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NOTE

Isolation and characterization of 23-*O*-mycinosyl-20-dihydro-rosamicin: a new rosamicin analogue derived from engineered *Micromonospora rosaria*

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Macrolides, including some of the most important antibiotics clinically used, contain deoxysugars attached to an aglycone core. These sugars often impart specific biological activity to the molecule or enhance this activity. Many genes involved in the biosynthesis of macrolide antibiotics have been cloned and sequenced; in addition, the functions of many proteins encoded by macrolide biosynthetic genes have been elucidated. With this information and experimental results, manipulation of the polyketide synthase and deoxysugar biosynthetic pathways to create novel macrolide antibiotics has become possible.[1] Therefore, a combined approach that uses genes involved in the biosynthesis of macrolactone rings and deoxysugars, and in the glycosylation of macrolactone rings has been used to modify the macrolide structure.[2]

Rosamicin (that is rosaramicin; Figure 1) is a 16-membered macrolide antibiotic produced by *Micromonospora rosaria* IFO13697 (that is NRRL 3718).[3] It contains a branched lactone and deoxyhexose sugar D-desosamine at the C-5 position. The engineered strain *M. rosaria* TPMA0001 carries genes involved in the D-mycinose biosynthetic pathway of *Micromonospora griseorubida* A11725, namely, *mycCI*, *mycCII*, *mycD*, *mycE*, *mycF*, *mydH* and *mydI*; this engineered strain was found to produce a mycinosyl rosamicin derivative IZI.[4] *M. griseorubida* A11725 produces the 16-membered macrolide antibiotic mycinamicin II, which comprises a branched lactone and two different deoxyhexose sugars—D-desosamine and D-mycinose—at the C-5 and C-21 positions, respectively. All the genes involved in D-mycinose biosynthesis lie on the mycinamicin biosynthetic gene cluster.[5] The functions of these gene products have been elucidated through chemical, genetic and enzymatic analyses. The genes *mycCI* and *mycCII* encode a cytochrome P450 enzyme and ferredoxin, respectively, which function in combination with ferredoxin reductase to mediate the hydroxylation of mycinamicin VIII at the C-21 methyl group. On completion of this hydroxylation reaction, MycD transfers 6-deoxy-D-allose to the C-21 hydroxyl group by using dTDP-6-deoxy-D-allose as a substrate; dTDP-6-deoxy-D-allose is synthesized from dTDP-4-keto and 6-deoxy-D-glucose by MydH and MydI. The methyltransferases MycE and MycF convert the resulting compound mycinamicin VI to mycinamicin IV, which has D-mycinose attached at the C-21 position. In particular, we have recently elucidated the biochemical functions of MycCI, MycCII, MycE and MycF by using the purified form of these proteins, which were overexpressed in *Escherichia coli*.[6,7] In our earlier study, when EtOAc extracts obtained from culture broths of the wild-strain *M. rosaria* IFO13697 and the engineered strain *M. rosaria* TPMA0001 were compared using HPLC, two additional peaks—IZI and IZII—appeared in the chromatogram (at 285 nm) of the extract from the engineered strain. IZI was identified as a mycinosyl rosamicin derivative, 23-*O*-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxyrosamicin.[4] Moreover, our detailed studies showed that another metabolite (designated as IZIII) was produced by the engineered strain. Here, we report the isolation and characterization of a novel mycinosyl rosamicin derivative IZIII; that is, 23-*O*-mycinosyl-20-dihydro-rosamicin (Figure 1), produced by *M. rosaria* TPMA0001. Moreover, the structure of IZII was also determined as 23-*O*-mycinosyl-20-dihydro-12,13-deepoxyrosamicin (Figure 1).

