INACTIVATED PRODUCTS OF RIFAMPICIN BY PATHOGENIC *Nocardia* spp.: STRUCTURES OF GLYCOSYLATED AND PHOSPHORYLATED METABOLITES OF RIFAMPICIN AND 3-FORMYLRIFAMYCIN SV

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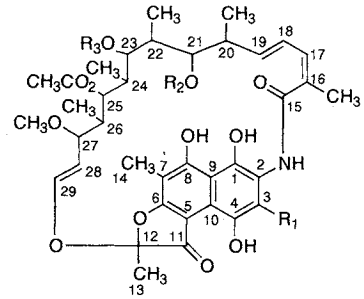
Rifampicin (**1**) was converted into four inactivated products by pathogenic *Nocardia*, RIP-1 and RIP-2 by *N. brasiliensis* and RIP-3 and RIP-4 by *N. otitidiscaviarum*. MS and NMR analysis showed the compounds to be 3-formyl-23-[*O*-(β-D-glucopyranosyl)]rifamycin SV (**2**), 23-[*O*-(β-D-glucopyranosyl)]rifampicin (**3**), 21-(*O*-phosphoryl)rifampicin (**4**) and 3-formyl-21-(*O*-phosphoryl)-rifamycin SV (**5**), respectively.

Most pathogenic *Nocardia* are found to be resistant to rifampicin (**1**),[1~3] a semisynthetic antibiotic widely used as a valuable chemotherapeutic agent.[4] During the studies on the mechanism of the resistance, four inactivated products of **1** were isolated, which show that the principal resistance mechanism of these organisms is the transformation of **1** to inactive compounds.[4,5]

In this paper we describe the structural elucidation of the four products, RIP-1 and RIP-2 inactivated by *N. brasiliensis*, and RIP-3 and RIP-4 by *N. otitidiscaviarum*. The spectral evidence indicates the structures of RIP-1, -2, -3 and -4 as 3-formyl-23-[*O*-(β-D-glucopyranosyl)]rifamycin SV (**2**), 23-[*O*-(β-D-glucopyranosyl)]rifampicin (**3**), 21-(*O*-phosphoryl)rifampicin (**4**) and 3-formyl-21-(*O*-phosphoryl)rifamycin SV (**5**), respectively (Fig. 1).

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



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

Experimental

^1H, ^{13}C and ^{31}P NMR spectra were measured in CD_3OD on a JEOL ALPHA-500 NMR spectrometer at 500, 125 and 202.35 MHz, respectively. chemical shifts of ^1H and ^{13}C NMR were recorded in δ units relative to internal tetramethylsilane (δ=0) and ^{31}P NMR were relative to external potassium phosphate (δ=0). FAB-MS and HRFAB-MS were

Table 1. Mass spectral data for RIPs.

Compounds	Method	Found (*m/z*)	Assignments
RIP-1	Positive FAB-MS	910	M+Na
	Negative FAB-MS	886	M−H
	HRFAB-MS	910.3518	Calcd for $C_{44}H_{57}NO_{18}Na$ (M+Na): 910.3474
RIP-2	Positive FAB-MS	1,007	N+Na
	Negative FAB-MS	983	M−H
	HRFAB-MS	1,007.4550	Calcd for $C_{49}H_{68}N_4O_{17}Na$ (M+Na): 1,007.4480
RIP-3	Positive FAB-MS	903	M+H
		925	M+Na
		947	M+2Na−H
		969	M+3Na−2H
	Negative FAB-MS	901	M−H
		923	M+Na−2H
	HRFAB-MS	947.3471	Calcd for $C_{43}H_{58}N_4O_{15}PNa_2$ (M+2Na−H): 947.3431
RIP-4	Positive FAB-MS	828	M+Na
		850	M+2Na−H
		872	M+3Na−2H
	Negative FAB-MS	804	M−H
		826	M+Na−2H
	HRFAB-MS	828.2602	Calcd for $C_{38}H_{48}NO_{16}PNa$ (M+Na): 828.2608

Table 2. ^{1}H NMR chemical shifts (δ, ppm) and coupling constants of rifampicin, RIP-1 and RIP-2 in CD_3OD.

