Structural Elucidation of Novel Phosphocholine-Containing Glycosylinositol-Phosphoceramides in Filamentus Fungi: (2). Spectral Analysis of the Sugar-Inositol Portion by 2D-NMR

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Received November 18, 2003; accepted January 27, 2004

The sugar-inositol portion of the novel glycosylinositol-phosphoceramides, ZGL1 and ZGL2, from the filamentus fungi, *Acremonium* sp., were elucidated by a combination of NMR techniques including ^{1}H–^{1}H (COSY and HOHAHA) and ^{1}H–^{13}C (HMQC and HMBC) spectroscopy. Further, examination of the ^{1}H–^{31}P HMQC spectrum showed connectivity of inositol and ceramide through phosphate.

Key words *Acremonium* sp.; glycosylinositol-phosphoceramide; ^{1}H–^{31}P HMQC; spectral analysis

As described in a previous paper,[1)] we isolated five novel glycosylinositol-phosphoceramides (ZGL1—ZGL5) from the filamentus fungi, *Acremonium* sp.,[2)] and elucidated their structures by chemical and spectroscopic analysis using 1D NMR, composition and linkage analysis, and partial acetolysis and exoglycosidase digestion. As a result, their structures were characterized to be: GlcNα1-2Ins1-P-1Cer (ZGL1), Manα1-6GlcNα1-2Ins1-P-1Cer (ZGL2), Manα1-6Manα1-6GlcNα1-2Ins1-P-1Cer (ZGL3), phosphocholine→6Manα1-6GlcNα1-2Ins1-P-1Cer (ZGL4), and phosphocholine→6Manα1-6Manα1-6GlcNα1-2Ins1-P-1Cer (ZGL5). The core structure of their oligosaccharides, GlcNα1-2Ins1-P-, is different from those of other fungi such as Manα1-6Ins1-P-, Manα1-2Ins1-P- and Manβ1-2Ins1-P-.[3—5)] The structure is rather similar to the GPI-anchored structure.[6)] However, the identification of the individual sugar components was not made.

In this paper, we describe the detailed spectral analysis of sugar-inositol portion about two glycosylinositol-phosphoceramides (ZGL1 and ZGL2). The complete structural assignments of them have been accomplished exclusively on the basis of two-dimensional proton–proton and proton–carbon chemical shift correlation spectroscopy. There are a few examples which were elucidated through the structural analysis by acetylated glycolipids or using the simple monosaccharide's glycolipids for the structural studies of glycosphingolipids by 2D-NMR.[7—10)] However, there are still few examinations of the complete structural assignment of oligosaccharides without adding chemical change.[11,12)] It is important to make full use of an NMR to process the data. Furthermore, at this time we used the ^{1}H–^{31}P HMQC experiment in order to confirm the connective position between inositol and phosphate. Although a few research groups have reported approaches to ^{1}H–^{31}P HMQC experiments to elucidate the structure of lipopolysaccharides[13)] and glycoglycerolipid[14)] having phosphocholine, this is the first report of the structural elucidation of glycosylinositol-phosphoceramides.

Results and Discussion

Structure of ZGL1 In the 600 MHz ^{1}H-NMR spectrum, a doublet peak at 5.13 ppm ($J_{1,2}$=3.3 Hz) was assigned to the anomeric proton resonances of α-2-amino-2-deoxy glucose residues from the chemical shift and spin coupling constant. A broad singlet peak at 4.08 ppm may be assigned to the H-2 of *myo*-inositol resonances. The only equatorial proton in *myo*-inositol is H-2, and its signal has a characteristic fine structure caused by two small (*ca.* 2 Hz) gauche couplings which result in a narrow triplet (or broad singlet peak), and the resonance of the sole equatorial proton (H-2) is shifted furthest downfield.[15)] The anomeric proton and H-2 of *myo*-inositol proton were used in the analysis of the COSY and HOHAHA spectra as starting points for the sequential assignments of all the proton resonances. Analysis of COSY data (Fig. 1) starting from the anomeric proton at δ=5.13 allowed us to identify the sugar unit as a 2-amino-2-deoxy-α-glucose (α-GlcN*p*). In fact, the small coupling constant between H-1 and H-2 indicated the equatorial position of H-1 and the axial position of H-2 (δ=2.80, br d). The H-3, H-4, H-5, H-6a and H-6b protons were proven in a similar manner. It was also deduced from a HOHAHA experiment (data not shown). On the other hand, starting with H-2 of *myo*-inositol (Ins), H-1 and H-3 were identified from the cross peak at δ=3.80 and 3.23, and the low field (3.80 ppm) was de-

