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Ribosylation by Mycobacterial Strains as a New Mechanism of Rifampin Inactivation

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Several fast-growing *Mycobacterium* strains were found to inactivate rifampin. Two inactivated compounds (RIP-Ma and RIP-Mb) produced by these organisms were different from previously reported derivatives, i.e., phosphorylated or glucosylated derivatives, of the antibiotic. The structures of RIP-Ma and RIP-Mb were determined to be those of 3-formyl-23-[*O*-(α-D-ribofuranosyl)]rifamycin SV and 23-[*O*-(α-D-ribofuranosyl)]rifampin, respectively. To our knowledge, this is the first known example of ribosylation as a mechanism of antibiotic inactivation.

Rifampin is an important chemotherapeutic agent for use against tuberculosis, leprosy, and infections by organisms related to those causing these diseases (3, 7). The antimicrobial activity of rifampin is due to its inhibition of DNA-dependent RNA polymerase, and most rifampin-resistant bacteria have been reported to have an alteration in the β-subunit of this enzyme (2). Such a resistance mechanism has been reported for *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium africanum*, and *Mycobacterium leprae* (14, 16). During our studies of fast-growing mycobacterial strains, we found that several strains inactivate rifampin (1, 5). We analyzed the inactivated antibiotics and found them to differ in mass spectrum and chromatographic mobility from those previously reported, i.e., the glycosylated (glucosylated) or phosphorylated compounds produced by pathogenic *Nocardia* spp. (11, 17). These results prompted us to determine the detailed structures of the inactivated compounds. In this paper the isolation, structures, and antimicrobial activities of the inactivated compounds are reported.

Rifampin was generously provided by CIBA-GEIGY Pharmaceuticals, Basel, Switzerland. MICs were determined by an agar dilution method with brain heart infusion agar (Difco Laboratories, Detroit, Mich.) medium. The inoculum size of each test organism was adjusted to 10^6 CFU/ml. The plates were spotted with a multipoint inoculator (A 400; Denly Instruments, Ltd., Sussex, England) that delivered 0.005 ml of inoculum, resulting in a spot inoculum of approximately 5×10^4 CFU. Inactivation of rifampin was monitored by a bioassay method with *Bacillus subtilis* PCI 219 as a test organism (17). Inactivated compounds were monitored with a thin-layer chromatography scanner (CS-910; Shimadzu Seisakusho, Kyoto, Japan) (17, 18) at 238 nm.

One loopful of the culture from slant cultures of *Mycobacterium smegmatis* DSM 43756 was inoculated into a 100-ml Erlenmeyer shake flask containing 20 ml of a seed culture medium (2% glycerol-enriched brain heart infusion medium; Difco Laboratories) with 4-mm-diameter glass beads to reduce aggregation of the mycobacterial cells (18). The inoculated flasks were shaken at 250 rpm (5.8-cm stroke) for 4 days at 33°C. For large-scale preparation of the inactivation products, the seed culture was used as an inoculum. Ten milliliters of seed culture was inoculated into 500-ml shake flasks containing 100 ml of the same medium. After 24 h of incubation at 33°C, rifampin (stock solution in methanol at 100 mg/ml) was added to a final concentration of 50 μg/ml, and the mixture was incubated for 3 days. Following separation from mycelia by centrifugation at 6,000 rpm ($5,800 \times g$), the supernatant was adjusted to pH 2.0 with 1 N HCl and extracted three times with an equal volume of ethylacetate. The extracts were combined, dehydrated with Na_2SO_4, and concentrated in vacuo to dryness. The dried material was purified by silica gel column chromatography (column size, 3.5 by 15 cm) by using 75 g of Wakogel C-200 (Wako Pure Chemicals, Osaka, Japan) and a mixture of $CHCl_3$ and MeOH (95:5) as the solvent. Preparative silica gel thin-layer chromatography with $CHCl_3$-MeOH (4:1) for RIP-Mb and $CHCl_3$-MeOH (3:1) for RIP-Ma was performed. Sephadex LH-20 column chromatography (column size, 2 by 40 cm) with MeOH as the solvent was also used for further purification of both compounds. Throughout these steps, inactivated compounds to be isolated were monitored by thin-layer chromatography. From 100 mg of starting rifampin (in 2 liters of the culture broth), 6.5 mg of purified RIP-Ma and 11.2 mg of RIP-Mb were obtained. The R_f values of RIP-Ma, RIP-Mb, and rifampin developed with $CHCl_3$-MeOH (3:1) were 0.18, 0.33, and 0.56, respectively. These thin-layer-chromatography R_f values were different from those of the formerly reported phosphorylated and glucosylated compounds RIP-1, -2, -3, and -4, whose R_f values in the above-mentioned developing-solvent system were 0.08, 0.26, 0, and 0, respectively (11, 17). Control experiments demonstrated that removal of the side chain at position 3 was not an artifact of the preparative technique in the present experimental conditions.

