Pholipeptin, a Novel Cyclic Lipoundecapeptide from *Pseudomonas fluorescens*

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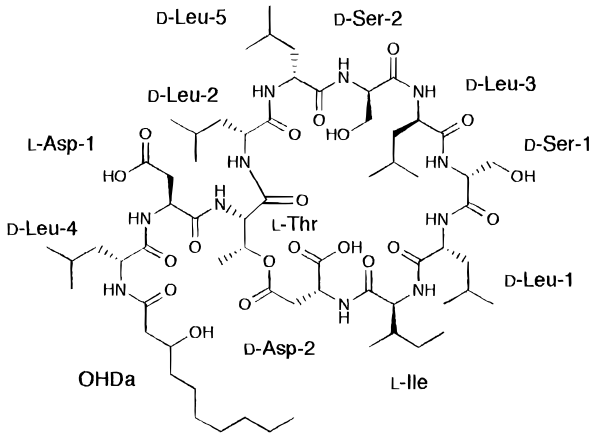
Received February 27, 1996 (Revised Manuscript Received October 28, 1996[⊗]*)*

An inhibitor of phosphatidylinositol-specific phospholipase C (PI-PLC), pholipeptin (**1**), was purified from the culture broth of *Pseudomonas* sp. by solvent extraction and column chromatography. Acid hydrolysis of **1** gave Leu, Ile, Ser, Thr, and Asp moieties. Although **1** was a peptide compound, fragmentation by mild hydrolysis was not accomplished under any conditions. So, we performed the structure elucidation using various 2D NMR techniques. In the NMR studies, the addition of a small amount of trifluoroacetic acid gave relatively sharp and resolved signals, such that the structure of this novel cyclic lipodepsipeptide consisting of 11 amino acids and a 3-hydroxydecanoic acid moiety could be determined. Chirality of the constituent amino acids was analyzed by chiral HPLC, but two Asp residues could not be distinguished because they were contained as a racemic mixture. Finally, their chiralities were determined by NMR analysis of ^{13}C-labeled **1** into which [L-^{13}C]Asp had been biosynthetically incorporated.

Phosphatidylinositol-specific phospholipase C (PI-PLC) is involved in signal transduction of growth factors and hormones[1,2] and in some transformed cells.[3] Therefore, inhibitors of PI-PLC will be useful tools for exploring the mechanism of intracellular signal transduction systems. In the course of our screening for an inhibitor of PI-PLC, we isolated the novel cyclic lipodepsipeptide pholipeptin (**1**) from the culture broth of *Pseudomonas* sp. In this paper, we report the isolation and structural elucidation of **1**.



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The culture filtrate of *Pseudomonas fluorescens* BMJ279-76F1 was absorbed with Diaion HP-20, and the MeOH eluate was successively extracted with EtOAc/H_2O. The organic layer was then taken to dryness and dissolved in MeOH. The MeOH-soluble fraction was further purified by two passages through a silica gel column followed by Sephadex LH-20 column chromatography to yield **1**.

Pholipeptin (**1**) was isolated as a white powder with mp 220−224 °C dec, and its UV spectrum showed only end-absorption. The ^{1}H and ^{13}C NMR spectra were indicative of a peptide, and the structure was also suggested by the positive response to the Rydon−Smith color reaction. The IR spectrum of **1** exhibited typical absorption bands of peptide at 3295, 1657, and 1547 cm^{-1}. In the positive- and negative-ion FAB-MS spectra, the molecular ion of **1** appeared at *m*/*z* 1376 and 1352, indicating $(M + Na)^+$ and $(M - H)^-$, respectively. The negative ion FAB-HRMS of **1** showed the peak at *m*/*z* 1352.7922 (calcd for $C_{64}H_{110}O_{20}N_{11}$ *m*/*z* 1352.7929). In FAB-MS, fragmentation peaks commonly observed for acyclic peptides were not noted. Therefore, a cyclic rather than linear oligopeptide structure was suggested for this compound. Furthermore, **1** could not be analyzed by amino acid sequencer, indicating a cyclic peptide or blockage of the N-terminus; this probability was supported by negative responses to a ninhydrin test. Standard amino acid analysis revealed the presence of 11 or 12 amino acids consisting of 2 mol of Asp and/or Asn, 2 mol of Ser, 1 mol each of Thr and Ile, and 5 or 6 mol of Leu. Asn and Asp could not be distinguished by the retention time, and the molecular ratio of Leu also could not be determined exactly from the peak area in this analysis. Moreover, the molecular weight calculated for a peptide comprising these 11 or 12 amino acids did not fulfill the observed values. So **1** was suggested to contain some other component in addition to these amino acids.

