# Cytotoxicity and Antimicrobial Activity of Some Naphthol Derivatives

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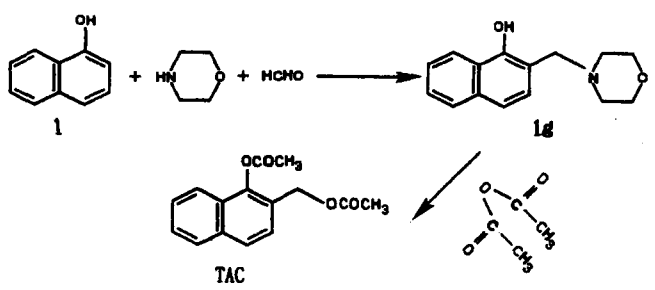
2-Hydroxymethyl-1-naphthol diacetate (TAC) and sixteen *Mannich* base derivatives of naphthol were prepared and examined for cytotoxicity and antimicrobial activity. Cytotoxicity was examined against four human carcinoma cell lines. Several derivatives were effective at concentrations < 4 μg/ml. TAC showed the highest cytotoxicity. Inhibition of DNA-, RNA-, and protein synthesis by TAC was also studied and discussed. TAC also exhibits potent antimicrobial activity against *Enterobacter clocae* 23355, *Klebsiella pneumonia* 13883, *Proteus vulgaris* 13315, *Pseudomonas aeruginosa* 27853, *Candida parapsilosis*, *Candida tropicalis*, *Trichosposon beigelli*, and *Rhodotorul spp.* with minimum inhibitory concentrations of 0.1 ~ 0.4 μM. These results indicate that esterification by *Bruson* reaction of 1-naphthol *Mannich* base to TAC enhances the cytotoxicity and antimicrobial activity.

**Zytotoxische und antimikrobielle Wirkung von Naphthol-Derivaten**
2-Hydroxymethyl-1-naphthol (TAC) und die Naphtholderivate **1-1i** und **2-2i** wurden hergestellt und auf ihre zytotoxische und antimikrobielle Wirkung geprüft. Die Zytotoxizität wurde an vier Karzinom-Zellinien des Menschen untersucht. Einige Verbindungen sind bei < 4 μg/ml wirksam, TAC zeigte die stärkste Zytotoxizität. Die Hemmwirkung von TAC auf die DNA-, RNA- und Protein-Synthese wurde untersucht. TAC wirkt auch antimikrobiell auf *Enterobacter clocae* 23355, *Klebsiella pneumonia* 13883, *Proteus vulgaris* 13315, *Pseudomonas aeruginosa* 27853, *Candida parapsilosis*, *Candida tropicalis*, *Trichosposon beigelli*, und *Rhodotorul spp.*. Die kleinsten Hemmkonzentrationen betragen 0.1-0.4 μM

Chemotherapy plays an important role in treating cancer, although its use is often palliative rather than curative[1)]. The basic limitation of chemotherapy is the physiological similarity between normal and tumor cells. 1-Naphthol has been shown to exhibit selective toxicity in short-term organ cultures of human colonic tumor tissue as compared to normal tissue from the same patients[2,3)]. A significant increase in life span was also observed in animals bearing *Ehrlich* ascites tumor cells after 1-naphthol treatment[4)]. A possible mechanism of 1-naphthol action may involve the formation of naphthoquinone which is known to be a cytotoxic agent and has been used as an antitumor agent[2,5)].

*Mannich* bases of several compounds exhibit antifungal or antitumor activities[6-8)]. These findings encouraged us to prepare the *Mannich* bases of 1-naphthol and 2-naphthol, and to investigate the role of the 1- and 2-positions on the naphthalene molecule. In addition, 1-naphthol is known to be metabolized at the 1-hydroxy group to form conjugates with sulfate ester and glucuronic acid[3,9-12)]. Protection of the hydroxy group by esterification may increase the duration and activity of the modified compound. This was examined by synthesizing 2-hydroxymethyl-1-naphthol diacetate (TAC) as well as several *Mannich* bases of 1-naphthol. The cytotoxicity of the derivatives to different cancer cell lines was investigated as well as their effects on cellular synthesis of protein, RNA and DNA.