Proton	Rifampicin	RIP-1	RIP-2
13	1.71 (3H, s)	1.70 (3H, s)	1.69 (3H, s)
14	2.02 (3H, s)	2.02 (3H, s)	2.00 (3H, s)
17	6.35 (1H, br d, J=10.5 Hz)	6.30 (1H, br d, J=11.0 Hz)	6.40 (br d, J=10.0 Hz)
18	7.25 (1H, dd, J=15.8, 10.5 Hz)	7.44 (1H, dd, J=16.0, 11.0 Hz)	7.20 (1H, m)
19	6.08 (1H, dd, J=15.8, 7.0 Hz)	6.14 (1H, dd, J=16.0, 7.5 Hz)	6.04 (1H, dd, J=16.0, 6.5 Hz)
20	2.31 (1H, m)	2.32 (1H, m)	2.30 (1H, m)
21	3.87 (1H, dd, J=10.0, 1.0 Hz)	3.88 (1H, d, J=9.5 Hz)	3.87 (1H, d, J=9.0 Hz)
22	1.74 (1H, m)	1.92 (1H, br q, J=7.0 Hz)	1.83 (1H, br q, J=7.0 Hz)
23	3.08 (1H, dd, J=10.5, 2.0 Hz)	3.59 (1H, d, J=9.0 Hz)	3.56 (1H, d, J=9.0 Hz)
24	1.48 (1H, m)	1.54 (1H, m)	1.56 (1H, m)
25	5.16 (1H, d, J=10.5 Hz)	5.39 (1H, d, J=10.5 Hz)	5.34 (1H, d, J=10.5 Hz)
26	1.24 (1H, m)	1.02 (1H, m)	1.01 (1H, m)
27	3.38 (1H, d, J=8.0 Hz)	3.34 (1H, d, J=8.5 Hz)	3.34 (1H, dd, J=8.5, 1.5 Hz)
28	5.07 (1H, dd, J=12.7, 8.0 Hz)	5.09 (1H, dd, J=12.5, 8.5 Hz)	5.14 (1H, dd, J=12.5, 8.5 Hz)
29	6.26 (1H, d, J=12.7 Hz)	6.31 (1H, d, J=12.5 Hz)	6.27 (1H, d, J=12.5 Hz)
30	2.02 (3H, s)	1.98 (3H, s)	2.02 (3H, s)
31	0.93 (3H, d, J=7.0 Hz)	1.01 (3H, d, J=7.0 Hz)	0.94 (3H, d, J=7.0 Hz)
32	0.99 (3H, d, J=7.0 Hz)	1.08 (3H, d, J=7.0 Hz)	1.03 (3H, d, J=7.0 Hz)
33	0.61 (3H, d, J=7.0 Hz)	0.60 (3H, d, J=7.0 Hz)	0.54 (3H, d, J=7.0 Hz)
34	−0.21 (3H, d, J=7.0 Hz)	−0.11 (3H, d, J=7.0 Hz)	0.03 (3H, d, J=7.0 Hz)
36	2.02 (3H, s)	2.02 (3H, s)	2.02 (3H, s)
37	3.00 (3H, s)	3.01 (3H, s)	3.01 (3H, s)
N−CH_3	2.78 (3H, s)	—	2.42 (3H, s)
PhCH=N−N	8.32 (1H, s)	—	8.19 (1H, s)
PhCH=O	—	10.52 (1H, s)	—
CH_2N	3.30 (4H, br m)[a],	—	3.18 (4H, br m),
	3.18 (4H, br m)	—	2.72 (4H, br m)
Glu-1′		4.42 (1H, d, J=7.5 Hz)	4.40 (1H, d, J=7.8 Hz)
Glu-2′		2.97 (1H, dd, J=9.5, 7.5 Hz)	2.99 (1H, dd, J=9.0, 7.8 Hz)
Glu-3′		3.28 (1H, dd, J=9.5, 9.0 Hz)	3.26 (1H, dd, J=9.5, 9.0 Hz)
Glu-4′		3.15 (1H, dd, J=9.0, 8.0 Hz)	3.16[a]
Glu-5′		3.17 (1H, ddd, J=8.0, 4.5, 1.5 Hz)	3.16[a]
Glu-6′		3.58 (1H, dd, J=12.5, 4.5 Hz),	3.59 (1H, dd, J=12.5, 5.0 Hz),
		3.81 (1H, dd, J=12.5, 1.5 Hz)	3.81 (1H, dd, J=12.5, 1.5 Hz)

[a] Obscured by other resonances.

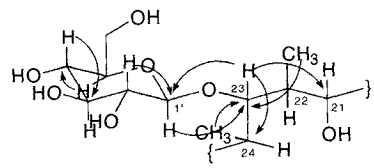
Table 3. ^{13}C NMR chemical shifts (δ, ppm) of rifampicin, RIP-1 and RIP-2 in CD_3OD.

