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Preparation and Antibacterial Activity of 3-Methyl-1-*p*-substituted Phenylpyrazole-5-thiol

3-Methyl-1-phenylpyrazole-5-thiol (**3a**) and its *p*-nitro- (**5**) and *p*-fluorophenyl (**8**) derivatives were prepared as potential antimicrobial agents in relatively good yields. Compounds **3a** and **8** showed good antibacterial activities against MRSA, *S. aureus, S. epidermidis, E. faecalis, E. faecium,* and *S. pyogenes.* Moreover, compound **3a** also showed a synergistic effect with some aminoglycosides.

Keywords: 3-Methyl-1-*p*-substituted phenylpyrazole-5-thiol; Antibacterial; Synergistic effect; MRSA; Tautomerism

Received: September 25, 2001 [FP635]

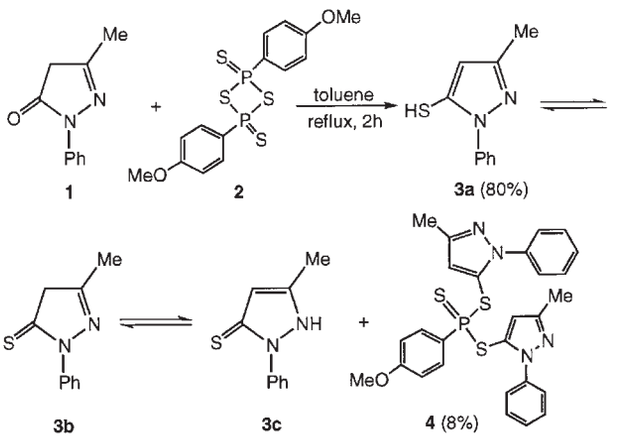
Introduction

Pyrazolone derivatives such as antipyrine, aminopyrine, or sulpyrine have been used clinically for a long time as antipyretic analgesics which affect the central nervous system; however, some serious allergenic and carcinogenic side effects have been found. Recently, through computationally directed broad screening, a novel 1,5-diphenylpyrazole class of HIV-1 non-nucleoside reverse transcriptase inhibitors has been discovered [1]. Thus, we were interested in developing new analogues of pyrazolone or pyrazole derivatives. Among them, 3-methyl-1-*p*-substituted phenylpyrazole-5-thiol was selected on the basis of the random screening using high throughput screening (HTS) techniques for new good antibacterial agents. We now report the preparation of some 3-methyl-1-*p*-substituted phenylpyrazole-5-thiols and the related compounds, and the results of the minimal inhibitory concentration (MIC) and synergy testing for gram-positive bacteria.

Results and Discussion

Chemistry

The most basic structure of this series, 3-methyl-1-phenylpyrazole-5-thiol (**3a**) was prepared in 80% yield by the reaction of commercially available 3-methyl-1-phenyl-5-pyrazolone (**1**) with Lawesson's reagent (**2**), along with (4-methoxyphenyl)-phosphonotrithioic acid bis-(5-methyl-2-phenyl-2*H*-pyrazol-3-yl) ester (**4**) in 8% yield as by-product (Scheme 1).



Scheme 1

In this reaction, the existence of 3 kinds of tautomers **3a**–**3c** is possible, and the ^{1}H- and ^{13}C-NMR spectra in various solvents ($CDCl_3$, C_6D_6, and DMSO-d_6) indicate very strongly that it exists predominantly as the SH form, **3a** [2]. Furthermore, D_2O exchange experiments show that the hydrogen of 4-position has high acidity, in keeping with the experimental finding that sulfonation, bromination, and chlorination of 1-phenylpyrazolin-5-ones all involve 4-substitution before attack on the phenyl ring [3]. The optimal reaction conditions for preparing **3a** were investigated by varying the solvent, reaction temperature, and reaction time: **3a** was obtained in the highest yield (85%) when the reaction mixture was heated at 60 °C for 1 h in benzene.