M. rosaria TPMA0001 and the wild-type strain *M. rosaria* IFO13697 were cultured at 27 °C in 5 ml of trypticase soy broth (Becton, Dickinson and Company, Sparks, MD, USA) for 8 days. The broth was adjusted to pH 9–11 with 28% ammonia solution and extracted with an equal volume of EtOAc; the extract was then concentrated *in vacuo*. The crude extracts were dissolved with EtOAc, and then an equal volume of 0.1% trifluoroacetic acid (TFA) was added. The water layer containing rosamicins was adjusted to pH 9–11 with 28% ammonia solution and extracted with an equal volume of EtOAc. The organic layer was concentrated *in vacuo*, and

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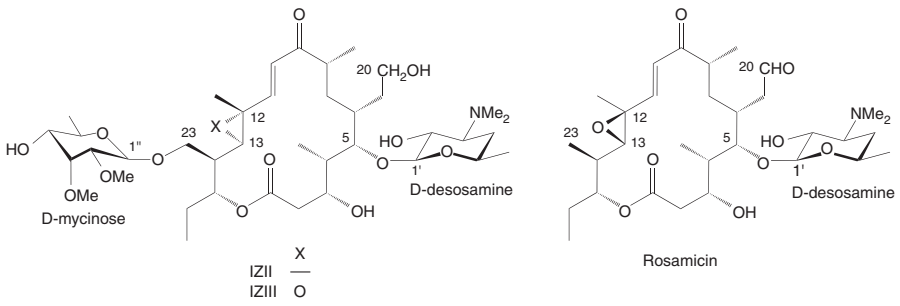

Figure 1 Structures of IZII, IZIII and rosamicin.

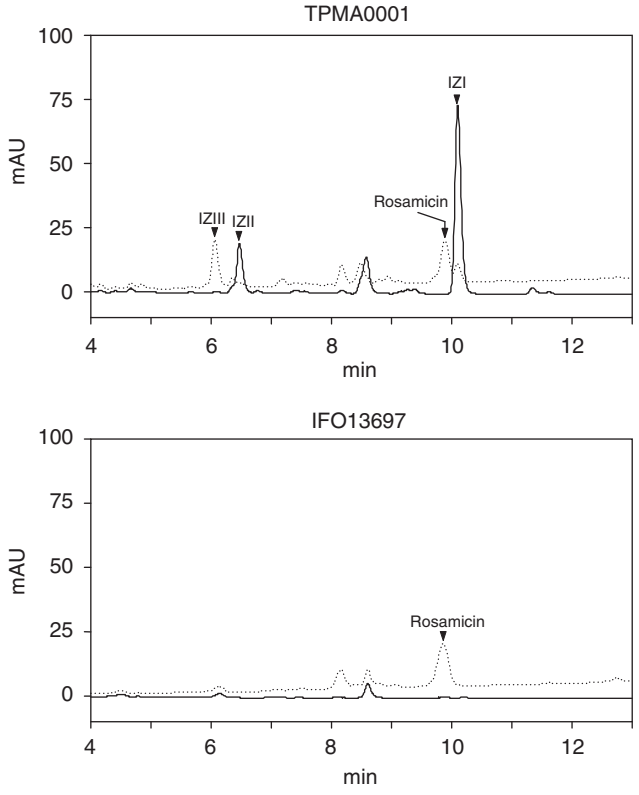

Figure 2 HPLC chromatograms of the EtOAc extract obtained from *M. rosaria* TPMA0001and *M. rosaria* IFO13697. Solid line, at 285 nm; dashed line, at 240 nm.

each residue was dissolved in MeOH for HPLC and LC-MS analyses. HPLC analysis scanning with a diode array detector model L-2450 (Hitachi, Tokyo, Japan) was performed on the following condition: ODS-80TM, i.d.=150×4.6 mm (Toso, Tokyo, Japan); mobile phase, 25–60% solvent B over 15 min (A=deionized water+0.1% TFA, B=acetonitrile+0.1% TFA); flow rate, 1.0 ml min^{-1}; UV wavelength, 200–300 nm. In HPLC chromatograms recorded at 240 nm, rosamicin was detected at 11.16 min in the samples from both strains, TPMA0001 and IFO13697 (rosamicin productivity, 0.3 and 0.1 mg l^{-1}, respectively), and a new peak of IZIII also appeared at 6.08 min in the chromatogram of TPMA0001 (Figure 2). Moreover, additional two peaks of IZI and IZII appeared at 10.11 and 6.49 min, respectively, in the chromatogram of TPMA0001 recorded at 285 nm (Figure 2). LC-MS analysis was also performed for the extract to measure the MW of these metabolites [IZI, *m/z* 742 (M+H)+; IZII, *m/z* 758 (M+H)+ and IZIII, *m/z* 774 (M+H)+] on LCMS2010 (Shimadzu, Kyoto, Japan) by using reverse-phase HPLC.