**Scheme 1**

In a precedent work, we have investigated the *Mannich* base derivatives of naphthol as potential antimicrobial agents[13)]. In order to determine whether esterification by *Bruson* reaction of 1-naphthol *Mannich* base to 2-hydroxymethyl-1-naphthol diacetate (TAC) would ameliorate the pharmacological response, the antibacterial and antifungal activity of this compound was also examined.

## Results and Discussion

The cytotoxic activities of the *Mannich* bases **1a-i**, **2a-i**, and TAC (Scheme 1) against *Colo 205*, *Hela*, *Hep-2*, and *Kb cell* lines are given in Table 1. According to *Geran*[14)], results are significant when the $IC_{50}$ value is lower than 4 μg/ml. Highest activities against *Colo 205* were obtained with TAC and compounds **2a**, **1h**, whereas TAC and compounds **1g**, **1h**, **2b**, **2h**, **2i**, showed activity against *Hela cells*. Only TAC was effective against *Kb cells*. *Hep-2* was resistant to all compounds, although TAC showed the highest activity with an $IC_{50}$ value of 6.0 μg/ml. These data indicate that naphthol cytotoxicity towards *Colo 205* and *Hela cells* is enhanced by esterification by *Bruson* reaction of naphthol to TAC or with the *Mannich* bases of naphthol. The cytotoxicities of the naphthol derivatives against *Hep-2* and *Kb* were not greatly increased compared to the parent compound 1-naphthol. Overall, TAC is more cytotoxic to all cells as compared with the *Mannich* bases of naphthol.

Two metabolites of 1-naphthol, 1,2-naphthoquinone or 1,4-naphthoquinone, showed greater cytotoxicity than 1-

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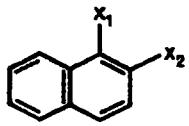
**Table 1:** Cytotoxicity of 1-naphthol (**1**) and 2-naphthol (**2**) derivatives



| Compound | | | $IC_{50}$($\mu$g/ml) | | | |
|---|---|---|---|---|---|---|
| NO | $X_1$ | $X_2$ | Colo 205 | Hela | Hep-2 | KB |
| **1** | OH | H | 28.0 | 10.7 | 13.2 | 13.0 |
| **1a** | OH | $CH_2N(CH_3)_2$ | 6.9 | 8.2 | 14.0 | 14.0 |
| **1b** | OH | $CH_2N(C_2H_5)_2$ | 9.1 | 7.3 | 26.0 | 16.2 |
| **1c** | OH | $CH_2N(C_3H_7)_2$ | 11.0 | 22.0 | 29.2 | 12.6 |
| **1d** | OH | $CH_2N(C_4H_9)_2$ | 18.0 | 15.1 | 28.0 | 20.3 |
| **1e** | OH | $CH_2N(CH_2CH{=}CH_2)_2$ | 10.0 | 18.9 | ND | 20.5 |
| **1f** | OH | $CH_2N$  $\cdot H_2O$ | 19.2 | 30.5 | 36.5 | ND |
| **1g** | OH | $CH_2N$  | 10.0 | 1.2 | 12.6 | 20.6 |
| **1h** | OH | $CH_2N$  | 3.0 | 4.1 | 11.4 | ND |
| **1i** | OH | $CH_2N$  | 10.0 | 7.7 | 12.7 | 21.0 |
| TAC | $OCOCH_3$ | $CH_2OCOCH_3$ | 2.7 | 3.1 | 6.0 | 3.0 |
| **2** | H | OH | 20.0 | 19.9 | 18.9 | 10.3 |
| **2a** | $CH_2N(CH_3)_2$ | OH | 4.0 | 10.7 | 18.7 | 13.1 |
| **2b** | $CH_2N(C_2H_5)_2$ | OH | 15.2 | 4.0 | 18.2 | 10.7 |
| **2c** | $CH_2N(C_3H_7)_2$ | OH | 18.0 | 9.7 | 13.7 | 26.4 |
| **2d** | $CH_2N(C_4H_9)_2$ | OH | 18.5 | 19.0 | 31.0 | 22.6 |
| **2g** | $CH_2N$ | OH | 8.0 | 7.1 | 19.5 | 17.6 |
| **2h** | $CH_2N$ | OH | 14.0 | 4.0 | ND | ND |
| **2i** | $CH_2N$ | OH | 7.0 | 4.0 | 12.2 | 27.0 |

ND: not determine

*naphthol* to colon cell and hepatocytes[3,15]. Naphthoquinones may mediate toxicity by direct covalent binding or by acting as semiquinone radicals to the cell[16]. Likewise, naphthalene is metabolized *in vivo* to 1,2-dihydroxynaphthalene[17] which may be oxidized to 1,2-naphthoquinone[17,18].