For the structure determination of cyclic peptides such as arthrofactin,[4] mild hydrolysis and subsequent MS or HPLC analysis have been successfully performed. But mild hydrolysis of **1**, including acid or alkaline hydrolysis,

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[⊗] Abstract published in *Advance ACS Abstracts*, December 15, 1996.
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S0022-3263(96)00399-4 CCC: $14.00

Table 1. ^{1}H and ^{13}C NMR Assignments of 1 in DMSO-d_6 (0.02 M, pH 4, Adjusted with CF_3COOH)

		^{13}C	^{1}H[a]				^{13}C	^{1}H[a]	
residue	position	δ (ppm)	δ (ppm)	mult, J (Hz)	residue	position	δ (ppm)	δ (ppm)	mult, J (Hz)
OHDa	1	171.0			Leu-5	δ	a 22.2*3	0.79[d]	6H
	2	43.4	a 2.215	dd, 7.5, 14	(L5)		b 22.4*3		
			b 2.249	dd, 6, 14		α-CO	171.5		
	3	67.6	3.790	m		α-NH		8.00[c]	
	4	36.6	a 1.32[b]		Ser-2	α	54.5	4.50[d]	
			b 1.34[b]		(S2)	β	62.2	a 3.48[d]	
	5	24.8	a 1.23[b]					b 3.58[d]	
			b 1.37[b]			β-OH		4.716	br s
	6	29.0	1.22[b]	2H		α-CO	170.2		
	7	28.6	1.24[b]	2H		α-NH		8.064	br d, 8
	8	31.2	1.24[b]	2H	Leu-3	α	51.2	4.25[d]	
	9	22.0	a 1.25[b]		(L3)	β	40.2*	1.52[d]	
			b 1.28[b]			γ	24.0*2	1.52[d]	
	10	13.8	0.86[b]	3H		δ	a 22.8*3	0.86[d]	6H
	3-OH		3.344				b 22.9*3		
Leu-4	α	50.8	4.28[c]			α-CO	171.95		
(L4)	β	40.1*	1.44[d]			α-NH		7.80[d]	
	γ	23.9*2	1.62[d]		Ser-1	α	56.5	4.104	dd, 5.5, 12.5
	δ	a 21.3*3	a 0.84[d]	3H	(S1)	β	60.6	a 3.55[d]	
		b 21.4*3	b 0.88[d]	3H				b 3.59[d]	
	α-CO	172.4				β-OH		4.849	
	α-NH		8.02[c]			α-CO	169.9		
Asp-1	α	49.6	4.481[d]			α-NH		8.28[d]	
(D1)	β	35.4	a 2.49[d]		Leu-1	α	51.6	4.19[d]	
			b 2.66[d]	dd, 5, 16	(L1)	β	40.6*	1.47[d]	
	α-CO	170.2				γ	24.1*2	1.55[b]	
	β-CO	171.7				δ	a 22.9*3	a 0.84[d]	3H
	α-NH		8.27[d]	d, 7			b 23.0*3	b 0.88[d]	3H
Thr	α	55.3	4.47[d]			α-CO	171.3		
(T)	β	70.6	4.849	dq, 8, 6		α-NH		8.01[d]	
	γ	16.3	1.054	3H, d, 6	Ile	α	56.4	4.33[d]	
	α-CO	168.6			(I)	β	35.3	1.910	m
	α-NH		7.77[d]	d, 9		γ	24.2	1.10[b]	
Leu-2	α	51.0	4.22[d]			β-CH_3	15.1	0.86[b]	3H
(L2)	β	40.1*	1.44[d]			δ	10.6	0.81[b]	3H
	γ	24.0*2	1.57[d]			α-CO	170.7		
	δ	a 21.4*3	a 0.82[d]	3H		α-NH		7.80[d]	
		b 21.9*3	b 0.87[d]	3H	Asp-2	α	47.9	5.049	br m
	α-CO	172.01			(D2)	β	36.6	a 2.448	dd, 4, 17
	α-NH		8.106	br d, 7				b 2.981	br d, 17
Leu-5	α	51.4	4.29[c]			α-CO	~172		
(L5)	β	40.2*	1.53[d]			β-CO	169.6		
	γ	24.0*2	1.55[b]			α-NH		7.893	br d, 8

[a] Chemical shifts taken from the 1D proton spectrum whenever possible: s, singlet; d, doublet; q, quartet; m, multiplet; br, broad. Multiplicities are not shown for overlapped signals. [b] Taken from the HMQC spectrum. [c] Taken from the ROESY spectrum. [d] Taken from the HOHAHA spectrum. *,*2,*3 Interchangeable with similar symbols.

methanolysis, and proteolysis, did not give the suitable fragments for the structural analysis because of its severe response to the change in the hydrolyzing conditions. In addition, pholipeptin was only obtained as an amorphous powder, unsuitable for X-ray analysis.

