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# Inactivation of Rifampin by *Nocardia brasiliensis*

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**Rifampin was glycosylated by a pathogenic species of *Nocardia*, i.e., *Nocardia brasiliensis*. The structures of two glycosylated compounds (RIP-1 and RIP-2) isolated from the culture broth of the bacterium were determined to be 3-formyl-23-(*O*-[β-D-glucopyranosyl])rifamycin SV and 23-(*O*-[β-D-glucopyranosyl])rifampin, respectively. Both compounds lacked antimicrobial activity against other gram-positive bacteria as well as the *Nocardia* species.**

Rifamycins are members of the naphthalenic ansamycin group of antibiotics characterized by a 17-membered ansa chain (1, 5, 11, 17). Tolypomycin Y, halomicin B, and streptovaricin A belong to this group (11). A vast number of chemical modifications to the structures of the rifamycins have been made to obtain substances possessing greater activity. Consequently, rifampin (Fig. 1), which is now used as a valuable chemotherapeutic agent, was introduced (2, 3, 6). Rifampin has activity against a great variety of organisms, such as bacteria, viruses, and eukaryotes. It is one of the principal agents used to combat tuberculosis (5, 9, 14). The antimicrobial activity of rifampin against bacteria is due to its inhibition of DNA-dependent RNA polymerase (2). Most rifampin-resistant bacteria have been reported to have an alteration in β-subunits of DNA-dependent RNA polymerase (13). Contribution of such a resistance mechanism has been reported in the in vitro-developed mutant of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (18). In 1987, Dabbs (7) suggested the existence of a new rifampin resistance mechanism, i.e., an inactivation enzyme in *Rhodococcus* spp., but no detailed chemical studies of the inactivation products were reported then (7). Quite recently Andersen and Dabbs (1) succeeded in cloning of DNA conferring the ability to inactivate rifamycin from *Rhodococcus* spp., but neither the analysis of the inactivation product nor the sequencing of the gene has yet been done.

During our studies on the susceptibility of *Nocardia* spp. to various antibiotics (19–21), we found that most pathogenic *Nocardia* spp., i.e., *N. asteroides*, *N. brasiliensis*, *N. farcinica*, *N. nova*, and *N. otitidiscaviarum*, were resistant to rifampin. Our continued studies on the mechanisms of the resistance indicated that most of the resistance is due to the inactivation of rifampin. In this paper, we report the susceptibility of pathogenic *Nocardia* spp. to rifampin and full structures of two new products inactivated by *N. brasiliensis*.

## MATERIALS AND METHODS

**Drug and inoculum.** Rifampin was generously provided by CIBA-GEIGY Pharmaceuticals, Basel, Switzerland. All bacteria, including the pathogenic *Nocardia* spp. and their type strains, were maintained on slants of Mueller-Hinton II agar (Becton Dickinson, Cockeysville, Md.). One loopful of spores or mycelial fragments from the slant cultures of *Nocardia* spp. was inoculated into a 10-ml Erlenmeyer shake flask containing 5 ml of a seed medium (2% glucose-enriched brain heart infusion medium; Difco Laboratories, Detroit, Mich.) with 4-mm glass beads intended to reduce aggregation of the nocardial cells (21). The inoculated flasks were shaken at 250 rpm (5.8-cm stroke) for 4 days at 27°C. The mature seed cultures were used as inocula for the inactivation or MIC experiments. Other bacterial inocula were prepared by the method previously described (21).

**MIC determination.** The MIC, defined as the lowest drug concentration causing complete inhibition of visible growth, was determined by an agar dilution method using Mueller-Hinton II agar medium to give final concentrations from 0.39 to 400 μg/ml. Drug-free plates were included as a bacterial growth control. The inoculum size of a test organism was adjusted to $10^7$ CFU/ml for *Nocardia* spp. and $10^6$ CFU/ml for other bacteria. The plates were spotted with a multipoint inoculator (A 400; Denly Instruments, Ltd., Sussex, England) that delivered 0.005 ml, resulting in a spot inoculum of approximately $5 \times 10^4$ CFU.