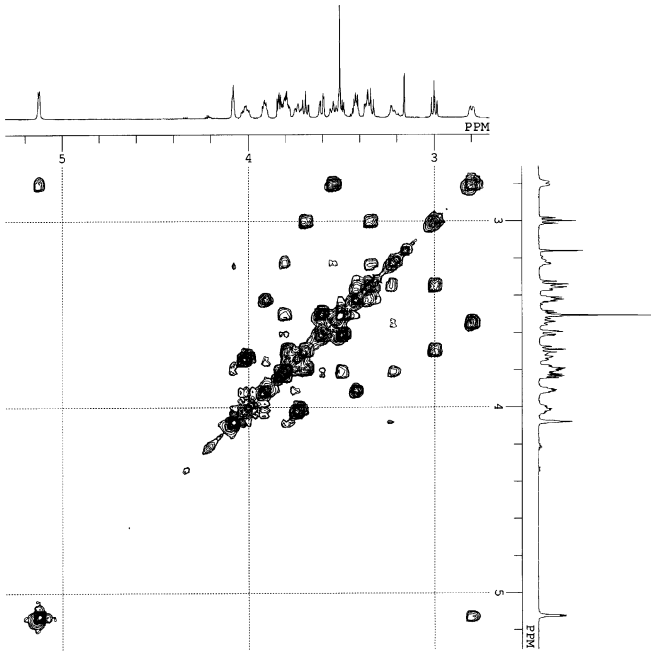

Fig. 1. A Part of COSY Spectrum of ZGL1

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Table 1. ^{13}C- and ^{1}H-NMR Data of ZGL1 and ZGL2 in DMSOd_6–D_2O (49 : 1) at 60 °C

		^{1}H		^{13}C	
		ZGL1	ZGL2	ZGL1	ZGL2
FA	1	—	—	173.0	173.3
	2	3.83 (dd : 4.4, 8.0)	3.84 (dd : 4.4, 7.0)	71.0	71.1
	3a	1.31[a)]	1.30[a)]	31.0	31.0
	3b	1.48[a)]	1.47[a)]	—	—
	4a	1.47[a)]	1.45[a)]	34.1	34.1
	4b	1.58 (m)	1.58 (m)	—	—
	5	1.31[a)]	1.30[a)]	24.3	24.3
	6—23	1.23[a)]	1.25[a)]	21—31	21—31
	24	0.84 (t: 7.1)	0.84 (t: 6.9)	13.6	13.6
LCB	1a	3.73 (m)	3.73 (m)	63.9	64.1
	1b	4.01 (m)	4.01 (m)	—	—
	2	3.91 (m)	3.92 (m)	50.2	50.2
	3	3.42 (m)	3.42[a)]	73.2	73.3
	4	3.37[a)]	3.35[a)]	70.4	70.5
	5—17	1.23[a)]	1.25[a)]	21—31	21—31
	18	0.84 (t: 7.1)	0.84 (t: 6.9)	13.6	13.6
Ins	1	3.80[a)]	3.79[a)]	76.0	76.3
	2	4.08 (br s)	4.11 (br s)	81.8	81.9
	3	3.23[a)]	3.23[a)]	70.1	70.2
	4	3.34 (t: 9.9)	3.33 (t: 7.4)	71.8	71.7
	5	3.00 (t: 8.8)	3.01 (t: 8.8)	74.8	74.6
	6	3.69 (t: 8.8)	3.72 (t: 9.0)	71.8	71.7
αGlcN	1	5.12 (d: 3.3)	5.17 (d: 2.2)	98.4	97.8
	2	2.80 (dd: 2.7, 10.4)	2.89 (br d: 8.2)	54.8	54.4
	3	3.54 (t: 9.3)	3.60[a)]	71.0	70.2
	4	3.20[a)]	3.28[a)]	69.7	69.9
	5	3.80[a)]	3.97[a)]	72.5	70.8
	6a	3.51 (dd: 5.0, 11.5)	3.54 (m)	60.1	64.7
	6b	3.60 (dd: 2.1, 11.5)	3.77[a)]	—	—
αMan	1	—	4.65 (d: 1.4)	—	99.8[b)]
	2	—	3.67 (dd: 1.6, 3.7)	—	69.9
	3	—	3.51 (m)	—	70.8
	4	—	3.40[a)]	—	67.0
	5	—	3.75[a)]	—	71.7
	6a	—	3.46 (dd: 5.0, 11.3)	—	61.1
	6b	—	3.62[a)]	—	—

