Regular Article

Synthesis and Antimicrobial Activity of Nitrobenzyl-oxy-phenol Derivatives

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Two hydroquinone derivatives were prepared and their antimicrobial activity evaluated. Their minimum inhibitory concentrations (MICs) were determined using a broth dilution method. Gentamycin and ciprofloxacin were used as reference antibiotics. The antimicrobial activity of 4-(benzyloxy)phenol (monobenzone) was also evaluated based on its structural similarity to the new compounds; activity was comparable to that of 3,5-dimethyl-4-((4-nitrobenzyl)oxy)phenol (4a). 2,3,5-Trimethyl-4-((4-nitrobenzyl)oxy)phenol (4b) exhibited the best antibacterial activity against both clinical isolates and type strain of *Moraxella catarrhalis* (*M. catarrhalis*), with a MIC value of 11 μM, comparable to ciprofloxacin 9 μM.

Key words phenol; monobenzone; *Moraxella catarrhalis*; antimicrobial activity

The chemistry and biological properties of phenolic compounds are of profound interest. Phenolic compounds are a very large and diverse that includes phenols, hydroquinones, coumarins, flavonoids, tannins and polymers.[1] They are amongst the most widespread classes of metabolite in nature.[2,3] They are not only found in bacteria, plants and arthropods but are also identified in the active site of lysyl oxidase from bovine aorta.[4] In this context, chemical and biological studies have been reported so far. A model compound for hydrogen bonded phenol and its electrochemical properties was utilized to understand proton coupled electron transfer, which is considered as one of the key biological processes involving phenols.[5] Furthermore, the photocleavable property of acylphenol derivatives is applied for semi-quantitative probes for laser desorption ionization mass spectrometry.[6,7] In addition, there are large numbers of synthetic phenol derivatives with various pharmacological applications. For instance, synthetic hydroquinone dimethyl ethers showed potential anticancer and antimicrobial activities,[8] substituted phenols and quinones have potential anticancer activity.[9] Some phenolic derivatives such as caffeic acid and *p*-coumaric acid show antioxidant properties and 1,5-dicaffeoylquinic acid was found to be a hepatoprotector.[10]

The antioxidant efficiency of mono-phenols has been increased by the introduction of a second, *ortho*- or *para*-hydroxyl, or by one or two *ortho*-methoxy substitutions.[11,12] Antioxidant activity of phenols is attributed to their ability to chelate metal ions involved in the production of free radicals,[13] or might be due to the fact that phenolic hydroxyl groups are good hydrogen atom donors that can quench reactive oxygen and nitrogen species.[14–16] Phenolic compounds are also responsible for other interesting biological activities including antibacterial, antiviral, anti-inflammatory, antithrombogenic, anti-diabetic, cardio-protective, antitumor, inhibit low density lipoprotein (LDL) oxidation and immune-modulatory actions. They have been reported to modulate intracellular signalling pathways, altering the activity of target enzymes and affecting gene expression.[17–19]

Many antimicrobial agents have been synthesised for use against a wide range of infectious diseases. However, the emergence and spread of antimicrobial resistance is now a serious health threat across the world. Strains with multiple resistance to antibiotics such as staphylococci, enterococci and pneumococci have been widely disseminated.[20] Despite the advances in medicinal chemistry and biological sciences that have resulted in the discovery of many new antimicrobials, infectious diseases such as lower respiratory tract infections, remain among the major killers.[21] The rapid spread of antibiotic resistance, accompanied with low success rate of developing new antimicrobials, pose a serious challenge to those committed to global health and highlight the urgent demand for new effective antimicrobial agents.

We have synthesized many phenolic and related compounds to exploit new functional molecules.[5–7] On the course of our study, we investigated the antibacterial activities for our chemicals. In this report, two new nitrobenzyl-oxy-phenol derivatives were synthesized, their structures were confirmed based on their spectral data and their antibacterial activities determined.

MATERIALS AND METHODS

General All chemicals were purchased from TCI (Tokyo, Japan) and used without further purification. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) and precoated RP-18 F_{254s} plates (Merck, Germany) were used for TLC analysis. Sephadex LH-20 (Pharmacia Fine Chemical Co., Ltd., Tokyo, Japan) was used for column chromatography. Melting points were uncorrected. IR spectra (KBr) were recorded on a Nexus670NT Fourier transform (FT)-IR and are reported in frequency of absorption (cm^{-1}). High resolution (HR)-FAB-MS and electrospray ionization (ESI)-MS were recorded on JEOL JMS700N and JMS-100TD spectrometers respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a Unity plus 500 spectrometer (Varian Inc., U.S.A.) operating at 500 MHz and referenced to internal residual solvent. NMR data were pro-

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cessed using the acid citrate dextrose (ACD)/NMR Processor Academic Edition software (Advanced Chemistry Development, Inc., Canada).