The nitration of the *para*-position of the phenyl ring at **3a** seems to be difficult because of the above-mentioned highly acidic hydrogen at the 4-position of the pyrazole ring; actually, nitration of 1-phenylpyrazolin-5-ones using nitric acid alone only gives the 4-nitropyrazolinone products [3]. However, we selectively obtained 3-methyl-1-*p*-

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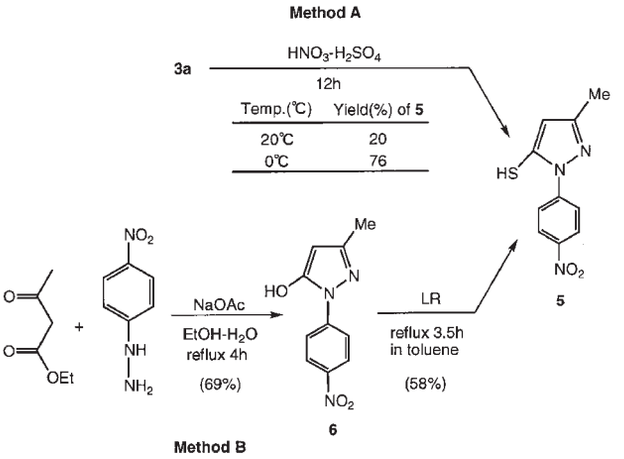
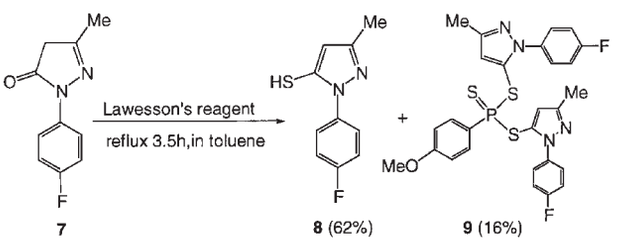


Scheme 2

nitrophenylpyrazole-5-thiol (**5**) in 76% yield by using nitric acid-sulfuric acid at 0°C (Method A) (Scheme 2).

Compound **5** was also obtained in 58% yield by the reaction of Lawesson's reagent with 3-methyl-1-*p*-nitrophenylpyrazol-5-ol (**6**) (69%), which was prepared via ring closure reaction of ethyl acetoacetate and *p*-nitrophenylhydrazine (Method B) (Scheme 2).

We are also interested in the biological activity of the *p*-fluorophenyl derivative of **3a**, so we synthesized 3-methyl-1-*p*-fluorophenylpyrazole-5-thiol (**8**) by the reaction of Lawesson's reagent with 3-methyl-1-*p*-fluorophenyl-5-pyrazolone (**7**), which was obtained via ring closure reaction of ethyl acetoacetate and *p*-fluorophenylhydrazine, along with (4-methoxyphenyl)-phosphonotrithioic acid bis[2-(4-fluorophenyl)-5-methyl-2*H*-pyrazol-3-yl]-ester (**9**) (Scheme 3).



Scheme 3

Pharmacology

The compounds **3a**, **5**, and **8** prepared above were tested *in vitro* against several gram-positive bacterial strains as compared with linezolid (Pharmacia) and vancomycin. MIC values were determined using arranged broth microdilution method and are shown in Table 1. Compounds **3a** and **8** showed an MIC range of 6.3–25 µg/mL which is comparable with that of linezolid. We found that **3a** showed low cytotoxicity of HeLa and COS7 cells by standard MTS assay [4] (IC_{50} > 240 µg/mL) [5]. On the other hand, **3a**, **5**, and **8** showed hardly any antibacterial activity against gram-negative bacteria such as *E. coli* MC1061, *Klebsiella pneumoniae, Proteus vulgaris,* and *Serratia marcescens* (all >100 µg/mL).

Table 1. Antibacterial activity (MIC, µg/mL) of **3a**, **5**, **8**, linezolid and vancomycin.

