PRADIMICIN S, A NEW PRADIMICIN ANALOG

II. ISOLATION AND STRUCTURE ELUCIDATION

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Pradimicin S was isolated from the culture filtrate of *Actinomadura spinosa* AA0851. NMR and MS analyses proved that pradimicin S is the 3'-*O*-(3''-*O*-sulfo-β-D-glucopyranosyl) analog of pradimicin A, a new member of the pradimicin family of antibiotics. Stereochemical assignment was made by correlating pradimicin S with pradimicin L.

A directed search for rare actinomycetes capable of producing antibiotics active in an assay which was designed to detect inhibitors of syncytium formation resulted in the isolation of *Actinomadura spinosa* AA0851 producing pradimicin S, a new member of the pradimicin family of antibiotics[1]. Herein, we describe the isolation and structure elucidation of pradimicin S. The taxonomy and fermentation of *A. spinosa* AA0851 and biological activities of pradimicin S are described in another paper[1].

Results

Isolation

Strain *A. spinosa* AA0851 produced pradimicin S as the major product along with pradimicins L[2] and B[3]. The majority of these pradimicin analogs produced by this strain could be recovered by treatment of the centrifuged fermentation broth with Diaion HP-20. The highly hydrophilic by-products were removed by washing the resin with water and the pradimicin analogs extracted into 80% aqueous acetone. The crude extract was then applied to a column of YMC ODS-A60 using acetonitrile - 0.01 M phosphate buffer (22.5 : 77.5, pH 3.5) as eluent. Pradimicin S eluted first followed by the two minor components, pradimicins L and B. Homogeneous pradimicin S was obtained by chromatography on a column of Lichroprep RP-18 using acetonitrile - 0.01 M phosphate buffer (24 : 76, pH 3.5) as eluent. Crystallization from 0.1 N NaOH - ethanol - ethyl acetate (20 : 40 : 7) provided pradimicin S sodium salt as dark red needles. A small amount of sample for elemental analysis was obtained by recrystallization from water at pH 3.0.

Physico-chemical Properties

The physico-chemical properties of pradimicin S are summarized in Table 1. Pradimicin S is an acidic antibiotic, readily soluble in aqueous media (>20 mg/ml at physiological pHs), dimethyl sulfoxide and dimethylformamide, slightly soluble in ethanol, methanol and acetone, but practically insoluble in other common organic solvents and aqueous acidic media (<pH 3.0).

Structure Studies

The UV-vis spectra of pradimicin S are almost identical with those of the other pradimicins including

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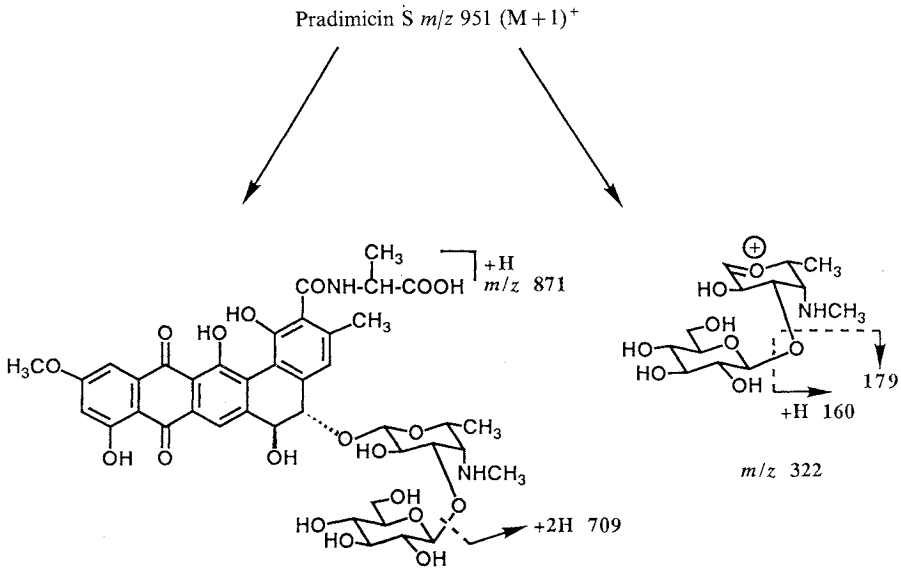
Table 1. Physico-chemical properties of pradimicin S.