Synthetic Procedure for 3,5-Dimethyl-4-((4-nitrobenzyl)-oxy)phenol (4a) To a solution of 2,6-dimethylbenzene-1,4-diol (**1a**) (5.0 mmol) in acetic acid (2 mL) was added acetic anhydride (5.5 mmol). The solution was refluxed for 4 h. After cooling down, the reaction solution was evaporated *in vacuo* and the residue was purified by silicagel column chromatography (hexane–EtOAc=95 : 5 to 50 : 50). 4-Hydroxy-3,5-dimethylphenyl acetate (**2a**) was obtained as a white solid (55%), mp 90–91°C. ^{1}H-NMR (500 MHz, $CDCl_3$, tetramethylsilane (TMS), room temperature (r.t.)) δ (ppm): 2.14 (6H, s), 2.25 (3H, s), 5.00 (1H, br s), 6.65 (2H, s); ^{13}C-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 16.0, 21.0, 120.9, 124.4, 143.1, 150.0, 170.6.

To compound **2a** (0.36 g, 2.0 mmol) in dry *N*,*N*-dimethylformamide (DMF) was added 4-nitrobenzyl bromide (0.48 g, 2.2 mmol) and K_3CO_3 (0.55 g, 4.0 mmol) at r.t. The suspension was stirred for 16 h, poured into water and EtOAc (50 mL) added. The organic layer was washed with 10% aqueous $NaHSO_4$ solution, and dried over $MgSO_4$ for 10 min. After removal of $MgSO_4$ by filtration, the EtOAc was evaporated *in vacuo*. The residue was purified by silica gel column chromatography (*n*-hexane–EtOAc=95 : 5 to 50 : 50) and 3,5-dimethyl-4-((4-nitrobenzyl)oxy)phenyl acetate (**3a**) was obtained as white solid in 91% yield, mp 89–91°C. ^{1}H-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 2.28 (6H, s), 2.28 (3H, s), 4.91 (2H, s), 6.78 (1H, s), 7.65 (2H, d, J=8.6 Hz), 8.27 (2H, d, J=8.6 Hz); ^{13}C-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 16.4, 21.0, 72.4, 121.6, 123.7, 127.5, 132.0, 144.9, 146.5, 147.5, 152.9, 169.8.

Compound **3a** (1 mmol) was treated with a solution of HCl in methanol (2 mol/L, 5 mL) for 5 h at r.t. After evaporation under reduced pressure, 3,5-dimethyl-4-((4-nitrobenzyl)oxy)-phenol (**4a**) was obtained as yellow solid (>99%).

4a: mp 15–17°C; IR (KBr, cm^{-1}): 3374, 2922, 1601, 1521, 1476, 1344, 1317, 1200, 1013, 857, 828, 742; ^{1}H-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 2.20 (6H, s), 4.86 (2H, s), 5.11 (1H, br s), 6.52 (2H, s), 7.64 (2H, d, J=8.3 Hz), 8.22 (2H, 1/2 AB, J=8.3 Hz); ^{13}C-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 16.3, 72.6, 115.2, 123.6, 127.7, 131.8, 145.1, 147.2, 148.8, 151.7; HR-MS *m*/*z* Calcd for $C_{15}H_{16}NO_4$ 274.1049. Found 274.10793.

Synthetic Procedure for 2,3,5-Trimethyl-4-((4-nitrobenzyl)oxy)phenol (4b) To a solution of 2,3,5-trimethylbenzene-1,4-diol (**1b**) (5 mmol) in acetic acid (2 mL) was added acetic anhydride (5.5 mmol). The solution was heated under reflux for 4 h. After cooling, the solution was evaporated *in vacuo* and the residue was purified by silicagel column chromatography (hexane–EtOAc=95 : 5 to 50 : 50). 4-Hydroxy-2,3,5-trimethylphenyl acetate (**2b**) was obtained as a white solid (25%), mp 120–121°C. ^{1}H-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 2.03 (3H, s), 2.14 (3H, s), 2.17 (3H, s), 2.30 (3H, s), 4.83 (1H, br s), 6.63 (1H, s), ^{13}C-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 12.2, 12.8, 15.9, 20.8, 120.6, 121.2, 123.4, 127.0, 142.18, 149.8, 170.2.

