Pradimicin S, A New Pradimicin Analog

III.[†] Application of the Frit-FAB LC/MS Technique to the Elucidation of the Pradimicin S Biosynthetic Pathway

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The biosynthetic pathway of pradimicin S (PRM-S) was investigated by using sinefungin and bioconversion experiments with aglycones of pradimicin A (PRM-A) and *Actinomadura spinosa* AA0851, a PRM-S producer. Addition of sinefungin to the strain inhibited the formation of 11-*O*-demethyl-7-*O*-methylpradinone II (11dM-7M-PNII) as also determined to occur with its addition to the PRM-A producer. In feeding PRM-A aglycone and its analogs to the strain early in PRM-S biosynthesis, good identifications of bioconverted products were obtained by frit-FAB LC/MS as follows: 11-*O*-demethylpradinone II (11dM-PNII), 11dM-7M-PNII, 11-*O*-demethylpradinone I (11dM-PNI), 11-*O*-demethylpradimicinone I (11dM-PMNI) and pradimicinone I (PMNI) were converted to PRM-S. Pradimicin B (PRM-B) and pradimicin L (PRM-L) were converted to PRMs-L and -S and PRM-S, respectively. A biosynthetic pathway for PRM-S is proposed.

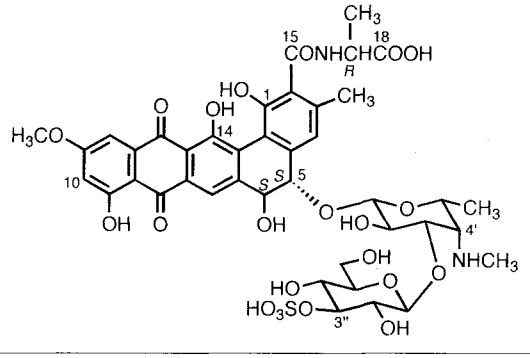
The pradimicins and benanomicins are a new dihydrobenzo[a]naphthacenequinone family of antifungal antibiotics produced by actinomycetes[3~9]. Pradimicin S (PRM-S), a new member of the pradimicin family of antibiotics, is produced by *Actinomadura spinosa* AA0851[1] and contains a dihydrobenzo[a]-naphthacenequinone, D-alanine and disaccharide with a sulfate moiety[2]. The structure of PRM-S was determined as shown in Fig. 1 by NMR and mass spectral analyses and chemical degradation[2].

Biosynthetic studies of the dihydrobenzo[a]naphthacenequinone antibiotics pradimicin and benanomicin have established that their aglycones are of polyketide origin, apparently derived from a dodecaketide and an amino acid[10,11]. The biosynthetic pathways of pradimicins A (PRM-A, 3′-*O*-(β-D-xylopyranosyl)-PRM-B) and T were elucidated by using sinefungin[12~16] and bioconversion experiments with aglycones of PRM-A and their nonproducing mutants[9,17~19].

Subsequently, we isolated pradimicin L (PRM-L)[20] and PRM-S from the fermentation broth of *A. spinosa* AA0851. They were determined to be 3′-*O*-(β-D-glucopyranosyl) pradimicin B (PRM-B) and 3″-*O*-sulfo-PRM-L, the 3′-*O*-(3″-*O*-sulfo-β-D-glucopyranosyl) analog of PRM-A, respectively. Since PRM-S shows better anti-HIV activity than PRM-A, we were very interested in elucidating of the biosynthetic pathway of PRM-S. Since the biosynthetic pathway of PRM-S was theorized as similar to that of PRM-A, sinefungin and the biosynthetic intermediates of PRM-A were utilized in the present study.