Therefore, we tried a variety of homo- and heteronuclear 2D NMR techniques. The ^{1}H and ^{13}C NMR spectra of **1** did not give sharp signals when DMSO-d_6 was used as a solvent. Although measurement in DMSO-d_6 at 70 °C improved the resolution of ^{1}H signals, **1** decomposed during the prolonged 2D experiments. A solvent system with $CDCl_3/CD_3OD$ (10/1) slightly improved the resolution. We finally found that the addition of a small amount of trifluoroacetic acid[5] to the DMSO-d_6 solution afforded relatively sharp NMR signals at pH 4, which allowed us to make the following structural elucidation:

Proton and ^{13}C signal assignments (Table 1) were made by standard 1D and 2D NMR techniques, such as DEPT,[6] COSY,[7] HOHAHA,[8] C−H COSY,[9] HMQC,[10] HMBC,[11] and ROESY[12] experiments. The numerals attached to amino acid residues were used for discrimination of the same type of amino acid residues according to their chemical shift order of α-protons.

As described above, amino acid analysis of the acid hydrolysate of **1** had revealed the constituents as Asp and/or Asn, Thr, Ser, Ile, and Leu in the molar ratio of

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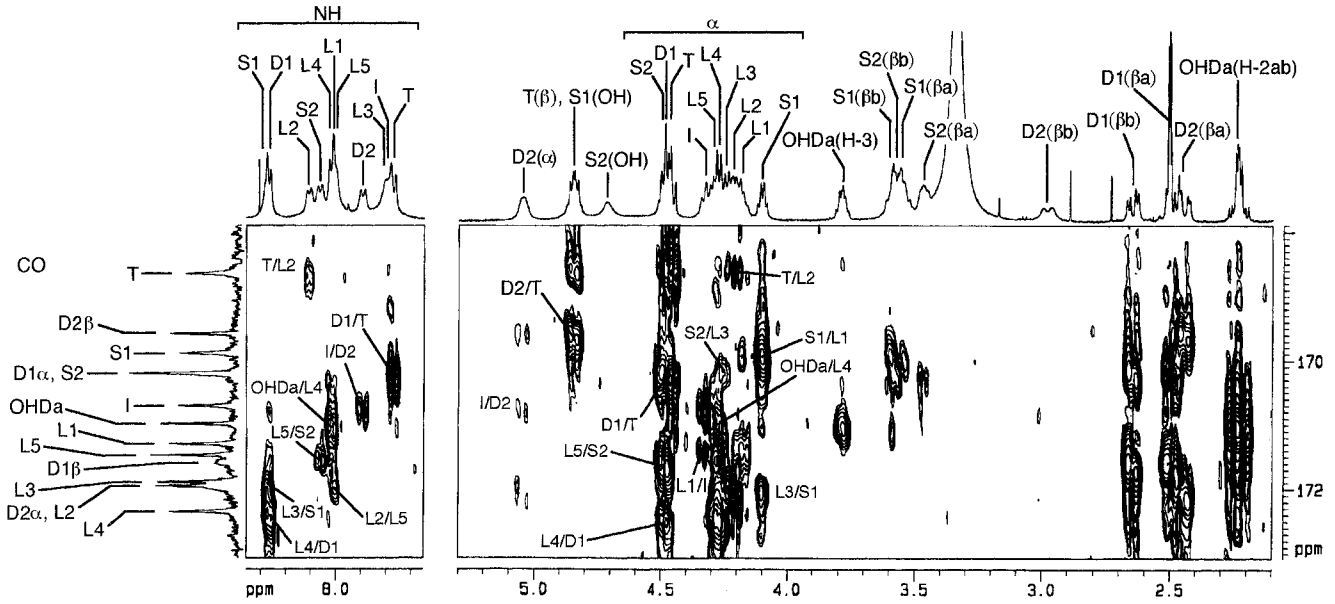

Figure 1. CO signal region of the HMBC spectrum of **1** in DMSO-d_6 (0.02M, pH 4, adjusted with CF_3COOH). Component amino acids are abbreviated using the usual one letter code. Labeled cross peaks were used for determination of interresidue sequences.