a) Submerged by other signals. *b*) J_{CH}=167 Hz.

cided on H-1 because the resonance of the proton at the position phosphorylated is shifted downfield. The H-4, H-5 and H-6 signals were assigned in a similar manner from H-1 and H-3. Furthermore, we examined the HMQC spectra in order to assign the carbon signals. As a result, all proton and carbon signals were assigned as shown in Table 1. For the purpose of clarifying the linkage of α-GlcN*p* and Ins, we examined the HMBC spectrum of ZGL1 (Fig. 2). The $^{3}J_{CH}$ correlation between C-1 of α-GlcN*p* (δ_C=98.4) and H-2 of Ins (δ_H=4.08) was clearly observed. Furthermore, we also investigated the ^{1}H–^{31}P HMQC spectrum (Fig. 3). The $^{3}J_{PH}$ correlation between P (δ_P=1.13) and H-1 of Ins (δ_H=3.80), H-1 of long chain base (LCB) (δ_H=3.73, 4.01) was indicated. The linkage of α-GlcN*p*-Ins was also confirmed by NOESY spectral data. Accordingly, the structure of ZGL1 was elucidated to be GlcNα1-2Ins1-P-1Cer as shown in Chart 1.

Structure of ZGL2 The proton and carbon chemical shifts of ZGL2 were determined in the same manner as ZGL1. In the 600 MHz ^{1}H-NMR spectrum, doublets at 5.17 ppm ($J_{1,2}$=2.7 Hz) and 4.65 ppm ($J_{1,2}$=1.4 Hz) were assigned to the anomeric proton resonances of 2-amino-2-deoxy-α-glucose (α-GlcN*p*) and α-mannopyranose (α-Man*p*) residues from the chemical shifts and spin coupling

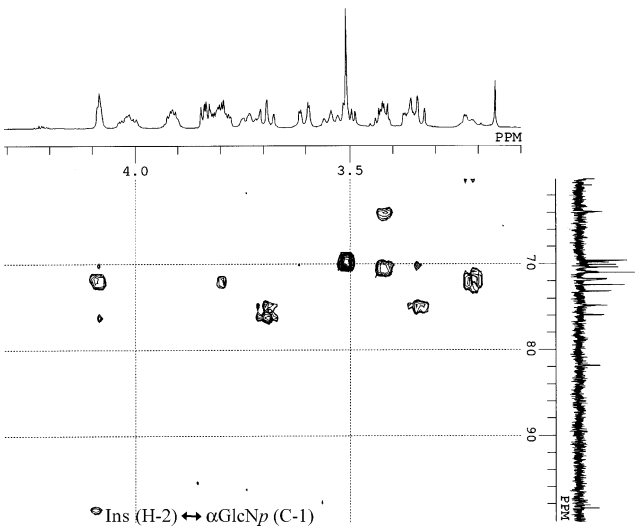

Fig. 2. A Part of HMBC Spectrum of ZGL1

constants. The α-configuration of the Man*p* was indicated by the J_{CH} value of 167 Hz in the ^{13}C-NMR spectrum. The determination of all proton and carbon chemical shifts of