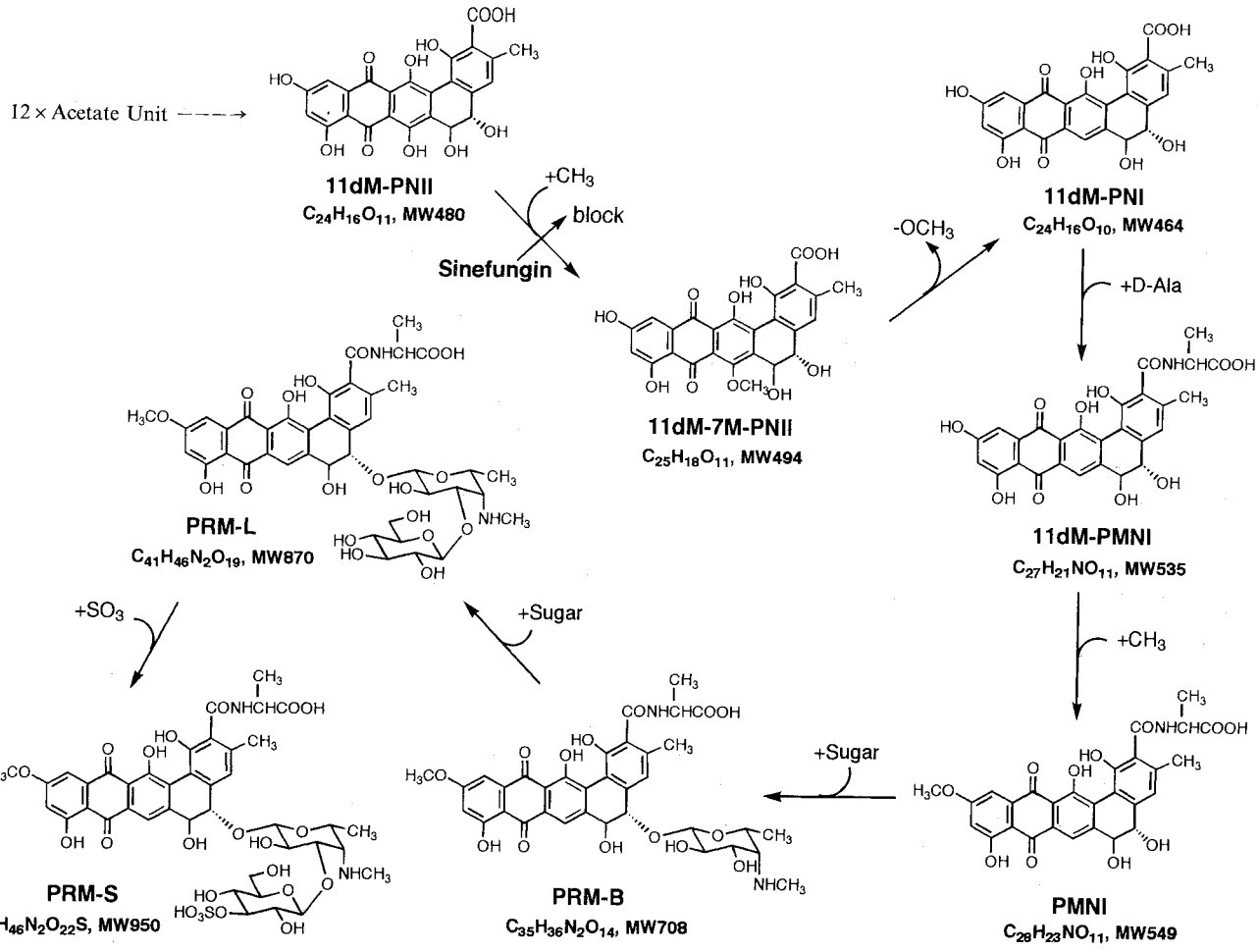
In this type of study, sensitive and specific analytical methods for bioconverted products are necessary if the identification of all products is to be followed during the course of a bioconversion reaction. For this reason the frit-FAB LC/MS[21] seems to be useful because the technique does not require a typical isolation process

Fig. 1. Structure of pradimicin S.



† See ref.[1,2].

