Synthesis and Biological Activity of 5′-Aminobenzoxazinorifamycin Derivatives

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Benzoxazinorifamycin reacted with various secondary amines to yield various 5′-substituted aminobenzoxazinorifamycin derivatives. The derivatives exhibited potent activities against gram-positive bacteria and mycobacteria. The antimicrobial activities of these compounds against *Mycobacterium tuberculosis* and *Mycobacterium intracellulare* were superior to those of rifampicin. Some of these compounds showed good plasma levels after oral administration in rats.

Keywords rifamycin; 5′-piperidinylbenzoxazinorifamycin; benzoxazinorifamycin; antimicrobial activity; gram-positive bacteria; mycobacteria; *Mycobacterium tuberculosis*; *Mycobacterium intracellulare*; plasma level; rat

Since the discovery of rifamycins by Sensi *et al.* in 1959,[1] there have been reports on many chemical modifications of rifamycins.[2] Among these compounds, rifampicin (RFP) is the most representative derivative with potent activities against gram-positive bacteria and mycobacteria, and it has been widely used in the treatment of mycobacterial infections, particularly *Mycobacterium tuberculosis*.

Recently, infection with nontuberculous mycobacteria, especially *Mycobacterium avium–intracellulare* complex (MAC), is becoming a serious problem in immunocompromised hosts, such as patients with acquired immunodeficiency syndrome (AIDS), because the incidence of MAC infections is increasing in AIDS patients and known antimicrobial drugs, including RFP, have clinically limited efficacy against MAC infections. Several rifamycin derivatives, such as rifabutin (RFB),[3] rifapentine[4] and CGP-7040,[5] which are now under investigation, have been reported to be active *in vitro* against MAC.[6] RFB has been well investigated, and although a clinical trial of RFB alone or in combination with other drugs for MAC infection associated with AIDS patients has been done, its efficacy was unsatisfactory.[7] This prompted us to synthesize a new series of rifamycin derivatives, to search for compounds with more potent activity against MAC.

In the search studies on new rifamycin derivatives, we found that benzoxazinorifamycin (I),[8,9a] which has been reported to have antimicrobial activity against *Mycobacterium tuberculosis*,[9b] reacted with piperidine and gave a blue-colored product. This product was found to have potent antimicrobial activity against *Mycobacterium intracellulare*. The structure of the blue-colored product was identified as 5′-piperidinylbenzoxazinorifamycin. Using this reaction, several hundreds of 5′-substituted aminobenzoxazinorifamycins including alkyl, hydroxyl and other functional groups at 3′-, 4′- and 6′-positions were synthesized, and evaluated by the tests of antimicrobial activity *in vitro* and gastrointestinal absorption in laboratory animals.[10] In this paper, we describe the chemical synthesis (Chart 1) and biological evaluation of 5′-amino-