To isolate IZII and IZIII, *M. rosaria* TPMA0001 was cultured in two 300-ml Erlenmeyer flasks, each containing 50 ml of trypticase soy broth. The flasks were incubated on a rotary shaker (120 r.p.m.) at 27 °C for 3–5 days. Further, 4 ml of the culture was transferred into each of 18 500-ml Sakaguchi flasks, each containing 300 ml of trypticase soy broth; these flasks were incubated on a rotary shaker (120 r.p.m.) at 27 °C for 14 days. The culture filtrate (5 l) was passed through a column of HP20 sorbent (Mitsubishi Chemical, Tokyo, Japan). After washing with water, rosamicin derivatives were eluted from the column with MeOH and the eluate was concentrated to a pasty residue *in vacuo*. This residue was resuspended in 300 ml of water; the pH of this suspension was adjusted to 9–11 with 28% ammonia solution and extracted twice with equal volumes of EtOAc. The organic extracts were combined and concentrated to dryness *in vacuo*. The crude product was dissolved in chloroform-methanol-28% ammonia solution (100:10:1), applied to a silica gel column (28×3 cm, silica gel 60; Merck, Darmstadt, Germany), and then eluted using the same mixture. The fractions containing the antibacterial metabolites were applied to a preparative HPLC system [Shim-pack PREP-ODS, internal diameter (i.d.)=250×20 mm, Shimadzu; mobile phase, acetonitrile—0.06% TFA (25:75); flow rate, 20 ml min^{-1}]. The pH of two fractions, which contained IZII or IZIII, was adjusted to 9–11 with 28% ammonia solution, and these fractions were extracted with an equal volume of EtOAc. After concentrating the organic solution to dryness *in vacuo*, IZII (16.6 mg) and IZIII (10.9 mg) were obtained.

The molecular formula of IZIII was established as $C_{39}H_{67}NO_{14}$ on the basis of HR-FAB-MS (*m/z* 796.4465, calcd for $C_{39}H_{67}NO_{14}Na$, *m/z* 796.4460). UV λ_{max} of IZIII was at 240 nm from the UV spectrum data of HPLC analysis. The structure of IZIII was elucidated by NMR spectroscopic studies. Chemical-shift assignments for IZIII were determined on the basis of the DQF-COSY, HMQC, HMBC and NOESY spectral data. The ^{1}H- and ^{13}C-NMR data of IZIII were compared with those of rosamicin and tylosin, as shown in Table 1.[8–10] We found that ^{13}C-NMR spectral data of IZIII were considerably similar to those of rosamicin, except that IZIII did not contain an aldehyde carbon at 202.9 p.p.m. (C-20) or a methyl carbon at 14.5 p.p.m. (C-23) as seen in rosamicin. Instead, IZIII contained an alcohol carbon and an ether carbon at 61.1 and 67.4 p.p.m., respectively. The aldehydic proton H-20 (9.74 p.p.m., s) found in rosamicin was not found in IZIII; instead, a pair of methylene proton H-20 (3.6 and 3.7 p.p.m., m) was observed. The signal detected at 67.4 p.p.m. in IZIII was assigned to C-23, after comparison with the ^{13}C-NMR data of tylosin. Therefore, IZIII was thought to be a structural derivative of rosamicin; in the former compound, the aldehyde group at C-20 is reduced to alcohol and the methyl group at