Fig. 2. Biosynthetic pathway of pradimicin S.



prior to analysis. We were very interested in applying this specific technique to elucidate the biosynthetic pathway of PRM-S. In this paper, we describe the biosynthetic pathway of PRM-S as summarized in Fig. 2.

Materials and Methods

Bacterial Strain

Actinomadura spinosa AA0851 (ATCC 55138) used in this report has been described in a preceding paper[1].

Time Course Study on Production of PRM-S

Strain AA0851 was transferred into a 500 ml flask containing 100 ml of sterile seed medium consisting of sucrose 1%, Pharmamedia (Traders Protein) 0.5%, yeast extract 0.5% and $CaCO_3$ 0.1% (pH was adjusted to 7.5 before autoclaving), and the flask was incubated at 32°C for 5 days on a rotary shaker (200 rpm). The seed culture (5 ml) thus obtained was transferred into 500-ml flask containing 100 ml of the production medium, and the fermentation was carried out for 12 days at 28°C on a rotary shaker (200 rpm). For the time-course study, fermentation broths were sampled (1 ml each) on days 1, 3, 5, 7, 9 and 12, and metabolites in the broth supernatant were analyzed by HPLC with absorbance at 500 nm[1].

Effect of Sinefungin on the Production of PRM-S

Strain AA0851 was transferred into a 500-ml flask containing 100 ml of seed medium and incubated at 32°C for 4 days on a rotary shaker (200 rpm). The fermentations were carried out at 28°C for 9 days on a rotary shaker (200 rpm) in 50 ml flasks containing 10 ml of production medium, with various concentrations of sinefungin. To confirm the effect of sinefungin, products in each broth supernatant were submitted for antibiotic assay[1].

Bioconversion of Biosynthetic Intermediates and PRMs-B and -L

Seed and production fermentations of strain AA0851 were carried out under the fermentation conditions described above. Cultured broth (5 ml) after 3 days of production fermentation was transferred into each of eight 50-ml flasks. Seven substrates (11dM-PNII, 11dM-7M-PNII, 11dM-PNI, 11dM-PMNI, PMNI, PRM-B and PRM-L, 1 mg each) were added into each broth and fermented for another 18 hours. After fermentation, each whole broth was centrifuged (3,000 rpm, 15 minutes) to obtain broth supernatant, followed by membrane filtration (0.45 μm). Supernatants obtained were adsorbed to an ODS cartridge (Bond Elut, 1 g, Waters). The cartridges were washed with water and bioconversion

products were eluted from each cartridge with 5 ml of acetonitrile - 0.005 N HCl (7 : 3). The solvent was removed *in vacuo*, and the aq residue was lyophilized to yield 5 mg of a crude complex containing bioconversion products. These bioconversion products were analyzed and identified by HPLC and frit-FAB LC/MS.

The HPLC Analysis of the Seven Substrates and PRM-S, and Bioconversion Products

Analytical HPLC chromatography was carried out on a LC-100P (Yokogawa Electric, Japan) apparatus using a column of TSKgel 80Ts (5 mm, 150 × 4.6 mm i.d., Tosoh, Japan) at a flow-rate 0.5 ml/minute. A LC-100U UV-detector (Yokogawa Electric) was used for HPLC measurement at the wavelength of 254 nm. A mobile phase was composed of 0.1% TFA (aq) as the solvent A and methanol - acetonitrile (4 : 1) as the solvent B, and the separation was accomplished by applying a linear solvent gradient from 40% the solvent B to 80% the solvent B in a time period of 50 minutes.

Frit-FAB LC/MS Condition of the Seven Substrates and PRM-S, and Bioconversion Products

For the separations, a LC-9A liquid chromatograph (Shimadzu, Japan) was used. Separation was performed on a TSKgel 80Ts column (5 mm, 150 × 4.6 mm i.d.) at 35°C. A mobile phase was composed of 0.1% TFA (aq) containing 1% *m*-nitrobenzyl alcohol (NBA) as the solvent A and methanol - acetonitrile (4 : 1) containing 1% NBA as the solvent B, and separation was accomplished by applying a linear solvent gradient from 40% the solvent B to 90% the solvent B in a time period of 50 minutes. The flow rate was set to 0.3 ml/minutes.