Nature	Dark red needles (sodium salt)
MP (dec.)	>180°C (sodium salt)
$[\alpha]_D^{26}$	+400° (*c* 0.02, H_2O)
FAB-MS (positive) *m/z*	951 $(M+H)^+$, 973 $(M+Na)^+$
FAB-MS (negative) *m/z*	949 $(M-H)^-$, 972 $(M-H+Na)^-$
HRFAB-MS, $(M+H)^+$ *m/z*	
Calcd	951.2341
Found	951.2323
Molecular formula	$C_{41}H_{46}N_2O_{22}S$
Analysis	
Calcd for $C_{41}H_{46}N_2O_{22}S \cdot H_2O$	C 50.82, H 4.99, N 2.89, S 3.31
Found	C 50.86, H 5.16, N 2.69, S 3.25
UV λ_{max} nm (ε)	
in 0.02 N NaOH - MeOH (1 : 1)	243 (34,900), 319 (15,900), 498 (16,000)
in 0.02 N HCl - MeOH (1 : 1)	234 (33,300), 300 (30,000), 460 (12,600)
IR ν_{max} (KBr) cm^{-1}	
Acidic form	3600~2500, 1733, 1623~1607, 1448, 1388, 1296, 1258, 1235, 1062, 970, 808
Sodium salt	3600~2500, 1625~1605, 1440, 1385, 1260, 1160, 1070, 970, 810
TLC[a] Rf	0.25
HPLC[b] Rt (minutes)	7.22

[a] Merck Kiesel gel $60F_{254}$; MeOAc - *n*-PrOH - 28% NH_4OH (45 : 105 : 60).
[b] YMC gel A301-3, C_{18}, 3 μm (4.6 mm i.d. × 100 mm); CH_3CN - 0.01 M phosphate buffer (30 : 70, pH 3.5); 1 ml/minute; detection, UV 254 nm.

Fig. 1. Fragmentation of pradimicin S in a positive ion FAB-MS.



Pradimicin L

pradimicins A and L, indicating that pradimicin S has a core structure of dihydrobenzo[*a*]naphthacenequinone. The IR spectrum of pradimicin S in acidic form showed the presence of carboxylic acid (1733 cm^{-1}), quinone carbonyl (1623 cm^{-1}) and sulfate (1258 cm^{-1}) functionalities. The molecular formula of

pradimicin S was determined as $C_{41}H_{46}N_2O_{22}S$ by elemental analysis and HRFAB-MS. The positive ion FAB-MS of pradimicin S showed a pseudomolecular ion peak at *m/z* 951 $(M+1)^+$, and prominent peaks at *m/z* 871, 709, 322 and 160. The B/E constant linked scan experiments performed on the pseudomolecular ion peak provided information about the fragmentation pattern as shown in Fig. 1. The pseudomolecular ion gave rise to daughter ions at *m/z* 871 $(951-SO_3)^+$, 322 (disaccharide)$^+$ and 160 (*N*-methylthomosamine)$^+$, suggesting that pradimicin S is a derivative of pradimicin L [*m/z* 871 $(M+1)^+$] having a sulfo substituent. The negative ion FAB-MS showed a pseudomolecular ion peak at *m/z* 949

Fig. 2. Fragmentation of pradimicin S in a negative ion FAB-MS.

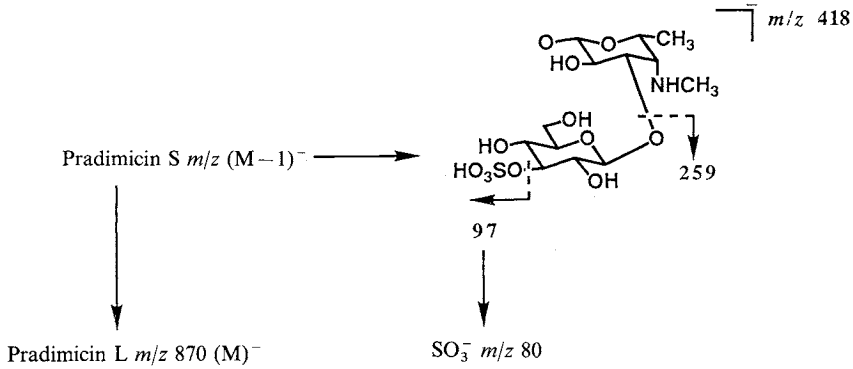


Table 2. ^1H and ^{13}C NMR assignments of pradimicin S in DMSO-d_6.

