PRADIMICINS FS AND FB, NEW PRADIMICIN ANALOGS: DIRECTED PRODUCTION, STRUCTURES AND BIOLOGICAL ACTIVITIES

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(Received for publication October 12, 1992)

Exogenous addition of D-serine to *Actinomadura spinosa* AA0851 resulted in directed production of pradimicin FS, a new D-serine analog of pradimicin S, together with pradimicin FB. Pradimicin FS was produced in higher yields by derivation of ferrous sulfate-resistant strains from strain AA0851. Pradimicin FB, a minor product, was identified as deglucosylpradimicin FL. Pradimicin FS was equivalent to pradimicin S in syncytium formation inhibition activity and *in vitro* and *in vivo* antifungal activities.

Normally, *Actinomadura spinosa* AA0851 produces pradimicin S together with pradimicins B and L as minor components[1,2]. This producer is clearly differentiated from known pradimicins-producing strains such as *Actinomadura hibisca* P157-2[3] and *Actinomadura verrucosospora* subsp. *neohibisca* R103-3[4] in their spore surface ornamentations, cellular fatty acid compositions and pradimicin S production.

Pradimicin S[2] differs from the other members of the pradimicin family in the sugar moiety, and more particularly in pradimicin S, the D-xylosyl-D-thomosamine of pradimicin A[5,6] is replaced by 3-*O*-sulfo-D-glucosyl-D-thomosamine at the C-5 position. It is active against human immunodeficiency virus (HIV) and pathogenic fungi and yeasts[1].

During the course of extensive studies on fermentation of the pradimicin-benanomicin family of antibiotics, we developed an approach to directed fermentation of the D-serine analogs[7,8] of pradimicin A and benanomicin A which have therapeutically useful properties. Consequently, our interest was focused on the generation of new D-serine analogs of pradimicin S under directed biosynthesis by *A. spinosa* AA0851, as pradimicin S seemed more potent in anti-HIV activity than other pradimicin analogs. The present paper describes directed fermentation of pradimicins FS and FB in strain AA0851 by exogenous addition of D-serine and their structures as well as biological activities of pradimicin FS.

Materials and Methods

Directed Fermentation

Actinomadura spinosa AA0851 was grown at 28°C for 3 weeks on YS agar medium consisting of soluble starch 1%, yeast extract (Difco) 0.2% and agar 1.8%. A loopful spores of strain AA0851 were cultivated for 5 days at 32°C and 200 rpm on a rotary shaker in a 500-ml of Erlenmeyer flask containing 100 ml of seed medium (sucrose 1%, Pharmamedia (The Procter & Gamble Oilseed Products Co.) 0.5%, yeast extract (Oriental Yeast Co.) 0.5% and $CaCO_3$ 0.1%; pH 7.0 before autoclaving). The vegetative seed culture (5 ml) was transferred into a 500-ml of Erlenmeyer flask containing 100 ml of FR20-1 medium

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(sucrose 3%, glucose 1%, Pharmamedia 3%, $FeSO_4 \cdot 7H_2O$ 0.1% and $CaCO_3$ 0.3%; pH 7.5 before autoclaving) supplemented with 0.2% amino acid and 10 μg/ml D-cycloserine (separately sterilized). The fermentation was carried out for 14 days at 28°C and 200 rpm on a rotary shaker. Products in the broth supernatant were analyzed by HPLC.

HPLC Analysis of Pradimicin Analogs

After fermentation, the supernatant of broth was diluted ten-fold with DMSO and passed through a membrane filter (Gelman Science Japan, Ltd., Ekicrodisc 13CR, pore size: 0.45 μm). The filtrate was analyzed with a Waters M600 HPLC system. Conditions employed for analysis of pradimicin analogs were as follows: Reversed phase Cosmosil $5C_{18}$-AR column (4.6 i.d. × 100 mm, particle size 5 μm, Nacalai Tesque Inc.), using a 27:73 mixture of CH_3CN-0.01 M KH_2PO_4 (pH 3.5) as a mobile phase and a flow rate of 1 ml/minute under spectrophotometric monitoring at 460 nm. Retention times for pradimicins FL, FB, FS, L, B and S were 4.0, 5.2, 5.4, 7.7, 9.8 and 11.6 minutes, respectively.