Compound	*S. aureus*	*S. epidermidis*	*E. faecalis*	*E. faecium*	*S. pyogenes*	MRSA
3a	6.3	6.3	25.0	12.5	12.5	6.3
5	100	>100	>100	>100	>100	>100
8	6.3	25.0	12.5	25.0	25.0	12.5
Linezolid[a)]	3.2	3.2	3.2	12.5	3.2	3.2
Vancomycin	1.6	0.8	1.6	0.8	0.2	1.6

[a] (*S*)-*N*-[3-[3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidiniyl]methylacetamide (Pharmacia).

As **3a** gave the best antibacterial activity result compared to **5** and **8** (Table 1), taking the structural similarity of these 3 compounds into consideration, only the synergistic effect of **3a** in combination with known drugs active against a variety of bacteria was further investigated.
The effects of combinations of the known drugs with **3a** were evaluated by the checkerboard method [6]. As defined by Elion et al. [7], the fractional inhibitory concentration (FIC) is the ratio of the MIC of a drug in the combination to the MIC of the drug alone expressed as a decimal fraction. The FIC index (FICI) is the sum of the FICs for each of the drugs in a particular combination. Drug interaction was classified as "synergism (FICI ≤ 0.5)" and "partial synergism (0.5 < FICI < 1)". When the FICI is less than unity, synergy is suggested [8].
The synergistic activity of **3a** with the known drugs, such as cephems, aminoglycosides, glycopeptide, tetracycline, macrolides, quinolones, and so on against MRSA was examined and **3a** indicated the strong synergy effect with several aminoglycosides (Table 2).
Furthermore, we examined the effects of combination of 4 kinds of aminoglycosides and tetracycline with **3a** against gram-positive bacteria as shown in Table 3.
Compound **3a** has a good synergistic effect against gram-positive bacterial strains tested as shown in

Table 2. FICIs for **3a** and the known drugs in combination against MRSA.

Drugs	FICI
Vancomycin	0.66
Streptomycin	0.69
Gentamycin	0.50
Dibekacin	0.50
Amikacin	0.44
Arbekacin	0.39
Isepamicin	0.31
Tetracycline	0.88
Levofloxacin	0.75
Rifampicin	0.63

Table 3. FICIs for the combination of 4 kinds of aminoglycosides and tetracycline with **3a** against gram-positive bacteria.

Strain	A	B	C	D	E
S. aureus	0.63	0.50	0.50	0.57	0.75
S. epidermidis	0.53	0.52	0.60	0.60	0.57
E. faecalis	0.63	–	–	–	0.63
E. faecium	0.51	0.38	0.57	0.50	1.0
S. pyogenes	0.60	0.42	0.29	0.57	0.63

A = **3a** + Gentamycin, B = **3a** + Amikacin, C = **3a** + Arbekacin, D = **3a** + Isepamicin, E = **3a** + Tetracycline.

Table 3, but had low or no synergistic activity against gram-negative bacterial strains.

Acknowledgement

The authors are grateful to Ms. M. Nakashima, Mr. K. Atarashi, and Dr. K. Ikesue (Fundamental Research Laboratory of Hisamitsu Pharmaceutical Co., Inc.) for their excellent technical assistances.

Experimental

Chemistry

Melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. Spectral data were recorded on the following spectrometers: IR spectra, JASCO FT/IR-470 Plus; ^{1}H-NMR spectra, JEOL GX-400 (400 MHz) and JEOL A-500 (500 MHz); ^{13}C-NMR spectra, JEOL GX-400 (100 MHz) and JEOL A-500 (125 MHz); MS spectra, JMS-HX100 for FAB-MS. The H-COSY, DEPT, and HMQC experiments were also used for the assignments of the structures. The chemical shifts are given on the δ scale. Elemental analyses were performed on a Yanaco CHN CORDER MT-6 instrument. Medium pressure liquid chromatography (MPLC) was carried out with Yamazen 540 FMI-C pump and Wakogel FC-40 (20–40 μm, Wako). Column chromatography was carried out with Kieselgel 60 (70–230 mesh, Merck). High-performance thin layer chromatography (HPTLC) for the yields shown in Table 1 was conducted on Shimadzu high speed thin layer chromatoscanner (CS-9300PC) with the detector set at λ = 254 nm.