Carbon	Rifampicin	RIP-1	RIP-2	Carbon	Rifampicin	RIP-1	RIP-2
1~10	184.0	185.3	185.6	25	75.6	77.3	77.3
	175.8	174.2	174.7	26	41.7	42.8	42.8
	149.3	152.3	149.0	27	78.5	78.7	78.9
	147.9	148.8	147.1	28	120.1[b]	119.9	120.0
	119.7[b]	120.3	118.8	29	144.7	145.4	145.1
	118.2	120.2	117.9	30	20.8	20.3	20.7
	116.1	119.2	116.9	31	18.2	18.3	18.4
	116.0	115.3	115.9	32	11.0	11.9	12.3
	105.0	105.7	104.3	33	9.4	10.9	10.9
	101.9	101.7	101.3	34	9.7	9.5	9.8
11	189.0	188.6	187.3	35	172.4	172.9	172.9
12	110.6	110.6	110.5	36	20.8	21.0	21.0
13	22.4	22.4	22.3	37	56.7	56.4	56.4
14	7.5	7.4	7.4	NCH_3	43.7	—	45.4
15	171.1	171.6	174.7	PhCH=N–N	138.8	—	136.8
16	133.1	133.3	133.2	PhCH=O	—	193.1	—
17	134.8	134.1	135.1	CH_2N	53.2	—	54.6
18	129.0	129.7	128.6		49.5[a]	—	50.9
19	140.7	140.0	141.0	Glu-1′		104.0	103.9
20	39.1	39.3	40.3	Glu-2′		76.0	75.9
21	75.2	76.0	74.0	Glu-3′		78.1[c]	78.1[d]
22	34.4	36.0	36.4	Glu-4′		71.3	71.4
23	78.2	88.9	88.5	Glu-5′		78.2[c]	78.2[d]
24	39.4	39.6	39.5	Glu-6′		62.7	62.8

[a] Obscured by other resonances.
[b~d] Interchangeable.

measured on a JEOL HX110 instrument. Biological transformation of rifampicin to RIPs, the isolation and purification are described elsewhere.[4,5]

Fig. 2. Partial structure of RIP-1 (**2**) derived from ^1H-^{13}C correlation by HMBC experiment.



Structure

The structures of RIPs were determined spectroscopically by comparison with the data of rifampicin.

Positive FAB-MS of RIP-1 showed a peak at m/z 910 $(M+Na)^+$, and negative FAB-MS at m/z 886 $(M-H)^-$. The molecular formula was determined by HRFAB-MS to be $C_{44}H_{57}NO_{18}$ (Table 1).

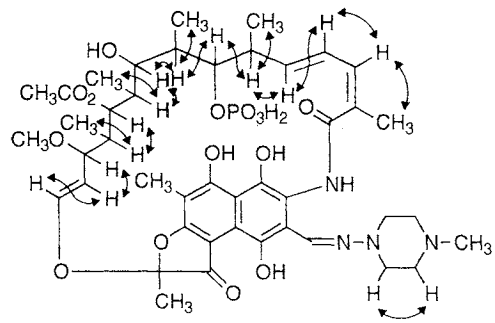
The structure of RIP-1 was elucidated to be 3-formyl-23-[*O*-(β-D-glucopyranosyl)]rifamycin SV (**2**) by ^1H NMR, ^{13}C NMR, COSY, C-H COSY and HMBC experiments. ^1H NMR spectrum of RIP-1 (Table 2) showed a singlet due to a formyl proton at δ 10.52 instead of the signals of *N*-methyl piperazine moiety (δ 2.78, 3.30, 3.18) and of an olefinic proton on the carbon adjacent to C-3 (δ 8.32) present in rifampicin,[6] indicating the 1-amino-4-methylpiperazine moiety has been cleaved off by hydrolysis. The formyl carbon signal was also shown at δ 193.1 in ^{13}C NMR spectrum (Table 3). Protons 17-H through 21-H and 25-H through 29-H were correlated by COSY spectrum and carbon signals of C-15 through C-29 were assigned by HMBC experiment. The presence of D-glucose was shown by signals at δ 104.0 (C-1′), 76.0 (C-2′), 78.1 (C-3′), 71.3 (C-4′), 78.2 (C-5′) and 62.7 (C-6′) in the ^{13}C NMR spectrum.[7] The site of glycosylation was determined by HMBC experiment (Fig. 2) to correlate 23-H (δ 3.59) with C-1′, and 1′-H (δ 4.42) with C-23 (δ 88.9). Down field shifts of the 23-H signal ($\Delta\delta$ 0.51 ppm) and C-23 signal ($\Delta\delta$ 10.7 ppm) relative to those of rifampicin also indicated the glycosylation of the OH group at C-23. The coupling constant of the anomeric proton at δ 4.42 (d, $J=7.5$ Hz) is consistent with the β-glucoside. No other significant differences were observed in the NMR and UV spectra of RIP-1 and rifampicin, indicating that the rest of the structures of these two compounds are the same.