The 1-hydroxyl group of 1-naphthol forms a sulfate and conjugates with glucuronic acid. The selective toxicity of 1-naphthol may be mediated in part through accumulation of unconjugated 1-naphthol in tumor tissue with impaired xenobiotic detoxification function[3,10-12]. The esterification of the hydroxyl group should impair the conjugation of 1-naphthol to the sulfate ester or glucuronic acid and may increase passage through membranes due to increased lipophilicity. Thus, TAC is expected to accumulate in tumor cells. Hydrolysis of the ester group and subsequent formation of naphthoquinones in tumor cells could explain the increased potency of TAC compared to 1-naphthol *in vitro*. The isomers of 1-naphthol derivatives, namely the *Mannich* bases of 2-naphthol, show similar cytotoxic effects (Table 1). 1-Naphthol derivatives may metabolize to 1,4-naphthoquinones instead of 1,2-naphthoquinones because the 2-position is blocked by the basic side chain. 2-Naphthol derivatives, in contrast, are unable to produce 1,2-naphthoquinone or 1,4-naphthoquinone. This suggests that the cytotoxic effects of 2-naphthol derivatives are not mediated through quinone metabolites, and act by a different mechanism.

Earlier work from *Wilson et al.*[3] showed that 1-naphthol inhibited protein synthesis in *Colo 206* and *Lovo* cells. In the present study, TAC was the most potent compound against colon carcinoma cells (Table 1). TAC was further examined for its ability to inhibit DNA, RNA and protein synthesis in *Colo 205*, *Hep G2* and *CHA* cells.

The effect of 1-naphthol, **1a**, and TAC on the incorporation of [$^3$H]leucine into protein is summarized in Table 2. TAC almost completely inhibited protein synthesis in *Colo 205* cells at 56 µM (14.5 µg/ml). 1-Naphthol and **1a**, however, did not inhibit protein synthesis even at 500 µM. This result confirms the high potency of TAC as compared to 1-naphthol.

DNA damage induced by naphthol has never been reported. DNA determination closely reflects the number of cells undergoing growth inhibition either due to an inhibition of cell division or cell death[19]. The results showed that 50% DNA synthesis in *Colo 205* cells was inhibited by 222 µM (32 µg/ml) of 1-naphthol and 64.6 µM (16.6 µg/ml) of TAC. *CHA* rat hepatoma cells were more sensitive to TAC with $IC_{50}$ for DNA synthesis inhibition of 18.3 µM (4.7 µg/ml) compared to 64.6 µM (16.6 µg/ml) for *Colo 205*

**Table 2:** Effect of 1-naphthol, **1a** and TAC on protein synthesis in Colo 205 cells

| | Protein Synthesis[a] (% control) | | |
|---|---|---|---|
| Dose ($\mu$M) | 1-naphthol | **1a** | TAC |
| 2 | 94±2 | 104±4 | 99±3 |
| 6 | 94±3 | 95±1 | 104±7 |
| 19 | 102±1 | 101±4 | 114±7 |
| 56 | 106±1 | 96±2 | 0.25±0 |
| 167 | 126±7 | 124±4 | 0 |

a: Each experiment was performed in triplicate. Values represent the mean ± S.D.

**Table 3:** The effect of TAC on DNA and RNA syntheses in CHA cells after different incubation times

| | $IC_{50}$[a] ($\mu$M) | | |
|---|---|---|---|
| | 24hr | 48hr | 72hr |
| DNA synthesis | 18.3±1.2 | 45.1±2.6 | 32.5±1.9 |
| RNA synthesis | 17.4±1.8 | 19.9±1.4 | 20.4±0.8 |

a: Each experiment was performed in triplicate. Values represent the mean ± S.D.

after exposure to drug for 24 h. Increasing the drug exposure time to 48 h or 72 h in *CHA* cell had little effect on inhibition of DNA- or RNA-synthesis by TAC (Table 3). Overall, these data suggest that modification of 1-naphthol by esterification by *Bruson* reaction improved drug cytotoxicity and may explain that the cytotoxicity of TAC was mediated in the inhibition of nucleoside synthesis in *CHA* cell. It may also imply an alternative mechanism of action for the compounds tested since the $IC_{50}$ values for these activities are higher than the $IC_{50}$ values against *Colo 205* cells.

