

## Antibacterial Activities and Inhibitory Effects of Sitafloxacin (DU-6859a) and Its Optical Isomers against Type II Topoisomerases

TAKA AKI AKASAKA,\* SEIKO KUROSAKA, YOKO UCHIDA, MAYUMI TANAKA,  
KENICHI SATO, AND ISAO HAYAKAWA

*New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan*

Received 29 December 1997/Returned for modification 12 February 1998/Accepted 12 March 1998

**The in vitro inhibitory effects of sitafloxacin (DU-6859a) and its three stereoisomers on bacterial DNA gyrase from *Escherichia coli*, topoisomerase IV from *Staphylococcus aureus*, and topoisomerase II from human placenta were compared. No correlation was observed between the inhibitory activities of quinolones against bacterial type II topoisomerases and those against human topoisomerase II. Sitafloxacin showed the most potent inhibitory activities against bacterial type II topoisomerases and the lowest activity against human type II topoisomerase.**

Quinolone antibacterial agents have been used in therapy for various bacterial infections. The enzymic targets of quinolones are considered to be DNA gyrase and topoisomerase IV (6, 7), which are essential enzymes responsible for controlling the topological state of DNA in DNA replication and transcription (16). DNA gyrase has ATP-dependent DNA supercoiling activity (8) and is a primary target of quinolones in the gram-negative species, such as *Escherichia coli* and *Neisseria gonorrhoeae* (3, 9, 15). In contrast, topoisomerase IV has an essential role in partitioning replicated chromosomes (13) and is more sensitive than DNA gyrase to some quinolones, such as levofloxacin and ciprofloxacin, in the gram-positive species, such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (5, 19, 22, 30).

Certain quinolones have been shown to interfere with the activity of eukaryotic type II topoisomerase (topoisomerase II) (23, 24), and their inhibitory potencies against topoisomerase II might be correlated with their cytotoxicities (11). Actually, Akahane et al. (1) reported that quinolones inhibited the proliferation of murine cells in a dose-dependent manner, and the cytotoxicities of quinolones correlated well with their inhibitory potencies against topoisomerase II. Therefore, it is justified to compare the inhibition by quinolones of the activity of DNA gyrase or topoisomerase IV with their inhibition of topoisomerase II activity. Our previous study demonstrated that levofloxacin, the *l*-isomer of ofloxacin, possessed a higher selectivity than ofloxacin and its *d*-isomer (11).

Sitafloxacin (also known as DU-6859a) {(-)-7-[(7*S*)-amino-5-azaspiro(2,4)heptan-5-yl]-8-chloro-6-fluoro-1-[(1*R*,2*S*)-*cis*-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid sesquihydrate}, a newly developed quinolone antibacterial agent, had more potent activity against a wide spectrum of bacteria (20, 25, 29) than levofloxacin and ciprofloxacin. Our previous studies demonstrated that DNA gyrase in *E. coli* is a primary target of sitafloxacin and that sitafloxacin recognizes topoisomerase IV preferentially over DNA gyrase in *S. aureus* (10, 30). In this study we focused on the inhibitory activities of

sitafloxacin and its optical isomers DU-6856 {(-)-7-[(7*S*)-amino-5-azaspiro(2,4)heptan-5-yl]-8-chloro-6-fluoro-1-[(1*S*,2*R*)-*cis*-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxoquinoline-3-carboxylic acid}, DU-6857 {(-)-7-[(7*R*)-amino-5-azaspiro(2,4)heptan-5-yl]-8-chloro-6-fluoro-1-[(1*R*,2*S*)-*cis*-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxoquinoline-3-carboxylic acid}, and DU-6858 {(-)-7-[(7*R*)-amino-5-azaspiro(2,4)heptan-5-yl]-8-chloro-6-fluoro-1-[(1*S*,2*R*)-*cis*-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxoquinoline-3-carboxylic acid} (14) against DNA gyrase from *E. coli*, topoisomerase IV from *S. aureus*, and topoisomerase II from human placenta.