Position	δ_H	δ_C	Position	δ_H	δ_C
Chromophore			Ala		
1		151.8 (s)	$N^{16}H$	8.32 (d, 7.3)	
2		127.6 (s)	17	4.46 (m)	47.4 (d)
3		137.3 (s)	17-CH_3	1.37 (d, 7.3)	17.0 (q)
3-CH_3	2.34 (s)	19.3 (q)	18		174.0 (s)
4	7.17 (d)	119.0 (d)	Gal		
4a		137.6 (s)	1′	4.80 (d, 7.7)	104.0 (d)
5	4.64 (d, 9.8)	80.9 (d)	2′		69.6 (d)
6	4.66 (d, 9.8)	71.6 (d)	3′	4.01 (dd, 4.3, 9.8)	80.1 (d)
6a		147.7 (s)	4′	3.52∼3.59	63.3 (d)
7	8.07 (s)	114.4 (d)	4′-NCH_3	2.75 (bs)	36.1 (q)
7a		131.4 (s)	5′	3.92 (q, 6.8)	67.3 (d)
8		185.1 (s)	6′	1.30 (d, 6.8)	16.2 (q)
8a		110.1 (s)	Glu		
9		164.7 (s)	1″	4.60 (d, 7.7)	104.2 (d)
10	6.93 (d, 2.6)	106.6 (d)	2″		72.0 (d)
11		166.0 (s)	3″	4.06 (t, 8.5)	82.3 (d)
11-OCH_3	3.97 (s)	56.4 (q)	4″	3.29∼3.35	68.8 (d)
12	7.32 (d, 2.6)	107.5 (d)	5″		76.6 (d)
12a		134.6 (s)	6″-Hax	3.49 (dd, 5.8, 11.6)	60.6 (t)
13		186.8 (s)	6″-Heq	3.76 (dd, 4.3, 11.6)	
13a		116.0 (s)			
14		157.7 (s)			
14a		126.5 (s)			
14b		115.8 (s)			
15		167.1 (s)			

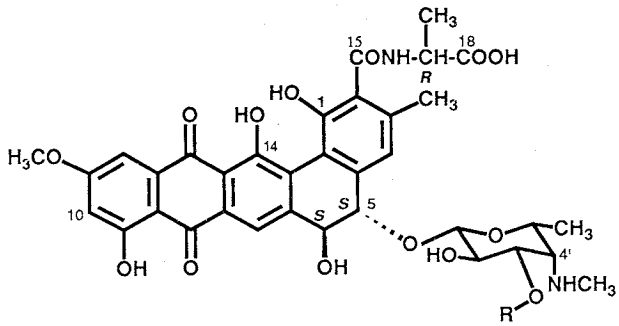
ppm (multiplicity, J=Hz).

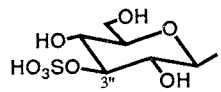
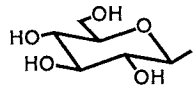
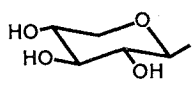
$(M-1)^-$, and the B/E constant linked scan spectrum revealed that the ions at m/z 870 (pradimicin L)$^-$, 418 (disaccharide + SO_3)$^-$, 259 (glucose + SO_3)$^-$ and 97 $(HSO_4)^-$ were derived from the pseudomolecular ion as shown in Fig. 2. These results indicated that pradimicin S is a derivative of pradimicin L with a sulfo substituent attached to the glucose moiety.

In order to determine the location of the sulfo substituent, we compared the ^{13}C NMR spectra of pradimicins S and L. The spectra of both compounds contained 41 carbon signals with the same multiplicity as determined by DEPT experiments. Chemical shift differences were observed for 3 carbon signals assignable to C-2″, C-3″ and C-4″ of the glucose moiety; the NMR spectrum of pradimicin S showed the C-3″ signal at 82.3 ppm which is 6.4 ppm more deshielded and the C-2″ and C-4″ signals 1.1 ~ 1.5 ppm more shielded compared to the corresponding signals of pradimicin L, indicating that the sulfo substituent is located at C-3″ of the glucose moiety. This assignment is consistent with the result of combination experiments using double quantum filtered COSY, relayed COSY and NOESY where the proton signal (4.06 ppm) assignable to H-3″ was 0.8 ppm more deshielded than the corresponding proton of pradimicin L. The ^1H and ^{13}C NMR data and full assignments thus obtained are summarized in Table 2.

In order to determine the absolute configuration of pradimicin S, the following degradation study was conducted with a hope of converting pradimicin S into pradimicin L directly. Although pradimicin S proved to be extremely difficult to selectively hydrolyze under various alkaline conditions, hydrolysis under mild acidic conditions (1 N HCl, 70°C) gave rise to a single compound which was identical in all respects including the sign and magnitude of optical rotation with an authentic sample of pradimicin L. Based on these experiments, we propose the structure of pradimicin S with absolute stereochemistry as depicted in Fig. 3.

Fig. 3. Structures of pradimicins S, L and A.



	R
Pradimicin S	
Pradimicin L	
Pradimicin A	

Discussion

The present study demonstrated that pradimicin S, the major antibiotic produced by *A. spinosa* AA0851, is 3″-*O*-sulfopradimicin L, the 3′-*O*-(3″-*O*-sulfo-β-D-glucopyranosyl) analog of pradimicin A. This is the first example of natural pradimicin analogs having a sulfate functionality. It is interesting to note that introduction of a sulfo substituent into pradimicin L by the action of sulfur trioxide-trimethylamine afforded 6″-*O*-sulfopradimicin L selectively. Recently, KONDO *et al.*[4] reported that a similar reaction on benanomicin A afforded a benanomicin A derivative having a sulfo substituent at C-4″ position of the xylose moiety.