Reaction of **1** *with* **2**

A mixture of 3-methyl-1-phenyl-5-pyrazolone (0.5 g, 2.87 mmol) and Lawesson's reagent (0.68 g, 1.69 mmol) in dry toluene (30 mL) was refluxed for 2 hours and the solvent was evaporated. The resulting residue was chromatographed on MPLC to give 3-methyl-1-phenylpyrazole-5-thiol (**3a**) (with the mixed solvent of *n*-hexane:AcOEt = 50:1) and (4-methoxyphenyl)-phosphonotrithioic acid bis-(5-methyl-2-phenyl-2*H*-pyrazol-3-yl) ester (**4**) (with the mixed solvent of *n*-hexane:AcOEt = 1:1). Compound **3a** was recrystallised from *n*-hexane-Et_2O to give colorless needles, mp 111–112 °C. 0.44 g (80%). Anal. Calcd. for $C_{10}H_{10}N_2S$: H, 5.30; C, 63.13; N, 14.72. Found: H, 5.37; C, 63.03; N, 14.60. IR (KBr): ν (cm^{-1}) 3141, 2539, 1559, 1495, 1359, 692. ^{1}H-NMR ($CDCl_3$): δ 2.30 (3H, s, CH_3), 3.36 (1H, s, SH), 6.25 (1H, s, H-4), 7.36–7.39 (1H, m, Ar-H), 7.44–7.47 (2H, m, Ar-H), 7.48–7.52 (2H, m, Ar-H). (C_6D_6): δ 2.21 (3H, s, CH_3), 2.87 (1H, s, SH), 6.01 (1H, s, H-4), 6.96–6.97 (1H, m, Ar-H), 7.04–7.07 (2H, m, Ar-H), 7.48–7.50 (2H, m, Ar-H). (DMSO-d_6): δ 2.21 (3H, s, CH_3), 3.35 (1H, s, SH), 6.41 (1H, s, H-4), 7.21–7.51 (5H, m, Ar-H). ^{13}C-NMR ($CDCl_3$): δ 13.5 (q, CH_3), 111.7 (d, C-4), 125.4 (d, Ar), 127.2 (s, Ar), 127.9 (d, Ar), 128.9 (d, Ar), 139.3 (s, Ar), 149.6 (s, Ar). (C_6D_6): δ 13.6 (q, CH_3), 112.4 (d, C-4), 125.4 (d, Ar), 125.7 (s, Ar), 127.4 (d, Ar), 128.7 (d, Ar), 128.8 (d, Ar), 140.2 (s, Ar), 149.6 (s, Ar). (DMSO-d_6): δ 13.2 (q, CH_3), 114.7 (d, C-4), 124.6 (d, Ar), 125.2 (d, Ar), 125.3 (d, Ar), 127.8 (d, Ar), 128.6 (d, Ar), 128.9 (d, Ar), 138.5 (s, Ar), 148.7 (s, Ar). MS (FAB^+); 191 (M^+ + H). Compound **4** was recrystallised from *n*-hexane-AcOEt to give pale pink prisms, mp 172.5–173.5 °C. 0.06 g (8%). Anal. Calcd. for $C_{27}H_{25}N_4OPS_3$: H, 4.59; C, 59.10; N, 10.21. Found: H, 4.68; C, 59.09; N, 10.04. IR (KBr): ν (cm^{-1}) 3071, 2918, 2360, 1589, 1518, 1496, 1261, 1096. ^{1}H-NMR ($CDCl_3$): δ 2.30 (6H, s, CH_3 × 2), 3.85 (3H, s, OCH_3), 6.30 (2H, s, H-4 × 2), 6.74–6.76 (2H, dd, *J* = 3.4 Hz, Ar-H), 7.31–7.33 (10H, m, Ar-H), 7.48–7.53 (2H, dd, *J* = 9.0 Hz, Ar-H). ^{13}C-NMR ($CDCl_3$): δ 13.7 (q, CH_3), 55.5 (q, OCH_3), 113.9 (d, Ar), 114.0 (d, Ar), 116.2 (d, C-4), 116.3 (d, C'-4), 124.7 (d, Ar), 125.8 (d, Ar), 125.8 (d, Ar), 126.1 (d, Ar), 127.8 (d, Ar), 128.5 (d, Ar), 133.2 (d, Ar), 133.3 (d, Ar), 139.0 (s, Ar), 149.5 (s, Ar), 163.2 (s, Ar). MS (FAB^+); 549 (M^+ + H).