Table 1 ^{13}C- and ^{1}H-NMR data of IZIII, rosamicin and tylosin

	IZIII			*Rosamicin*[a]		*Tylosin*[b]		
Position	*^{13}C (p.p.m.)*	*^{1}H (p.p.m.)*	*J (Hz)*	*^{13}C (p.p.m.)*	*^{1}H (p.p.m.)*	*^{13}C (p.p.m.)*	*^{1}H (p.p.m.)*	*J (Hz)*
1	173.7	—	—	173.5		173.9	—	—
2	39.7	2.08 (d)	16.5	39.7	2.1 (d)	39.4	1.94 (dd)	16.5, 0.8
		2.57 (dd)	16.7, 10.6		2.65 (dd)		2.47 (dd)	16.5, 10.3
3	67.4	3.85 (br d)	10.8	68	3.91 (br d)	71.1	3.82 (br d)	10.3
4	40.9	1.75[c]	—	45.1		45.1	1.59 (dq)	9.1, 6.8
5	81.4	3.76 (br d)	9.4	81.3	3.71 (br d)	81.6	3.71 (dd)	9.1, 1.3
6	31.9	1.65[c]	—	31.4		32.3	2.21	
7	33.2	1.55[c]	—	31.8		32.9	1.48	
		1.69[c]	—				1.52	
8	45.2	2.7 (m)	—	37.9	2.55 (m)	40.3	2.62	
9	201.4	—	—	200.9		202.8	—	—
10	123.2	6.43 (d)	15.8	122.8	6.46 (d)	118.8	6.25 (d)	15.5
11	150.5	6.55 (d)	15.8	150.9	6.53 (d)	148	7.28 (d)	15.5
12	59.4	—	—	59.7		143.9	—	—
13	64.4	3.17 (d)	9.8	66.8	2.82 (d)	142.2	5.88 (d)	10.5
14	43.5	1.71[c]	—	41.3		44.7	2.95	
15	74.0	5.3 (td)	9.4, 2.7	76.8	4.89 (d)	75.3	4.99 (ddd)	9.4, 2.7
16	24.8	1.55 (m)	—	24.7		25.5	1.56	
		1.89 (m)	—				1.89	
17	9.2	0.89 (td)	7.4	9.1		9	0.94 (t)	7.3
18	9.2	1.13 (d)	6.8	9		9.6	0.99 (d)	6.8
19	31.9	1.96 (m)	—	43.8	2.46 (m)	43.9	2.36	
					3.08 (m)		2.86	
20	61.1	3.66 (m)	—	202.9	9.73 (s)	203	9.68 (dd)	1.9, 1.0
		3.7 (m)	—					
21	17.4	1.51 (d)	6.8	17.4		17.4	1.21 (d)	6.8
22	14.9	1.42 (s)	—	15		13	1.79 (d)	1.2
23	67.4	3.64 (dd)	10.6, 2.7	14.5		68.2	3.56 (dd)	9.7, 6.2
		4.14 (dd)	10.1, 3.2				3.96 (dd)	9.7, 4.2
1′	104.8	4.31 (d)	7.3	104.5	4.22 (d)	—		
2′	70.4	3.23 (dd)	10.0, 7.3	70.4	3.2 (dd)	—		
3′	65.8	2.53 (br t)	10.7	65.8	2.49 (m)	—		
4′	28.6	1.26[c]	—	28.4		—		
		1.68[c]	—					
5′	69.6	3.5 (m)	—	67.7	3.46 (m)	—		
6′	21.1	1.22 (d)	6.1	21.2		—		
7′	40.3	2.32 (s)	—	40.5	2.29 (s)	—		
8′	40.3	2.32 (s)	—	40.5	2.29 (s)	—		
1″	100.9	4.56 (d)	7.7	—		101.1	4.56 (d)	7.8
2″	82.0	3.09 (dd)	7.6, 2.9	—		82	3.01 (dd)	7.8, 3.0
3″	79.7	3.77 (t)	3	—		79.9	3.74 (t)	3
4″	72.7	3.21 (dd)	9.4, 3.2	—		72.9	3.15 (44)	9.4, 3.0
5″	70.8	3.52 (m)	—	—		70.6	3.53	
6″	17.8	1.26 (d)	6	—		17.8	1.25 (d)	6.2
7″	59.7	3.56 (s)	—	—		59.6	3.48 (s)	
8″	61.7	3.62 (s)	—	—		61.7	3.60 (s)	

[a]Data from Nakajima *et al*.[8]
[b]Data from Ōmura *et al*.[9] and Simova and Inanova.[10]
[c]Overlapping.

