immediately before use. Injection volumes were 5 mL/kg, and injection solutions had approximately neutral pH.

Biochemistry. Brain levels of DOPA and 5-HTP were analyzed by HPLC with electrochemical detection.[2e] For biochemical results and experimental details, see Table III and footnote a in Table III.

Locomotor Activity. The motor activity was measured by means of photocell recordings (M/P 40 Fc Electronic Motility Meters, Motron Products, Stockholm, Sweden) as previously described.[22] For experimental details, see footnotes a and b in Table IV. Each box was equipped with a semitransparent mirror that allowed gross behavior observations of the animals during the experiments. The motor activity results are shown in Table IV.

Binding Experiments. Membrane Preparation. Calf striatal tissue was obtained from a local slaughter house and stored at −80 °C until use. The tissue was homogenized with 40 v/v of ice-cold salt buffer (50 mM TRIS, 1 mM EDTA, 5 mM HCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4) with use of an Ultraturrax (1000 rpm). The homogenate was centrifuged at 43000*g* for 20 min at 5 °C. The pellet was resuspended in 40 v/v of salt buffer and preincubated for 30 min at 35 °C, centrifuged at 43000*g* for 20 min at 5 °C and resuspended and centrifuged once more. The pellet thus obtained was resuspended in 10 v/v of salt buffer. Aliquots of 2.35 mL of tissue homogenate were frozen in plastic tubes (liquid nitrogen) and stored at −20 °C.

[^{3}H]Spiroperidol Binding. The tissue homogenate was suspended in 3 v/v of ice-cold incubation buffer (50 mM Tirs-HCl, 1 mM EDTA, 50 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, pH 7.2). Triplicate determinations were conducted in borosilicate glass tubes. Each tube (final volume 1 mL) included 100 μL of 10 nM [^{3}H]spiroperidol (20 Ci/mmol, NEN) and the competitor drug (50–100 μL) both in Tris-salt buffer. Nonspecific binding was defined by using 1 μM (+)-butaclamol (Ayerst) in buffer. In saturation experiments, the specific binding of [^{3}H]-spiroperidol was obtained as a function of its concentration (0.025–1.5 nM). Nonspecific binding was defined as binding in the presence of 1 μM (+)-butaclamol. Eadie–Hofstee plots indicated a single binding site with a K_D of 0.16 ± 0.006 nM and a capacity of 21.5 ± 0.8 pmol/g tissue.

The reaction was initiated by the addition of 100 μL of the membrane suspension (1.5–2 mg/tube) and incubated at 37 °C for 40 min. Bound ligand was separated from free by rapid vacuum filtration over GF/B filters with 4 × 3.5 mL washes of the filters with ice-cold Tris-salt buffer. The filters were placed in glass vials with 6 mL of Plasmasol (Packard). After at least 6 h of equilibration, the vials were counted by liquid scintillation spectroscopy using a Beckman LS 1800 (47% efficiency).

[^{3}H]NPA Binding. This assay ([^{3}H]NPA, 55.8 Ci/mmol; NEN) was performed essentially as described above for the [^{3}H]spiroperidol binding. The buffer used was a 50 mM Tris-HCl buffer with 1 mM EDTA, 4 mM $MgCl_2$, and 0.01% ascorbic acid (pH 7.2). In the assay, each tube (1-mL final volume) included 100 μL of 5 nM radioligand, 50–100 μL of the competitor drug, and 100 μL of membrane suspension (1.5–2 mg/tube), all dissolved in buffer. Nonspecific binding was defined by using 1 μM (+)-butaclamol and the incubation was performed at 25 °C for 45 min, followed by vacuum filtration and scintillation counting as described above. Saturation experiments were performed by using 0.1–3 nM of [^{3}H]NPA. Nonspecific binding was defined as binding in the presence of 1 μM (+)-butaclamol. Eadie–Hofstee plots indicated a single binding site with a K_D of 0.71 ± 0.02 nM and a capacity of 18.3 ± 2.1 pmol/g tissue. The in vitro binding data are shown in Table V.

Acknowledgment. We thank Lucia Gaete and Boel Göransson for skillful assistance in the pharmacological testing. Support for this study was provided by a grant from the Swedish Natural Science Research Council.

Supplementary Material Available: Positional and thermal parameters, bond lengths, and bond angles (2 pages). Ordering information is given on any current masthead page.