**Time course of rifampin inactivation.** The mature seed cultures were inoculated at a 10% rate into 100-ml Erlenmeyer flasks containing 50 ml of brain heart infusion medium. Methanol-sterilized rifampin was added aseptically at a final concentration of 20 or 200 μg/ml. For determination of the rifampin concentration, 2 ml of the flask cultures was harvested at various time intervals and filtered. The pH of the filtrate was adjusted to 2.0 with 2 N HCl, and the filtrate was then extracted with 2 ml of ethyl acetate. After concentration of the extract in vacuo to dryness, 100 μl of methanol was added and 50 μl of the mixture was tested for antibacterial activity. The rifampin concentration was measured by the size of the inhibition zone on a lawn of *Bacillus subtilis* PCI 219 grown on nutrient agar (Difco) (15). Inactivation products were monitored by thin-layer chromatography (TLC) using $CHCl_3$-MeOH (4:1) as a developing solvent. Although rifampin or its inactivation products were easily detected by their characteristic reddish brown spots on untreated TLC plates, the inactivation products were usually monitored additionally by a dual-wavelength TLC scanner (CS-910M; Shimadzu Seisakujyo, Tokyo, Japan) at 238 nm. Growth was determined by measuring the dry weight of mycelia: specifically, 5 ml of the culture was harvested at

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Rifamycin S

Rifampin $R_1$ =CH=N-N N-CH$_3$
$R_2$ = H

RIP-1 $R_1$ = CHO, $R_2$ = β-D-glucose

RIP-2 $R_1$ =CH=N-N N-CH$_3$
$R_2$ = β-D-glucose

FIG. 1. Structures of rifamycin S, rifampin, and inactivation products, RIP-1 and RIP-2.

various time intervals and filtered through a Whatman glass disk filter (GF/C2) with suction. After the mycelia on the filter were washed with cold distilled water, they were dried in an oven at 120°C to ensure a constant weight.

**Large-scale preparation of the inactivation products.** For large-scale preparation of the inactivation products, the described seed culture was used as an inoculum. The seed culture was inoculated into 100-ml shake flasks at a 2% rate. After 24 h of incubation at 30°C, rifampin was added to a final concentration of 400 μg/ml and the mixture was incubated for 2 days. After separation from the mycelia by centrifugation at 6,000 rpm (5,800 × *g*), the supernatant (ca. 50 ml) was extracted three times with an equal amount of $CHCl_3$. The extracts were combined and washed with 1/15 M phosphate buffer (pH 7.3). After dehydration with $Na_2SO_4$, the extract was concentrated in vacuo to dryness. The dried material was further purified by means of silica gel column chromatography using a mixture of $CHCl_3$ and MeOH (19:1) as the solvent. Preparative silica gel TLC using $CHCl_3$-MeOH (4:1) and Sephadex LH-20 column chromatography with MeOH as a solvent were also used for purification. Subsequently, 47.2 mg of RIP-2 was obtained. For the purification of RIP-1, the pH of the 1/15 M phosphate buffer was adjusted to 2.0 with 2 N HCl and RIP-1 was extracted with ethyl acetate. The extract was further purified by preparative TLC and Sephadex LH-20 column chromatography, and 27.0 mg of RIP-1 was obtained.

**Spectroscopic methods.** Nuclear magnetic resonance (NMR) spectra were measured on a JEOL GX-500 FT-NMR spectrometer, and chemical shifts were recorded in δ units relative to internal tetramethylsilane (δ = 0) in $CD_3OD$. Fast-atom bombardment mass spectra and high-resolution fast-atom bombardment mass spectra were measured on a JEOL JMS-HX110 instrument.

## RESULTS

**Antinocardial activity of rifampin.** The MICs of rifampin for five species of *Nocardia* (160 strains) are shown in Table 1. Most *Nocardia* species are resistant to rifampin and required mean MICs of >100 μg/ml. Among the *Nocardia* species tested, *N. brasiliensis* and *N. otitidiscaviarum* were found to be resistant. Although *N. nova* and *N. farcinica* were for the most part resistant, there were a few susceptible strains of these species detected. On the other hand, the MICs of rifampin for *N. asteroides* ranged from >400 μg/ml to less than 0.39 μg/ml. The metabolic fate of added rifampin was studied to determine its possible role in the resistance mechanism. One hundred sixty strains of *Nocardia* were incubated with rifampin, and inactivation was followed by

TABLE 1. Antimicrobial activity of rifampin against five pathogenic species of *Nocardia*