C-23 is modified. Tylosin has a mycinose moiety at C-23, and the ^{1}H- and ^{13}C-NMR data of IZIII coincided well with those of this mycinose moiety. On the basis of these results, the structure of IZIII was determined as 23-*O*-mycinosyl-20-dihydro-rosamicin.

UV λ_{max} of IZII [*m/z* 758 (M+H)+] were at 287 nm from UV spectrum data of HPLC analysis. To estimate the structure of IZII, the ^{1}H- and ^{13}C-NMR data of IZII was compared with those of IZIII (Supplementary Table S1). The ^{1}H- and ^{13}C-NMR spectra of IZII resembled those of IZIII except for the proton and carbon signals of C-12 and C-13. The chemical shifts of C-12 and C-13 of IZII were 135.0 and 141.8 p.p.m., respectively, and indicated the alkene carbons. Also, the chemical shifts of H-13 of IZII and IZIII were 5.88 p.p.m. (d) and 3.17 p.p.m. (d), respectively, and H-13 in IZII was an alkene proton. On the basis of these results, the structure of IZII was

Table 2 Antibacterial activity of rosamicin, IZI, IZII and IZIII

	MIC ($\mu g\,ml^{-1}$)			
Microorganism	*Rosamicin*	*IZI*	*IZII*	*IZIII*
S. aureus ATCC 25923	0.20	0.20	0.39	1.56
M. luteus ATCC 9341	0.048	0.048	0.097	0.20
S. enterica serovar Typhimurium ATCC 14028	6.25	100	>100	>100
E. coli ATCC 25922	3.12	50.0	>100	>100

estimated as 23-*O*-mycinosyl-20-dihydro-12,13-deepoxyrosamicin, and the compound has been already reported as a bioconversion compound, which was converted from 20-deoxo-20-dihydro-12,13-deepoxy-12,13-dihydrorosaranolide by the mycinamicin non-producing mutant GS-9001.[11]

The antibacterial activity of IZII and IZIII, which was tested on *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 9341, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *E. coli* ATCC 25922, was lower than that of rosamicin and IZI (Table 2). The antibacterial activity of IZI against gram-positive bacteria (*S. aureus* ATCC 25923 and *M. luteus* ATCC 9341) was very similar to that of rosamicin; however, the activity of IZII and IZIII was lower than that of rosamicin and IZI.

Two types of cytochrome P450 enzymes are considered to be responsible for C-20 oxidation and C-12/13 epoxidation in the rosamicin biosynthetic pathway of *M. rosaria* IFO13697.[4] The rosamicin biosynthetic pathway was elucidated with reference to the tylosin biosynthetic pathway because the structure of rosamicin is very similar to that of *O*-mycaminosyltylonolide, an intermediate in the tylosin biosynthetic pathway.[12,13] In the tylosin biosynthetic pathway, the cytochrome P450 protein TylI has been proposed to convert 5-mycaminosyl-tylactone to 23-deoxy-*O*-mycaminosyl-tylonolide through 20-dihydro-23-deoxy-*O*-mycaminosyl-tylonolide; that is, TylI is believed to catalyze both hydroxylation and dehydrogenation at C-20 of 5-mycaminosyl-tylactone. Moreover, the TylI protein has been thought to catalyze hydroxylation and dehydrogenation at the C-20 position of 5-mycaminosyl tylacone.[13,14] Therefore, the hydroxyl group at the C-20 position in IZII and IZIII was probably generated by the action of the cytochrome P450 enzyme, which is responsible for C-20 formylation in rosamicin; further, this could be attributed to the conserved biosynthetic pathway of rosamicin in *M. rosaria* TPMA0001. Moreover, the epoxy group at C-12/13 in IZIII was probably generated by the action of another cytochrome P450 enzyme, which is responsible for C-12/13 epoxidation in rosamicin. However, a mycinosyl rosamicin derivative with formylated C-20 has not yet been detected in the culture broth of *M. rosaria* TPMA0001.

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