(22) Engström, G.; Svensson, T. H.; Waldeck, B. *Brain Res.* **1974**, *77*, 471.

Synthesis of Antimicrobial Agents. 3. Syntheses and Antibacterial Activities of 7-(4-Hydroxypiperazin-1-yl)quinolones

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A series of novel pyridone carboxylic acids having a 4-hydroxypiperazinyl group at the 7-position of norfloxacin and ciprofloxacin were prepared. The in vivo antibacterial efficacies of these compounds were superior to those of corresponding piperazinyl derivatives. From the results of the studies on the pharmacokinetic profile and toxicity, the 4-hydroxypiperazinyl derivatives were confirmed to be pharmacologically superior to corresponding piperazinyl derivatives. Thus, a 4-hydroxypiperazinyl group was revealed to be a beneficial substituent for potential use in future quinolone antibacterials.

Introduction

Since norfloxacin (NFLX, 1) was reported by Koga et al.,[1] many analogues having a fluorine atom and piperazinyl moiety attached to the quinoline or naphthyridine ring have been synthesized. Among these compounds, perfloxacin (PFLX, 2),[2] ciprofloxacin (CPFX, 3),[3] enoxacin (ENX, 4),[4] and ofloxacin (OFLX, 5)[5] have been introduced into clinical use.

These drugs, on the basis of the nature of the 4-nitrogen atom of piperazinyl group, can be classified into two types:

(1) Koga, H.; Itoh, A.; Murayama, S.; Suzue, S.; Irikura, T. *J. Med. Chem.* **1980**, *23*, 1358.
(2) Goueffon, Y.; Montay, G.; Roquet, F.; Pesson, M. *C. R. Hebd. Seances Acad. Sci.* **1981**, *292*, 37.
(3) Wise, R.; Andrews, J. M.; Edwards, L. *J. Antimicrob. Agents Chemother.* **1983**, *23*, 559.
(4) Matsumoto, T.; Miyamoto, T.; Minamida, A.; Nishimura, Y.; Egawa, H. *J. Med. Chem.* **1984**, *27*, 292.
(5) Sato, K.; Matsuura, Y.; Inoue, M.; Une, T.; Osada, Y.; Ogawa, H.; Mitsuhashi, S. *Antimicrob. Agents Chemother.* **1982**, *22*, 548.

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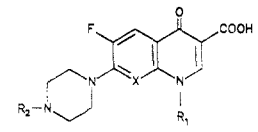
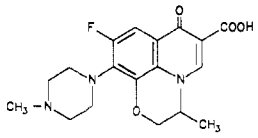
Chart I. Clinically Significant Quinolone Antibacterials

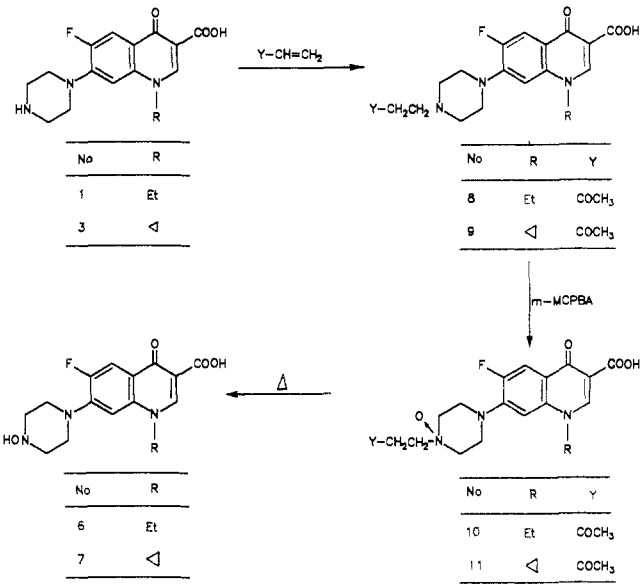


compd	No	X	R_1	R_2
norfloxocin	1	CH	Et	H
pefloxocin	2	CH	Et	CH_3
ciprofloxocin	3	CH	◁	H
enoxocin	4	N	Et	H



