Structure Determination of Ribosylated Rifampicin and Its Derivative: New Inactivated Metabolites of Rifampicin by Mycobacterial Strains

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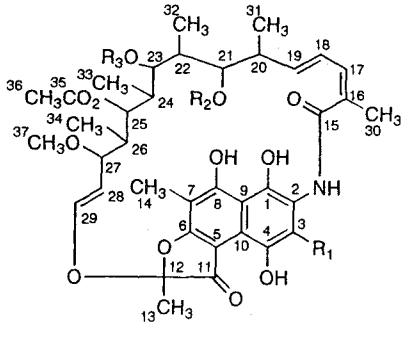
Rifampicin (**1**) was converted into two inactivated products RIP-Ma and RIP-Mb by *Mycobacterium smegmatis* DSM43756. MS, NMR and chromatographic analysis showed the compounds to be 3-formyl-23-[*O*-(α-D-ribofuranosyl)]rifamycin SV (**6**) and 23-[*O*-(α-D-ribofuranosyl)]rifampicin (**7**), respectively.

A semisynthetic antibiotic rifampicin (**1**) is an important chemotherapeutic agent for tuberculosis and leprosy.[1~3] As the inactivated product of rifampicin by *Nocardia* spp., most of which are resistant to rifampicin, we isolated four metabolites RIP-1, -2, -3 and -4, indicating the major resistance mechanism of these organisms is the transformation of **1** to the inactive compounds.[4~6] The inactivation pattern is genus specific[7]: *N. brasiliensis* glycosylate **1** at 23-OH to form 3-formyl-23-[*O*-(β-D-glucopyranosyl)]rifamycin SV (RIP-1, **2**) and 23-[*O*-(β-D-glucopyranosyl)]rifampicin (RIP-2, **3**), and *N. otitidiscaviarum* phosphorylate **1** at 21-OH to form 21-(*O*-phosphoryl)rifampicin (RIP-3, **4**) and 3-formyl-21-(*O*-phosphoryl)rifamycin SV (RIP-4, **5**) (Fig. 1). *Bacullus* species also inactivate rifampicin by phosphorylation to RIP-3 and RIP-4, or decolorization.[8]

During our studies on rifampicin inactivation by fast

Fig. 1. Structures of rifampicin (**1**), RIP-1 (**2**), RIP-2 (**3**), RIP-3 (**4**), RIP-4 (**5**), RIP-Ma (**6**) and RIP-Mb (**7**).



Rifampicin (**1**) R_1 = CH=N-N⟨piperazine⟩N-CH_3; R_2 = H, R_3 = H

RIP-1 (**2**) R_1 = CHO, R_2 = H, R_3 = β-D-glucose

RIP-2 (**3**) R_1 = CH=N-N⟨piperazine⟩N-CH_3; R_2 = H, R_3 = β-D-glucose

RIP-3 (**4**) R_1 = CH=N-N⟨piperazine⟩N-CH_3; R_2 = -PO_3H_2, R_3 = H

RIP-4 (**5**) R_1 = CHO, R_2 = -PO_3H_2, R_3 = H

RIP-Ma (**6**) R_1 = CHO, R_2 = H, R_3 = α-D-ribose

RIP-Mb (**7**) R_1 = CH=N-N⟨piperazine⟩N-CH_3; R_2 = H, R_3 = α-D-ribose

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growing mycobacterial strains,[9,10] we found that several of them are resistant to rifampicin. From the culture broth of rifampicin resistant *M. smegmatis* DSM43756 grown in the presence of rifampicin, two new inactivated metabolites, RIP-Ma (**6**) and RIP-Mb (**7**), were isolated. This paper reports on the structure determination of **6** and **7** (Fig. 1). Biological transformation of rifampicin to RIP-Ma and RIP-Mb, isolation and antimicrobial activities are described elsewhere.[11]

Experimental

General

^1H and ^{13}C NMR spectra were measured in CD_3OD on a JEOL ALPHA-500 NMR spectrometer at 500 and 125 MHz, respectively. Chemical shifts of ^1H and ^{13}C NMR were recorded in δ units relative to internal tetramethylsilane ($\delta=0$). FAB-MS and HRFAB-MS were measured on a JEOL JMS-HX110 instrument. HPLC was performed on a Tosoh CCPP-M apparatus.