2:1:2:1:5−6. Therefore, our attention was first focused on signal assignments of each amino acid residue. The presence of five residues of Leu was primarily confirmed by the observation of five separate signals for the α-carbons in the ^{13}C NMR spectrum. These five ^{13}C signals were assigned by inspection of the C−H COSY spectrum. In this case, the HMQC spectrum was not useful because of the poor resolution of the F_1 domain. Other ^{13}C signals were assigned from the HMQC spectrum, since some weak ^{13}C signals did not show their correlations in the C−H COSY spectrum. From COSY and HOHAHA experiments, proton spin systems were separately established for each amino acid residue. The existence of two residues of Asp was clarified by the lack of ^1H-signals for NH_2. The chemical shifts of amide and α-protons of Leu-4 and 5 were obtained from their interresidue correlations in the ROESY spectrum because of their mostly overlapped α-H−NH correlations in the COSY and HOHAHA spectra. The hydroxyl signals of Ser-1 (δ 4.849, completely overlapping the β-H signal of Thr) and Ser-2 (δ 4.716) were assigned by examination of the COSY spectrum. The β-protons of Ser-1 and -2 showed cross peaks with respective hydroxyl protons as well as with water (δ 3.344) in the HOHAHA spectrum, which indicated that these hydroxyl protons and water were chemically exchanging at relatively slow rates on the ^1H NMR time scale.

The signal assignments of amino acid moieties left nine carbons comprising one methyl, seven methylenes, and one oxymethine in the DEPT spectrum. From HOHAHA and HMQC experiments, these carbons could be assembled into a sole straight chain. The chain was linked to a carbonyl group to construct a portion of 3-hydroxydecanoic acid (OHDa), the structure of which was corroborated by the HMBC correlations of the methylene and oxymethine protons (H-2ab and 3, respectively) with the carbonyl carbon at δ 171.0. Since the resonances of methylene carbons of OHDa were well resolved, the HMQC correlations served to decipher the chemical shifts of mostly overlapped methylene protons. In contrast to the hydroxyl protons of Ser-1 and 2, the COSY and HOHAHA spectra did not provide any evidence of a 3-hydroxyl proton. The ROESY spectrum, however, showed a correlation peak, having the same phase as the diagonal resonances, between H-3 of OHDa and a water signal. Similar correlations were also detected between water and β-protons of Ser-1 and -2. Saturation transfer undoubtedly contributes to these cross peaks via chemical exchange processes. Thus, the presence of the free hydroxyl proton at C-3, exchanging with water at a relatively fast rate, was proved by these experiments.

Table 2. HMBC Correlations for Carbonyl Carbons of 1 ($^nJ_{CH}$ = 5.6 Hz)

		correlation with		
residue	C=O	NH	α-H	β-H and others
OHDa	1	Leu-4	Leu-4	H-2,3 (OHDa)
Leu-4	α	Asp-1	Asp-1, Leu-4	
Asp-1	α	Thr	Asp-1, Thr	βa,b (Asp-1)
	β		Asp-1	βa,b (Asp-1)
Thr	α	Leu-2	Thr, Leu-2	β (Thr)
Leu-2	α	Leu-5	Leu-2	
Leu-5	α	Ser-2	Ser-2, Leu-5	
Ser-2	α		Ser-2, Leu-3	βa,b (Ser-2)
Leu-3	α	Ser-1	Ser-1	
Ser-1	α		Leu-1, Ser-1	βa,b (Ser-1)
Leu-1	α		Ile, Leu-1	
Ile	α	Asp-2	Asp-2, Ile	
Asp-2	α		Asp-2	βa (Asp-2)
	β		Asp-2	β (Thr), βa (Asp-2)

The sequence of the residues of amino acids and OHDa was determined by interpretation of HMBC (Figure 1 and Table 2) and ROESY spectra (Figure 2 and Table 3). In order to avoid being misled by long-range ROE correlations, we initially tried to assign the through-bond HMBC correlations. A complication in the HMBC spectrum arose from spectral crowding in the region of carbonyl carbons. We found that the signals of α-carbonyl carbons of Asp-1 and Ser-2 were coincident at δ 170.2. The signal of the α-carbonyl carbon of Asp-2 was hidden in the vicinity of two close signals of α-carbonyl carbons of Leu-2 (δ 172.01) and Leu-3 (δ 171.95). In addition, an intraresidue correlation between the α-carbonyl carbon and α-H of Leu-3 could not be detected due to severe signal overlapping. Therefore, the HMBC experiments using several mixing times only allowed construction of three partial structures of OHDa-Leu-4-Asp-1, Thr-Leu-2-Leu-5-Ser-2, and Ser-1-Leu-1-Ile-Asp-2. These sequences

