

## Antimycobacterial Activities of Novel Levofloxacin Analogues

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In order to investigate structure-activity relationships between antimycobacterial activities and basic substituents at the C-10 position of levofloxacin (LVFX), we synthesized a series of pyridobenzoxazine derivatives by replacement of the *N*-methylpiperazinyl group of LVFX with various basic substituents. A compound with a 3-aminopyrrolidinyl group had one-half the activity of LVFX against *Mycobacterium avium*, *M. intracellulare*, and *M. tuberculosis*. Mono- and dimethylation of the 3-amino moiety of the pyrrolidinyl group increased the activities against *M. avium* and *M. intracellulare* but not those against *M. tuberculosis*. On the other hand, dialkylation at the C-4 position of the 3-aminopyrrolidinyl group enhanced the activities against *M. avium*, *M. intracellulare*, and *M. tuberculosis*. Thus, introduction of an *N*-alkyl or a *C*-alkyl group(s) into the 3-aminopyrrolidinyl group may contribute to an increase in potency against *M. avium*, *M. intracellulare*, and/or *M. tuberculosis*, probably through elevation of the lipophilicity. However, among the compounds synthesized, compound VII, which was a 2,8-diazabicyclo[4.3.0]nonanyl derivative with relatively low lipophilicity, showed the most potent activity against mycobacterial species: the activity was 4- to 32-fold more potent than that of LVFX and two to four times as potent as that of gatifloxacin. These results suggested that an increase in the lipophilicity of LVFX analogues in part contributed to enhancement of antimycobacterial activities but that lipophilicity of the compound was not a critical factor affecting the potency.

During the past decade, an increase in the number of patients with tuberculosis has been one of the most serious health problems in many countries (2, 27). In particular, the now pandemic combination of tuberculosis with human immunodeficiency syndrome (2, 3, 20) and the appearance of multi-drug-resistant *Mycobacterium tuberculosis* (6, 10, 32) have aggravated attempts to treat these patients. In addition, the number of patients infected with *Mycobacterium avium*-*M. intracellulare* complex (MAC) is on the increase (5, 9). However, an effective therapy for MAC infection has not yet been established. Given these observations, the development of effective drugs for the mycobacterial infections described above has been keenly desired.

Recently, new quinolone antibacterial agents have been developed and marketed and are widely used clinically. They have potent and broad activities against both gram-negative and gram-positive pathogens. These agents also have been evaluated and shown to have potent activities against certain types of mycobacterial species in *in vitro* tests and in experimental animals (ofloxacin [26, 29, 31], levofloxacin [LVFX] [18, 22, 33], ciprofloxacin [4, 34], sparfloxacin [12, 21, 30], gatifloxacin [GFLX, formerly AM-1155] [28], and sitafloxacin [formerly DU-6859a] [25]).

LVFX is a representative new quinolone which is characterized by its potency, safety, and good pharmacokinetic profiles in humans. This agent has a unique pyridobenzoxazine structure. In the previous paper, members of our group reported the synthesis of pyridobenzoxazines bearing a series of 3-aminopyrrolidinyl substituents at the C-10 position and evaluated their activities against gram-negative and -positive bacteria (13). In this paper, we report the *in vitro* activities of novel pyridobenzoxazine derivatives having various basic substituents against *M. avium*, *M. intracellulare*, and *M. tuberculosis* and the structure-activity relationships (SARs) between basic substituents and antimycobacterial activities.

### MATERIALS AND METHODS

**Organisms.** *M. avium* (four strains), *M. intracellulare* (four strains), and *M. tuberculosis* (12 strains) were grown in MYCOBACTERIA 7H11 agar medium (Difco Laboratories, Detroit, Mich.) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Difco Laboratories).

**Drugs.** Rifampin (RFP; Sigma-Aldrich Japan, Tokyo, Japan) and isoniazide (INH; Sigma-Aldrich Japan) were obtained commercially and were used as potent drugs. LVFX was synthesized at New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan, as was GFLX. The synthesis of pyridobenzoxazine derivatives I, IV, V, and VI has been reported previously (13); other compounds were newly prepared, and brief descriptions of the synthetic method as well as the physical properties of the compounds are given below. The structures of all the compounds synthesized are shown in Fig. 1.