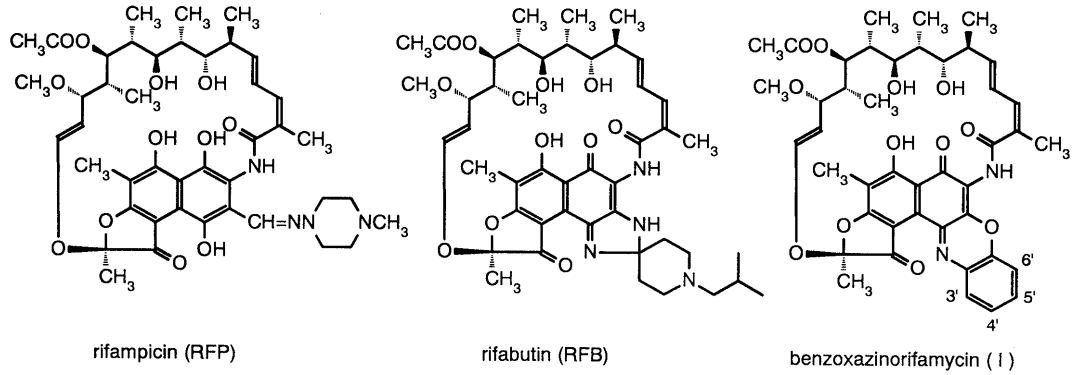


Fig. 1. Structure of Rifamycin Derivatives

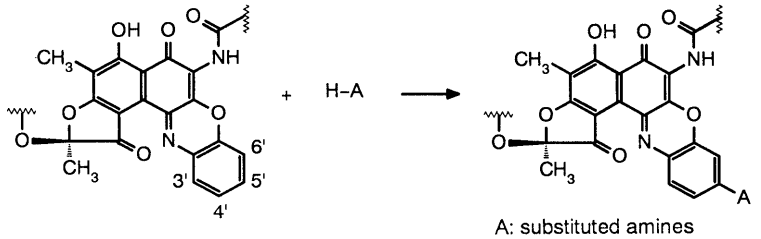
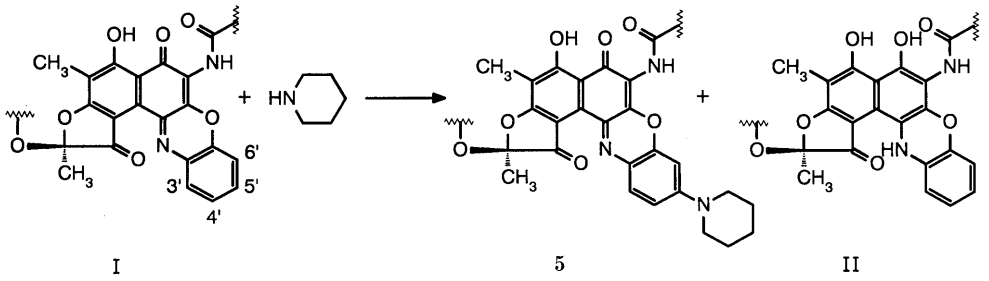


Chart 1



I 5 II

Chart 2

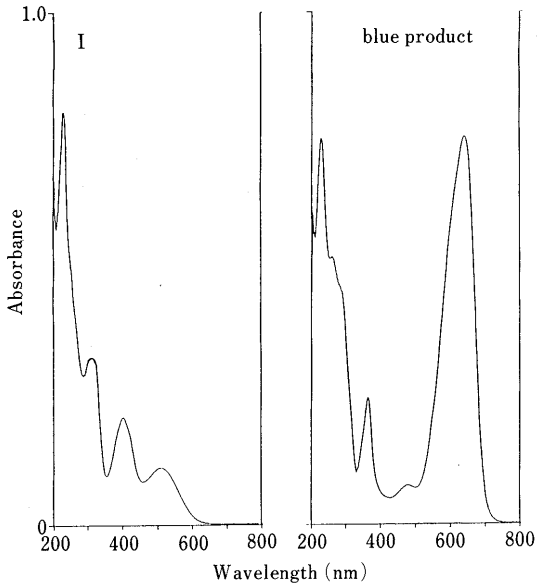


Fig. 2. UV-Vis Spectra of I and Blue Product in Methanol

Concentration (μg/ml); (I) 13.3, blue product 14.1.

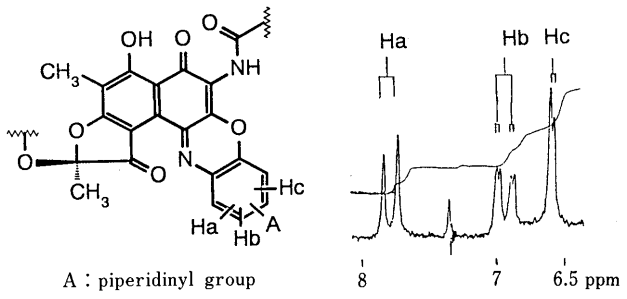
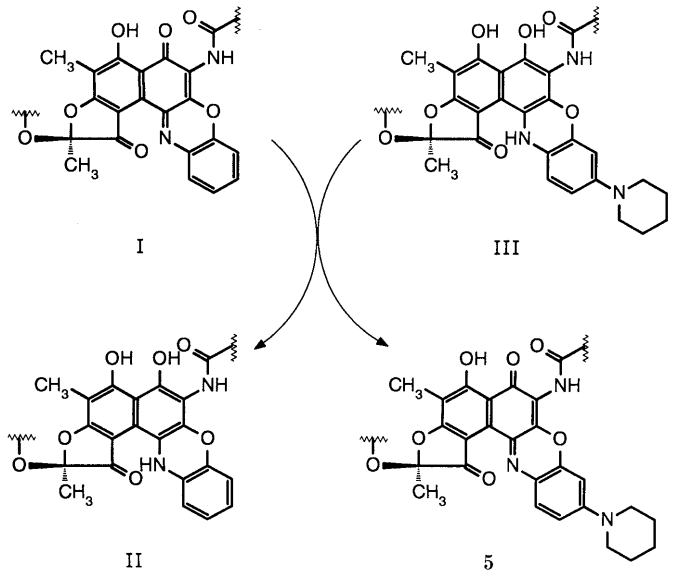