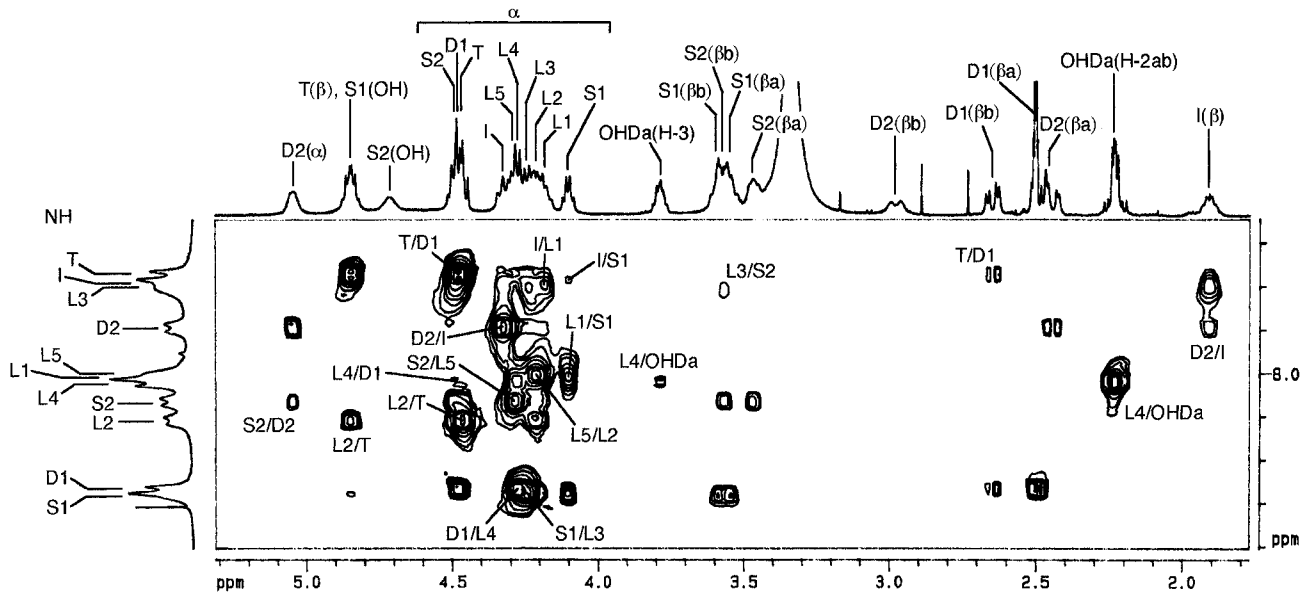

Figure 2. NH signal region of the ROESY spectrum of **1** in DMSO-d_6 (0.02M, pH 4, adjusted with CF_3COOH). Component amino acids are abbreviated using the usual one letter code. Labeled cross peaks were used for determination of interresidue sequences.

Table 3. Interresidue ROE Correlations[a] of 1

		correlation with	
residue	proton	sequentially adjacent residues	other residues
OHDa	H-2	α-H, NH (Leu-4, w, s)	*β*-H (Thr, w), α-H (Asp-1, w)
	H-3	α-H, NH (Leu-4, w, w)	*β*-H (Thr, m)
Leu-4	α-H	NH (Asp-1, s), H-2,3 (OHDa, w, w)	
	NH	H-2,3 (OHDa, s, w), α-H (Asp-1, w)	
Asp-1	α-H	NH (Thr, s), NH (Leu-4, w)	H-2 (OHDa, w)
	NH	α-H (Leu-4, s)	
Thr	α-H	NH (Leu-2, s)	
	β-H	α-H, NH (Leu-2, w, m), α-H (Asp-2, w)	H-2,3 (OHDa, w, m)
	NH	α-H, *β*b-H (Asp-1, s, m)	
Leu-2	α-H	NH (Leu-5, s), *β*-H (Thr, w)	
	NH	α-H, *β*-H (Thr, s, m)	
Leu-5	α-H	NH (Ser-2, s)	α-H (Asp-2, m)
	NH	α-H (Leu-2, s)	
Ser-2	α-H		α-H (Asp-2, m)
	NH	α-H (Leu-5, s)	α-H (Asp-2, w)
Leu-3	α-H	NH (Ser-1, s)	
	NH	*β*b-H (Ser-2, w)	
Ser-1	α-H	NH (Leu-1, s)	NH (Ile, w)
	NH	α-H (Leu-3, s)	
Leu-1	α-H	NH (Ile, m)	
	NH	α-H (Ser-1, s)	
Ile	α-H	NH (Asp-2, s)	
	NH	α-H (Leu-1, m)	α-H (Ser-1, w)
Asp-2	α-H	*β*-H (Thr, w)	α-H, NH (Ser-2, m, w), α-H (Leu-5, m)
	*β*a-H		*β*b-H (Ser-2, w)
	NH	α-H, *β*-H (Ile, s, m)	