Determination of Ribose Configuration

The configuration of ribose in RIP-Mb (**7**) was determined on a chiral HPLC column after hydrolysis and derivatization. One mg of **7** was hydrolyzed with 2 M-CF_3COOH (0.2 ml) at 120°C for 1 hour and, after the removal of the solvent with N_2 stream, the reaction product was dried *in vacuo*. The crude ribose was converted to oxime with $HONH_2 \cdot HCl$ (1 mg), CH_3COONa (2 mg) and methanol (0.2 mg) at 60°C for 1 hour. After removal of the solvent, the product was treated with $(CH_3CO)_2O$ (0.2 ml) at 120°C for 1 hour. Removal of the residual reagent followed by dissolving the products in $CHCl_3$ and washing with H_2O gave crude aldononitrile acetate (**8**). After purifying by HPLC (ODS column, mobile phase: 45% CH_3CN - H_2O), the ribose derivative **8** was analyzed on a chiral column (Chiralcel OD-H, 4.6 mm i.d. × 250 mm, Daicel Chemical Ind., Ltd., mobile phase: 2% isopropanol - *n*-hexane, 0.5 ml/minute).

Results and Discussion

Structure Determination

The structures of RIP-Ma and RIP-Mb were determined by comparison with the spectroscopic data of rifampicin, RIP-1 and RIP-2.[4]

The molecular weights and molecular formulae of RIP-Ma ($C_{43}H_{55}NO_{17}$: 857) and RIP-Mb ($C_{48}H_{66}N_4O_{16}$: 954) were determined by positive and negative FAB-MS and HRFAB-MS (Table 1). These data suggested that RIP-Ma and RIP-Mb are the derivatives of 3-formylrifamycin SV and rifampicin glycosylated with a pentose, respectively.

The structure of RIP-Ma (**6**) was elucidated by NMR data. ^1H and ^{13}C NMR chemical shifts were assigned by COSY, C-H COSY and HMBC experiments (Tables 2 and 3). Signals due to a formyl group at δ_H 10.53 ppm and δ_C 193.1 ppm were shown in the expence of the signals due to N-methylpiperazine moiety, indicating that 1-amino-4-methylpiperazine has been cleaved off. The presence of ribofuranosyl moiety was indicated by the ^1H and ^{13}C NMR signals[12~14]: δ_H 5.24 ppm (H-1′), 3.93 ppm (H-2′), 3.87 ppm (H-3′), 4.01 ppm (H-4′) and 3.52 ppm (H-5′ × 2): ^1H-^1H couplings. $J_{1'-2'}=4.5$ Hz, $J_{2'-3'}=5.5$ Hz, $J_{3'-4'}=1.5$ Hz, $J_{4'-5'}=4.0$ Hz: δ_C 105.1 ppm (C-1′), 73.2 ppm (C-2′), 71.6 ppm (C-3′), 86.7 ppm (C-4′) and 63.4 ppm (C-5′). The glycosylation site was determined by HMBC experiment to correlate H-23 with C-1′, and H-1′ with C-23 (Fig. 2). Down field shifts of the H-23 signal ($\Delta\delta_H$ 0.14 ppm) and C-23 signal ($\Delta\delta_C$ 9.9 ppm) relative to those of rifampicin were also in accord with the ribosylation of the 23-OH.

The structure of RIP-Mb (**7**) was similarly determined by NMR data (Tables 2 and 3). ^1H and ^{13}C NMR spectra of **7** exhibited the signals due to ribosyl group: δ_H 5.22 ppm (H-1′), 3.93 ppm (H-2′), 3.88 ppm (H-3′), 4.00 ppm (H-4′) and 3.51 ppm (H-5′): δ_C 105.1 ppm (C-1′),

Table 1. Mass spectral data of RIP-Ma (**6**) and RIP-Mb (**7**).

	Molecular formula & molecular weight	Positive FAB-MS	Negative FAB-MS	HRFAB-MS
Rifampicin	$C_{43}H_{58}N_4O_{12}$ 822			
RIP-Ma	$C_{43}H_{55}NO_{17}$ 857	m/z 857 (M) 858 (M+H) 880 (M+Na)	m/z 856 (M-H)	Calcd. for $C_{43}H_{55}NO_{17}Na$ (M+Na) 880.3368 Found 880.3449 (M+Na)
RIP-Mb	$C_{48}H_{66}N_4O_{16}$ 954	m/z 954 (M) 977 (M+Na) 993 (M+K)	m/z 953 (M-H)	Calcd. for $C_{48}H_{66}N_4O_{16}Na$ (M+Na) 977.4372 Found 977.4459

Table 2. ^{1}H NMR chemical shifts (δ, ppm), multiplicity and coupling constants (J, Hz) of rifampicin (**1**), RIP-Ma (**6**) and RIP-Mb (**7**) in CD_3OD.