The structures of inactivated compounds (RIP-Ma and RIP-Mb) were deduced from the information based on the following mass spectrometry data. The molecular formulae and molecular weights of RIP-Ma ($C_{43}H_{55}NO_{17}$ and 857, respectively) and RIP-Mb ($C_{48}H_{66}N_4O_{16}$ and 954, respectively) were deter-

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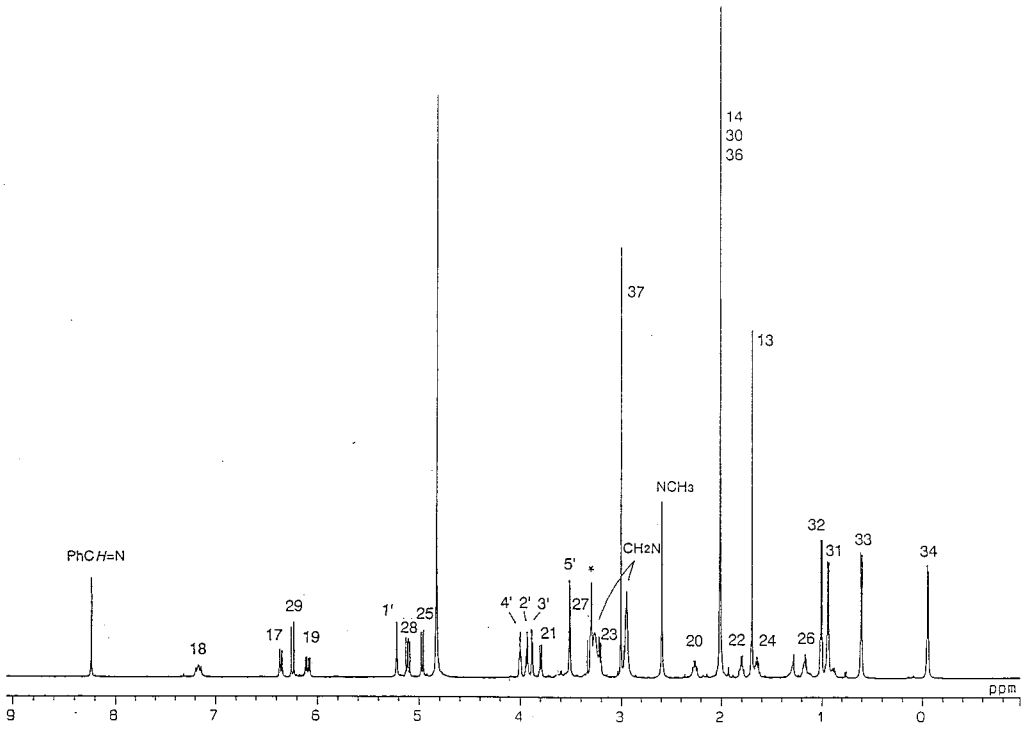

FIG. 1. ^{1}H NMR spectrum of RIP-Mb and assignment of each peak in CD_3OD. *, peak due to methanol.

mined by positive and negative fast atom bombardment-mass spectrometry and high-resolution fast atom bombardment-mass spectrometry. These molecular formulae of RIP-Ma and RIP-Mb suggested that they are monoglycosylated ($C_5H_{10}O_5$)-3-formyl-rifamycin SV and ($C_5H_{10}O_5$)-3-formyl-rifampin, respectively. The molecular formulae were different from those of the glucosylated ($C_6H_{12}O_6$)-3-formyl-rifamycin SV and ($C_5H_{12}O_6$)-3-formyl-rifampin, which we have reported in our previous papers (11, 17). The ^{1}H nuclear magnetic resonance (NMR) spectrum of RIP-Mb and the complete assignments of each signal are shown in Fig. 1. In a comparison of the NMR data with those of the reference sugars, such as D-arabinose (6, 12), D-lyxose (12), D-xylose (12), and D-ribose (6, 12, 13), the presence of α-D-ribofuranosyl moiety was suggested by ^{1}H signals appearing at δH 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) and their ^{1}H-^{1}H couplings ($J_{1'-2'}$ = 4.5 Hz, $J_{2'-3'}$ = 5.5 Hz, $J_{3'-4'}$ = 1.5 Hz, and $J_{4'-5'}$ = 4.0 Hz), together with ^{13}C signals (δC 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′]). Ribosylation of 23-OH of rifampin in RIP-Ma was indicated by correlation of H-1′-C-23 (δC 88.1 ppm) and H-23 (δH 3.22 ppm)-C-1′ by heteronuclear multiple-bond correlation experiments. Finally, the structure of RIP-Ma (Fig. 2) was determined to be that of 3-formyl-23-[*O*-(α-D-ribofuranosyl)]rifamycin SV from the NMR spectral data. Hydrolytic elimination of the *N*-amino-*N*-methylpiperazine moiety of rifampin was confirmed by the lack of its ^{1}H and ^{13}C signals and the appearance of formyl signals (δH 10.53 ppm and δC 193.1 ppm in CD_3OD) (10). The molecular formula and ^{1}H NMR data indicated that RIP-Mb (Fig. 2) was 23-[*O*-(α-D-ribofuranosyl)]rifampin.