| Species | No. of strains with rifampin inactivation activity/no. of strains tested | No. of strains (no. of rifampin-inactivated strains) for which MIC is (μg/ml): | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 400 | <400, >200 | 200 | <200, >50 | 50 | <50, >12.5 | 12.5 | <12.5, >3.13 | 3.13 | <3.13, >0.78 | 0.78 | ≤0.39 |
| *N. asteroides* | 29/37 | 9 (7) | 3 (2) | 4 (2) | 6 (6) | 4 (3) | 2 (2) | 6 (6) | 1 (1) | 0 (0) | 1 (0) | 0 (0) | 1 (0) |
| *N. farcinica* | 36/38 | 26 (26) | 1 (1) | 6 (5) | 1 (1) | 2 (2) | 1 (1) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (0) |
| *N. nova* | 20/21 | 8 (8) | 8 (8) | 2 (2) | 0 (0) | 0 (0) | 0 (0) | 2 (2) | 0 (0) | 0 (0) | 0 (0) | 1 (0) | 0 (0) |
| *N. brasiliensis* | 44/45 | 40 (39) | 2 (2) | 1 (1) | 0 (0) | 1 (1) | 0 (0) | 1 (1) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *N. otitidiscaviarum* | 18/19 | 13 (12) | 3 (3) | 2 (2) | 0 (0) | 1 (1) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

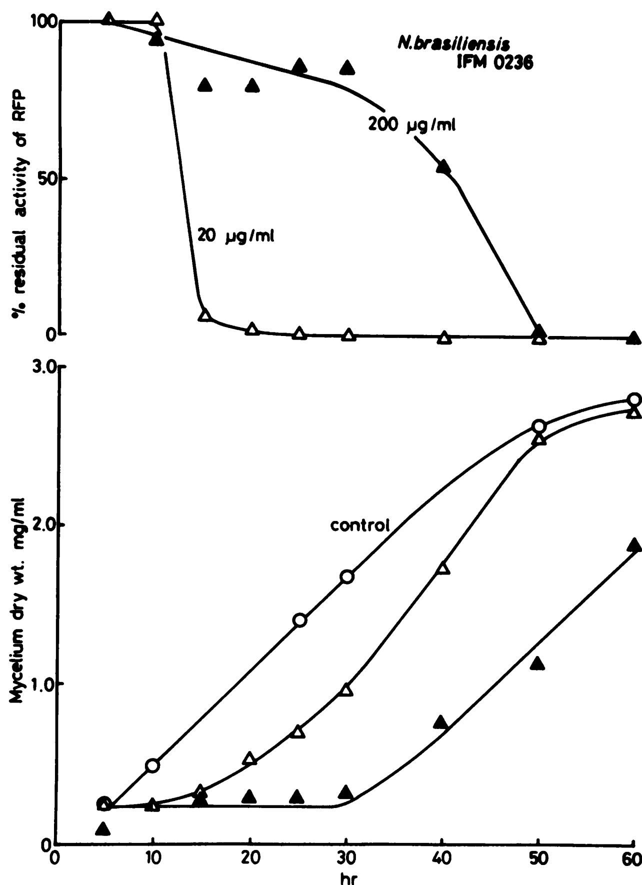


FIG. 2. Time course of rifampin inactivation in the culture broth and growth rate of *N. brasiliensis* IFM 0236. Rifampin (20 or 200 µg/ml, final concentration) was added at 0 h.

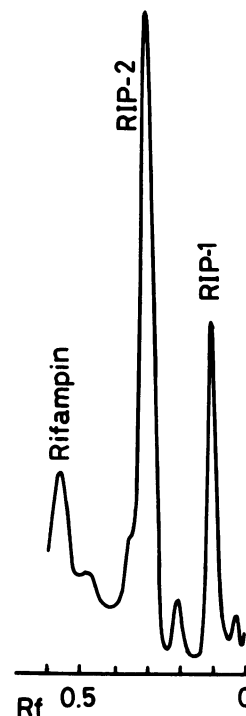


FIG. 3. Chromatography of rifampin and inactivation products by silica gel TLC. Inactivation products RIP-1 and RIP-2 were monitored by a TLC chromatoscanner at 238 nm.

residual antibacterial activity against *B. subtilis* PCI 219. As shown in Table 1, most of the nocardial strains were found to inactivate rifampin, but such inactivation was not confirmed in susceptible strains requiring MICs of <1.25 µg/ml. Interestingly, there were several exceptional strains even among the resistant strains (MIC > 100 µg/ml) showing no inactivation activity.

*N. brasiliensis* IFM 0236, which required the highest MICs, was selected to further analyze the inactivation mechanism. In these studies, the time course of rifampin inactivation was determined. As shown in Fig. 2, when 20 µg of rifampin per ml was added to the culture medium, rifampin activity diminished within 20 h, and when 200 µg of rifampin per ml was used, 50 h was required to inactivate the antibiotic. In both experiments, the growth was inhibited in the presence of rifampin, but as rifampin inactivation progressed, growth began. When the inactivation process was monitored by TLC plates, new inactivation products, designated RIP-1 and RIP-2, were observed. The TLC $R_f$ values of RIP-1 and RIP-2 when developed with $CHCl_3$-MeOH (4:1) were 0.12 and 0.31, respectively (Fig. 3). The new products were purified by the isolation method described in Materials and Methods. Finally, RIP-1 and RIP-2 were obtained as pure compounds. Since the purified compounds showed the same TLC $R_f$ values in various developing solvents (data not shown), they were considered to be identical to the inactivation products initially recognized and their structures were investigated.