All melting points (mp) were taken on a micro-mp apparatus (MP-500D; Yanagimoto Co., Kyoto, Japan) and are uncorrected. Proton nuclear magnetic resonance spectra (<sup>1</sup>H-NMR) were recorded at 400 MHz with a JNM-EX400 spectrometer (JEOL, Tokyo, Japan) in 0.1 N NaOD. Chemical shifts are expressed in ppm (δ) with sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. Elemental analyses were indicated only by the symbols of the elements; analytical results were within ±0.4% of the theoretical values unless otherwise noted. Optical rotation ([α]<sub>D</sub>) was measured at 589 nm with a SEPA-300 polarimeter (Horiba Co., Kyoto, Japan).

**Representative procedure:** 10-[(*S,S*)-2,8-diazabicyclo[4.3.0]nonan-8-yl]-9-fluoro-2,3-dihydro-3(*S*)-methyl-7-oxo-7H-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid (VII). A solution of 9,10-difluoro-2,3-dihydro-3(*S*)-methyl-7-oxo-7H-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid difluoroborate chelete (329 mg, 1.0 mmol), (*S,S*)-2-*tert*-butoxycarbonyl-2,8-diazabicyclo[4.3.0]nonan (452 mg, 2.0 mmol), and triethylamine (Et<sub>3</sub>N) (0.5 ml) in dimethyl sulfoxide (4.0 ml) was stirred at room temperature for 30 min. After evaporation of the Et<sub>3</sub>N, water was added to the residue with ice-water cooling, and then the mixture was stirred at room temperature for 30 min. The precipitate was washed with water, collected by filtration, and then dissolved in 80% aqueous methanol (20 ml). Et<sub>3</sub>N (5.0 ml) was added to the solution, and the mixture was refluxed for 5 h. After concentration, the residue was dissolved in chloroform (CHCl<sub>3</sub>), which was washed with 10% aqueous citric acid and brine, dried over anhydrous sodium sulfate (Na<sub>2</sub>SO<sub>4</sub>), and then evaporated to dryness. The residue was dissolved in concentrated HCl (10 ml) with ice-water cooling and stirred for 5 min at room temperature. The mixture was adjusted to pH 11 with 20% aqueous NaOH with ice-water cooling and then was neutralized with 10% aqueous HCl to pH 7.4, which was extracted with CHCl<sub>3</sub>. The extract was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness to yield a crude VII, which was recrystallized from ethanol-28% NH<sub>4</sub>OH to yield VII (260 mg, 67%) as slightly yellow needles. mp, 296 to

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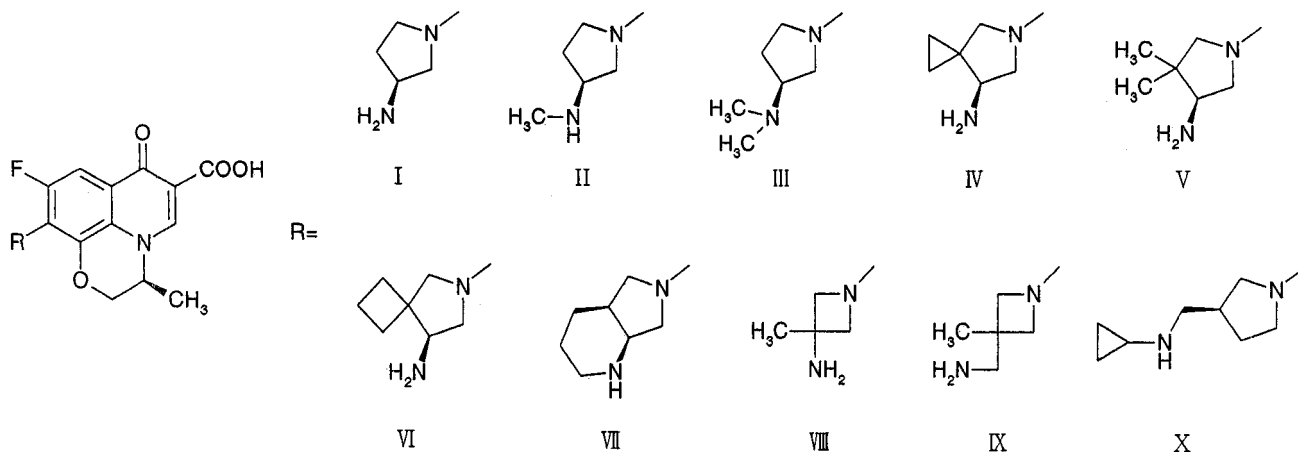


FIG. 1. Structures of pyridobenzoxazine derivatives.