Table 4. ^{1}H NMR chemical shifts (δ, ppm) and coupling constants of RIP-3 and RIP-4 in CD_3OD[a].

Proton	RIP-3	RIP-4
13	1.69 (3H, s)	1.71 (3H, s)
14	1.99 (3H, s)	1.99 (3H, s)[d]
17	6.33 (1H, br m)	6.30 (1H, d, J=11.0 Hz)
18	6.98 (1H, br m)	7.16 (1H, br m)
19	5.83 (1H, br dd, J=15.0, 9.0 Hz)	5.92 (1H, dd, J=15.0, 8.0 Hz)
20	2.22 (1H, br m)	2.30 (1H, br m)
21	4.28 (1H, br m)	4.23 (1H, br m)
22	1.81 (1H, br m)	1.88 (1H, br m)
23	2.81 (1H, dd, J=9.5, 3.0 Hz)	2.84 (1H, dd, J=10.0, 2.5 Hz)
24	1.43 (1H, br m)	1.52 (1H, m)
25	5.01 (1H, br d, J=9.5 Hz)	5.08 (1H, d, J=11.0 Hz)
26	1.0[b] (1H, br m)	0.93 (1H, br m)
27	3.3[b]	3.3[b]
28	5.25 (1H, dd, J=12.5, 8.0 Hz)	5.27 (1H, dd, J=12.5, 7.5 Hz)
29	6.16 (1H, d, J=12.5 Hz)	6.19 (1H, d, J=12.5 Hz)
30	1.97 (3H, s)[c]	2.02 (3H, s)[d]
31	1.13 (3H, d, J=7.0 Hz)	1.12 (3H, d, J=7.0 Hz)
32	0.91 (3H, d, J=7.0 Hz)	0.97 (3H, d, J=7.0 Hz)
33	0.21 (3H, d, J=7.0 Hz)	0.22 (3H, d, J=7.0 Hz)[e]
34	0.31 (3H, br m)	0.23 (3H, d, J=7.0 Hz)[e]
36	2.01 (3H, s)[c]	2.02 (3H, s)[d]
37	3.02 (3H, s)	3.02 (3H, s)
NCH_3	2.49 (3H, s)	—
PhCH=N–N	8.17 (1H, s)	—
PhCH=O	—	10.50 (1H, s)
CH_2N	3.18 (4H, br m), 2.71 (4H, br m)	—

[a] The data of rifampicin is shown in Table 2.
[b] Obscured by other resonances.
[c~e] Interchangeable.

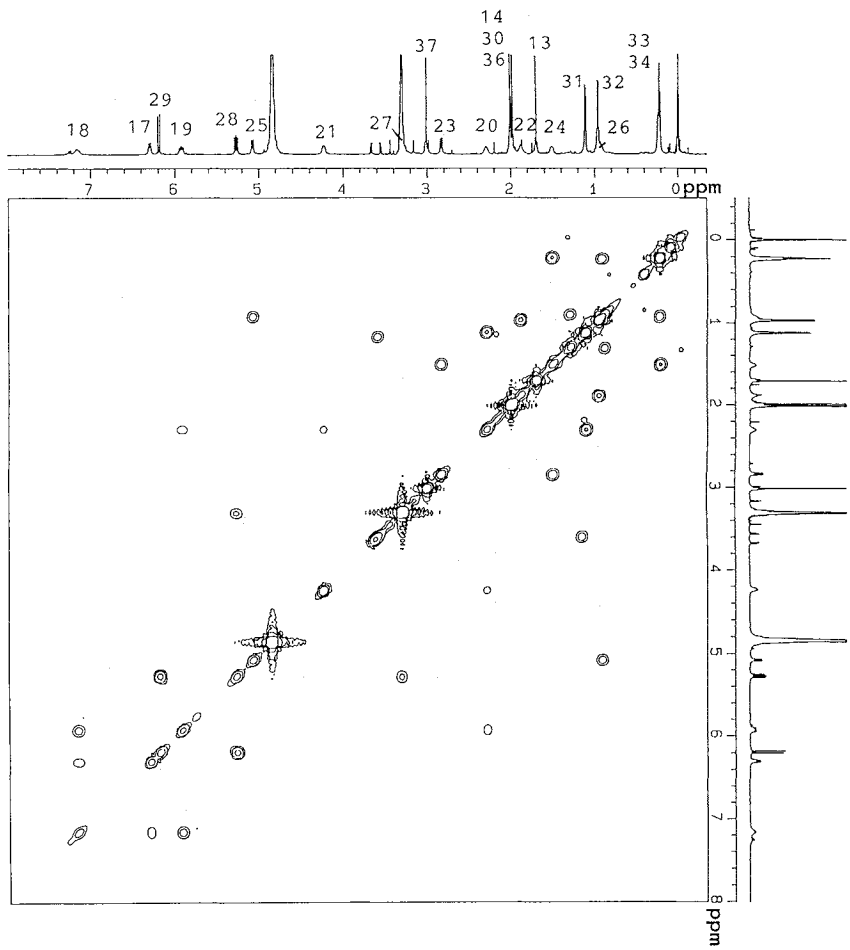
Fig. 3. ^{1}H-^{1}H correlation of RIP-3 (**4**) by COSY experiment.