**Structure elucidation of RIP-1 and RIP-2.** The structures of RIP-1 and RIP-2 were determined spectroscopically by comparison with the data for rifampin obtained with a standard specimen and the reported assignments for rifampin (14) and for rifamycin S (8). The molecular formulae were determined by high-resolution fast-atom bombardment mass spectrometry to be $C_{44}H_{57}NO_{18}$ for RIP-1 ($Na^+$ adduct ion, *m/z* 910.3518; calculated for $C_{44}H_{57}NO_{18}Na$ [M + Na], 910.3474) and $C_{49}H_{68}N_1O_{17}$ for RIP-2 ($Na^+$ adduct ion, *m/z* 1007.4550, calculated for $C_{49}H_{68}N_4O_{17}Na$ [M + Na], 1007.4480). The structure of RIP-1 was elucidated to be 3-formyl-23-(*O*-[β-D-glucopyranosyl])rifamycin SV by $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$H correlated spectroscopy, $^1$H-$^{13}$C correlated spectroscopy, and heteronuclear multiple-bond correlation experiments. The $^1$H-NMR spectrum of RIP-1 showed a singlet due to a formyl proton at δ 10.52 instead of the signals of the *N*-methyl piperazine moiety present in rifampin. The formyl carbon signal was also shown at δ 193.1 in the $^{13}$C-NMR spectrum. The presence of D-glucose was determined 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′) (16). The site of glycosylation was determined by a heteronuclear multiple-bond correlation experiment, which demonstrated the couplings between H-23 (signal at δ 3.59) and C-1′ and between H-1′ (signal at δ 4.42) and C-23 (signal at δ 88.9). On comparison of $^1$H- and $^{13}$C-NMR spectra of RIP-1 and rifampin, downfield shifts of the signals due to H-23 (0.51 ppm) and C-23 (10.7 ppm) of RIP-1 were observed, and this indicated that the glycosylation occurred on the OH group at C-23. The coupling constant of the anomeric proton at δ 4.42 (d, $J$ = 7.5 Hz) was consistent with the β configuration of a

TABLE 2. MICs of rifampin, RIP-1, and RIP-2[a]

| Organism | MIC (μg/ml) of: | | |
|---|---|---|---|
| | Rifampin | RIP-1 | RIP-2 |
| *Staphylococcus aureus* IFM 2014 | ≤0.2 | 50 | 50 |
| *Micrococcus luteus* IFM 2066 | ≤0.2 | >100 | 50 |
| *Mycobacterium* sp. strain 607 IFM 2051 | 6.25 | >100 | >100 |
| *Bacillus subtilis* PCI 219 | ≤0.2 | >100 | >100 |
| *Corynebacterium xerosis* IFM 2057 | ≤0.2 | >100 | >100 |
| *Nocardia asteroides* IFM 0319 | 50 | >100 | >100 |
| *Nocardia farcinica* IFM 0284 | >100 | >100 | >100 |
| *Nocardia nova* IFM 0290 | >100 | >100 | >100 |
| *Nocardia brasiliensis* IFM 0236 | >100 | >100 | >100 |
| *Nocardia otitidiscaviarum* IFM 0239 | >100 | >100 | >100 |
| *Rhodococcus bronchialis* IFM 0150 | 0.39 | >100 | >100 |

[a] MICs were determined by the agar dilution method using Mueller-Hinton II agar at 37°C for 2 days.

glucose. No other significant differences were observed between the NMR spectra of RIP-1 and rifampin, which indicates that the rest of the structures of these two compounds are the same. The molecular formula of RIP-2 suggested that it is a monoglycosylated rifampin. The structure of RIP-2 was confirmed to be 23-(*O*-[β-D-glucopyranosyl])rifamycin by the same procedures as those used for RIP-1. In a heteronuclear multiple-bond correlation experiment, it was verified that H-23 (signal at δ 3.56) coupled with the C-1′ of glucose (δ 103.9) and H-1′ (signal at δ 4.40) coupled with C-23 (signal at δ 88.5). Both the H-23 signal and the C-23 signal shifted downfield (0.48 and 10.3 ppm, respectively) compared with those signals of rifampin. The hydroquinone form of the naphthalene ring in RIP-1 and RIP-2 was confirmed by the comparison of the $^{13}$C-NMR spectra of RIP-1 and RIP-2 with that of rifampin.