299°C (decomposition). <sup>1</sup>H-NMR δ: 1.40 to 1.78 (4H, m), 1.46 (3H, d, *J* = 6.35 Hz), 2.12 to 2.22 (1H, m), 2.48 to 2.59 (1H, m), 2.84 to 2.91 (1H, m), 3.23 to 3.28 (1H, m), 3.30 to 3.39 (2H, m), 3.76 to 3.90 (2H, m), 4.19 and 4.39 (each 1H, d, *J* = 11.72 Hz), 4.46 to 4.45 (1H, m), 7.39 (1H, d, *J* = 14.65 Hz), 8.34 (1H, s). Elemental analysis results were as follows. Calculated for C<sub>20</sub>H<sub>22</sub>FN<sub>3</sub>O<sub>4</sub>: C, 62.01; H, 5.72; N, 10.85. Found: C, 62.00; H, 5.93; N, 10.82. [α]<sub>D</sub><sup>20</sup>, -221.81° (concentration, 0.550 in 1N NaOH). Analogous procedures were used to obtain other compounds, for which physical, analytical, and <sup>1</sup>H-NMR spectral data are described below.

**9-Fluoro-2,3-dihydro-3(S)-methyl-10-[3(S)-N-methylamino-1-pyrrolidinyl]-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (II).** mp, 147 to 156°C (decomposition). <sup>1</sup>H-NMR δ: 1.46 (3H, d, *J* = 6.83 Hz), 1.72 to 1.82 (1H, m), 2.13 to 2.20 (1H, m), 2.34 (3H, s), 3.24 to 3.37 (2H, m), 3.54 to 3.60 (2H, m), 3.67 to 3.73 (1H, m), 4.30 and 4.45 (each 1H, d, *J* = 11.23 Hz), 4.52 to 4.60 (1H, m), 7.44 (1H, d, *J* = 14.16 Hz), 8.31 (1H, s). Elemental analysis results were as follows. Calculated for C<sub>18</sub>H<sub>20</sub>FN<sub>3</sub>O<sub>4</sub> · 1.0H<sub>2</sub>O: C, 56.99; H, 5.84; N, 11.08. Found: C, 57.20; H, 5.88; N, 11.37. [α]<sub>D</sub><sup>20</sup>, -32.62° (concentration, 0.802 in 1 N NaOH).

**9-Fluoro-2,3-dihydro-3(S)-methyl-10-[3(S)-N,N'-dimethylamino-1-pyrrolidinyl]-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (III).** mp, 220 to 224°C (decomposition). <sup>1</sup>H-NMR δ: 1.48 (3H, d, *J* = 6.35 Hz), 1.72 to 1.82 (1H, m), 2.16 to 2.21 (1H, m), 2.26 (6H, s), 2.86 to 2.94 (1H, m), 3.45 to 3.60 (3H, m), 3.67 to 3.73 (1H, m), 4.30 and 4.45 (each 1H, d, *J* = 11.23 Hz), 4.51 to 4.58 (1H, m), 7.43 (1H, d, *J* = 14.16 Hz), 8.30 (1H, s). Elemental analysis results were as follows. Calculated for C<sub>19</sub>H<sub>22</sub>FN<sub>3</sub>O<sub>4</sub>: C, 60.79; H, 5.91; N, 11.19. Found: C, 60.08; H, 5.97; N, 11.16. [α]<sub>D</sub><sup>20</sup>, +40.51° (concentration, 0.669 in 1 N NaOH).