Fig. 3. ^{1}H-NMR Spectrum of Benzoxazine Ring of the Blue Product

benzoxazinorifamycin derivatives.

Chemistry Benzoxazinorifamycin (I) was prepared by the method described by Kump and Bickel.[9a] The color of I is reddish-purple. I reacted with piperidine to give a blue-colored product. The structure of this product was assumed that piperidine was introduced into the benzoxazine ring of I, because the ultraviolet and visible (UV-Vis) spectrum pattern changed greatly (Fig. 2). The proton-nuclear magnetic resonance (^{1}H-NMR) spectrum of the blue product showed that one piperidinyl group was contained in I. Figure 3 shows the ^{1}H-NMR spectrum of the blue product in the lower magnetic field (6.5—8 ppm, solvent: 10% CD_3OD in $CDCl_3$). In the lower magnetic field, there were three signals derived from the protons in

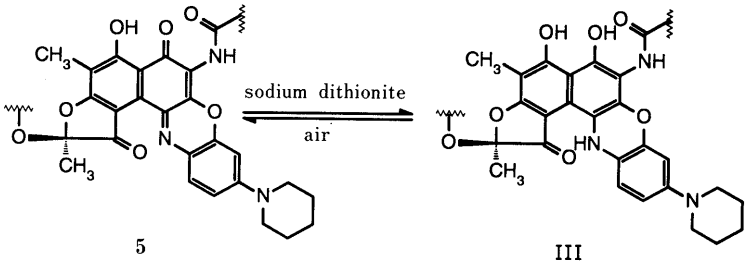


I III II 5

Chart 3

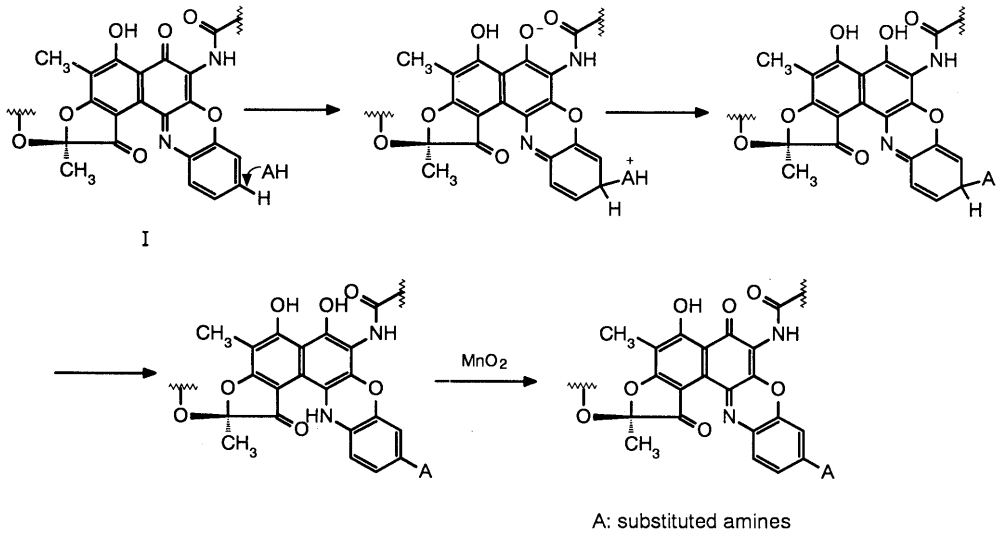
the benzoxazine ring (Ha, Hb and Hc). The aromatic protons Hb and Hc showed smaller chemical shifts than Ha, because an electron-donative piperidinyl-group was introduced in the adjacent carbon of the protons Hb and Hc. The coupling constant between Ha and Hb is 9 Hz, which is a value typical of aromatic *ortho*-coupling constants.[11] The coupling constant between Hb and Hc is 3 Hz, which is also a value typical of aromatic *meta*-coupling constants.[11] Judging from the ^{1}H-NMR spectrum pattern, the product was assumed to be 4′- or 5′-piperidinylbenzoxazinorifamycin. The results that are described below distinguished between the 4′-position and 5′-position in which piperidine was introduced. 4′- and 5′-methylbenzoxazinorifamycin were prepared according to the literature.[9a] 4′-Methylbenzoxazinorifamycin reacted with piperidine to give a blue product, however, 5′-methylbenzoxazinorifamycin did not give a blue product. These results showed that 5′-hydrogen was susceptible to nucleophilic attack using piperidine, but neither 5′-methyl nor 4′-hydrogen were susceptible. We concluded that the blue-colored product was 5′-piperidinylbenzoxazinorifamycin (**5**).