Proton	Rifampicin*1	RIP-Ma	RIP-Mb
13	1.71(3H, s)	1.70(3H, s)	1.70(3H, s)
14	2.02(3H, s)	2.01(3H, s)	2.02(3H, s)*4
17	6.35(1H, br d, 10.5)	6.28(1H, br d,11.0)	6.36(1H, d,11.0)
18	7.25(1H, dd, 15.8, 10.5)	7.40(1H, dd,16.0, 11.0)	7.17(1H, dd,15.5, 11.0)
19	6.08(1H, dd, 15.8, 7.0)	6.14(1H, dd,16.0, 7.5)	6.10(1H, dd,15.5, 7.0)
20	2.31(1H, m)	2.27(1H, ddq, ca. 9.0, 7.0,7.0)	2.27(1H, m)
21	3.87(1H, dd, 10.0, 1.0)	3.84(1H, dd, 9.5, 1.0)	3.80(1H, brd, 9.0)
22	1.74(1H, m)	1.87(1H, brq, 7.0)	1.80(1H, m)
23	3.08(1H, dd, 10.5, 2.0)	3.22(1H, dd, 9.5,1.0)	3.21(1H, dd, 9.0,1.5)
24	1.48(1H, m)	1.62(1H, dq, 9.5, 7.0)	1.65(1H, m)
25	5.16(1H, d, 10.5)	4.96(1H, d,10.5)	4.97(1H, d,10.5)
26	1.24(1H, m)	1.15(1H, ddq,10.5, 1.0, 7.0)	1.17(1H, m)
27	3.38(1H, d, 8.0)	ca. 3.34*2	3.30(1H, d, ca.8.0)*3
28	5.07(1H, dd,12.7, 8.0)	5.08(1H, dd,12.5, 8.0)	5.11(1H, dd,12.5, 8.0)
29	6.26(1H, d,12.7)	6.25(1H, d, 12.5)	6.24(1H, d, 12.5)
30	2.02(3H, s)	1.99(3H, s)	2.01(3H, s)*4
31	0.93(3H, d, 7.0)	1.01(3H, d, 7.0)	0.94(3H, d, 7.0)
32	0.99(3H, d, 7.0)	1.03(3H, d, 7.0)	1.01(3H, d, 7.0)
33	0.61(3H, d, 7.0)	0.64(3H, d, 7.0)	0.61(3H, d, 7.0)
34	-0.21(3H, d, 7.0)	-0.12(3H, d,7.0)	-0.05(3H, d,7.0)
36	2.02(3H, s)	2.01(3H, s)	2.02(3H, s)*4
37	3.00(3H, s)	3.00(3H, s)	3.00(3H, s)
N-C*H*$_3$	2.78(3H, s)	-----	2.60(3H, s)
PhC*H*=N-N	8.32(1H, s)	-----	8.24(1H, s)
PhC*H*=O	-----	10.53(1H, s)	-----
C*H*$_2$N	3.30(4H, br m)*3	-----	3.26(4H, brm)
	3.18(4H, br m)	-----	2.95(4H, brm)
1'		5.24(1H, d, 4.5)	5.22(1H, d, 4.5)
2'		3.93(1H, dd, 5.5, 4.5)	3.93(1H, dd, 5.5, 4.5)
3'		3.87(1H, dd, 5.5, 1.5)	3.86(1H, dd, 5.5, 2.0)
4'		4.01(1H, ddd, ca.4.0, 4.0, 1.5)	4.00(1H, m)
5'		3.52(2H, d, 4.0)	3.51(2H, d, 4.0)

*1 N. MORISAKI *et al.*, J. Antibiotics, 46: 1605~1610, 1993.
*2 Overlapping with other signals.
*3 Obscured by other resonances.
*4 Interchangeable.

73.2 ppm (C-2'), 71.7 ppm (C-3'), 86.7 ppm (C-4') and 63.5 ppm (C-5'), that were absent in the spectra of rifampicin (**1**). Down field shifts of H-23 ($\Delta\delta_H$ 0.13 ppm) and C-23 signal ($\Delta\delta_C$ 9.7 ppm) of **7** relative to those of rifampicin indicated the ribosylation of the OH group on C-23.