**10-(3-Amino-3-methyl-1-azetidiny)-9-fluoro-2,3-dihydro-3(S)-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (VIII).** mp, 300 to 302°C (decomposition). <sup>1</sup>H-NMR δ: 1.47 (3H, s), 1.50 (3H, d, *J* = 7.00 Hz), 4.07 to 4.11 (2H, m), 4.21 to 4.23 (2H, m), 4.27 and 4.43 (each 1H, d, *J* = 11.00 Hz), 4.56 to 4.58 (1H, m), 7.49 (1H, d, *J* = 13.00 Hz), 8.30 (1H, s). Elemental analysis results were as follows. Calculated for C<sub>17</sub>H<sub>18</sub>FN<sub>3</sub>O<sub>4</sub> · 0.5H<sub>2</sub>O: C, 57.29; H, 5.37; N, 11.79. Found: C, 57.48; H, 5.41; N, 11.73. [α]<sub>D</sub><sup>20</sup>, -74.48° (concentration, 0.827 in 1 N NaOH).

**10-(3-Aminomethyl-3-methyl-1-azetidiny)-9-fluoro-2,3-dihydro-3(S)-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (IX).** mp, 276 to 278°C (decomposition). <sup>1</sup>H-NMR δ: 1.29 (3H, s), 1.49 (3H, d, *J* = 7.00 Hz), 2.81 (2H, s), 4.00 to 4.02 (2H, m), 4.11 to 4.12 (2H, m), 4.26 to 4.29 (2H, m), 4.39 to 4.44 (2H, m), 4.56 (1H, broad), 7.44 to 7.52 (1H, m), 8.32 (1H, s). Elemental analysis results were as follows. Calculated for C<sub>18</sub>H<sub>20</sub>FN<sub>3</sub>O<sub>4</sub> · 0.75H<sub>2</sub>O: C, 57.67; H, 5.78; N, 11.21. Found: C, 57.38; H, 5.75; N, 11.23. [α]<sub>D</sub><sup>20</sup>, -68.28° (concentration, 0.700 in 1 N NaOH).

**10-[3-(R)-N-Cyclopropylaminomethyl-3-pyrrolidinyl]-9-fluoro-2,3-dihydro-3(S)-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (X).** mp, 173 to 176°C (decomposition). <sup>1</sup>H-NMR δ: 0.35 to 0.45 (2H, m), 0.47 to 0.56 (2H, m), 1.48 (3H, d, *J* = 6.83 Hz), 1.48 to 1.58 (1H, m), 2.05 to 2.24 (2H, m), 2.37 to 2.46 (1H, m), 2.69 to 2.76 (2H, m), 3.22 to 3.34 (1H, m), 3.38 to 3.70 (3H, m), 4.27 and 4.44 (each 1H, d, *J* = 9.28 Hz), 4.54 to 4.61 (1H, m), 7.44 (1H, d, *J* = 14.16 Hz), 8.36 (1H, s). Elemental analysis results were as follows. Calculated for C<sub>23</sub>H<sub>24</sub>FN<sub>3</sub>O<sub>4</sub> · 0.25H<sub>2</sub>O: C, 62.14; H, 6.08; N, 10.35. Found: C, 62.13; H, 6.01; N, 10.16. [α]<sub>D</sub><sup>20</sup>, -114.70° (concentration, 0.544 in 1 N NaOH).

**Determination of apparent partition coefficients (P').** The apparent partition coefficients of the compounds synthesized were measured according to the method reported previously (1).

**Susceptibility testing.** The MICs of the drugs for mycobacteria were measured by the twofold agar dilution method reported by Saito et al. with MYCOBACTERIA 7H11 agar supplemented with 10% OADC (25). The MICs were deter-

mined after 14 days (*M. avium* and *M. intracellulare*) or 21 days (*M. tuberculosis*) of incubation at 37°C.