This regioselective reaction which furnished **5** proceeded less than 50% in the theoretical yield without an oxidizing reagent. A redox reaction occurred in the same reaction system and the starting material (I) changed to the corresponding reduced product: reduced-benzoxazinorifamycin (II, Chart 2). The addition of an oxidizing reagent, such as manganese dioxide, into the reaction system



5 III

Chart 4



I

Chart 5

TABLE I. 5′-Substituted Aminobenzoxazinorifamycin Derivatives

Compd. No.	5′-Amino group	Reaction time (h)	Yield[a] (%)	Crystal. solvent	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	TLC[b] *Rf*
1	$N(CH_3)_2$	24	49.6	Ethyl acetate	$C_{45}H_{53}N_3O_{12}\cdot H_2O$	63.89	6.55	4.97	63.83	6.54	4.60	0.55
2	$N(C_2H_5)_2$	72	25.9	Ethyl acetate hexane	$C_{47}H_{57}N_3O_{12}\cdot H_2O$	64.59	6.80	4.81	64.56	6.64	4.85	0.56
3	$N(C_2H_4OC_2H_5)_2$	144	10.6	Ethyl acetate hexane	$C_{51}H_{65}N_3O_{14}\cdot H_2O$	63.67	7.02	4.37	63.84	6.92	4.37	0.57
4		16	45.3	Ethyl acetate	$C_{47}H_{55}N_3O_{12}\cdot H_2O$	64.73	6.59	4.82	64.82	6.52	4.83	0.56
5		17	67.0	Ethyl acetate hexane	$C_{48}H_{57}N_3O_{12}$	66.42	6.62	4.84	66.14	6.72	4.78	0.57
6		4	41.3	Ethyl acetate hexane	$C_{49}H_{59}N_3O_{12}\cdot H_2O$	65.39	6.83	4.67	65.62	6.79	4.58	0.60
7		6	15.5	Ethyl acetate hexane	$C_{49}H_{59}N_3O_{12}\cdot H_2O$	65.39	6.83	4.67	65.31	6.58	4.50	0.57

a) The yield was not optimized. *b*) Chloroform–methanol (95 : 5, v/v).

increased the yield from 23% to 67%. These results suggested that the redox reaction occurred between piperidinyladduct (III) and I (Chart 3). 5′-Piperidinylbenzoxazinorifamycin (**5**) reacted with a strong reducing reagent, sodium dithionite, to give the corresponding reduced product (III). This reduced product was re-oxidized to **5** by bubbling air through the solution (Chart 4). From these results, the assumed reaction mechanism of the blue product is shown in Chart 5.