[a] Observed in the ROESY spectrum, spin-locking time 200 ms: w, weak; m, medium; s, strong.

were also supported by strong interresidue ROE correlations (Table 3). Furthermore, the ROESY spectrum showed a strong cross peak between the α-H of Asp-1 and NH of Thr, which would allow the combination of the first two partial sequences to give the sequence of OHDa-Leu-4-Asp-1-Thr-Leu-2-Leu-5-Ser-2. A strong ROE correlation between the α-H of Leu-3 and the NH of Ser-1 indicated that the Leu-3 residue was linked to the third substructure. Three interresidue ROE correlations from α-H, NH, and *β*b-H of Ser-2 to α-H, α-H, and *β*a-H of Asp-2, respectively, hampered an unambiguous conjunction between Ser-2 and Leu-3, although a weak ROE correlation between *β*b-H of Ser-2 and NH of Leu-3 was observed. However, HMBC correlation between a carbonyl carbon at δ 170.2 (Asp-1 or Ser-2) and the α-H of Leu-3, and the linkage of Asp-1-Thr obtained from ROESY experiments, enabled us to connect Ser-2 and Leu-3. Finally the whole peptide sequence was established as OHDa-Leu-4-Asp-1-Thr-Leu-2-Leu-5-Ser-2-Leu-3-Ser-1-Leu-1-Ile-Asp-2.

The ester linkage between the residues of Thr and Asp-2 was confirmed by the HMBC correlations between a carbonyl carbon at δ 169.6 and the *β*-H of Thr and between it and α- and *β*a-H of Asp-2. The absence of the NMR signal for the *β*-hydroxyl proton of Thr also supported the structure. However, there still remained the problem as to whether the carbonyl carbon was attached to the α- or *β*-carbon of Asp-2. During signal assignment, we found that the addition of trifluoroacetic acid to a sample solution not only improved the resolution but also caused the α-H signal of Asp-2 to shift downfield from δ 4.77 (pH 7) to δ 5.05 (pH 4), whereas the chemical shift of the α-proton of Asp-1 was only slightly shifted from δ 4.42 (pH 7) to δ 4.48 (pH 4). A similar downfield shift was reported[13] for a *β*-proton of a *β*-hydroxyaspartic acid residue that has a free *β*-carboxylic acid. Therefore, the α-carboxylic acid of Asp-2 was considered to be free, as shown in structure **1**. Two other findings substantiating this lactone structure were as follows: Firstly, a larger chemical shift difference was observed between *β*a- and *β*b-H of Asp-2 (0.53 ppm) than between those of Asp-1 (0.17 ppm). Secondly, the residue of Asp-2 only gave one intraresidue ROE correlation between the NH and *β*b-H, whereas Asp-1 afforded two correlations between the NH and both *β*a- and *β*b-H with similar intensity. These two observations suggested the mobilities of *β*-protons of Asp-2 were more restricted in a cyclic structure than those of Asp-1. Thus, on the basis of the NMR spectroscopic studies, pholipeptin was determined as the lipoundecapeptide lactone **1**.

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Several long-range interresidue ROEs (Table 3) provided information on the conformation of the molecule. The peptide chain of OHDa-Leu-4-Asp-1 must be folded toward the Thr residue, the branching point of the cyclic peptide lactone, based on the ROE correlations from a β-H of Thr to H-2 and 3 of OHDa. Regarding the cyclic moiety, the Asp-2 residue was considered to be located spatially in the vicinity of Leu-5 and Ser-2, according to the ROE correlations from α-H of Asp-2 to NH and α-H of Ser-2 and to α-H of Leu-5 and from βa-H of Asp-2 to βb-H of Ser-2.