The structures of sitafloxacin and its isomers are shown in Fig. 1. The quinolones tested in this study were synthesized at Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. Other chemicals were purchased from their respective manufacturers. The bacterial strains used in this study were reference strains and clinical isolates from several hospitals and laboratories in Japan. The MICs were determined by the microbroth dilution method recommended by the National Committee for Clinical Laboratory Standards (21). The inoculum size was approximately 10<sup>5</sup> CFU/ml. The MIC was defined as the lowest drug concentration that prevented visible bacterial growth of the inoculum after incubation for 18 h at 37°C.

The in vitro activities of sitafloxacin, its optical isomers (DU-6856, DU-6857, and DU-6858), levofloxacin, ofloxacin, and ciprofloxacin against a variety of reference strains and clinical isolates are given in Table 1. Among the quinolones tested, sitafloxacin had the greatest activity against members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *S. pneumoniae*, and *Haemophilus influenzae*.

Subunits A and B of DNA gyrase were purified separately from *E. coli* KL-16 by using novobiocin-epoxy-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) column chromatography, as described previously (12). The specific activities of purified subunits A and B of DNA gyrase were 10<sup>4</sup> U/mg of protein. Subunits A and B of *S. aureus* topoisomerase IV were purified as fusion proteins separately with maltose-binding protein by the method described previously (30). Human topoisomerase II was purchased from TopoGEN, Inc. (Columbus, Ohio). Inhibitory activities of quinolones against type II topoisomerases were assayed electrophoretically as described previously (12, 30) with minor modifications. The gel was

\* Corresponding author. Mailing address: New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., 16-13, Kitakasai 1-Chome, Edogawa-ku, Tokyo 134-8630, Japan. Phone: 81-3-3680-0151. Fax: 81-3-5696-8344. E-mail: XLX06563@niftyserve.or.jp.

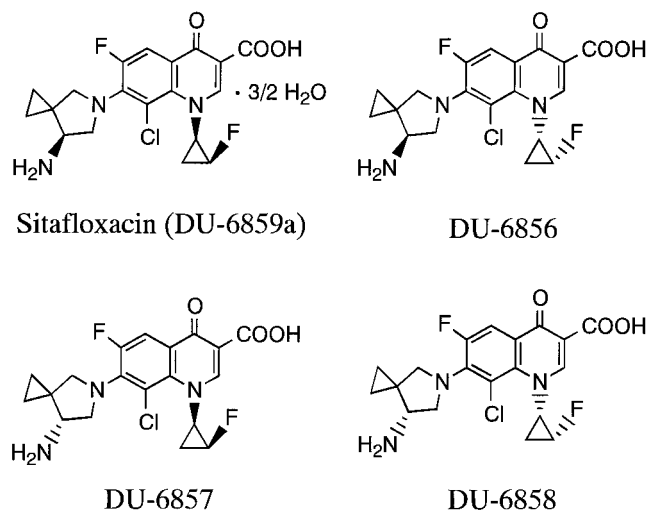


FIG. 1. Structures of sitafloxacin and its stereoisomers.

stained with ethidium bromide and photographed by using a UV light (302 nm) with a Gel Print 2000i/VGA (Bio Image Co., Ann Arbor, Mich.), and the brightness of bands was traced with an Image Analyzer (Bio Image). For the supercoiling assay of DNA gyrase, the reaction mixture (20  $\mu$ l), containing subunits A and B (1 U of each, which brought 50% of the pBR322 plasmid to the supercoiled form), drug solution, 20 mM Tris hydrochloride (pH 7.5), 20 mM KCl, 4 mM MgCl<sub>2</sub>, 1 mM spermidine hydrochloride, 1 mM ATP, 1 mM dithiothreitol, 20  $\mu$ g of bovine serum albumin per ml, and 0.2  $\mu$ g of relaxed pBR322 plasmid DNA, was incubated at 37°C for 1 h. Each band was quantified, and the amount of supercoiled plasmid DNA treated with each concentration of quinolone was measured to determine the 50% inhibitory concentration (IC<sub>50</sub>) against DNA gyrase. The IC<sub>50</sub>s against topoisomerase IV were determined as the drug concentrations that reduced the decatenation activity seen with drug-free controls by 50%. Additionally, for the relaxing assay of topoisomerase II, a reaction mixture (20  $\mu$ l) containing 1 U of topoisomerase II (which brought 50% of the pBR322 plasmid to the relaxed form), 50 mM Tris hydrochloride (pH 7.5), 100 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30  $\mu$ g of bovine