The HPLC column was connected *via* a fused silica capillary tubing (100 cm × 0.06 mm i.d.) and a frit-FAB LC/MS interface (JEOL, Japan) to the ion source of a JEOL HX110 spectrometer. Injected samples were introduced into the ion source at a split ratio of 1/125 with a flow splitter. The temperature of the ion source was kept at 55°C, and neutral xenon beam was used as a primary beam for the ionization of samples by FAB. The acceleration voltages of the primary and secondary beams were adjusted to 6 and 8 kV, respectively. FAB mass spectra were obtained with injection of 800 μg of the mixture of 8 components, and 100 μg of the bioconversion products obtained after 17 hours. All data were processed by a JEOL JMS DA5000 data system.

Results

Effect of Sinefungin on the Production of PRM-S

A. spinosa AA0851 produces PRM-S as a major component, together with several minor components containing PRMs-B and -L. As PRM-S has a methoxy group at C-11 in the aglycone moiety, the effect of sinefungin was investigated on the production of PRM-S by strain AA0851 in the same way as that of PRM-A. As shown in Table 1, the production of PRM-S depends on the concentration of sinefungin, and increased addition of sinefungin inhibits the formation of 11dM-7M-PNII, PRMs-S, -B and -L and causes the accumulation of 11dM-PMNII and PMNII as shunt products. The results showed that effect of sinefungin on the production of PRM-S was almost the same as that of PRM-A[19], suggesting that it is possible to elucidate the biosynthetic pathway of PRM-S by identification of bioconversion products derived from addition of the seven biosynthetic intermediates (11dM-PNII, 11dM-7M-PNII, 11dM-PNI, 11dM-PMNI, PMNI, PRM-B and PRM-L) on the production of PRM-S.

Time Course Study on Production of PRM-S

Normally, the production of PRM-S with the strain AA0851 increases with an increase of the pH of the fermentation, and reaches a maximum at day 12. In order to determine an appropriate addition time, the producing strain was fermented, and its pH and the production of PRMs-B, -L and -S were carefully observed. As shown in Table 2, the production of PRM-S by growing cultures of the strain increased expectedly along with a rise in pH value. On day 3, the total production (PRMs-B and -S) was quite low: 107 mcg/ml, and reached 2,232 mcg/ml (PRMs-B, -L and -S) by day 12. From these results, we concluded that the 3rd day is suitable for addition of the substrates for bioconversion. We attempted to elucidate the biosynthetic pathway of PRM-S by feeding pradimicin aglycones and PRMs-B and -L into growing cultures of the strain and subsequent identification of bioconversion products derived using frit-FAB LC/MS and quantitative HPLC analysis.

Table 1. Effect of sinefungin on the production of pradimicin S by *A. spinosa* AA0851.

Sinefungin (mM)	Metabolite (μg/ml)					
	11dM-7M-PMNII	11dM-PMNII	PMNII	PRM-B	PRM-L	PRM-S
0	0	0	0	319	202	1,000
0.114	0	0	0	300	140	880
0.342	75	75	727	0	0	38
0.684	68	206	470	0	0	12

Table 2. Time course study on production of pradimicin S by growing cultures of strain AA0851.

Day	pH	UV potency (mcg/ml)	HPLC content (%)		
			PRM-B	PRM-L	PRM-S
1	7.1	23	—	—	—
3	7.2	107	52.6	—	47.4
5	7.5	599	47.2	9.1	43.7
7	7.9	1,148	34.6	9.0	56.4
9	8.3	1,807	28.8	10.9	60.3
12	8.8	2,232	29.3	3.7	67.0

Fig. 3. HPLC chromatogram of seven substrates and pradimicin S.