3-Methyl-1-(p-nitrophenyl)pyrazole-5-thiol (**5**) – *method A*

Compound **3a** (0.5 g, 2.63 mmol) was dissolved in sulfuric acid (20 mL) and the solution was cooled to –10 °C. Then nitric acid (0.24 g, 3.73 mmol) was added to the solution and the reaction mixture was stirred 12 hours at 0 °C. After the reaction, the mixture was added to ice to give the yellow precipitate which was recrystallised from *n*-hexane-AcOEt to give yellow powder, mp 165 °C, 0.47 g (76%). Anal. Calcd. for $C_{10}H_9N_3O_2S$: H, 3.86; C, 51.05; N,17.86. Found: H, 3.64; C, 51.14; N, 17.84. IR (KBr): ν (cm^{-1}) 3125, 2360, 1594, 1519, 1340, 854. ^{1}H-NMR ($CDCl_3$):

δ 1.55 (1H, s, SH), 2.28 (3H, s, CH_3), 6.35 (1H, s, H-4), 7.67 (2H, d, *J* = 9.2 Hz, Ar-H), 8.26 (2H, d, *J* = 9.2 Hz, Ar-H). ^{13}C-NMR ($CDCl_3$): δ 13.5 (q, CH_3), 117.5 (d, C-4), 124.3 (d, Ar), 124.7 (d, Ar), 133.5 (s, Ar), 143.8 (s, Ar), 146.4 (s, Ar), 151.1 (s, Ar). MS (FAB$^+$); 236 (M^+ + H).

Method B

A mixture of 3-methyl-1-(*p*-nitrophenyl)pyrazol-5-ol (**6**) (0.5 g, 2.28 mmol) and Lawesson's reagent (0.54 g, 1.35 mmol) in dry toluene (20 mL) was refluxed for 3.5 hours and the solvent was evaporated. The residue was chromatographed on MPLC to give **5** (with the mixed solvent of *n*-hexane :AcOEt = 10:1), 0.31 g (58%).

3-Methyl-1-(p-nitrophenyl)pyrazol-5-ol (**6**)

A mixture of ethyl acetoacetate (2.55 g, 19.6 mmol), 4-nitrophenylhydrazine (3.0 g, 19.6 mmol) and sodium acetate (1.0 g, 12.1 mmol) in 50% ethanol (90 mL) was refluxed for 4 hours. After the reaction, on cooling to room temperature a brown precipitate appeared. The precipitate was filtered off and recrystallised from methanol to give **6**, yellow prisms, mp 223–224 °C, 2.96 g (69%). Anal. Calcd. for $C_{10}H_9N_3O_3$: H, 4.14; C, 54.79; N, 19.17. Found: H, 4.30; C, 54.61; N, 18.93. IR (KBr): ν (cm^{-1}) 3124, 2360, 1627, 1580, 1507, 1333, 786. ^{1}H-NMR (DMSO-d_6): δ 2.16 (3H, s, CH_3), 5.43 (1H, s, H-4), 8.06 (2H, d, *J* = 9.2 Hz, Ar-H), 8.30 (2H, d, *J* = 9.2 Hz, Ar-H), 10.5–12.0 (1H, OH). ^{13}C-NMR (DMSO-d_6): δ 13.9 (q, CH_3), 89.1 (d, C-4), 117.2 (d, Ar), 118.9 (d, Ar), 124.8 (d, Ar), 143.2 (s, Ar), 143.8 (s, Ar). MS (FAB$^+$); 220 (M^+ + H).