serum albumin per ml, and 0.2  $\mu$ g of supercoiled pBR322 was incubated at 37°C for 1 h. The IC<sub>50</sub> of each quinolone against the relaxing activity of topoisomerase II was calculated by the same method as that for DNA gyrase.

MICs and inhibitory effects on topoisomerases are shown in Table 2. Sitafloxacin was twice as active against *E. coli* KL-16 as DU-6856, four times more active than DU-6857, and eight times more active than DU-6858. Against the supercoiling activity of *E. coli* DNA gyrase, the IC<sub>50</sub>s of sitafloxacin, DU-6856, DU-6857, and DU-6858 were 0.13, 0.18, 0.42, and 0.69  $\mu$ g/ml, respectively. Thus, the anti-DNA gyrase activity of sitafloxacin was highest, followed by those of DU-6856, DU-6857, and DU-6858, in that order. There is a high correlation between the inhibitory effects of the quinolones on bacterial growth and the inhibition of DNA gyrase; the correlation coefficient, calculated by using a transformation of the data points of all the compounds tested, was 0.941.

Against *S. aureus* FDA 209-P, sitafloxacin was twice as active as DU-6856 and eight times more active than DU-6857 and DU-6858. Against the decatenation activity of topoisomerase IV, the inhibitory activity of sitafloxacin was about twice as potent as that of DU-6856. DU-6857 and DU-6858 were about three times less active than sitafloxacin. There was also a significant correlation between the inhibitory effects on *S. aureus* growth and the inhibition of topoisomerase IV (correlation coefficient, 0.986).

On the other hand, the IC<sub>50</sub>s of four isomers against topoisomerase II from human placenta ranged from 1,147 to 2,369  $\mu$ g/ml, and the inhibitory potency was lowest for sitafloxacin, followed by DU-6857, DU-6856, and DU-6858 in increasing order of potency. There was no correlation between the IC<sub>50</sub> for any bacterial topoisomerase and that for mammalian topoisomerase II.

Eukaryotic topoisomerase II is known as an essential enzyme for cell growth (4). Because of the similarities in the biochemical mechanisms and amino acid sequences between DNA gyrase and mammalian topoisomerase II (17), the inhibition of human topoisomerase II by quinolones would be an undesirable side effect of antibacterial chemotherapy, and information on the relative selectivity toward bacterial target enzyme versus human topoisomerase II would be of significance. Fortunately, the compounds tested required higher concentrations to inhibit human topoisomerase II than to inhibit DNA gyrase or topoisomerase IV, and all the selectivity values