Antimicrobial activity of the *Mannich* base of naphthols was tested[13]. Morpholino derivatives (**1g**, **2g**) of the naphthols were the most active compounds with MIC of around $10^{-7}$M against three to seven strains of the bacteria tested. **2g** was sent to the National Cancer Institute, USA, for anti-HIV using NCI protocol[20] test. This compound is moderately active (Fig. 1). The therapeutic index range was 2.5-3.3. The antibacterial activity of TAC was similar to **2g** against gram-negative strains of *Enterobacter clocae* 23355, *Klebsiella pneumonia* 13883, *Proteus vulgaris* 13315, and *Pseudomonas aeruginosa* 27853 which is an important nosocomial pathogen and resistant to many antimicrobial agents (Table 4). The number of organisms inhibited by the compounds with MIC from 0.1 to 0.5 μM is shown as the top right under the compounds. Comparison of the activities of TAC with 1-naphthol, ampicillin (AM) and kanamycin (KM) is shown in Table 4. The result showed that esterification by *Bruson* reaction of 1-naphthol to TAC increase antibacterial activity. Probably the hydrophobicity of TAC would relate to its permeability through higher lipid content of cell wall by gram-negative bacteria and might be associated with the potent antibacterial activity. Furthermore, the inhibition of opportunistic mycoses of TAC was examined (Table 5). Both, 1-naphthol and TAC are quite active against four and five different strains of the fungi tested, while the positive control amphotericin B inhibitd only three of the organisms. If cell-mediated immunity is impaired, opportunistic mycoses produce progressive systemic disease[21], *e.g.* in patients with AIDS. These

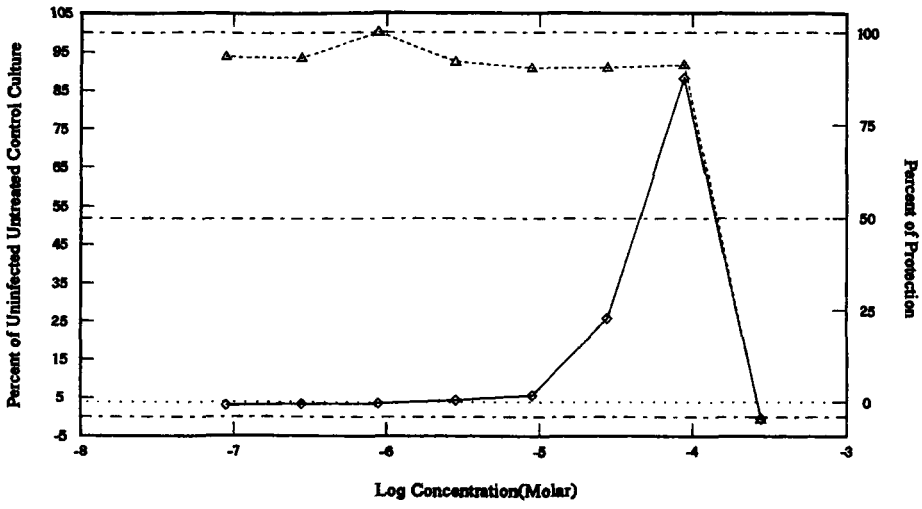



**Fig. 1.** Dose response curves of anti-HIV activity by **2g**. The solid line connecting the diamond symbols (◇-◇) depicts the percentage of surviving HIV-infected cells (CEM-IW) treated with **2g** relative to uninfected untreated controls. The dashed line connecting the triangular symbols (Δ···Δ) depicts the percentage of surviving uninfected cells treated with **2g** relative to uninfected, untreated controls. A dotted reference line (····) indicates the viral cytopathic effect. -·-·- indicates the reference line. Testing was performed at the Developmental Therapeutics Program, National Cancer Institute U.S.A.