Using this unique reaction, we synthesized a series of 5′-substituted aminobenzoxazinorifamycin derivatives. Table I summarizes the introduced 5′-amino group, reaction time, isolated yield, solvent for crystallization, elementary analyses and *Rf* values by thin layer chromatography (TLC) of the various 5′-aminobenzoxazinorifamycins. Other nucleophiles, such as aromatic amines and alkyl thiols, could also react with I, however these products did not show good bioavailability. Oxygen nucleophile, for example methoxide anion, could not give a reaction product, because the basicity of oxygen nucleophile was too strong, so decomposition of the rifamycin skeleton predominantly occurred.

TABLE II. *In Vitro* Antimicrobial Activities (MIC, μg/ml)

Compd. No.	*Ml*[a] IFO 12708	*Bs*[b] IFO 3134	*Sa*[c] IFO 12732	*Ec*[d] IFO 12734	*Kp*[e] IFO 3512	*Ms*[f] ATCC 607	*Mt*[g] H37Rv	*Mi*[h] Shibazaki
1	0.01	0.01	0.005	1.25	2.5	1.25	0.07	1.25
2	0.01	0.02	0.005	2.5	5	1.25	0.07	1.25
3	0.02	0.02	0.005	5	10	1.25	0.15	0.63
4	0.01	0.01	0.005	5	2.5	0.63	0.035	1.25
5	0.005	0.01	0.005	10	5	0.63	0.07	0.63
6	0.02	0.04	0.02	>10	>10	1.25	0.07	1.25
7	0.02	0.04	0.04	1.25	>10	0.63	0.07	2.5
RFP	0.01	0.02	0.005	10	5	10	>0.3	>10
RFB	0.01	0.02	0.01	2.5	5	1.25	0.07	10

a) Micrococcus luteus. b) Bacillus subtilis. c) Staphylococcus aureus. d) Escherichia coli. e) Klebsiella pneumoniae. f) Mycobacterium smegmatis. g) Mycobacterium tuberculosis. h) Mycobacterium intracellulare.

TABLE III. MICs for 16 Strains[a] of *M. intracellulare*

Compd. No.	MIC_{50}	MIC_{90}
4	0.3≧	2.5
5	0.6	2.5
RFP	>5	>5

a) Batty, HB2898, HB3024, 100616, Wodli, Gamoh, Ueda, Wakamatsu, Yamawaki, Abe, Itoh, Tomioka, Isozaki, Tashiro, Takeuchi, Butsuryoh.

TABLE IV. Plasma Levels after Oral Administration in Rats

Compd. No.	Dose (mg/kg)	Plasma level (μg/ml)[a] 1	3	5 (h)	*AUC*[b] (μg·h/ml)
1	20	1.2	2.8	1.8	9.2
2	20	1.8	3.3	1.3	10.6
3	20	2.1	5.4	1.8	15.8
4	20	2.0	4.5	3.6	15.6
5	20	2.8	5.0	2.9	17.1
6	20	2.1	6.1	5.2	20.6
7	20	3.2>	5.7	4.9	16.3
I	100	ND[c]	ND	ND	
RFP	20	11.7	12.3	12.5	54.7
RFB	20	1.2	1.7	1.5	6.7

a) Arithmetic mean of 2 animals. *b)* Area under the plasma concentration curve within 5 h. *c)* Not detected.

Biological Result and Discussion

The minimal inhibitory concentrations (MICs) of newly synthesized 5′-aminobenzoxazinorifamycin derivatives (compounds **1**—**7**) were determined by the twofold serial dilution method.[12] The MIC values of these compounds against gram-positive bacteria, gram-negative bacteria and mycobacteria were compared with RFP and RFB. The results are summarized in Table II.

All of the synthesized compounds showed almost equally strong activity against gram-positive bacteria, *i.e. M. luteus*, *B. subtilis* and *S. aureus*, and their potency was comparable to RFP and RFB, while they showed weak activity against gram-negative bacteria, *i.e. E. coli* and *K. pneumoniae*, as did RFP and RFB. The activity of the compounds against *M. smegmatis* and *M. tuberculosis* was more potent than RFP but similar to RFB. It is noteworthy that all of the compounds exhibited good activity against *M. intracellulare*, while RFP and RFB exhibited weak activity. With regard to antimicrobial activities, their activities were not influenced by the kinds of the introduced amino components. In addition, the structure–activity relationship of these compounds was unclear. Among these compounds having activities against both *M. tuberculosis* and *M. intracellulare*, compound **5** (5′-piperidinylbenzoxazinorifamycin) seemed to be the most active derivative. Compounds **4** and **5** were further evaluated for *in vitro* activity against strains of *M. intracellulare*. As shown in Table III, the MIC_{50} and MIC_{90} values (MICs at which 50 or 90% strains are inhibited) of both compounds for 16 *M. intracellulare* strains were lower than those of RFP.