Furthermore, absolute configurations of the constituent amino acids were analyzed by chiral HPLC. Peaks of hydrolysate were identified by coinjection of each authentic optically pure sample. The configuration of Leu was detected with a Crownpak CR(+) column, and those of other amino acids were detected with a Chiralpak WH column. The configurations of D- and L-Leu could not be distinguished by the latter column. The molar ratio of amino acids was calculated from the peak area from the Chiralpak WH column. All of the Leu and Ser moieties were shown to be in the D-configuration, and both Ile and Thr were in the L-configuration. The peak area of D-Leu was analyzed as 77.2 pmol × 5 mol/100 pmol pholipeptin, because no L-Leu was detected when the Crownpak CR-(+) column was used. The absolute intensity of L-Thr was low (57.7 pmol for 100 pmol pholipeptin), as reported for arthrofactin.[4] The fact that the intensity became lower as time passed (data not shown) suggests the instability of Thr in 0.02 N HCl solution. Two Asp moieties were contained as a racemic mixture, 76.8 and 90.5 pmol for 100 pmol of D-Asp and L-Asp, respectively, so the configurations of Asp-1 and Asp-2 could not be determined simply.

We tried to determine the absolute configurations of Asp-1 and -2 by a biosynthetic method using ^{13}C-labeled material. We fed L-Asp-4-^{13}C to the production medium and then subjected the purified **1** to ^{13}C NMR analysis. As a result, the β-carbonyl carbon signal of Asp-1 was observed to be stronger than any other carbonyl carbon signals including the β-carbonyl carbon of Asp-2. The intensity was 1.4 times as strong as that of the adjacent carbonyl signal at δ 168.6 (Thr). Therefore, L-Asp-4-^{13}C was concluded to be incorporated selectively into the position of Asp-1, and chirality of Asp-1 was determined as the L-configuration. Thus, optical configurations of all amino acids in **1** were determined as shown. Related peptides include arthrofactin,[4] acyclic peptide isolated from *Arthrobacter* sp. in which, however, the ester bond was formed between the C-terminal Asp and OHDa.

Pholipeptin (**1**) inhibited PI-PLC of human carcinoma A431 cells with an IC_{50} of 5.8 μM. It inhibited the enzyme noncompetitively with the substrate. The phospholipase C inhibitor (PCI) peptide was also reported to inhibit the enzyme in a noncompetitive manner.[14] The structure of PCI peptide is Tyr-Arg-Lys-Met-Arg-Leu-Arg-Tyr, in which Tyr residues of the N- and C-terminus are necessary for the inhibition. But there was no Tyr residue in **1**. Moreover, this peptide is rich in basic amino acids, such as Arg and Lys, while **1** does not contain any basic amino acid. The mechanism of PI-PLC inhibition for **1** is being studied by preparation of the partial structure. Other PI−PLC inhibitors include fluvirucin B_2[15] and plipastatin.[16] The former is a macrolide, and the latter, being a detergent peptide, inhibits phospholipase both A and C. The inhibitory activity of **1** on PI-PLC is comparable to that of fluvirucin B_2. The structure of **1** is completely different from those of other PI-PLC inhibitors. So **1** would be a new guide compound for preparation of more potent PI-PLC inhibitors.

Experimental Section

Measurement of PI-PLC Activity. PI-PLC activity was assayed as described before[15] by the method of Wahl et al.[2] with slight modifications.

Isolation of Pholipeptin (1). The producing strain has been deposited in the National Institute of Bioscience and Human Technology Agency, Ministry of International Trade and Industry, Tsukuba, Japan, under the accession number FERM P-14943. *P. fluorescens* BMJ279-76F1 was cultured in production medium (1.5% glycerol, 1.5% soluble starch, 0.5% soy bean meal, 1.5% fish meal, and 0.2% $CaCO_3$, pH 7.4 before autoclaving) at 27 °C for 96 h. The culture broth (2 L) was centrifuged at 10 000 rpm, and the filtrate was applied onto a Diaion HP-20 column. The column was washed with H_2O and 50% MeOH, and the active fraction was eluted with 100% MeOH. After addition of water to the eluate, evaporation was carried out *in vacuo* to remove the MeOH. The resultant solution was adjusted to pH 2 and extracted with an equal volume of EtOAc. The organic extract was concentrated to dryness and dissolved in MeOH. The MeOH-insoluble fraction was removed by filtration, and the soluble fraction was fractionated on a silica gel column (stepwise gradient elution, 100% $CHCl_3$ to $CHCl_3$/MeOH (10/5)). The active fraction was further chromatographed on a silica gel column (stepwise gradient elution, BuOAc/BuOH/MeOH/H_2O (6/4/1/1) to (2/4/1/1)), again, and followed by Sephadex LH-20 column chromatography (MeOH) to yield **1** (20 mg).