The structure of RIP-2 was elucidated to be 23-[*O*-(β-D-glucopyranosyl)]rifampicin (**3**) by the same procedures as used for RIP-1. Positive FAB-MS showed a peak at m/z 1,007 $(M+Na)^+$, and negative FAB-MS at m/z 983 $(M-H)^-$. Its molecular formula was determined by HRFAB-MS to be $C_{49}H_{68}N_4O_{17}$ (Table 1) which is in accord with a monoglucosylated rifampicin, and this was confirmed by its ^{1}H NMR and ^{13}C NMR data (Tables 2 and 3). The site of glycosylation was similarly determined by HMBC spectrum to correlate 23-H (δ 3.56) with C-1′ (δ 103.9) and 1′-H (δ 4.40) with C-23 (δ 88.5). Both the 23-H signal and the C-23 signal shifted to downfield ($\Delta\delta$ 0.48 ppm and $\Delta\delta$ 10.3 ppm, respectively) compared with those signals of rifampicin. Thus, the structure of RIP-2 was established to be 23-[*O*-(β-D-glucopyranosyl)]rifampicin (**3**).

Positive and negative FAB-MS data of RIP-3 and RIP-4 indicated their molecular weight to be 902 and 805, respectively. The molecular formulae were determined by HRFAB-MS to be $C_{43}H_{59}N_4O_{15}P$ for RIP-3 and $C_{38}H_{48}NO_{16}P$ for RIP-4 (Table 1). ^{31}P NMR spectrum of RIP-3 and of RIP-4 showed a signal at δ 2.05 and δ 1.79, respectively, due to a phosphoric acid ester. These data suggest that RIP-3 and RIP-4 are *O*-phosphorylated rifampicin and phosphorylated 3-formylrifamycin SV, respectively, instead

Fig. 4. COSY spectrum of RIP-4 (**5**).



of the glycosylation in the case of RIP-1 and RIP-2.

The structure of RIP-3 was determined by ^{1}H NMR (Table 4), COSY (Fig. 3) and ^{31}P NMR experiments. In COSY experiment, ^{1}H signals of 17-H through 24-H and of 25-H through 29-H were correlated, and the signals at δ 4.28 and at δ 2.81 were assigned to 21-H and 23-H, respectively. Comparing the chemical shifts of ^{1}H-signals with those of rifampicin (Table 2), prominent down field shift caused by phosphorylation was observed only for 21-H ($\Delta\delta$ 0.41 ppm).[8,9] In a {H}-P selective decoupling experiment, a doublet at δ 2.05 ($J_{21\text{-H,P}}=8.4$ Hz)[10] due to ^{31}P callapsed into a sharp singlet by irradiation at δ 4.28 (21-H). These facts indicate that 21-OH of rifampicin was phosphorylated in RIP-3 (**4**).

^{1}H NMR spectrum of RIP-4 lacked the signals of *N*-methyl piperazine moiety present in rifampicin and, instead, exhibited a signal due to a formyl proton at δ 10.50 (Table 4). The signals of 21-H (δ 4.23) and 23-H (δ 2.84) were assigned based on the correlation of 19-H through 24-H in COSY experiments (Fig. 4). Down field shift of 21-H (Δ 0.36 ppm) indicates that phosphorylation occured, like RIP-3, on the 21-OH.

No other significant differences was observed in the ^{1}H NMR spectra of RIP-3, RIP-4 and rifampicin, indicating that the rest of the structures of these compounds are the same. Thus, the structures of RIP-3 and RIP-4 were determined to be 21-(*O*-phosphoryl)rifampicin (**4**) and 3-formyl-21-(*O*-phosphoryl)-rifamycin SV (**5**), respectively.

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