TABLE 1. Antimicrobial activities of quinolones<sup>a</sup>

Organism	MIC ( $\mu$ g/ml)						
	STFX	DU-6856	DU-6857	DU-6858	LVFX	OFLX	CPFX
<i>E. coli</i> KL-16	0.008	0.016	0.031	0.063	0.031	0.063	0.008
<i>Citrobacter freundii</i> IID 976	0.008	0.016	0.031	0.063	0.031	0.031	$\leq$ 0.004
<i>Proteus vulgaris</i> 08601	$\leq$ 0.004	0.016	0.016	0.031	0.016	0.031	0.008
<i>Proteus mirabilis</i> 08103	0.008	0.016	0.031	0.063	0.031	0.063	0.013
<i>Klebsiella pneumoniae</i> type 1	0.031	0.031	0.063	0.125	0.063	0.125	0.031
<i>Enterobacter cloacae</i> 03400	$\leq$ 0.004	0.008	0.031	0.063	0.031	0.031	0.016
<i>Serratia marcescens</i> 10100	0.031	0.063	0.063	0.125	0.125	0.25	0.063
<i>P. aeruginosa</i> PAO1	0.125	0.25	0.25	0.5	0.5	1	0.125
<i>Bacterium anitratum</i> ATCC 19606	0.063	0.125	0.25	0.5	0.25	0.5	1
<i>H. influenzae</i> 033803 960614	0.004	0.008	0.031	0.031	0.031	0.063	0.031
<i>S. aureus</i> FDA 209-P	0.008	0.016	0.063	0.063	0.25	0.5	0.25
<i>S. epidermidis</i> 56500	0.063	0.063	0.25	0.25	0.5	1	0.5
<i>S. pyogenes</i> G-36	0.063	0.125	0.5	0.5	2	>2	2
<i>Enterococcus faecalis</i> ATCC 19433	0.25	0.25	1	1	2	>2	2
<i>S. pneumoniae</i> J24	0.031	0.063	0.5	0.25	1	2	1

<sup>a</sup> STFX, sitafloxacin; LVFX, levofloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin.

TABLE 2. Inhibitory effects of quinolones against type II topoisomerases

Compound	IC <sub>50</sub> ± SD (MIC) for <sup>a</sup> :			Selectivity	
	Gyr	Topo IV	Topo II	Topo II/Gyr	Topo II/Topo IV
Sitafloxacin	0.13 ± 0.01 (0.008)	0.39 ± 0.15 (0.008)	2,369 ± 101	18,221	6,022
DU-6856	0.18 ± 0.02 (0.016)	0.86 ± 0.22 (0.016)	1,854 ± 20	10,298	2,164
DU-6857	0.42 ± 0.01 (0.031)	1.44 ± 0.07 (0.063)	1,939 ± 147	4,616	1,346
DU-6858	0.69 ± 0.10 (0.063)	1.21 ± 0.06 (0.063)	1,147 ± 80	1,663	951
Levofloxacin	0.39 ± 0.00 (0.031)	2.36 ± 0.41 (0.25)	1,854 ± 35	4,754	786
Ofloxacin	0.71 ± 0.07 (0.063)	4.17 ± 1.25 (0.5)	2,221 ± 48	3,129	532
Ciprofloxacin	0.32 ± 0.02 (0.008)	2.83 ± 1.14 (0.25)	325 ± 44	1,016	115

<sup>a</sup> IC<sub>50</sub>s and MICs are expressed as micrograms per milliliter. Gyr, DNA gyrase from *E. coli* KL-16; Topo IV, topoisomerase IV from *S. aureus* FDA 209-P; Topo II, topoisomerase II from human placenta.

of the compounds tested were greater than 100 (Table 2). Sitafloxacin proved to possess the highest relative selectivity for bacterial target enzyme versus human topoisomerase II among the compounds tested in this study.

It has been proposed that quinolones bind to a specific site on DNA in the DNA-enzyme complex (26–28). According to this model, differences in enzyme inhibitory potency are mainly determined by the strength of drug binding to a DNA receptor site on the enzyme-substrate complex, while the interaction of the C-7 substituent with the enzyme plays a supporting role (18). Yoshida et al. (31) have provided evidence showing that C-7 is in close contact with the so-called quinolone pocket in GyrB. Against DNA gyrase or topoisomerase IV, sitafloxacin and its C-7-7'(*S*)-isomer DU-6856 were more active than its C-7-7'(*R*)-isomers DU-6857 and DU-6858. These results suggested that C-7-7'(*S*)-isomers were more potent in the interaction between the C-7 substituent and the enzyme, compared to C-7-7'(*R*)-isomers. In the stereoisomeric derivatives of the N-1 substituent, *cis*-isomers were more potent than *trans*-isomers, as previously reported (2). In this study, sitafloxacin {C-1-([1*R*,2*S*]-*cis*-2-fluorocyclopropyl)} showed more-potent antibacterial activities against the bacterial enzymes, DNA gyrase and topoisomerase IV, than its C-1-([1*S*,2*R*]-*cis*-2-fluorocyclopropyl) isomer DU-6856. This result suggested that the C-1-([1*R*,2*S*]-*cis*-2-fluorocyclopropyl) moiety has interactive potential with the receptor site on the DNA-enzyme complex. We plan to pursue further study of the difference in the ratios of binding of the isomers to DNA-topoisomerase II complex.