Fermentation and Isolation of Pradimicins FS and FB

Five-ml seed culture each of strain AA0851 described above was transferred into one hundred 500-ml Erlenmeyer flasks containing 100 ml of production medium composed of sucrose 3%, glucose 1%, Pharmamedia 3%, $FeSO_4 \cdot 7H_2O$ 0.1%, $CaCO_3$ 0.3%, D-serine 0.2% and D-cycloserine 10 μg/ml (separately sterilized); pH 7.5 before autoclaving, and the broths were fermented at 28°C and 200 rpm on a rotary shaker. After 12 days' fermentation, the combined broth (9 liters) was centrifuged, and the resulting supernatant was adsorbed on Diaion HP-20 (3 liters). After the resin was rinsed with water (50 liters), pradimicins were eluted with 80% aqueous acetone (2.6 liters). The acetone was evaporated *in vacuo*, and the aqueous concentrate was lyophilized to give a crude solid (6.2 g) which was estimated to contain pradimicins FL (24 mg), FB (0.92 g), FS (1.85 g), L (38 mg), B (1.08 g) and S (1.52 g) by HPLC analysis. The solid was dissolved in water (2 liters) at pH 7.5, and insoluble impurities were removed by filtration. The filtrate was adjusted to pH 2.5 with 0.1 N HCl to yield pradimicin FS precipitates. The resulting precipitates were washed with 0.001 N HCl (1.5 liters) and then dissolved in alkaline water (150 ml). The pradimicin FS solution was dropwise added to acetone (1.5 liters) to afford precipitates (7.2 g). The precipitates were dissolved in CH_3CN-0.01 M KH_2PO_4, pH 7 (15:85, 250 ml) and applied to a column of YMC ODS-A60 gel (Yamamura Chemical Lab. Ltd., 5 liters). The column was eluted with the same solvent mixture, and the eluate fractions were monitored by HPLC described above. The fractions (7.7 liters) containing pradimicin FS were combined and concentrated *in vacuo* for removal of CH_3CN. The concentrate was applied onto a column of Diaion HP-20 (250 ml) and eluted with acetone-H_2O, pH 2.5 (60:40, 1 liter). The pradimicin FS fraction was concentrated to 80 ml *in vacuo* and lyophilized to give pradimicin FS hydrochloride (409 mg). A portion of the preparation (250 mg) was dissolved in alkaline water (pH 7.5, 40 ml) and lyophilized to afford pradimicin FS sodium salt (257 mg).

The fractions (2.8 liters) containing pradimicin FB were similarly worked up to afford pradimicin FB hydrochloride (91 mg).

Isolation of High Producer Strains

Spore suspension (about 5×10^5 spores/ml) of strain AA0851 was spread onto YS agar plates containing 0.3% ferrous sulfate and incubated for 3 weeks at 28°C. Colonies grown on agar plates were confirmed for their ferrous sulfate resistance by repeated cultivation on 0.3% ferrous sulfate-containing agar plates. Twenty six ferrous sulfate-resistant strains thus obtained were subjected to fermentation studies.

Acid Hydrolysis of Pradimicin FS

Pradimicin FS (100 mg) was suspended in 6 N HCl (20 ml) and refluxed at 115°C for 16 hours. After cooling, the red precipitates were collected by filtration. The filtrate was diluted with water and neutralized with Amberlite IR-45 (OH^-). The solution was concentrated to 20 ml *in vacuo* and passed through a column of Diaion HP-20 (50 ml). Ninhydrin-positive fractions (8 ml) were concentrated *in vacuo* and lyophilized to yield a white powder of serine (3 mg), which was determined to have the *R*-configuration by HPLC (Excelpak SIL-C_{18} 5B column (4.6 i.d. × 150 mm, particle size 5 μm, Yokogawa Electric Co., Ltd.)), using MeOH-22 mM sodium phosphate buffer containing THF 1%, pH 7.0 (20:80) as a mobile

phase and a flow rate of 1.0 ml/minute under spectrophotometric monitoring at 344 nm. Retention times of L-serine and D-serine were 9.72 and 9.92 minutes, respectively.