3-Methyl-1-(p-fluorophenyl)-5-pyrazolone (**7**)

A mixture of ethyl acetoacetate (4.0 g, 30.7 mmol), 4-fluorophenylhydrazine hydrochloride (5.0 g, 30.7 mmol), and sodium acetate (1.55 g, 18.9 mmol) in 50% ethanol (150 mL) was refluxed for 3 hours. After the reaction, the solvent was evaporated and the residue was extracted with Et_2O. The residue from the Et_2O extract was recrystallised from *n*-hexane-Et_2O to give **7**, pale brown powder, mp 151–152 °C, 3.83 g (65%). Anal. Calcd. for $C_{10}H_9N_2OF$: H, 4.72; C, 62.49; N, 14.58. Found: H, 4.68; C, 62.47; N, 14.49. IR (KBr): ν (cm^{-1}) 1622, 1506, 1404, 1333, 1224, 823, 781. ^{1}H-NMR ($CDCl_3$): δ 2.18 (3H, s, CH_3), 3.41 (2H, s, H-4), 7.05–7.08 (2H, m, Ar-H), 7.81–7.84 (2H, m, Ar-H). ^{13}C-NMR ($CDCl_3$): δ 16.9 (q, CH_3), 43.0 (t, C-4), 115.4 (d, Ar), 115.5 (d, Ar), 120.6 (d, Ar), 120.6 (d, Ar), 134.3 (s, Ar), 134.3 (s, Ar), 156.3 (s, Ar), 158.9 (s, Ar), 160.9 (s, Ar), 170.3 (s, Ar). MS (FAB$^+$); 193 (M^+ + H).

Reaction of **7** *with Lawesson's reagent*

A mixture of **7** (0.62 g, 3.23 mmol) and Lawesson's reagent (0.77 g, 1.91 mmol) in dry toluene (18 mL) was refluxed for 3.5 hours and the solvent was evaporated. The residue was chromatographed on MPLC to give 3-methyl-1-(*p*-fluorophenyl)pyrazole-5-thiol (**8**) (with the mixed solvent of *n*-hexane: AcOEt = 20:1) and (4-methoxyphenyl)-phosphonotrithioic acid bis[2-(4-fluorophenyl)-5-methyl-2*H*-pyrazol-3-yl] ester (**9**) (with the mixed solvent of *n*-hexane:AcOEt = 1:1). Compound **8** was distilled under reduced pressure to give dark yellow viscous oil, 0.42 g (62%). Anal. Calcd. for $C_{10}H_9N_2SF$: H, 4.36; C, 57.68; N, 13.45. Found: H, 4.15; C, 57.76; N, 13.43. IR (neat): ν (cm^{-1}) 2928, 1511, 1365, 1226, 839, 605. ^{1}H-NMR ($CDCl_3$): δ 2.28 (3H, s, CH_3), 3.34 (1H, s, SH), 6.25 (1H, s, H-4), 7.05–7.16 (2H, m, Ar-H), 7.26–7.49 (2H, m, Ar-H). ^{13}C-NMR ($CDCl_3$): δ 13.5 (q, CH_3), 111.9 (d, C-4), 114.9 (d, Ar), 115.7 (d, Ar), 115.8 (d, Ar), 127.2 (d, Ar), 127.3 (d, Ar), 133.7 (s, Ar), 135.1 (s, Ar), 135.3 (s, Ar), 135.4 (s, Ar), 149.5 (s, Ar), 161.0 (s, Ar), 162.9 (s, Ar). MS (FAB$^+$); 209 (M^+ + H). Compound **9** was recrystallised from Et_2O to give colorless prisms, mp 121–122 °C, 0.25 g (16%). Anal. Calcd. for $C_{27}H_{23}F_2N_4OPS_3$: H, 3.97; C, 55.47; N, 9.58. Found: H, 4.09; C, 55.39; N, 9.40. IR (KBr): ν (cm^{-1}) 3122, 2928, 2361, 1592, 1509, 1229, 1101, 835. ^{1}H-NMR ($CDCl_3$): δ 2.30 (6H, s, CH_3 × 2), 3.86 (3H, s, OCH_3), 6.31 (2H, s, H-4 × 2), 6.78–6.80 (2H, dd, *J* = 3.7 Hz, Ar-H), 6.98–7.00 (4H, m, Ar-H), 7.26–7.31 (4H, m, Ar-H), 7.50–7.55 (2H, dd, *J* = 9.0 Hz, Ar-H). ^{13}C-NMR ($CDCl_3$): δ 13.7 (q, CH_3), 55.5 (q, OCH_3), 114.0 (d, Ar), 115.3 (d, Ar), 115.4 (d, Ar), 116.4 (d, C-4), 116.4 (d, C'-4), 124.7 (s, Ar), 125.4 (s, Ar), 126.0 (s, Ar), 128.0 (d, Ar), 133.2 (d, Ar), 135.0 (s, Ar), 149.6 (s, Ar), 161.0 (s, Ar), 162.9, (s, Ar), 163.4 (s, Ar). MS (FAB$^+$): 585 (M^+ + H).