#### REFERENCES

- Akahane, K., K. Hoshino, K. Sato, Y. Kimura, T. Une, and Y. Osada. 1991. Inhibitory effects of quinolones on murine hematopoietic progenitor cells and eukaryotic topoisomerase II. *Chemotherapy (Tokyo)* **37**:224–226.
- Atarashi, S., M. Imamura, Y. Kimura, A. Yoshida, and I. Hayakawa. 1993. Fluorocyclopropyl quinolones. 1. Synthesis and structure-activity relationships of 1-(2-fluorocyclopropyl)-3-pyridonecarboxylic acid antibacterial agents. *J. Med. Chem.* **36**:3444–3448.
- Belland, R. J., S. G. Morrison, C. Ison, and W. M. Huang. 1994. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. *Mol. Microbiol.* **14**:371–380.
- Drlica, K. 1984. Biology of bacterial deoxyribonucleic acid topoisomerases. *Microbiol. Rev.* **48**:273–289.
- Ferrero, L., B. Cameron, and J. Crouzet. 1995. Analysis of *gyrA* and *gltA* mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **39**:1554–1558.
- Ferrero, L., B. Cameron, B. Mansse, D. Lagneau, J. Crouzet, A. Famechon, and F. Blanche. 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. *Mol. Microbiol.* **13**:641–653.
- Gellert, M. 1981. DNA topoisomerases. *Annu. Rev. Biochem.* **50**:879–910.
- Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash. 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. *Proc. Natl. Acad. Sci. USA* **73**:3872–3876.
- Heisig, P. 1996. Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* **40**:879–885.
- Hoshino, K., A. Kitamura, I. Morrissey, K. Sato, J. Kato, and H. Ikeda. 1994. Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. *Antimicrob. Agents Chemother.* **38**:2623–2627.
- Hoshino, K., K. Sato, K. Akahane, A. Yoshida, I. Hayakawa, M. Sato, T. Une, and Y. Osada. 1991. Significance of the methyl group on the oxazine ring of ofloxacin derivatives in the inhibition of bacterial and mammalian type II topoisomerases. *Antimicrob. Agents Chemother.* **35**:309–312.
- Hoshino, K., K. Sato, T. Une, and Y. Osada. 1989. Inhibitory effects of quinolones on DNA gyrase of *Escherichia coli* and topoisomerase II of fetal calf thymus. *Antimicrob. Agents Chemother.* **33**:1816–1818.
- Kato, J., Y. Nishimura, R. Imamura, H. Niki, S. Hiraga, and H. Suzuki. 1990. New topoisomerase essential for chromosome segregation in *E. coli*. *Cell* **63**:393–404.
- Kimura, Y., S. Atarashi, K. Kawakami, K. Sato, and I. Hayakawa. 1994. (Fluorocyclopropyl)quinolones. 2. Synthesis and stereochemical structure-activity relationships of chiral 7-(7-amino-5-azaspiro[2,4]heptan-5-yl)-1-(2-fluorocyclopropyl)quinolone antibacterial agents. *J. Med. Chem.* **37**:3344–3352.
- Kumagai, Y., J. Kato, K. Hoshino, T. Akasaka, K. Sato, and H. Ikeda. 1996. Quinolone-resistant mutants of *Escherichia coli* DNA topoisomerase IV *parC* gene. *Antimicrob. Agents Chemother.* **40**:710–714.
- Luttinger, A. 1995. The twisted 'life' of DNA in the cell: bacterial topoisomerases. *Mol. Microbiol.* **15**:601–660.
- Lynn, R., G. Gaever, S. L. Swanberg, and J. C. Wang. 1986. Tandem regions of yeast DNA topoisomerase II share homology with different subunits of bacterial gyrase. *Science* **233**:647–649.