ofloxocin 5

Scheme I



No	R
1	Et
3	◁

No	R	Y
8	Et	$COCH_3$
9	◁	$COCH_3$

No	R
6	Et
7	◁

No	R	Y
10	Et	$COCH_3$
11	◁	$COCH_3$

a secondary amine type (A) such as NFLX, CPFX, and ENX, containing a piperazinyl group at the 7-position, and a tertiary amine type (B) such as PFLX and OFLX, having a 4-methylpiperazinyl group, as shown in Chart I. The type A analogue NFLX has been reported to exhibit 2.2–7-fold lower in vivo activities than PFLX, the corresponding type B analogue, against experimentally induced infections of mice after oral administration, though they showed almost equal in vitro antibacterial activities.[2] This interesting result was considered to be due to the enhancement of oral absorbability of PFLX, and the enhancement seemed to be a result of conversion of the secondary amine of NFLX to a tertiary amine.

In the course of our search for more potent in vivo analogues, our interest was directed to the substituent on the 4-nitrogen atom of the piperazinyl group.

In this paper, we report the syntheses and evaluations of two 7-(4-hydroxypiperazin-1-yl)quinolones, N-hydroxylated NFLX **6** and N-hydroxylated CPFX **7**, which have excellent in vivo antibacterial activities with low toxicities.

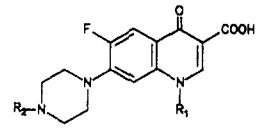
Chemistry

NFLX[1] and CPFX[3] were prepared according to the references.

7-(4-Hydroxypiperazin-1-yl) derivatives **6** and **7** were synthesized from the corresponding 7-(piperazin-1-yl) derivatives (**1** and **3**), respectively, as shown in Scheme I.

Treatment of **1** and **3** with methyl vinyl ketone in chloroform provided the corresponding 7-[4-(3-oxobutyl)piperazin-1-yl] derivatives **8**[6] and **9**[7] in good yields.

Table I. 6-Fluoro-7-(4-substituted-piperazin-1-yl)quinolones



no.	R_1	R_2	% yield[a]	mp, °C	formula[b]
6	Et	OH	60.9	260–264	$C_{16}H_{18}FN_3O_4$
7	◁	OH	55.0	261–265	$C_{17}H_{18}FN_3O_4$
8	Et	$CH_2CH_2COCH_3$	68.9	187–193	$C_{20}H_{24}FN_3O_4$
9	◁	$CH_2CH_2COCH_3$	86.0	185–187	$C_{21}H_{24}FN_3O_4$

[a] Yields were not optimized. [b] Carbon, hydrogen, and nitrogen analyses were within ±0.3% of the theoretical values.

They were then oxidized with *m*-chloroperbenzoic acid in chloroform at room temperature to their *N*-oxides **10** and **11**, which spontaneously rearranged to **6** and **7**, respectively, by a reverse Michael addition.[8] Therefore, the intermediates **10** and **11** were not isolated.

The physical properties of these compounds are listed in Table I.

In Vitro Antibacterial Activity

According to the method of Goto et al.,[9] the MICs of compounds were determined by the agar dilution method, using Mueller–Hinton agar. The results are summarized in Table II.

In Vivo Antibacterial Activity

The in vivo assay was carried out according to the method which was reported in a previous paper.[10] The test compounds were suspended in 0.5% sodium (carboxymethyl)cellulose (CMC) and administered orally at 1 h after infection.

ED_{50} values were calculated from the cumulative mortalities on the 7th day after infection by using the shortened version of the Weil method.[11] The results are summarized in Table III.

Blood Levels after Oral Administration in Mice

Test compounds suspended in 0.5% CMC were administered orally to ddY strain, male mice (20–25 g, five per group) at a dose of 50 mg/kg. After 30, 60, 120, and 240 min, mice were killed by bleeding. Blood samples were centrifuged at 3000 rpm for 20 min at 4 °C, and the serum was collected.