## RESULTS

The MICs for *M. avium*, *M. intracellulare*, and *M. tuberculosis* are shown in Table 1. The data for RFP and INH are included for comparison. Among the compounds synthesized, compound VII, bearing a 2,8-diazabicyclo[4.3.0]nonanyl group at the C-10 position, showed the most potent activity against both MAC and *M. tuberculosis*. Compound VII was four to eight times as potent as LVFX and two to four times as potent as GFLX, which showed more potent activity than standard antituberculosis agents RFP and INH against RFP-susceptible and -resistant *M. tuberculosis*. Compound I, having a 3-aminopyrrolidinyl group, had one-half the activity of LVFX against both MAC and *M. tuberculosis*. Compound II, with a 3-methylaminopyrrolidinyl group, was two times more active than I against MAC. Compound III, with a 3-dimethylaminopyrrolidinyl group, was furthermore two times more active than II against MAC, and its potency was higher than that of INH. However, compounds II and III were not superior to RFP, INH, and compound I in activities against some *M. tuberculosis* strains. Compounds IV to VI, bearing 4,4-dialkylated 3-aminopyrrolidinyl moieties, showed more potent activities than non-alkylated compound I against both MAC and *M. tuberculosis*; in particular, compound VI was two to eight times as potent as LVFX, and its potency was comparable to that of GFLX. Compound X, with a 3-aminomethylpyrrolidinyl moiety, was two times as potent as LVFX in activity against both MAC and *M. tuberculosis*. Compound VIII, with a 3-aminoazetidiny moiety substituted, showed activity equipotent to that of LVFX, and 3-aminomethylazetidiny derivative IX was less active than LVFX against MAC.

## DISCUSSION

In order to investigate SARs between antimycobacterial activities and basic substituents at the C-10 position of the pyridobenzoxazine nucleus, we modified LVFX by replacement of the *N*-methylpiperazine with various basic substituents. As shown in Table 1, the activity order of pyridobenzoxazine derivatives was *M. tuberculosis* > *M. avium* > *M. intracellulare*; e.g., the most potent compound, VII, had MIC ranges of 0.05 to 0.78, 0.20 to 1.56 and 0.39 to 3.13 μg/ml for *M. tuberculosis*, *M. avium*, and *M. intracellulare*, respectively. In addition, these

TABLE 1. MICs of pyridobenzoxazine derivatives and other drugs for MAC and *M. tuberculosis* strains

Drug	<i>P'</i> <sup>a</sup>	MIC ( $\mu\text{g/ml}$ ) <sup>b</sup>							
		<i>M. avium</i>		<i>M. intracellulare</i>		RFP-s <i>M. tuberculosis</i> <sup>c</sup>		RFP-r <i>M. tuberculosis</i> <sup>d</sup>	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
I <sup>e</sup>	0.3	6.25–25	15.6	6.25–50	39.1	0.39–12.5	3.5	1.56–25	9.4
II	0.7	3.13–50	17.2	6.25–25	14.1	0.78–6.25	3.2	1.56–25	9.7
III	17.7	1.56–25	9.0	3.13–12.5	7.0	0.39–6.25	3.0	0.78–25	9.5
IV	1.8	1.56–6.25	3.1	0.78–6.25	4.1	0.10–1.56	1.0	0.39–6.25	2.4
V	10.7	1.56–12.5	5.5	1.56–6.25	4.3	0.10–3.13	1.4	0.78–6.25	3.1
VI	16.6	0.78–6.25	2.3	0.78–3.13	2.2	0.05–1.56	0.6	0.20–1.56	1.1
VII	2.7	0.20–1.56	0.7	0.39–3.13	1.7	0.05–0.39	0.2	0.10–0.78	0.4
VIII	NT <sup>f</sup>	3.13–50	20.3	6.25–25	17.2	NT	NT	NT	NT
IX	NT	25–100	50.0	25–100	62.5	NT	NT	NT	NT
X	14.7	1.56–25	7.8	3.13–6.25	7.0	0.10–3.13	0.9	0.20–3.13	1.7
LVFX	5.1	1.56–50	16.8	6.25–25	14.1	0.20–3.13	1.3	0.39–6.25	3.4
GFLX	5.9	0.78–6.25	2.3	0.78–6.25	3.3	0.10–0.39	0.2	0.20–3.13	1.1
RFP	NT	6.25–100	45.3	0.78–3.13	1.8	0.05–12.5	4.3	>100	>100
INH	NT	3.13–6.25	4.7	6.25–12.5	10.9	0.05–>100	>33	0.78–100	22.0

<sup>a</sup> *P'*, apparent partition coefficient, chloroform (CHCl<sub>3</sub>)/0.1 mole/liter of phosphate buffer (pH 7.4).

<sup>b</sup> The numbers of strains tested for the species listed were as follows: *M. avium*, 4; *M. intracellulare*, 4; RFP-s *M. tuberculosis*, 7; RFP-r *M. tuberculosis*, 5.

