqueous layer was then extracted three times with 1-butanol. The 1-butanol extract was concentrated under reduced pressure. The residue was subjected to HPLC (RP-18, acetonitrile–0.01 M CF_3CO_2H (pH 2.5)=1 : 4), giving a fraction containing 2,4-dinitrophenyl derivative of *meso*-A_2pm, the retention time (12.4 min) of which was identical with that of authentic (2*R*,6*S*)-2-(2,4-dinitrophenyl)amino-6-aminopimelic acid (12.4 min). The positive Cotton effect at λ_{ext} 402 nm in the CD spectrum of the 2,4-dinitrophenyl derivative of *meso*-A_2pm coincided with that (λ_{ext}+402 nm) of the authentic (2*R*,6*S*)-2-(2,4-dinitrophenyl)amino-6-aminopimelic acid.[4] These findings indicated that the chiral center of the *S* configuration constituted the peptide skeletal chain in **1**.

Absolute Stereochemistry of 1,6-Anhydro-*N*-acetylmuramic Acid A part of the above-mentioned acid hydrolysates of **1** for the detection of A_2pm was subjected to HPLC [TSKgel Amide-80 (Toso), acetonitrile–10 mM NH_4OAc solution (4 : 1)], to give a peak with a retention time of 18.5 min which was identical with that (18.4 min) of authentic muramic acid with D-glucosamine and (*R*)-lactic acid. The CD spectrum of the compound [HR-ESI-MS *m/z* 252.1091 $[M+H]^+$ (Calcd for $C_9H_{18}NO_7$ 252.1083)] from **1** showed two negative Cotton effects at λ_{ext} 206 and 271 nm, and a positive one at λ_{ext} 243 nm which were almost identical with those of the authentic sample.[5]

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