The red precipitates obtained were washed with water and dried to afford pradimicinone Is[8] (62 mg): MP (dec.) 220°C; FAB-MS (positive, *meta*-nitrobenzylalcohol), *m/z* 566 $(M+H)^+$, (negative, *meta*-nitrobenzylalcohol), *m/z* 565 (M^-); molecular formula, $C_{28}H_{23}NO_{12}$; UV (0.02 N NaOH-MeOH = 1 : 1), λ_{max} nm (ε) 242 (28,300), 319 (14,000) and 497 (11,500).

Mild Acid Hydrolysis of Pradimicin FL

Pradimicin FL hydrochloride (50 mg) was hydrolyzed as described in a previous paper[4], resulting in deglucosylpradimicin FL (20 mg): MP >195°C (dec.); FAB-MS (*meta*-nitrobenzylalcohol), *m/z* 725 $(M+H)^+$; molecular formula, $C_{35}H_{36}N_2O_{15}$; UV (0.02 N NaOH-MeOH = 1 : 1) λ_{max} nm (ε) 300 (24,500), 458 (10,200); IR ν_{max} (KBr) 3400, 1720, 1620~1610, 1390, 1340, 1295, 1260, 1130, 1065 cm^{-1}.

General

MP's were measured with a Yanaco MP-3S micromelting point apparatus and are uncorrected. Spectra data were recorded with the following instruments: IR, JASCO IR-810; UV-vis, JASCO UVIDEC-610 spectrometer; ^1H and ^{13}C NMR, JEOL JMN-GX400 spectrometer; FAB-MS, JEOL JMS-AX 505H spectrometer.

Results and Discussion

Directed Biosynthesis

A. spinosa AA0851 usually produces pradimicin S together with pradimicins B and L as minor components[1]. Structurally, pradimicin S has D-alanine as the amino acid side chain on the 5,6-dihydrobenzo[*a*]naphthacenequinone chromophore[2]. As *A. spinosa* strain AA0851 differs from other pradimicins producers in taxonomic characteristics and pradimicin S productivity, the D-alanine moiety of pradimicin S seemed to be susceptible to substitution through the exogenous addition of a amino acid to the fermentation medium. No congener of pradimicin S was produced by supplementing L-amino acids as the case in other pradimicins producers. Subsequently, the effects of D-amino acids were examined on the production of pradimicins by strain AA0851. As the D-alanine moiety of pradimicin S seemed to be biosynthesized from L-alanine by alanine racemase, D-cycloserine, and alanine racemase inhibitor, was added at 10 μg/ml to FR20-1 supplemented with 0.2% D-amino acids or glycine. As shown in Table 1, the production yield of pradimicins in strain AA0851 is clearly decreased by the addition of D-amino acids and glycine. Among the D-amino acids so far tested, only D-serine results in the formation of new analogs of pradimicins S and B, named pradimicins FS and FB, respectively, together with known pradimicin FL[4].

Table 1. Effects of D-amino acids on the production of pradimicins by strain AA0851.

Amino acid (0.2%)	Pradimicin component (μg/ml)					
	FL	FB	FS	L	B	S
D-Aspartic acid				45	110	205
D-Leucine				110	266	562
D-Serine	35	176	212	60	136	324
D-Threonine				101	284	513
D-Valine				128	309	487
Glycine				176	326	542
None				182	514	735

Fermentation: 28°C, 14 days.
Basal medium: FR20-1 supplemented with 10 μg/ml D-cycloserine.

Fermentation and Isolation

Strain AA0851 was fermented at 28°C for 12 days in FR20-1 supplemented with 0.2% D-serine and 10 μg/ml D-cycloserine for isolation of pradimicins FS and FB. After fermentation, pradimicins FS and FB were isolated by a combination of Diaion HP-20 adsorption, acidic precipitation and reversed phase silica gel column chromatography. Pradimicin FL was unable to be isolated in a pure state because of low productibity, but HPLC analysis clearly allowed to identify it as pradimicin FL from *A. verrucosospora* subsp. *neohibisca*[4].