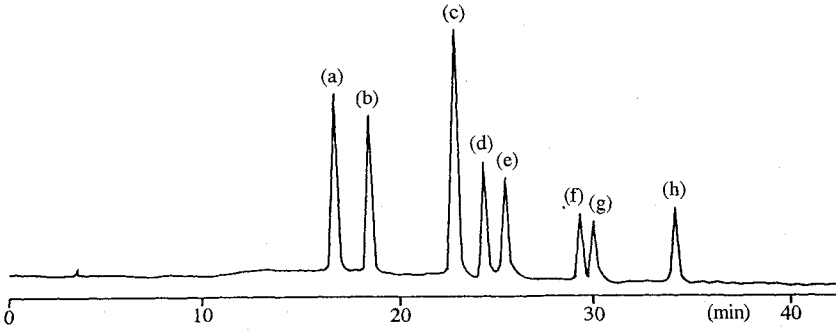
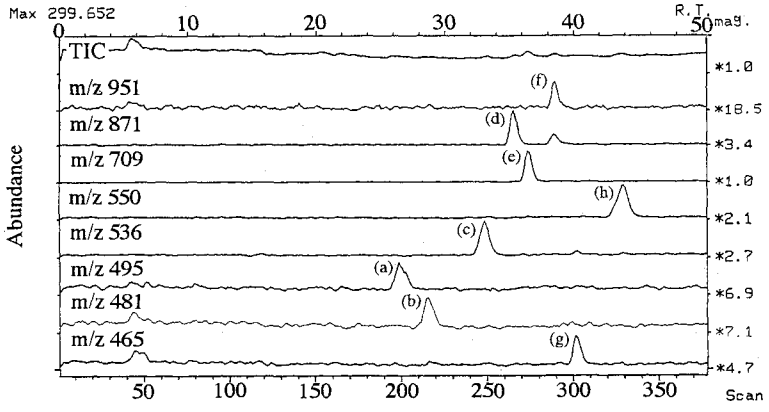


Fig. 4. Total ion chromatogram and mass chromatograms of seven substrates and pradimicin S monitored at *m/z* 465, 481, 495, 536, 550, 709, 871 and 951.



Bioconversion of PRM Aglycones and PRMs-B and -L by Growing Cultures of Strain AA0851

1. Identification of Bioconversion Products by Frit-FAB LC/MS

We used frit-FAB LC/MS for identification of bioconversion products, because this method can eliminate the requirement for separation of each product. Prior to frit-FAB LC/MS, the separation of a mixture of the seven substrates and PRM-S was investigated by HPLC. Methanol-acetonitrile (4:1) and 0.1% TFA (aq) were used as a mobile phase and it was clarified that these components were separated by a linear gradient elution of methanol-acetonitrile (4:1) from 40% to 80% for 50 minutes as shown in Fig. 3. Next, the identification of these components was attempted by using frit-FAB LC/MS in the positive ion mode. In this method, 1% NBA was added to methanol-acetonitrile (4:1) and 0.1% TFA (aq) as a matrix, respectively. The total ion chromatography (TIC) and mass chromatography (MC) monitored by molecular ion species of eight components are shown in Fig. 4. Although the peaks related to these components were not clearly given in the TIC, the peaks detected in the MC monitored at the molecular ion species showed excellent correlation with the HPLC separation. From the molecular ion species of each component, the elution order of these components was 11dM-7M-PNII (a)→11dM-PNII (b)→11dM-PMNI (c)→PRM-L (d)→PRM-B (e)→PRM-S (f)→11dM-PNI (g)→PMNI (h).

On the basis of these results, the identification of

Fig. 5. HPLC chromatogram of products derived from bioconversion of 11dM-PNII.