Serum concentrations of **6** and NFLX in mice treated with compound **6** and NFLX itself were determined by high-performance liquid chromatography (HPLC), after the serum specimens had been treated with 5% aqueous trichloroacetic acid and centrifuged to give protein-free specimens. The HPLC system was equipped with a Waters Model 6000A pump, a Waters U6K injector, a Shimazu SPD-6A spectrophotometric detector, and a YMC A-312 ODS column. A 5% Acetic acid–methanol (8:2) mixture (v/v) was used as the mobile phase and the flow rate was 1.0 mL/min. The specimens were assayed against standard solutions of **6** and NFLX prepared in mouse

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(7) CPFX type Japan Kokai 59-172471, 1984.
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Table II. In Vitro Antibacterial Activities of 7-(4-Hydroxypiperazin-1-yl) Derivatives (6 and 7)

	minimum inhibitory concentration (MIC),[a] μg/mL								
compd	Sa	Se	Bs	Ec	Kp	Pv	St	Sm	Pa
6	0.78	6.25	0.20	1.56	0.05	0.05	0.10	3.13	12.5
NFLX	0.39	3.13	0.39	0.20	0.10	0.10	0.05	0.39	1.56
7	0.39	1.56	0.39	0.10	0.025	0.05	0.025	0.78	1.56
CPFX	0.39	1.56	0.39	0.05	0.025	0.05	0.025	0.20	0.78

[a] The MICs were determined by the 2-fold agar dilution method on sensitivity test agar. Organisms selected for inclution in the table: Sa, *Staphylococcus aureus* FDA 209P JC-1; Se, *Staphylococcus epidermidis* IAM 1296; Bs, *Bacillus subtilis* ATCC 6633; Ec, *Escherichia coli* NIHJ JC-2; Kp, *Klebsiella pneumoniae* PCI-602; Pv, *Proteus vulgaris* OX-19; St. *Salmonella typhimurium* IID 971; Sm, *Serratia marcescens* IAM 1184; Pa, *Pseudomonas aeruginosa* IFO 3445.

Table III. In Vitro and in Vivo Antibacterial Activities and Acute Toxicities of 7-(4-Hydroxypiperazin-1-yl) Derivatives (6 and 7)

	St. aureus IID 803		*E. coli* KC-14		LD_{50},[b] mg/kg	
compd	MIC,[a] μg/mL	ED_{50},[b] mg/kg,[c] po	MIC,[a] μg/mL	ED_{50},[b] mg/kg,[c] po	po	iv
6	1.56	187 (133–262)	0.39	1.92 (1.46–2.53)	>4000	>500
NFLX	1.56	225 (100–503)	0.20	5.82 (4.15–8.18)	>4000	314
7	0.78	17.7 (11.9–26.2)	0.025	0.37 (0.27–0.52)	>4000	NT[d]
CPFX	0.78	46.7 (33.2–65.5)	0.0125	0.84 (0.64–1.17)	>4000	NT

[a] See Table II, footnote *a*. [b] See the Experimental Section. [c] 95% confidence limits. [d] Not tested.

Table IV. Concentration of Liberated NFLX in Serum and AUC after Oral Administration of 6 to Mice

	concentration in serum,[a] μg/mL				AUC (0–4 h), μg·h/mL
compd	30 min	60 min	120 min	240 min	
			6 (50 mg/kg po)		
6	ND[b]	ND	ND	ND	0
NFLX	4.14 ± 0.88	2.80 ± 1.64	1.09 ± 0.35	0.47 ± 0.16	6.5
			NFLX (50 mg/kg po)		
NFLX	1.41 ± 0.48	1.27 ± 0.20	0.73 ± 0.15	0.23 ± 0.07	2.3

[a] Mean ± SE. [b] Not detected.

serum and then treated by the above-mentioned method.

Stability Test

In Gastric or Intestinal Juice. The stability of **6** in artificial gastric juice (pH 1.2) or intestinal juice (pH 6.8) was determined. A solution of **6** (10 μg/mL, 100 μL) in $^1/_{15}$ M phosphate buffer (pH 7.4) was added to the above artificial juice (900 μL), individually, and the mixture was incubated at 37 °C.

After 30, 60, 120, and 240 min, 100 μL of incubated solution was taken periodically and added to the same volume of 5% aqueous trichloroacetic acid. The concentrations of each test sample was measured by HPLC. The HPLC conditions were the same as those mentioned above.

Liberation of NFLX

The liberation of NFLX after oral administration of **6** to mice was studied by incubation of **6**.

In Serum. A solution of **6** (10 μg/mL, 100 μL) in $^1/_{15}$ M phosphate buffer (pH 7.4) was added to the mouse serum (900 μL), and the mixture was incubated at 37 °C. Sample collection and determination of drug concentration were the same as those used in the case of the stability test.