The plasma concentrations of the compounds were determined by bioassay using *M. luteus*. As shown in Table IV, the plasma levels of benzoxazinorifamycin (I) were not detected (minimum detectable concentration, 0.05 μg/ml) when given orally, even at 100 mg/kg, in rats. Whereas its derivatives were absorbed and their plasma concentrations were more than 1 μg/ml after an oral dose of 20 mg/kg, suggesting that the amino components contributed to improved absorption from the gastrointestinal tract.

In conclusion, **5**, which was prepared from the reaction of benzoxazinorifamycin and piperidine, exhibited potent activities against gram-positive and mycobacteria and also showed good plasma levels after oral administration in rats.

Experimental Section

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Melting points were uncorrected. ^{1}H-NMR spectra were determined at 90 MHz on a Varian EM-390 NMR spectrometer. Chemical shifts are expressed in ppm values relative to tetramethylsilane as an internal standard. Significant ^{1}H-NMR data are described in the order number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; br, broad; dd, double doublet) and coupling constants in Hz. UV-Vis absorption spectra were measured with a Hitachi U-3200 or 323 spectrophotometer. Infrared (IR) spectra were recorded on a Hitachi IR 260-30 spectrophotometer. Column chromatography was done with a Wakogel C-200 (Wako Pure Chemical Industries Ltd.). General methods to prepare compounds (**1**—**7**) are described in **1** and **2** as typical examples. Spectra data are shown in other compounds (**3**—**7**).

5′-Dimethylaminobenzoxazinorifamycin (1) To a solution of 10.0 g, (12.7 mmol) of benzoxazinorifamycin (I)[9a] in 50 ml of dimethyl sulfoxide (DMSO) at room temperature was added 2.1 g (25.4 mmol) of dimethylamine hydrochloride, 3.6 ml (25.4 mmol) of triethylamine and 10.0 g (115 mmol) of manganese dioxide. After the addition the suspension was stirred for 24 h at room temperature, and then ethyl acetate was added and insoluble materials were filtered off. The filtrate was washed with water and brine, dried and evaporated to dryness. The residue was purified by silica-gel column chromatography (chloroform–acetone, 9 : 1,

v/v) and then crystallized from ethyl acetate, to yield 5.2 g of **1**, mp 190—195 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 0.55 (3H, br, $CHCH_3$), 0.80, 0.95 (each 3H, d, J=7 Hz, $CHCH_3$), 2.05, 2.10, 2.15, 2.20 (each 3H, s, CH_3), 3.05 (3H, s, OCH_3), 3.16 (6H, s, NCH_3), 6.58 (1H, d, J=3 Hz, arom H), 6.80 (1H, dd, J=3, 9 Hz, arom H), 7.77 (1H, d, J=9 Hz, arom H), 7.83 (1H, br, NH), 15.01 (1H, s, phenol OH). Vis spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 361 (154), 481 (51), 633 (436). IR (KBr): 3450, 2960, 2930, 2870, 1720, 1660, 1600, 1560, 1490, 1460, 1410, 1360, 1310, 1260, 1200, 1170, 1120, 1060, 1040, 980, 950, 910, 820, 770, 700 cm^{-1}.