**Antimicrobial activity of RIP-1 and RIP-2.** The antimicrobial activities of purified RIP-1 and RIP-2 were compared with that of rifampin (Table 2). All *Nocardia* species tested were resistant to RIP-1 and RIP-2, and their MICs against *Nocardia* were >100 μg/ml. Antimicrobial activities of RIP-1 and RIP-2 against other bacteria, such as *Staphylococcus aureus* IFM 2014, *Micrococcus luteus* IFM 2066, *Mycobacterium* sp. strain 607, and *Rhodococcus bronchialis* IFM 0150, were >50 μg/ml. These results indicated that rifampin was inactivated by the glycosylation of the 23-hydroxyl group. No significant differences in the activities of RIP-1 and RIP-2 were observed.

## DISCUSSION

Inactivating enzymes such as β-lactamase, acetyltransferase, phosphotransferase, adenyltransferase, and rRNA methylase have been demonstrated as antibiotic resistance mechanisms in pathogenic bacteria (4, 9, 10). Although there are many reports regarding microbial resistance to rifampin, only a single mechanism based on a mutational alteration of the target enzyme moiety, i.e., DNA-dependent RNA polymerase, has been investigated thoroughly (13, 18). However, Dabbs (7) and Andersen and Dabbs (1) suggested the existence of another inactivation mechanism for rifamycin on the basis of the following experimental data: (i) the disappearance of the 474-nm peak and (ii) a modest increase in adsorption at shorter wavelengths between 400 and 450 nm in the UV spectra of the inactivation compound (7). On the basis of the change in its UV absorption spectrum, Dabbs' report (7) suggested that this inactivation compound resulted from modifications in the skeleton of rifampin. No such changes were detected in the UV spectra of RIP-1 and RIP-2; therefore, RIP-1 and RIP-2 seem to be different from the inactivation product reported by Dabbs (7).

Another example of resistance relating to the glycosylation of an antibiotic has been reported. Recently, a new glycosylated compound, 2′-(*O*-[β-D-glucopyranosyl])-erythromycin, was isolated from the culture broth of *Streptomyces vendargensis* (10). However, it is not clear whether this example is relevant to drug resistance in pathogens, since *S. vendargensis* is not a pathogenic bacterium. Therefore, to our knowledge, ours is the first report demonstrating the existence of microbial glycosylation of antibiotics as a mechanism important to resistance of pathogenic bacteria.

*Nocardia mediterranea* is known to be a producer of rifamycin but was recently transferred to a new taxon, *Amycolatopsis mediterranei* (12). Despite its transfer to the new genus, it is closely related to *Nocardia* spp. in various aspects of its physiological and biochemical nature. It has been suggested that resistance genes of pathogenic bacteria may originate from antibiotic-producing microorganisms (4). It has been suggested also that there may be close similarity between the resistance of a producing organism to its own antibiotics and such resistance in pathogenic bacteria (4). Therefore, the present inactivation of rifampin may be correlated to rifamycin biosynthesis.

We were recently able to purify two different inactivation compounds from *N. otitidiscaviarum*. Structural studies of these compounds are in progress in our laboratory, and the results will be presented elsewhere. We also have observed a similar inactivation phenomenon in *N. asteroides*, *N. nova*, and *N. farcinica*; however, we did not succeed in isolating inactivation compounds from these species. The reason may have been related to the decomposition of rifampin. On the basis of the reported experiments, we propose that the ability to modify rifampin may be ubiquitous among the nocardioform microorganisms.

The contribution of altered RNA polymerase subunits to rifampin resistance in mycobacteria has been confirmed by many researchers (17), but the role of such alteration in *Nocardia* spp. remains obscure. Since pathogenic *Nocardia* spp. are closely related biochemically and physiologically to mycobacteria, further studies are warranted.

Based on reports of chemical modification of the structure of rifampin and the resulting structure-activity relationships (17), the following are considered the minimal structural requirements for rifamycin's activity: (i) two free hydroxyls at positions 21 and 23 of the ansa chain and (ii) the presence of a naphthoquinone ring carrying oxygen atoms at positions 1 and 8 in either the quinone or hydroquinone form (3). Of particular interest was a report that modifications of the ansa chain involving substitution or elimination of either 21- or 23-hydroxyls yielded inactive products (17). The present study confirmed one of these hypotheses, specifically, that the 23-hydroxyl group is a prerequisite to the exhibition of rifampin's antimicrobial activity.

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