The absolute configuration of the ribose was determined on a chiral HPLC column (Scheme 1). The ribose obtained by hydrolysis of RIP-Mb (**7**) was converted to the aldononitrile acetate **8**,[15] and **8** was analyzed on Chiralcel OD-H column.[16] Retention time of the derivatives **8** from the ribose of RIP-Mb, L-ribose and D-ribose were 60.0 minutes, 58.0 minutes and 59.6 minutes, respectively. Accordingly, the absolute configuration of ribose in RIP-Mb was assigned to be D.

The configuration at anomeric carbon of D-ribose in

Table 3. ^{13}C NMR chemical shifts (δ ppm) of rifampicin (**1**), RIP-Ma (**6**) and RIP-Mb (**7**) in CD_3OD.

Carbon	Rifampicin*1	RIP-Ma	RIP-Mb	Carbon	Rifampicin	RIP-Ma	RIP-Mb
1-10	184.0	185.2	184.8	25	75.6	75.4	75.3
	175.8	174.8	174.8	26	41.7	42.1	42.3
	149.3	152.5	149.2	27	78.5	78.4	78.5
	147.9	149.1	147.5	28	120.1*3	120.0	120.0
	119.7*3	120.7	119.5	29	144.7	144.7	144.7
	118.2	120.2	118.4	30	20.8	20.3	20.7
	116.1	119.4	116.5	31	18.2	18.1	18.3
	116.0	115.2	116.0	32	11.0	11.6	11.7
	105.0	105.7	104.6	33	9.4	10.3	10.2
	101.9	101.6	101.5	34	9.7	9.5	9.7
11	189.0	188.9	188.2	35	172.4	172.5	172.5
12	110.6	110.4	110.4	36	20.8	21.0	21.0
13	22.4	22.2	22.3	37	56.7	56.7	56.7
14	7.5	7.4	7.5	NCH_3	43.7	----	44.6
15	171.1	171.8	170.6	Ph*C*H=N-N	138.8	----	137.7
16	133.1	133.4	133.3	Ph*C*H=O	----	193.1	----
17	134.8	134.0	134.8	CH_2N	53.2	----	53.8
18	129.0	129.7	129.0		49.5*2	----	50.1
19	140.7	140.0	141.1	1'		105.1	105.1
20	39.1	38.9	39.4	2'		73.2	73.2
21	75.2	76.5	75.6	3'		71.6	71.7
22	34.4	34.8	35.0	4'		86.7	86.7
23	78.2	88.1	87.9	5'		63.4	63.5
24	39.4	39.6	39.6				

*1 N. MORISAKI *et al.*, J. Antibiotics, 46: 1605~1610, 1993.
*2 Obscured by other resonances.
*3 Interchangeable.

Scheme 1. Derivatization of ribose in RIP-Mb (**7**) for HPLC analysis.

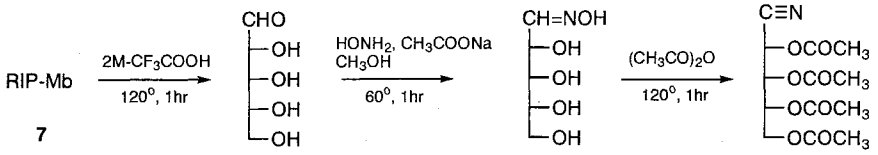
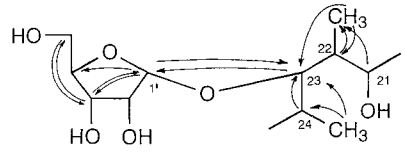


Fig. 2. Partial structure of RIP-Ma.



Arrows indicate ^1H-^{13}C correlation by HMBC experiment.

RIP-Ma and RIP-Mb was determined to be α from the reported ^{13}C NMR chemical shift data of methyl α- and β-furanosides of pentoses.[12~14] Thus, the structures of RIP-Ma (**6**) and RIP-Mb (**7**) were determined to be 3-formyl-23-[*O*-(α-D-ribofuranosyl)]rifamycin SV and 23-[*O*-(α-D-ribofuranosyl)]rifampicin, respectively. During the incubation period, the ratio of RIP-Ma and RIP-Mb in the culture medium was almost constant, and RIP-Mb did not decompose to RIP-Ma in the isolation

and purification procedure. Therefore, the imide group is considered to be biologically hydrolized.

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