In Liver. A solution of **6** (10 μg/mL, 0.1 mL) in aqueous dimethyl sulfoxide was added to rat liver microsomes[12] (0.9 mL), and the mixture was incubated at 37 °C as soon as possible. After 5, 15, 30, 60, and 120 min, incubated solution was taken periodically and added to the same volume of methanol. Each sample was centrifuged at 3000 rpm for 10 min at 5 °C, and the concentrations in each test sample (10 μL) was measured by HPLC. The HPLC conditions were the same as mentioned above, except for the mobile phase (0.025 M phosphate buffer–methanol, 7:3 v/v).

(12) Ohta, T.; Miyata, N.; Hirobe, M. *Chem. Pharm. Bull.* **1986**, *34*, 2494.

Acute Toxicity on Oral Administration and Intravenous Injection in Mice

Oral Administration. A suspension of each test compound in 0.5% CMC was administered orally to ddY strain, male mice (20–25-g body weight, five per group). Seven days later, LD_{50} values were determined by using the Weil method.

Intravenous Injection. Each of the compounds was dissolved in 1 N NaOH, and a phosphoric acid–saline buffer (pH 7.2) was added to prepare a test solution. The test solution was injected intravenously into ddY strain, male mice (20–25-g body weight, five per group). Seven days later, the LD_{50} was determined by using Weil method.

Results and Discussion

Table II summarizes the in vitro antibacterial activities of the 7-(4-hydroxypiperazin-1-yl) derivatives **6** and **7** against several organisms with the results for NFLX and CPFX.

The comparison of the in vitro antibacterial activities of hydroxypiperazinyl derivatives **6** and **7** with those of their piperazinyl derivatives **1** and **3** indicates that hydroxylation on the nitrogen atom of the piperazinyl group decreases the in vitro antibacterial activities about 2–8 times.

In vivo antibacterial activities of **6** and **7** against experimentally induced infection of mice after oral administration are given in Table III together with the in vitro activities of the infection strains. Unexpectedly, hydroxypiperazinyl derivatives **6** and **7** were found to be more potent in vivo by about 1.5–3.0 times than the corresponding piperazinyl derivatives **1** and **3** while in vitro activities of the former were equal to or lower than those of the latter.

In order to clarify the reason for increased in vivo activities of hydroxypiperazinyl derivatives, the disappearance of **6** was monitored in serum levels with HPLC after

oral administration to mice. From the studies with HPLC, we found that **6** was converted into NFLX within 30 min after oral administration to mice. The serum levels and area under the serum concentration of liberated NFLX when **6** was administered orally are listed in Table IV. The serum levels of liberated NFLX levels after oral administration of **6** were higher than those of NFLX itself, and the AUC of liberated NFLX from **6** was about 2.7 times more than that of NFLX itself.

On the basis of the above results, the superior in vivo potency of **6** compared to that of NFLX was likely due to the high blood concentration of liberated NFLX from **6**.

To clarify the mechanism of the liberation of NFLX when **6** was administered orally, the stabilities of **6** in artificial gastric juice (pH 1.2) and intestinal juice (pH 6.8) were estimated by HPLC. Then, the liberation of NFLX from **6** in mouse serum and in rat liver microsomes were also examined by HPLC. In gastric and intestinal juice and in mouse serum, a NFLX peak was not detected, in all runs. However, in rat liver microsomes **6** was found to produce NFLX, and the ratio of NFLX was gradually increased with the elapse of time. After a 30-min incubation, **6** was converted into NFLX completely.

From the above results, it can be presumed that the conversion of **6** into NFLX takes place in the liver after oral administration of **6**. But, the mechanism of liberation of NFLX from **6** in vivo is not elucidated in the present paper.

The acute toxicities of **6** and **7**, when administered orally to mice and injected intravenously in mice, are also summarized in Table III. From the comparison with NFLX and CPFX, it was confirmed that, at least, the hydroxylation of the piperazinyl group of NFLX or CPFX has not increased their acute toxicities.

From the microbiological, toxicological, and pharmacokinetic profile, the 4-hydroxypiperazinyl group was considered to be a beneficial group for use in other pyridone carboxylic acid antibacterial agents. Further application of 4-hydroxypiperazinyl group to another antibacterial agent will be discussed in another paper.[13]

Experimental Section

Melting points were determined on a Yanagimoto micro melting point apparatus, and all melting points are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined at 100 MHz on a Nihon Denshi PS-100 NMR spectrometer using tetramethylsilane as an internal standard. Mass spectra (MS) were measured with a Hitachi M-60.