Improvement of Pradimicin FS Productivity

As pradimicin S is a sulfated compound and the addition of ferrous sulfate is stimulatory to the pradimicin S production[1,2], selection of ferrous sulfate-resistant clones seemed to be a more rational approach to the improved production of pradimicin S (or pradimicin FS in the presence of D-serine). On the basis of this conjecture, strain AA0851 was subjected to the single spore selection for increased resistance to ferrous sulfate, providing 26 ferrous sulfate-resistance strains. They were subjected to fermentation studies in the presence or absence of D-serine and D-cycloserine. As expected, two ferrous sulfate-resistant strains (ASR-22 and ASR-25) among 26 strains show higher pradimicin FS productivities in the presence of D-serine and D-cycloserine and higher pradimicin S productivities in the absence of D-serine and D-cycloserine (Table 2). Interestingly, no pradimicin L production is observed in strain ASR-25. This result indicates that the natural population of highly productive clones in strain AA0851 is low and can easily be selected by ferrous sulfate resistance.

Physico-chemical Properties and Structures

Pradimicins FS and FB are acidic and possess physico-chemical properties as summarized in Table 3. Pradimicin FS was closely similar to pradimicin S in the UV-visible and IR spectra, indicating that pradimicin FS also has the chromophore of 5,6-dihydrobenzo[*a*]naphthacenequinone[2]. The molecular formula of pradimicin FS is determined to be $C_{41}H_{46}N_2O_{23}S$ by HRFAB-MS spectrometry. The pseudo-molecular ion peak of pradimicin FS is 16 mass units higher than that of pradimicin S. The ^1H and ^{13}C NMR spectra of pradimicin FS (Table 4) correspond well with those of pradimicin S[2], differing only in the C-17′-CH_3 signal (δ_H 1.37 and δ_C 17.0) in pradimicin S. That signal is replaced by a new signal

Table 2. Effects of D-serine and D-cycloserine on the production of pradimicins by strains ASR-22, ASR-25 and AA0851.

Strain	D-Ser[a]	D-CS[b]	Pradimicin component (μg/ml)					
			FL	FB	FS	L	B	S
ASR-22	+	−	12	491	601	10	520	916
	+	+	15	339	951	19	437	649
	−	−	—	—	—	25	936	1,717
ASR-25	+	−	0	521	518	0	577	811
	+	+	0	307	618	0	431	628
	−	−	—	—	—	0	871	1,740
AA0851	+	−	10	154	240	48	294	553
	+	+	25	179	287	57	203	441
	−	−	—	—	—	61	587	1,132

Fermentation: 28°C, 12 days.
[a] D-Serine (0.2%).
[b] D-Cycloserine (10 μg/ml).

Table 3. Physico-chemical properties of pradimicins FS and FB.

	Pradimicin FS	Pradimicin FB
Nature	Violet amorphous powder (sodium salt)	Dark red amorphous powder (sodium salt)
Solubility (covers both antibiotics)		
soluble in	Dimethyl sulfoxide, dimethylformamide, alkaline water	
slightly soluble in	Water, methanol, ethanol, acetone	
insoluble in	Acidic water, ethyl acetate, benzene, *n*-hexane, chloroform	
MP (°C, dec.)	>180 (sodium salt)	>195 (hydrochloride)
Molecular formula	$C_{41}H_{46}N_2O_{23}S$	$C_{35}H_{36}N_2O_{15}$
FAB-MS (Positive) *m/z*	967 $(M+H)^+$, 989 $(M+Na)^+$	725 $(M+H)^+$
HRFAB-MS (Positive) *m/z*	Obsd: 967.2267 $(M+H)^+$ Calcd for $C_{41}H_{46}N_2O_{23}S$: 967.2291	
UV λ_{max} nm (ε)		
in 0.02 N NaOH - MeOH (1 : 1)	240 (34,900), 320 (15,600), 498 (14,500)	244 (30,500), 320 (13,900), 498 (13,400)
IR (KBr) cm^{-1}		
Acidic form	3400, 3000~2900, 1735, 1640~1605, 1450, 1390, 1300, 1260, 1160, 1060, 970	3400, 1720, 1620~1610, 1390, 1340, 1295, 1260, 1130, 1065
Sodium salt	3410, 1625, 1610, 1445, 1390, 1260, 1160, 1060, 975	

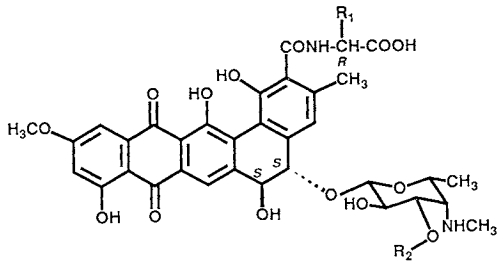
Table 4. ^1H and ^{13}C NMR assignments of pradimicin FS hydrochloride in DMSO-d_6.