NMR Spectroscopy. NMR spectra were recorded with a 5-mm $^{13}C/^{1}H/^{19}F$ triple-resonance probe[17] or a 5-mm inverse probe, operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C, and the sample temperature was maintained at 30 °C. Chemical shifts (δ) were reported in ppm relative to internal tetramethylsilane. Spectral simulations and determinations of the sign of couplings (J, Hz) were not carried out. HOHAHA spectra were recorded with mixing times of 55, 85, and 112 ms. ROESY spectra were measured with a spin-locking time of 200 ms. HMQC experiments were acquired for $^1J_{C-H} = 145$ Hz, whereas HMBC spectra were obtained for $^{2,3}J_{C-H} = 4.0$, 5.6, 7.0, 8.3 Hz.

Data for pholipeptin (1): white powder; UV (MeOH) end absorption; IR (KBr) 3295, 2957, 1657, 1547 cm^{-1}; ^1H NMR (DMSO-d_6 with TFA, pH 4) see Table 1; ^{13}C NMR (DMSO-d_6 with TFA, pH 4), see Table 1; FAB-MS, m/z = 1376 (M + Na + H), 1374 (M + Na − H), 1352 (M − H); FAB-HRMS calcd for $C_{64}H_{110}O_{20}N_{11}$ (M − H) 1352.7929, found 1352.7922.

Amino Acid Analysis. For standard amino acid analysis, 1 mg of **1** dissolved in 1 mL of 6 N HCl was placed in a sealed glass tube and heated at 110 °C for 20 h. After evaporation, the residue was dissolved in 1 mL of 0.02 N HCl and subjected to amino acid analysis on a Hitachi amino acid analyzer L-8500 system. Retention times in the amino acid analysis (min) were as follows: Asp (5.58), Thr (6.38), Ser (6.97), Ile (16.37), Leu (17.36).

Chiral HPLC Analysis. One mg of **1** was dissolved in 6 N HCl (1 mL) and heated at 120 °C for 20 h. The reaction mixture was evaporated *in vacuo*, and the residue was dissolved in 0.02 N HCl and analyzed by a Chiralpak WH (4.0 mm ϕ × 150 mm, Daicel Chemical Industries, LTD) column and a Crownpak CR(+) (4.6 mm ϕ × 250 mm, Daicel Chemical

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(17) For triple-resonance experiments using this probe, see: Miyake, T.; Koyama, Y. *Carbohydr. Res.* **1994**, *258*, 11−26.

Industries, LTD) column. For the Chiralpak WH column, the oven temperature was held at 45 °C, and elution was performed with 0.25 mM aqueous $CuSO_4$ (1 mL/min). Eluted amino acids were detected by absorption at 254 nm, and their retention times (min) were as follows: 29.22 (L-Ser), 20.90 (D-Ser), 30.73 (L-Thr), 22.96 (D-Thr), 44.17 (L-Ile), 34.84 (D-Ile), 58.49 (L-Asp), 38.85 (D-Asp), 35.57 (L-Leu), and 37.91 (D-Leu). Retention time (min) of the acid hydrolysate of **1** was as follows: 20.20 (D-Ser), 28.04 (L-Thr), 34.04 (D-, L-Leu), 39.11 (D-Asp), 44.99 (L-Ile), and 54.83 (L-Asp). For the Crownpak CR(+) column, each amino acid was eluted with aqueous perchloric acid (pH 1.5, 1 mL/min) and detected with absorption of 200 nm at room temperature. The retention time (min) for each amino acid residue was as follows: 4.93 (L-Ser), 4.89 (D-Ser), 5.36 (L-Thr), 5.05 (D-Thr), 11.37 (L-Ile), 10.40 (D-Ile), 5.83 (L-Asp), 5.64 (D-Asp), 17.28 (L-Leu), and 12.36 (D-Leu). The retention time (min) of acid hydrolysate of **1** was as follows: 4.76, 12.48 (D-Leu). The peak at 4.76 min was broad, and expected peaks were not detected separately.

Determination of Chirality by Biosynthetic Method. *P. fluorescens* BMJ279-76F1 was cultured as described above. After the bacteria had been inoculated into the production medium, 0.05 mg/mL of L-Asp-4-^{13}C (99 atom % ^{13}C; Isotec Inc.) was added; cultivation was then carried out for 96 h. **1** was isolated as above to yield 3.6 mg. It was then subjected to ^{13}C NMR analysis.

Acknowledgment. This work was partly supported by grants from the Ministry of Education, Science, and Culture and the Japan Owners Association.

JO9603993