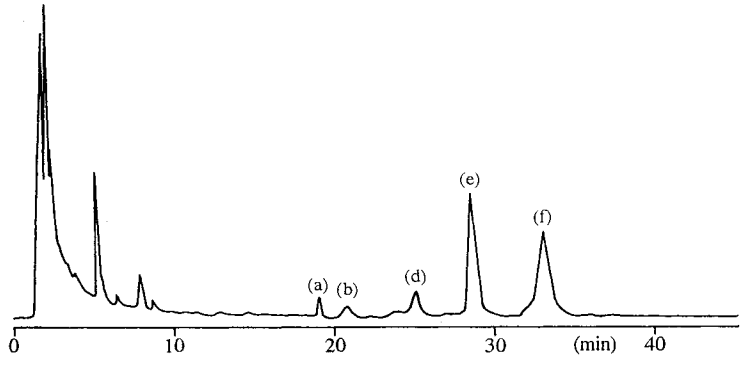
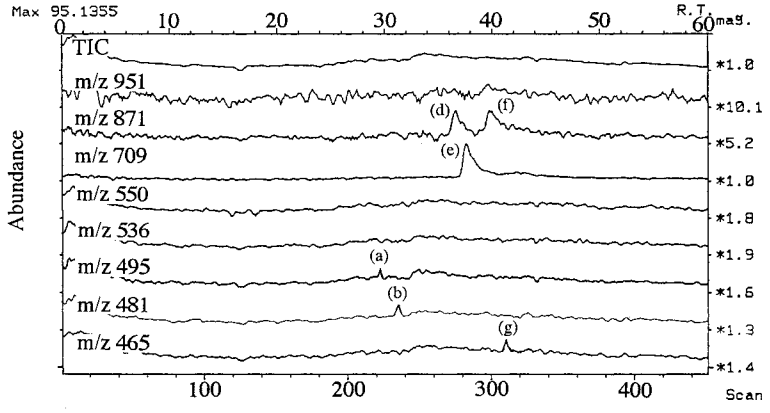


Fig. 6. Total ion chromatogram and mass chromatograms of bioconversion products of 11dM-PNII monitored at *m/z* 465, 481, 495, 536, 550, 709, 871 and 951.



bioconversion products in the production of PRM-S was attempted. The HPLC analysis of bioconversion products obtained after 17 hours from the addition of 11dM-PNII is shown in Fig. 5, and the TIC and MC monitored at the molecular ion species of eight components by frit-FAB LC/MS are shown in Fig. 6. The presence of 11dM-PNII (b), 11dM-PNI (g), 11dM-7M-PNII (a), PRM-B (e) and PRM-L (d) was confirmed by the peaks at *m/z* 465, 481, 495, 709 and 871, respectively. Since the peak at *m/z* 871 was assigned to be a fragment ion derived from PRM-S (*m/z* 951) as shown in Fig. 4, the presence of PRM-S was also confirmed. As it has been clarified that the addition of sinefungin inhibits the production of 11dM-7M-PNII, PRM-B, PRM-L and PRM-S, 11dM-PNII is a starting material for the biosynthesis of PRM-S after the formation of the aglycone moiety. In the same manner, the bioconversion products obtained after 17 hours from the addition of each of 11dM-7M-PNII, 11dM-PNI, 11dM-PMNI and PMNI were analyzed by frit-FAB LC/MS, and the productions of PRMs-B, -L and -S were confirmed. The results confirmed that all of the key bioconversion products used to elucidate the biosynthesis pathway of PRM-S were identified by frit-FAB LC/MS.

2. Quantitative Analysis of Bioconversion Products by HPLC

Table 3 shows the typical results of the bioconversion of probable biosynthetic intermediates by strain AA0851. After 17 hours, the following results were obtained: 11dM-PNII, 11dM-7M-PNII, 11dM-PNI, 11dM-PMNI and PMNI were converted to PRMs-B, -L and -S; in addition, 11dM-PNII and 11dM-PNI were also converted to 11dM-7M-PNII and 11dM-PNI, and 11dM-PMNI as the minor bioconversion products, respectively, besides the major products (PRM-B, PRM-L and PRM-S). PRM-B was converted to PRMs-L and -S, and PRM-L was converted to only PRM-S.