Position	δ_H	δ_C	Position	δ_H	δ_C
1		151.2 (s)	$N^{16}H$	8.26 (d, 5.1)	
2		127.1 (s)	17	4.47 (t, 5.1)	54.8 (d)
3		137.3 (s)	17′-CH_2	3.76 (dd, 5.1, 12.0)	61.2 (t)
3-CH_3	2.33 (s)	19.2 (q)	18		171.5 (s)
4	7.14 (br s)	119.4 (d)	1′	4.81 (d, 7.7)	103.8 (d)
4a		137.6 (s)	2′	3.25~3.34 (m)	69.4 (d)
5	4.60 (d, 10.7)	80.6 (d)	3′	4.00 (m)	79.8 (d)
6	4.62 (d, 10.7)	71.3 (d)	4′	3.50 (br d)	63.2 (d)
6a		147.7 (s)	4′-NCH_3	2.72 (br s)	35.9 (q)
7	8.04 (br s)	115.5 (d)	5′	3.91 (q, 6.8)	67.2 (d)
7a		131.2 (s)	5′-CH_3	1.27 (d, 6.8)	15.9 (q)
8		184.8 (s)	1″	4.58 (d, 7.7)	104.2 (d)
8a		110.0 (s)	2″	3.25~3.34 (m)	71.9 (d)
9		164.6 (s)	3″	4.04 (t, 8.8)	82.0 (d)
10	6.95 (d, 2.6)	106.8 (d)	4″	3.25~3.34 (m)	68.8 (d)
11		165.9 (s)	5″	3.25~3.34 (m)	76.5 (d)
11-OCH_3	3.96 (s)	56.2 (q)	6″ax	3.48 (br d)	60.6 (t)
12	7.30 (d, 2.6)	107.5 (d)	6″eq	3.75 (dd, 4.3, 11.1)	
12a		134.2 (s)			
13		187.3 (s)			
13a		116.2 (s)			
14		156.8 (s)			
14a		125.7 (s)			
14b		114.0 (s)			
15		167.0 (s)			

ppm (multiplicity, *J* in Hz).

(δ_H 3.76 and δ_C 61.2) which is assigned to 17′-CH_2OH in pradimicin FS. The C-17 signal is shifted 7.0 ppm downfield in pradimicin FS. These facts suggest that pradimicin FS has serine instead of alanine as the amino acid side chain. This conjection was confirmed by the following degradation study: Vigorous acid

Fig. 1. Structures of pradimicins FS, S, FB, B, FL and L.



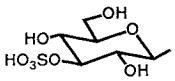
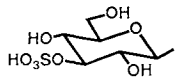
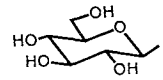
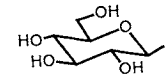
	R_1	R_2
Pradimicin FS	CH_2OH	
Pradimicin S	CH_3	
Pradimicin FB	CH_2OH	H
Pradimicin B	CH_3	H
Pradimicin FL	CH_2OH	
Pradimicin L	CH_3	

Table 5. Syncytium formation inhibitory activity.

Compound	ID_{50} (μg/ml)	TD_{50} (μg/ml)
Pradimicin FS	5.9	>100
Pradimicin S	5.0	>100
Dextran sulfate	18.0	>100

ID_{50}: Concentration to inhibit the syncytium formation by 50%.

TD_{50}: Concentration to inhibit the growth of host cells by 50%.

Table 6. Antiviral activity against herpes simplex virus type 1 and influenza virus A.