**Table 4:** Minimum Inhibitory Concentration (μM)[a] of Antibacterial Activity for Standard Drugs and TAC

| Strain | TAC | 1 | AM | KM |
|---|---|---|---|---|
| NO. Active (MIC= 0.1-0.5) | 4 | 0 | 4 | 3 |
| Bacillus subtilis 6633 | 145 | 173 | 1.11 | 10.73 |
| Staphylococcus aureus 25923 | 387 | 694 | 0.28 | 1.34 |
| Staphylococcus epidermidis 12228 | 96.99 | 43.40 | 0.54 | 0.17 |
| Escherichia coli 25922 | 24.22 | 86.80 | 17.88 | 0.32 |
| Escherichia coli 35218 | 193 | 434 | 17.88 | 0.32 |
| Enterobacter clocae 23355 | 0.34 | 21.73 | 143 | 85.91 |
| Enterobacter aerogenes 27853 | 1.55 | 347 | 143 | 85.91 |
| Klebsiella oxytoca 8724 | 6.05 | 520 | 8.92 | 42.95 |
| Klebsiella pneumonia 13883 | 0.19 | 13.54 | 17.88 | 85.91 |
| Proteus vulgaris 13315 | 0.38 | 374 | 17.88 | 21.47 |
| Pseudomonas aeruginosa 27853 | 0.19 | 13.54 | 8.92 | 21.47 |
| Serratia marcescens 8100 | 1.55 | 86.80 | 0.02 | 5.36 |
| Salmonella typhimurium 29629 | 193 | 43.40 | 0.02 | 2.68 |
| Shigella flexneri 12022 | 193 | 173 | 17.88 | 21.47 |

MICs expressed as μM. AM: Ampicillin. KM: Kanamycin. 1: Naphthol
a: Experiments were performed in duplicate and repeated

**Table 5:** Minimum Inhibitory Concentration (μM)[a] of Antifungal Activity for Standard Drugs and TAC

| Strain | TAC | 1 | AP |
|---|---|---|---|
| NO. Active (MIC= 0.1-0.5) | 5 | 4 | 3 |
| Candida albicans | 15.11 | 108 | 1.62 |
| Candida krusei | 15.11 | 108 | 1.62 |
| Candida parapsilosis | 0.11 | 0.20 | 3.25 |
| Candida tropicalis | 0.23 | 0.20 | 0.25 |
| Trichosporon beigelli ATCC 28592 | 0.19 | 0.20 | 0.06 |
| Trichosporon beigelli | 0.19 | 0.20 | 0.06 |
| Rhodotorula spp. | 0.34 | 0.83 | 3.25 |

MICs expressed as μM. AP: Amphotericin B.
a: Experiments were performed in duplicate and repeated

results encourage further studies of TAC or related compounds.

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## Experimental Part

Melting points: MP-3 micromelting apparatus, uncorrected.- IR spectra: Shimadzu IR-408, spectrophotometer.- NMR spectra: Varian T-300 spectrometer at the National Chum-Shan University, Kaohsiung, δ (ppm), with tetramethylsilane as internal standard.- Mass spectra: Jeol JMS-HX 110 mass spectrometer, National Chum-Shan University, Kaohsiung.- Elemental analysis: Heraeus Elemental Analyzer, National Cheng-Kung University, Tainan.- TLC: precoated silica gel $F_{254}$ plates (20 x 20 cm; 0.2 mm).

*Mannich bases* **1a-i, 2a-i**

Compounds were prepared by reaction of 1-naphthol or 2-naphthol with various secondary amines and formaldehyde in ethanol at room temp.[13].

*2-Hydroxymethyl-1-naphthol diacetate (TAC)*

10 g of 2-morpholinylmethyl-1-naphthol (**1g**) was dissolved in 30 ml of acetic anhydride and refluxed for 1.5 h[22]. Afterwards, the mixture was poured into 200 ml of cold water with stirring. Recrystallization from

ether/*n*-hexane gave 35% TAC, mp. 62~64°C.- IR (KBr): 3059; 2066; 1734; 1618; 844; 810; 621 $cm^{-1}$.- $^1$H-NMR ([$D_6$]DMSO): δ = 2.1 (s, 3H, $CH_2OCOC\underline{H}_3$), 2.4 (s, 3H, $ArOCOC\underline{H}_3$), 5.6 (s, 2H, Ar-$C\underline{H}_2$-O), 8.1 (m, 6H, Ar-H).- MS: m/z (%) = 258 (21; $M^{+\cdot}$), 216 (36), 156 (95), 128 (100).- $C_{15}H_{14}O_4$ (258.3) Calcd. C 69.8 H 5.50 Found C 69.8 H 5.43.