5′-Diethylaminobenzoxazinorifamycin (2) To a solution of 6.0 g (7.6 mmol) of I in 30 ml of DMSO at room temperature was added 1.6 ml (69 mmol) of diethylamine and 6.0 g (69 mmol) of manganese dioxide. After the addition the suspension was stirred for 72 h at room temperature, and then the reaction mixure was treated in the same manner described in the synthesis of **1** and crystallized from ethyl acetate–hexane, to yield 1.7 g of **2**, mp 200—205 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 0.55 (3H, br, $CHCH_3$), 0.76, 0.91 (each 3H, d, J=7 Hz, $CHCH_3$), 1.28 (6H, t, J=7 Hz, NCH_2CH_3), 1.79, 2.00, 2.10, 2.14 (each 3H, s, CH_3), 3.03 (3H, s, OCH_3), 3.54 (4H, q, NCH_2), 6.48 (1H, d, J=3 Hz, arom H), 6.80 (1H, dd, J=3, 9 Hz, arom H), 7.71 (1H, br, NH), 7.76 (1H, d, J=9 Hz, arom H), 15.13 (1H, s, phenol OH). Vis spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 363 (183), 480 (49), 642 (546). IR (KBr): 3450, 2970, 2940, 1715, 1665, 1650, 1600, 1560, 1490, 1470, 1405, 1380, 1355, 1310, 1260, 1185, 1175, 1120, 1080, 1040, 975, 945, 920, 900, 820, 765, 695, 445 cm^{-1}.

5′-Bis(2-ethoxyethyl)aminobenzoxazinorifamycin (3) mp 165—170 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 0.57 (3H, br, $CHCH_3$), 0.75, 0.92 (each 3H, d, J=7 Hz, $CHCH_3$), 1.18 (6H, t, J=7 Hz, OCH_2CH_3), 1.78, 2.00, 2.10, 2.16 (each 3H, s, CH_3), 3.05 (3H, s, OCH_3), 3.48 (4H, q, J=7 Hz, OCH_2CH_3), 3.67 (8H, br, NCH_2CH_2O), 6.57 (1H, d, J=3 Hz, arom H), 6.91 (1H, dd, J=3, 9 Hz, arom H), 7.55 (1H, br, NH), 7.76 (1H, d, J=9 Hz, arom H), 15.00 (1H, s, phenol OH). Vis spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 362 (156), 482 (54), 635 (460). IR (KBr): 3470, 2980, 2940, 2880, 1720, 1660, 1600, 1560, 1520, 1485, 1460, 1400, 1380, 1355, 1320, 1260, 1230, 1170, 1120, 1060, 1040, 980, 950, 915, 900, 810, 765, 695, 445 cm^{-1}.

5′-Pyrrolidinylbenzoxazinorifamycin (4) mp 208—218 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 0.52 (3H, br, $CHCH_3$), 0.80, 0.90 (each 3H, d, J=7 Hz, $CHCH_3$), 1.80, 2.00, 2.10, 2.18 (each 3H, s, CH_3), 2.11 (4H, br, pyrrolidinyl H), 3.02 (3H, s, OCH_3), 3.50 (4H, br, NCH_2), 6.58 (1H, br, arom H), 6.70 (1H, d, J=9 Hz, arom H), 7.70 (1H, d, J=9 Hz, arom H), 8.25 (1H, br, NH), 15.03 (1H, s, phenol OH). Vis spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 363 (138), 480 (43), 642 (409). IR (KBr): 3450, 2970, 2920, 2850, 1720, 1660, 1600, 1550, 1480, 1460, 1390, 1370, 1340, 1320, 1260, 1220, 1160, 1130, 1060, 1040, 980, 940, 920, 910, 860, 760, 600 cm^{-1}.

5′-Piperidinylbenzoxazinorifamycin (5) mp 204—210 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 0.53 (3H, br, $CHCH_3$), 0.76, 0.93 (each 3H, d, J=7 Hz, $CHCH_3$), 1.77 (6H, br, piperidinyl H), 1.81, 2.00, 2.09, 2.12 (each 3H, s, CH_3), 3.05 (3H, s, OCH_3),3.56 (4H, br, NCH_2), 6.68 (1H, d, J=3 Hz, arom H), 6.95 (1H, dd, J=3, 9 Hz, arom H), 7.70 (1H, br, NH), 7.73 (1H, d, J=9 Hz, arom H), 15.02 (1H, s, phenol OH). Vis spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 363 (160), 481 (55), 643 (488). IR (KBr): 3450, 2950, 2930, 2870, 1720, 1660, 1600, 1560, 1485, 1460, 1395, 1370, 1305, 1240, 1210, 1170, 1115, 1060, 1020, 980, 950, 920, 900, 850, 820, 770, 690, 640, 580 cm^{-1}.