A sequence from 11dM-PNII to PMNI in the biosynthesis of PRM-S is nearly identical to those observed in the formation of other pradimicins. However, trace amounts of PMNI were detected in the case of PRM-A by addition of 11dM-PNII and 11dM-7M-PNII into growing cultures of blocked mutant JN-213[17]. In the case of PRM-S, PMNI was not detected. The results on the bioconversion of PRMs-B and -L indicate that PRMs-B and -L are precursors for PRMs-L and -S and for PRM-S, respectively. In addition, productivity of PRMs-B, -L and -S in the bioconversion of added

Table 3. Bioconversion of pradimicin aglycones and pradimicins B and L by growing cultures of strain AA0851.

Added substrate	Hour	Substrate and products (mM)							
		11dM-PNII	11dM-7M-PNII	11dM-PNI	11dM-PMNI	PMNI	PRM-B	PRM-L	PRM-S
11dM-PNII	0	2.42							
	17	0.51	0.15	0.19			2.17	0.53	1.67
11dM-7M-PNII	0		3.14						
	17		1.65				2.02	0.53	1.76
11dM-PNI	0			1.23					
	17			0.11	0.27		1.55	0.61	1.68
11dM-PMNI	0				2.40				
	17				1.51		1.79	0.41	1.59
PMNI	0					3.03			
	17					1.77	1.76	0.50	1.67
PRM-B	0						0.36		
	17						1.86	0.58	1.57
PRM-L	0							0.19	
	17							0.55	1.58
None	17						1.43	0.34	1.36

substrates was as follows by HPLC analysis: PRM-B > PRM-S > PRM-L. The results suggest that the bioconversion from PRM-B to PRM-L does not progress smoothly in comparison with that of PRM-L to PRM-S. From the information obtained above, a biosynthetic sequence, 11dM-PNII → 11dM-7M-PNII → 11dM-PNI → 11dM-PMNI → PMNI → PRM-B → PRM-L → PRM-S, was determined as shown in Fig. 2.

Discussion

PRM-S having 3-*O*-sulfo-D-glucose instead of D-xylose for PRM-A showed more potent *in vitro* anti-HIV activity than PRM-A, suggesting that the difference of the activity between PRMs-A and -S depends on that of the carbohydrate unit. In view of the point above, we tried to elucidate the biosynthetic pathway of PRM-S using the same strategy with that of PRM-A as mentioned above. The results obtained showed that a biosynthetic sequence, 11dM-PNII → 11dM-7M-PNII → 11dM-PNI → 11dM-PMNI → PMNI → PRM-B, was quite identical to that for PRM-A, and PRMs-L and -S were biosynthesized by C-3′-glycosidation of PRM-B followed by C-3″-sulfonilation of PRM-L. These results indicate that both *Actinomadura hibisca* P157-2 (a PRM-A producer) and *A. spinosa* AA0851 have a common biosynthetic pathway to PRM-B (Fig. 2) and have different enzymes in the second glycosidation step. We have another interesting experimental result. To obtain new bioconversion products with better water-solubility than the parent antibiotics, PRMs-L and -S, a bioconversion of these antibiotics was carried out by adding exogenous D-serine into growing cultures of PRMs-L and -S producers to obtain two new bioconversion products, PRMs-FL[20] and -FS[22] having D-serine instead of D-alanine as an amino acid side chain, respectively. The results also support that pradimicins are biosynthesized in the manner shown in Fig. 2.

In the present study we used the LC/MS method for identification of bioconverted products, rather than a conventional procedure that includes isolation by chromatographic method and identification and/or structural characterization by MS and NMR. The LC/MS has the following advantages for the objective: it takes much less time to complete, requires a smaller scale fermentation, and does not need isolation. It is recommended to use a combination of LC/MS (identification) and HPLC (quantification) for biosynthetic study of antibiotics.

Acknowledgments

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