- Morrissey, I., K. Hoshino, K. Sato, A. Yoshida, I. Hayakawa, M. G. Bures, and L. L. Shen. 1996. Mechanism of differential activities of ofloxacin enantiomers. *Antimicrob. Agents Chemother.* **40**:1775–1784.
- Muñoz, R., and A. G. de la Campa. 1996. *ParC* subunit of DNA topoisomerase IV of *Streptococcus pneumoniae* is a primary target of fluoroquinolones and cooperates with DNA gyrase A subunit in forming resistance phenotype. *Antimicrob. Agents Chemother.* **40**:2252–2257.
- Nakane, T., S. Iyobe, K. Sato, and S. Mitsuhashi. 1995. In vitro antibacterial activity of DU-6859a, a new fluoroquinolone. *Antimicrob. Agents Chemother.* **39**:2822–2826.
- National Committee for Clinical Laboratory Standards. 1993. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A3, 3rd ed. National Committee for Clinical Laboratory Standards, Villanova, Pa.
- Pan, X. S., J. Ambler, S. Mehtar, and L. M. Fisher. 1996. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **40**:2321–2326.
- Robinson, M. J., B. A. Martin, T. D. Gootz, P. R. McGuirk, M. Moynihan, J. A. Sutcliffe, and N. Osheroff. 1991. Effects of quinolone derivatives on eukaryotic topoisomerase II. A novel mechanism for enhancement of enzyme-mediated DNA cleavage. *J. Biol. Chem.* **266**:14585–14592.
- Robinson, M. J., B. A. Martin, T. D. Gootz, P. R. McGuirk, and N. Osheroff. 1992. Effects of novel fluoroquinolones on the catalytic activities of eukaryotic topoisomerase II: Influence of the C-8 fluorine group. *Antimicrob. Agents Chemother.* **36**:751–756.
- Sato, K., K. Hoshino, M. Tanaka, I. Hayakawa, and Y. Osada. 1992. Antibacterial activity of DU-6859, a new potent fluoroquinolone, against clinical isolates. *Antimicrob. Agents Chemother.* **36**:1491–1498.
- Shen, L. L., J. Baranowski, and A. G. Pernet. 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: specificity and cooperativity of drug binding to DNA. *Biochemistry* **28**:3879–3885.
- Shen, L. L., W. E. Kohlbrenner, D. Weigl, and J. Baranowski. 1989. Mechanism of quinolone inhibition of DNA gyrase. Appearance of unique norfloxacin binding sites in enzyme-DNA complexes. *J. Biol. Chem.* **264**:2973–2978.
- Shen, L. L., L. A. Mitscher, P. N. Sharma, T. J. O'Donnell, D. W. Chu, C. S.

- Cooper, T. Rosen, and A. G. Pernet. 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: a cooperative drug-DNA binding model. *Biochemistry* **28**:3886-3894.
29. Tanaka, M., K. Hoshino, M. Hohmura, H. Ishida, A. Kitamura, K. Sato, I. Hayakawa, and T. Nishino. 1996. Effect of growth conditions on antimicrobial activity of DU-6859a and its bactericidal activity determined by the killing curve method. *J. Antimicrob. Chemother.* **37**:1091-102.
30. Tanaka, M., Y. Onodera, Y. Uchida, K. Sato, and I. Hayakawa. 1997. Inhibitory activities of quinolones against DNA gyrase and topoisomerase IV purified from *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **41**:2362-2366.
31. Yoshida, H., M. Nakamura, M. Bogaki, and S. Nakamura. 1993. Mechanism of action of quinolones against *Escherichia coli* DNA gyrase. *Antimicrob. Agents Chemother.* **37**:839-845.