Experimental

General

The melting points are uncorrected. Spectral data were recorded on the following instruments; ^{1}H and ^{13}C NMR, JEOL JNM-GX 400; IR, JASCO IR-810 spectrometer; UV-vis, JASCO UVIDEC-610 spectrometer; FAB-MS and HRFAB-MS, JEOL JMS-AX 505H spectrometer; optical rotation, JASCO DIP-140. For TLC, Merck precoated Kiesel gel $60F_{254}$ plates (0.25 mm) were used.

Isolation of Pradimicin S

The procedure for the fermentation of *A. spinosa* AA0851 was described in the preceding paper[1]. The whole fermented broth (11.5 liters) was centrifuged and the supernatant was mixed with 3.5 liters of Diaion HP-20. The resin was washed thoroughly with water and the pradimicin analogs were eluted with 2.5 liters of 80% aq acetone. The solvent was removed *in vacuo* and the aq residue was lyophilized to give 28 g of crude material which was estimated to contain 12 g of pradimicin S, 7 g of pradimicin B and 1.3 g of pradimicin L by HPLC analysis (YMC gel A301-3, C_{18}, 3 μm). This material was dissolved in 3.8 liters of acetonitrile - 0.01 M phosphate buffer (21 : 79, pH 3.5), adsorbed on 1.0 liter of reversed phase silica gel (YMC ODS-A60) and the gel applied to a column chromatography on YMC ODS-A60 (10 liters) pre-equilibrated with acetonitrile - 0.01 M phosphate buffer (22.5 : 77.5, pH 3.5). The column was eluted with the same solvent system and the eluate (120 liters) containing pradimicin S was concentrated. The aq residue (3 liters) was mixed with Diaion HP-20 (2.4 liters) and the resin was washed with water. The fraction (1.5 liters) eluted with acetone - 0.001 N HCl (60 : 40) was concentrated and the aq residue was lyophilized to afford 8.0 g of semipure pradimicin S hydrochloride. A portion (1.83 g) of this sample was dissolved in 340 ml of acetonitrile - 0.01 M phosphate buffer (18 : 82, pH 3.5) and applied to a column of Lichroprep RP-18 (4 liters). The column was eluted with acetonitrile - 0.01 M phosphate buffer (24 : 76, pH 3.5). The fraction (21 liters) containing pradimicin S was concentrated and the aq residue (12 liters) was mixed with Diaion HP-20 (800 ml). The resin was washed with water and pradimicin S was extracted into 400 ml of 80% aq acetone. The acetone was removed *in vacuo* and the aq residue was lyophilized to yield 1.5 g of pure pradimicin S. Crystallization from 800 ml of 0.1 N NaOH - EtOH - EtOAc (2 : 4 : 0.7) afforded 1.2 g of pure pradimicin S sodium salt as dark red needles. A small amount of sample for elemental analysis was obtained by recrystallization from water at pH 3.0; the solid was collected by filtration, washed with water and dried at 70°C under vacuum for 2 days.

Isolation of the Minor Components

The two minor components eluted from the column (YMC ODS-A60) were desalted in a similar fashion, and their structures were established by direct comparison with authentic samples of pradimicins B and L.

Acid Hydrolysis of Pradimicin S

A solution of pradimicin S (280 mg) in 20 ml of DMSO was mixed with 80 ml of 1 N HCl. The resulting suspension was heated at 70°C with stirring for 9.5 hours during which the starting material was consumed. The reaction mixture was cooled, adjusted to pH 6.0 with 6 N NaOH and diluted with 200 ml of water. The solution was mixed with Diaion HP-20 (100 ml) and the resin was washed with 800 ml of water. The product was extracted into 200 ml of acetone - 0.001 N HCl (60 : 40) and the acetone was removed *in vacuo*.

The aq residue was lyophilized to give 309 mg of amorphous material. Analysis by HPLC and TLC indicated that this material contained pradimicin L with traces of pradimicins S and B. A sample for direct comparison with pradimicin L was prepared as follows: Column chromatography on reversed phase silica gel (RP-18, E. Merck, 2.6 liters) using acetonitrile - 0.01 M phosphate buffer (24 : 76, pH 3.5) as eluent followed by adsorption on Diaion HP-20 (400 ml), elution from the resin with 200 ml of 80% aq acetone and (pH 3.0), concentration *in vacuo* and crystallization from the aq residue afforded a pure sample (147 mg, 57% yield from pradimicin S): MP >200°C (dec.); FAB-MS *m/z* 871 $(M+1)^+$; UV (0.02 N NaOH - MeOH, 1 : 1) λ_{max} nm (ε) 319 (12,500), 499 (12,200); $[\alpha]_D^{27}$ +437° (*c* 0.05, 0.1 N HCl).

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