<sup>c</sup> RFP-s, RFP-susceptible (RFP MIC,  $\leq 12.5 \mu\text{g/ml}$ ).

<sup>d</sup> RFP-r, RFP resistant (RFP MIC,  $> 100 \mu\text{g/ml}$ ).

<sup>e</sup> See Fig. 1 and Materials and Methods.

<sup>f</sup> NT, not tested.

pyridobenzoxazines had potent activities against RFP-resistant *M. tuberculosis*, demonstrating no cross-resistance to RFP.

At first, we synthesized pyridobenzoxazines substituted with 3-aminopyrrolidinyl groups at the C-10 position and evaluated the effects of these groups on the activities against mycobacteria. Compound I, having a 3-aminopyrrolidinyl group, was less active than LVFX against both MAC and *M. tuberculosis*. Methylation of the 3-amino moiety on the pyrrolidine ring of compound I enhanced the activity against MAC (most of all for compound III, then for compound II, and finally for compound I). This result substantiated the reports suggesting that alkylation of a terminal amino group enhanced the activities against MAC (8, 15, 16). However, *N*-methylation did not elevate the activities against *M. tuberculosis*. On the other hand, dialkylation of the fourth position on the 3-aminopyrrolidine ring yielded compounds IV to VI, having potent activities against both MAC and *M. tuberculosis* (IV to VI > LVFX > I to III). Compound VII showed the most potent activity against both MAC and *M. tuberculosis*. This compound had a 2,8-diazabicyclo[4.3.0]nonanyl group, which could be considered a hybrid structure of the *N*- and *C*-alkylation products of the original 3-aminopyrrolidinyl group, suggesting that ring formation by connecting the *N*-alkyl and *C*-alkyl terminals on the 3-aminopyrrolidinyl group enhances the activity against both MAC and *M. tuberculosis*. This result is consistent with the previous reports concerning the potent antimycobacterial activities of quinolones with this bicyclo substituent (17, 19).

It is well known that the mycobacterial cell wall contains unique lipophilic substances such as mycolic acid. This distinctive cell wall formation may play an important role in hindering drug penetration. Based on this assumption, it is expected that the more lipophilic compounds would have the advantage for penetration through the cell wall and exhibit potent antimycobacterial activities. Actually, some previous reports demonstrated that higher lipophilicity played an important role in the antimycobacterial activity (8). In comparing the activities of compounds I, II, and III, an increase in the lipophilicity of the compounds contributed to enhancement of the activities against MAC, but not *M. tuberculosis*. In a series of com-

pounds, I and IV to VI, the more lipophilic compounds had more potent activities against both MAC and *M. tuberculosis*. However, the most potent compound, VII, did not have the highest lipophilicity compared to compounds III, IV, V, and VI. These results suggested that an increase in lipophilicity by introduction of an *N*-alkyl or a *C*-alkyl group(s) into the 3-aminopyrrolidinyl group in part contributed to an increase in activities against MAC and/or *M. tuberculosis*, but the lipophilicity of the compound was not the critical factor affecting their potency. In this study, dealing with pyridobenzoxazines, the order of potency of basic substituents against mycobacteria (from the highest to the lowest) was 2,8-diazabicyclo[4.3.0]nonane > 3-aminomethylpyrrolidines > 3-aminopyrrolidines  $\cong$  piperazines  $\cong$  3-aminoazetidines > 3-aminomethylazetidines.

In conclusion, we have found that pyridobenzoxazine derivatives VI, VII, and X exhibited enhanced activities against mycobacteria compared with LVFX. These results suggested that 2,8-diazabicyclo[4.3.0]nonanyl, 3-aminomethylpyrrolidinyl, and 4,4-dialkyl-3-aminopyrrolidinyl groups were more effective for the activities against both MAC and *M. tuberculosis* than the piperazinyl group. There have been several reports demonstrating the SARs between antimycobacterial activity and the substituents of the N-1 and C-8 position of the 4-quinolone nucleus (7, 11, 14, 23, 24). In practice, a combination of the basic substituents and variations of the 4-quinolone nucleus leads to subtle changes in the intrinsic antibacterial activity. Consequently, the introduction of the basic substituents described above to the appropriate 4-quinolone nucleus could contribute to obtaining novel compounds possessing excellent antimycobacterial activities.

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