**Biological Studies**

*Reagents and cells:* [$^3$H]-leucine, [$^3$H]-thymidine, and [$^3$H]-uridine (50 Ci/mmol): ICN Biomedicals Inc., Costa Mesa, CA, USA.- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Sigma chemical company, St. Louis, MO, USA.- AS-30D rat hepatoma cell line[23] was generously provided by Dr. *J.P. Chang*, Institute of Zoology, Academia Sinica, R.O.C.- *Hep G2* human hepatoma and *Colo 205* human colon carcinoma cells: ATCC, Rockville, MD, USA.- *Hep-2*, *Kb* and *Hela* cells: National Cancer Institute of the United States.- Human cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. *CHA* cells were maintained in DMEM medium supplemented as above.

*Inhibition of protein, DNA and RNA synthesis:* Protein-, DNA-, and RNA synthesis of cell cultures were measured following the method of *Roffler et al.*[24]. Briefly, *Colo 205*, *Hep G2* or *CHA* cells were plated overnight in 96 well microtiter plates at 20,000 cells per well. Serial dilutions of TAC or 1-naphthol in medium containing 5% fetal calf serum were added to cells for 24 h at 37°C. Cells were subsequently washed once with sterile PBS and incubated for 2 h with [$^3$H]-leucine (1 µCi/well) in leucine-free medium for protein synthesis measurement or medium containing 1 µCi/well of [$^3$H]-thymidine or [$^3$H]-uridine for DNA and RNA synthesis determinations, respectively. Radioactivity of trichloroacetic acid precipitated and washed protein, RNA or DNA were measured in a Beckman LS 6000 series liquid scintilation counter.

*In vitro Cytotoxicity Testing:* Compounds were tested for their *in vitro* cytotoxicity against four different types of carcinoma cell lines derived from human carcinoma of the nasopharynx (*KB*), cervical carcinoma (*Hela*), colon carcinoma (*Colo-205*) and larynx carcinoma (*Hep-2*). The assay procedure has been described[25,26]. Tumor cells (1.6 x $10^4$ per ml) were cultured at 37°C in a 96-well microplate containing glutamine, 100 U/ml penicillin G potassium and 100 µg/ml streptomycin in a humidified atmosphere containing 5% $CO_2$. The cells were then treated with test compounds in graded concentrations, and incubated for 4 days. At the end of the incubation period, MTT (5 mg/ml) was added to each well and incubated for another 4 to 6 h. The optical density of each well was measured at 545 and 690 nm using a Titertek Multisk plate reader. The cytotoxicity index of the tested compounds were calculated as follows:

$$\text{Cytotoxicity index} = [1 - \frac{A_{545}(\text{tested}) - A_{690}(\text{tested})}{A_{545}(\text{control}) - A_{690}(\text{control})}] \times 100$$

$IC_{50}$ was determined by plotting the cytotoxicity index *versus* the concentration of the tested compound.

*Antimicrobial Activity:* Stock solutions were prepared in 95% ethanol. Control tests showed that the final content of ethanol did not interfere in the assay. Antibacterial and antifungal activities were assayed by the agar dilution method[27] using *Mueller-Hinton* agar (DIFCO, Michigan, USA) and yeast morphology agar (DIFCO, Michigan, USA), respectively.

Tested organisms: *Bacillus subtilis* (ATCC 6633), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *Escherichia coli* (ATCC 35218), *Enterobacter clocae* (ATCC 23355), *Enterobacter aerogenes* (ATCC 27853), *Klebsiella oxytoca* (ATCC 8724), *Klebsiella pneumonia* (ATCC 13883), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 27853), *Serratia marcescens* (ATCC 8100), *Salmonella typhimurium* (ATCC 29629), and *Shigella flexneri* (ATCC 12022).- Yeasts: *Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Trichosporon beigelli* (ATCC 28592), *Trichosporon beigelli*, and *Rhodotorula spp.*, generously provided by Dr. *C.F. Peng*, Kaohsiung Medical College, Department of Microbiology. Ampicillin (Sigma, USA) and kanamycin (Sigma, USA) were used as positive controls for gram positive and gram negative bacteria, respectively. Amphotericin B (Sigma, USA) acted as positive control for fungus assays. Minimal concentrations required to inhibit visible growth of an overnight inoculant of $10^8$/ml organisms was designated as the MIC expressed as µM.

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