Pharmacology

Antibacterial Agents

Streptomycin, Dibekacin, Amikacin, Tetracycline, Rifampicin were purchased from Sigma-Aldrich, Inc. Vancomycin, Gentamycin were purchased from Wako Pure Chemical Industries, Ltd. Arbekacin (Meiji Seika), Isepamicin (Schering Plough), Levofloxacin (Daiichi), were prepared from pharmaceutical solutions. Linezolid (Pharmacia), (*S*)-*N*-[3-[3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidiniyl]methyl-acetamide was synthesized.

Antibacterial Activity Assays

The synthesized pyrazole derivatives were screened for their *in vitro* activities against MRSA (methicillin-resistant *Staphylococcus aureus*) (Chiba University, Clinical isolate), *Staphylococcus aureus* (IFO127232 (ATCC6538P)), *Staphylococcus epidermidis* (JCM2414 (ATCC14990)), *Enterococcus faecalis* (JCM7738 (ATCC29212)), *Enterococcus faecium* (JCM5804 (ATCC19434)), *Streptococcus pyogenes* (JCM5674 (ATCC12344)) and *Escherichia coli* (IFO3972 (ATCC8739)), *Klebsiella pneumoniae* (JCM1662 (ATCC13883)), *Proteus vulgaris* (JCM1668 (ATCC13315)), *Serratia marcescens* (JCM1239 (ATTC13880)), *Pseudomonas aeruginosa* (IFO13275 (ATCC9027)). The MICs of the compounds were determined by the Japanese Society of Chemotherapy Standards broth microdilution method [9]. The compounds dissolved in DMSO (final concentration; 1%) were tested at different concentrations (from 100 to 0.05 μg/mL). The MICs were measured after 20 hours' incubation at 35 °C.

Checkerboard method

Interactions between antibacterial agents can be studied in vitro using checkerboard titrations with serial doubling dilutions. Antibacterial agents and **3a** dissolved in DMSO (final concentration; 1%) were tested at different concentrations (antibacterial agents: from 0.2 to 200 μg/mL; **3a**: from 3.2 to 200 μg/mL). Each was made to cross as **3a** in a vertical direction and the antibacterial agents in a horizontal direction. Then bacterial solution (ca. 107 cells/mL, 100 μL) was added. The FICIs were measured after 20 hours' incubation at 35 °C.

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