Glucosylation of antibiotics as a resistance mechanism against macrolides and lincomycin (4, 9, 10, 15) has been reported. Recently, we also identified a new type of rifampin

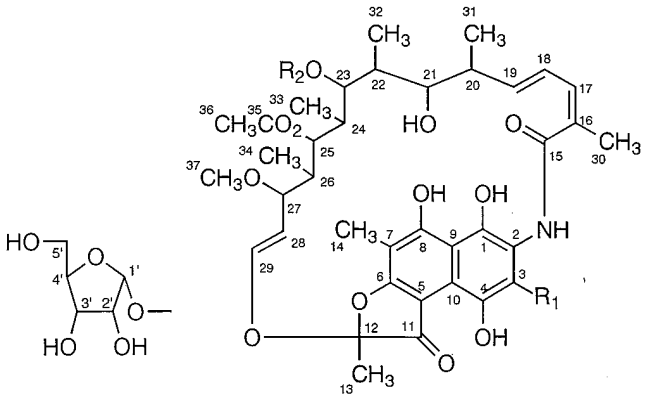

FIG. 2. Structures of rifampin and its inactivated products RIP-Ma and RIP-Mb.

TABLE 1. MICs for *Mycobacterium* spp. and their ribosylation activities with rifampin

Mycobacterium sp.	MIC (μg/ml)	Ribosylation activity
M. smegmatis DSM 43756	50	+
M. smegmatis ATCC 19420	50	+
Mycobacterium sp. strain 607	50	+
M. chelonae subsp. *abscessus* IFM 0359[a]	50	+
M. flavescens ATCC 14474	25	+
M. vaccae ATCC 15483	6.25	+
M. phlei ATCC 11758	0.6	−
M. parafortuitum ATCC 19686	50	+

[a] IFM, strain number of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University.

inactivation, inactivation by glucosylation (17). However, to our knowledge, inactivation of antibiotics by nonglucose sugars has not yet been described. Therefore, this is the first report of ribosylation. Details of structural determination will be given elsewhere.

The antimicrobial activities of purified inactivated compounds were compared with that of rifampin. MICs of both RIP-Ma and RIP-Mb for *M. smegmatis* DSM 43756 and *B. subtilis* PCI 219 were >100 μg/ml. On the other hand, MICs of rifampin for *M. smegmatis* DSM 43756 and *B. subtilis* PCI 219 were 25.0 and 0.02 μg/ml, respectively. These results indicated that RIP-Ma and RIP-Mb are inactivated forms of rifampin and that the 23-hydroxyl group is involved in the antimicrobial activity of this antibiotic. No significant differences in the activities of RIP-Ma and RIP-Mb were observed.

We previously reported two mechanisms of rifampin inactivation in pathogenic strains of *Nocardia*: *Nocardia brasiliensis* inactivated rifampin by glucosylation of the 23-OH group, and *Nocardia otitidiscaviarum* inactivated rifampin by phosphorylation of the 21-OH group. However, no ribosylation of rifampin in pathogenic *Nocardia* spp. was observed (17). Recent preliminary experiments with nocardioform bacteria of the genus *Rhodococcus* suggested that some have glycosylation or phosphorylation activities against rifampin. However, in *Rhodococcus* spp. ribosylation activity only and no glucosylation was observed. Therefore, these different profiles of inactivation activity may be characteristic of each genus. Fast-growing mycobacteria, including *M. smegmatis*, required relatively high MICs of rifampin (Table 1). Therefore, in addition to the mechanisms due to the *rpoB* mutations, whose existence in *M. smegmatis* as well as *M. tuberculosis* has been reported (8), the lower susceptibility of fast-growing *Mycobacterium* spp. to rifampin may be partly due to the inactivation mechanism we have identified. We also confirmed this ribosylation activity to occur in *M. smegmatis* ATCC 19420 and several other *Mycobacterium* spp. (Table 1). An active role for this mechanism in *M. tuberculosis* is not certain at this time.

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