5′-(3-Methylpiperidinyl)benzoxazinorifamycin (6) mp 195—201 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 0.47 (3H, br, $CHCH_3$), 0.77, 0.92 (each 3H, d, J=7 Hz, $CHCH_3$), 1.00 (3H, d, J=6 Hz, piperidinyl CH_3), 1.80, 2.00, 2.10, 2.14 (each 3H, s, CH_3), 3.03 (3H, s, OCH_3), 3.86 (4H, br, NCH_2), 6.67 (1H, d, J=3 Hz, arom H), 6.96 (1H, dd, J=3, 9 Hz, arom H), 7.77 (1H, d, J=9 Hz, arom H), 7.82 (1H, br, NH), 15.01 (1H, s, phenol OH). Vis spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 363 (163), 481 (52), 646 (498). IR (KBr): 3450, 2970, 2940, 2880, 1720, 1650, 1600, 1560, 1490, 1460, 1400, 1380, 1310, 1245, 1220, 1175, 1125, 1090, 1065, 1040, 970, 905, 860, 820, 770, 645, 590 cm^{-1}.

5′-Hexamethyleneiminylbenzoxazinorifamycin (7) mp 200—203 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 0.55 (3H, br, $CHCH_3$), 0.77, 0.93 (each 3H, d, J=7 Hz, $CHCH_3$), 1.65 (8H, br, hexamethyleniminyl H), 1.80, 2.00, 2.12, 2.13 (each 3H, s, CH_3), 3.05 (3H, s, OCH_3), 3.65 (4H, br, NCH_2), 6.58 (1H, d, J=3 Hz, arom H), 6.89 (1H, dd, J=3, 9 Hz, arom H), 7.67 (1H, br, NH), 7.79 (1H, d, J=9 Hz, arom H), 15.13 (1H, s, phenol OH). Vis spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 363 (178), 484 (55), 644 (539). IR (KBr): 3460, 2960, 2930, 1720, 1660, 1650, 1600, 1560, 1520, 1490, 1460, 1400, 1360, 1305, 1250, 1160, 1125, 1100, 1060, 1040, 1000, 975, 945, 900, 860, 820, 770, 690, 640, 445 cm^{-1}.

***In Vitro* Antimicrobial Activity**[12] The MIC (μg/ml) was determined by the twofold serial dilution technique using Mueller-Hinton (Difco) agar medium for gram-positive and gram-negative bacteria, glycerin-Czapek agar medium[13] for *Mycobacterium smegmatis* and Kirchner's semiliquid medium[14] for *M. tuberculosis* and *M. intracellulare*. The inoculum size was adjusted to 10^6 cfu/ml. MIC was defined as the lowest concentration of the compounds that inhibited growth of bacteria after incubation at 37 °C for 17 h for gram-positive and gram-negative bacteria, 41 h for *M. smegmatis*, 28 d for *M. tuberculosis* and 14 d for *M. intracellulare*.

Determination of Plasma Level Groups of 2 male Wistar rats (250—400 g) were used. Test compounds were suspended in 2.5% arabic gum containing 0.2% polyoxyethylene (20) sorbitan monooleate (Tween 80) and were administered orally to the animals under nonfasting conditions. Heparinized blood samples were collected at 1, 3 and 5 h after dosing and centrifuged to obtain plasma. Concentrations of test compounds in plasma were determined by bioassay employing *Micrococcus luteus* IFO 12708 as a test organism.

References and Notes

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14) 2% glycerin, 0.5% asparagine, 0.4% KH_2PO_4, 0.3% $Na_2HPO_4 \cdot 12H_2O$, 0.25% sodium citrate tribasic ($C_6H_5O_3Na_2 \cdot 10H_2O$), 0.06% $MgSO_4 \cdot 7H_2O$, 0.1% agar, 10% horse serum.