NFLX (**1**)[1] and CPFX (**3**)[3] were prepared according to the literature procedures.

1-Ethyl-6-fluoro-1,4-dihydro-7-[4-(3-oxobutyl)piperazin-1-yl]-4-oxoquinoline-3-carboxylic Acid (8). To a suspension of 1-ethyl-6-fluoro-1,4-dihydro-7-piperazin-1-yl-4-oxoquinoline-3-carboxylic acid (NFLX, **1**, synthesized in accordance with ref 1; 5.0 g) in $CHCl_3$ (150 mL) was added methyl vinyl ketone (2 g), and the mixture was stirred at room temperature for 5 h. The solvent was evaporated, and the resulting residue was recrystallized from a mixture of $CHCl_3$ and EtOH to give **8** (4.2 g) as colorless needles. ^{1}H NMR ($CDCl_3$) δ: 1.60 (3 H, t, J = 7.0 Hz, NCH_2CH_3), 2.10 (3 H, s, $CH_2CH_2COCH_3$), 2.60–2.80 (8 H, m, piperazine H), 3.24–3.44 (4 H, m, $CH_2CH_2COCH_3$), 4.34 (2 H, q, J = 7.0 Hz, NCH_2CH_3), 6.84 (1 H, d, J = 8.0 Hz, C_8-H), 8.02 (1 H, d, J = 1.35 Hz, C_5-H), 8.64 (1 H, s, C_2-H), 14.82 (1 H, br s, COOH). MS m/e: 389 (M^+).

1-Ethyl-6-fluoro-1,4-dihydro-7-(4-hydroxypiperazin-1-yl)-4-oxoquinoline-3-carboxylic Acid (6). To a solution of **8** (4.60 g) in $CHCl_3$ (150 mL) was added m-chloroperbenzoic acid (2.44 g) with ice cooling, and the reaction mixture was stirred at room temperature for 5 h. The reaction mixture was concentrated under reduced pressure to approximately half of the initial volume, and the resulting precipitate was filtered off and dried. Recrystallization from the mixture of DMF and water gave **6** (2.41 g) as pale yellow needles. ^{1}H NMR (DMSO-d_6) δ: 1.44 (3 H, t, J = 7.0 Hz, NCH_2CH_3), 7.16 (1 H, d, J = 8.0 Hz, C_8-H), 7.70 (1 H, d, J = 14.0 Hz, C_5-H), 8.18 (1 H, s, NOH), 8.88 (1 H, s, C_2-H), 15.04 (1 H, br, s, COOH). MS m/e: 335 (M^+).

1-Cyclopropyl-6-fluoro-1,4-dihydro-7-(4-hydroxypiperazin-1-yl)-4-oxoquinoline-3-carboxylic Acid (7). To a solution of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[4-(3-oxobutyl)piperazin-1-yl]quinoline-3-carboxylic acid (**9**, synthesized in accordance with ref 8; 6.8 g) in $CHCl_3$ (100 mL) was added m-chloroperbenzoic acid (3.5 g) with stirring. The reaction mixture was kept at room temperature overnight and was concentrated under reduced pressure to give a pale yellow residue. To the resulting residue was added a mixture of ethyl acetoacetate and $CHCl_3$ (1:3, v/v), and the mixture was stirred with ice cooling for 1 h. The insoluble substance was filtered off and dried. Recrystallization from a mixture of DMF and water gave **7** (3.1 g) as pale yellow needles. ^{1}H NMR (DMSO-d_6) δ: 1.05–1.50 (4 H, m, cyclopropyl H), 2.40–4.00 (9 H, m, piperazine H and cyclopropyl H), 7.55 (1 H, d, J = 8.0 Hz, C_8-H), 7.86 (1 H, d, J = 14.0 Hz, C_5-H), 8.22 (1 H, s, NOH), 8.62 (1 H, s, C_2-H), 14.91 (1 H, br s, COOH). MS m/e: 347 (M^+).

Acknowledgment. We are indebted to Dr. T. Nose, director of this laboratory, for his support and encouragement. We thank Dr. F. Sakamoto for helpful discussions and Miss S. Okazaki for hearty cooperation.

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