	Influenza virus-MDCK cell		HSV-Vero cell	
	ID_{50} (μg/ml)	TD_{50} (μg/ml)	ID_{50} (μg/ml)	TD_{50} (μg/ml)
Pradimicin FS	82	>100	>100	>100
Pradimicin S	22	>100	>100	>100
Acyclovir			0.09	>100
Ribavirin	9.5	>100		

hydrolysis (6N HCl, at 115°C for 16 hours) of pradimicin FS yielded pradimicinone Is together with D-serine, which was proved to have the *R*-configuration by a chiral HPLC[5]. From these results, the structure of pradimicin FS is concluded to be *N*-[[(5*S*,6*S*)-5-*O*-[4′,6′-dideoxy-4′-(methylamino)-3′-*O*-(3″-*O*-sulfo-β-D-glucopyranosyl)-β-D-galactopyranosyl]-5,6,8,13-tetrahydro-1,5,6,9,14-pentahydroxy-11-methoxy-3-methyl-8,13-dioxobenzo[*a*]naphthacene-2-yl]carbonyl]-D-serine as shown in Fig. 1.

The identity of pradimicin FB with the deglucosyl analog of pradimicin FL was established by direct comparison with the product obtained by acid hydrolysis of pradimicin FL[4].

The structures of pradimicins produced by *A. spinosa* AA0851 in the presence of D-serine are summarized in Fig. 1.

Biological Activities

As shown in Table 5, the syncytium formation inhibition activity of pradimicin FS was compared with those of pradimicin S and dextran sulfate as previously described[1], and pradimicin FS is equipotent to pradimicin S in this assay.

The *in vitro* antiviral activity of pradimicin FS was examined against influenza virus A (Victria strain) and herpes simplex virus (HSV) type 1 (KOS strain) as described in a previous paper[9]. As shown in Table 6, pradimicin FS appears to be less active than pradimicin S against influenza virus A.

In vitro antifungal activities of pradimicin FS were determined and compared with those of pradimicin S, amphotericin B and ketoconazole by the serial 2-fold agar dilution method using yeast morphology agar (pH 7.0). As shown in Table 7, pradimicin FS has good *in vitro* activity against *Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Trichophyton mentagrophytes* and seems equipotent to pradimicin S. The *in vivo* therapeutic efficacy of pradimicin FS

Table 7. *In vitro* antifungal activity.

Test organism	MIC (μg/ml)			
	Pradimicin FS	Pradimicin S	Amphotericin B	Ketoconazole
Saccharomyces cerevisiae ATCC 9763	6.3	3.1	0.2	100
Candida albicans A9540	12.5	25	0.4	25
C. albicans ATCC 38247	6.3	6.3	50	6.3
C. albicans ATCC 32354	12.5	6.3	0.2	50
C. albicans 83-2-14	12.5	25	0.4	25
C. tropicalis 85-8	25	50	0.4	100
C. tropicalis IFO 10241	25	50	0.4	50
Cryptococcus neoformans D49	6.3	1.6	0.4	6.3
Cryptococcus IAM 4514	6.3	1.6	0.4	6.3
Aspergillus fumigatus IAM 2034	6.3	3.1	0.4	3.1
Trichophyton mentagrophytes No. 4329	6.3	6.3	0.4	0.8

Medium: Yeast morphology agar + 1/15 M phosphate buffer (pH 7.0).
Inoculum size: 10^6 cells/ml (except for *T. mentagrophytes*: 10^7 cells/ml).
Incubation conditions: 28°C, 40 hours (60 hours for *T. mentagrophytes*).

was evaluated against systemic *C. albicans* A9540 infection in mice as previously described[10]. The PD_{50} value of pradimicin FS is equivalent to pradimicin S (Table 8).

Table 8. *In vivo* activity against systemic *C. albicans* A9540 infection in mice.

Compound	PD_{50} (mg/kg, single iv)
Pradimicin FS	18
Pradimicin S	20
Amphotericin B	0.31
Ketoconazole	>50

The acute LD_{50} of pradimicin FS in mice by single iv administration was 210 mg/kg.

It is important to note that pradimicin FS, the D-serine analog of pradimicin S, has the same *in vitro* and *in vivo* antifungal activities as those of parental pradimicin S, like pradimicins FA-1 and FA-2[7]. However, our current interest is focused on *in vivo* anti-HIV activity of this compound, because pradimicin S showed more selective and potent *in vitro* anti-HIV activity than other pradimicins[1].

Acknowledgments

The authors express their appreciation to Dr. S. MASUYOSHI and Mr. O. TENMYO for antifungal, syncytium formation inhibitory and antiviral activity test. We wish thank to Messrs. H. YAMAMOTO and S. OHTA for their helpful technical assistance. We also thank to Mr. Y. NARITA for FAB-MS measurement.

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