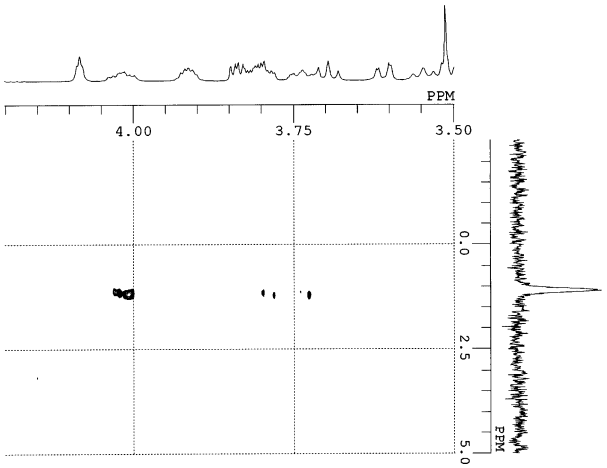

Fig. 3. ^{1}H–^{31}P HMQC Spectrum of ZGL1

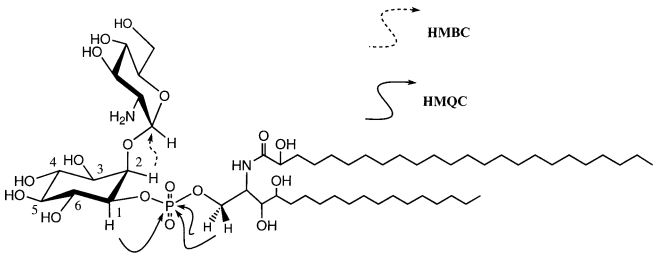

Chart 1. Structure of ZGL1

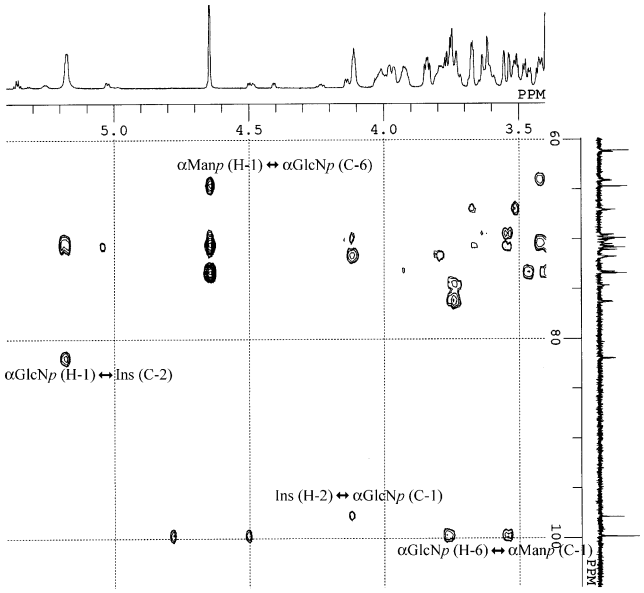

Fig. 4. A Part of HMBC Spectrum of ZGL2

αMan*p* residue was also made by the correlation from the above anomeric proton and carbon using COSY, HOHAHA and HMQC. For the purpose of clarifying the linkage of α-Man*p* and α-GlcN*p*, α-GlcN*p* and Ins, we examined the HMBC spectrum of ZGL2 (Fig. 4). The $^{3}J_{CH}$ correlation between C-1 of αMan*p* (δ_C=99.8) and H-6 of α-GlcN*p* (δ_H=3.54, 3.77), H-1 of αMan*p* (δ_H=4.65) and C-6 of α-GlcN*p* (δ_C=64.7), C-1 of α-GlcN*p* (δ_C=97.8) and H-2 of Ins (δ_H=4.11), H-1 of α-GlcN*p* (δ_H=5.17) and C-2 of Ins (δ_C=81.9) were clearly observed. Furthermore, we also investigated the ^{1}H–^{31}P HMQC spectrum of it (Fig. 5). The $^{3}J_{PH}$ correlation between P (δ_P=0.67) and H-1 of Ins (δ_H=3.79), H-1 of LCB (δ_H=3.76, 4.02) was indicated. Accordingly, the structure of ZGL2 was elucidated to be Manα1-6GlcNα1-2Ins1-P-1Cer as shown in Chart 2.

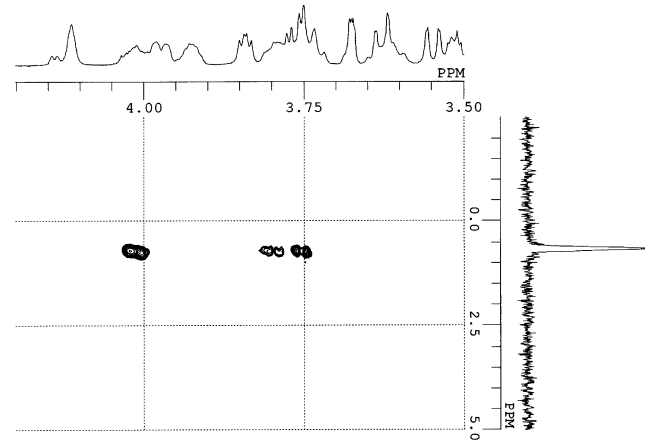

Fig. 5. ^{1}H–^{31}P HMQC Spectrum of ZGL2

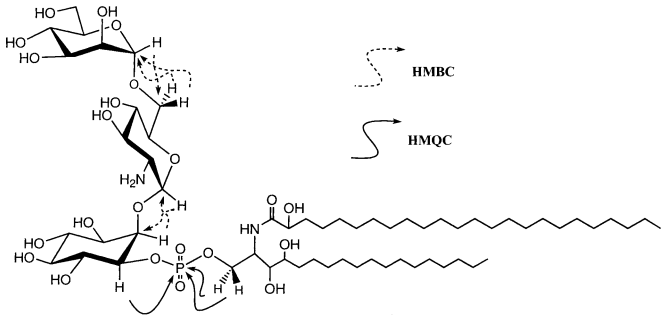

Chart 2. Structure of ZGL2

Conclusion

In this present paper, we have elucidated the structure of the novel glycosylinositol-phosphoceramides, ZGL1 and ZGL2, from the filamentus fungi, *Acremonium* sp. by 2D NMR analysis. Especially, ^{1}H–^{31}P HMQC was quite useful and noteworthy for the determination of the linkages position between inositol and ceramide through phosphorus.

Experimental

NMR: Purified ZGL1 (5 mg) and ZGL2 (5 mg) were analyzed in 0.6 ml of DMSO-d_6 containing 2% D_2O using a JEOL JNM-ECP600 spectrometer equipped with pulsed field gradients at 60 °C. All spectra were obtained at base frequency of 600 MHz for ^{1}H, 150.93 MHz for ^{13}C and 242.95 MHz for ^{31}P nuclei. For ^{1}H-NMR spectroscopy, the pulse sequence was with a delay and acquisition time of 3 and 1.8 s, respectively, using a spectral width of 9.0 kHz, 32 K data points and 256 scans. Chemical shifts were reported as δ values relative to residual DMSO-d_5 (δ_H 2.49) as an internal standard. For ^{13}C-NMR spectroscopy, the pulse sequence was with a delay and acquisition time of 1.5 and 0.9 s, respectively, using a spectral width of 37.7 kHz, 32 K data points and 66400—80000 scans. Chemical shifts were reported as δ values relative to DMSO-d_6 (δ_C 39.5) as an internal standard. For ^{31}P-NMR spectroscopy, the pulse sequence was with a delay and acquisition time of 3 and 0.4 s, respectively, using a spectral width of 172.4 kHz, 32—64 K data points and 320—400 scans. Chemical shifts were reported as δ values relative to H_3PO_4 (δ_P 0.0) as an external standard. Conventional pulse sequences were used for COSY, HOHAHA, NOESY, HMQC and HMBC. For HOHAHA experiments, spin lock times of 50, 100 and 150 ms were used. For a

NOESY experiment, 0.15 s mixing time was used. Two- and three-bond ^{1}H–^{13}C connectivities were determined by an HMBC experiment optimized for a J of 8 Hz. COSY, HOHAHA and NOESY experiments were acquired as 512×256 (real points) data matrixes, and zero filled to yield 512×512 spectra. HMQC and HMBC experiments were acquired as 1024×256 (real points) data matrixes, and zero filled to yield 1024×512 spectra. The ^{1}H–^{31}P HMQC experiments were acquired as 2048×64 (real points) data matrixes, and zero filled to yield 2048×128 spectra. Water suppression was carried out by selective pre-saturation placing the carrier on the solvent resonance.

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