To compound **2b** (1 mmol) in dry DMF was added 4-nitrobenzyl bromide (1.1 mmol) and K_3CO_3 (2 mmol) at r.t. The suspension was stirred for 16 h and poured into water. After addition of EtOAc (50 mL), the organic layer was washed with 10% aqueous $NaHSO_4$ solution (30 mL) three times, and dried over $MgSO_4$ for 10 min. After removal of $MgSO_4$ by filtration, the EtOAc was evaporated *in vacuo*. The residue was purified by silica gel column chromatography (*n*-hexane–EtOAc=95 : 5 to 50 : 50) and 2,3,5-trimethyl-4-((4-nitrobenzyl)oxy)phenyl acetate (**3b**) was obtained as a white solid (87%), mp 90–91°C. ^{1}H-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 2.05 (3H, s), 2.21 (3H, s), 2.25 (3H, s), 2.32 (3H, s), 4.86 (1H, s), 6.74 (1H, s), 7.65 (2H, d, J=8.6 Hz), 8.25 (2H, d, J=8.6 Hz); ^{13}C-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 12.8, 13.0, 16.2, 20.7, 72.5, 121.4, 123.6, 127.5, 128.8, 130.9, 144.9, 145.2, 147.4, 152.8, 169.6.

Compound **3b** (1 mmol) was treated with a solution of HCl in methanol (2 mol/L, 5 mL) for 5 h at r.t. After evaporation under reduced pressure, 2,3,5-trimethyl-4-((4-nitrobenzyl)-oxy)phenol (**4b**) was obtained as yellow solid in quantitative yield.

4b: Yellow solid; mp 97–99°C; IR (KBr, cm^{-1}): 3374, 2922, 1601, 1521, 1476, 1344, 1317, 1200, 1013, 857, 828, 742; ^{1}H-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 2.10 (3H, s), 2.20 (6H, s), 4.83 (2H, s), 6.49 (1H, s), 7.65 (2H, d, J=8.6 Hz), 8.25 (2H, d, J=8.6 Hz); ^{13}C-NMR (500 MHz, $CDCl_3$, TMS, r.t.) δ (ppm): 11.9, 12.4, 16.1, 72.9, 114.6, 121.6, 123.7, 127.6, 128.3, 130.6, 145.2, 147.4, 148.8, 149.9; HR-MS *m*/*z* Calcd for $C_{16}H_{18}NO_4$ 288.12358. Found 288.12484.

Antimicrobial Activity

Test Microorganisms

Moraxella catarrhalis (GTC 01544), *Moraxella catarrhalis* (Clinical strains), *Klebsiella pneumonia* (The American Type Culture Collection (ATCC) 13883), *Salmonella typhimurium* (ATCC 14028), *Streptococcus pyogenes* (ATCC 19615), *Neisseria lactamicus* (ATCC 23970), *Enterobacter cloacea*, (ATCC 23355), *Pseudomonas aeruginosa* (IFO 3445) *Vibrio cholera* (clinical strain) were used as test microorganisms.[22)]

Antibacterial Assay The antibacterial activity was measured as described previously.[22,23)] Bacterial suspensions were obtained from overnight cultures in Triptic Soy broth (Becton, Dickinson and Company, MD, U.S.A.), cultivated at 37°C for 24 h. The bacterial suspensions were adjusted to an inoculum size of 10^8 colony forming unit (cfu)/mL and 100 μL used for inoculation of the agar plates (Mueller–Hinton Agar, Becton, Dickinson and Company). Sterile paper discs (6 mm diameter) which were impregnated with the tested samples (10–100 μg) were placed aseptically over the bacterial culture on nutrient agar plates. After incubation at 37°C for 24 h, the zone of inhibition around the discs was measured on a millimetre scale. The experiment was replicated twice. Paper discs impregnated with only sterile solvents served as negative control.

Determination of MIC and Minimum Bactericidal Concentration (MBC) The MIC of the compound was defined as the lowest concentration that inhibited the visible bacterial growth and the MBC was defined as the lowest concentration that prevented the growth of the organism after subculture onto antibiotic-free plates. The MIC and MBC values were determined by the broth dilution method.[24)] Overnight broth culture was used to adjust the inoculum concentration to 2×10^3 cfu/mL in sterile distilled H_2O. Gentamycin and ciprofloxacin were used as positive controls, in addition to sterility control (broth+dimethyl sulfoxide (DMSO) 10% (v/v)+test compound) and negative control (broth+DMSO 10% (v/v)+test microorganism). Dilutions ranging from 0.05

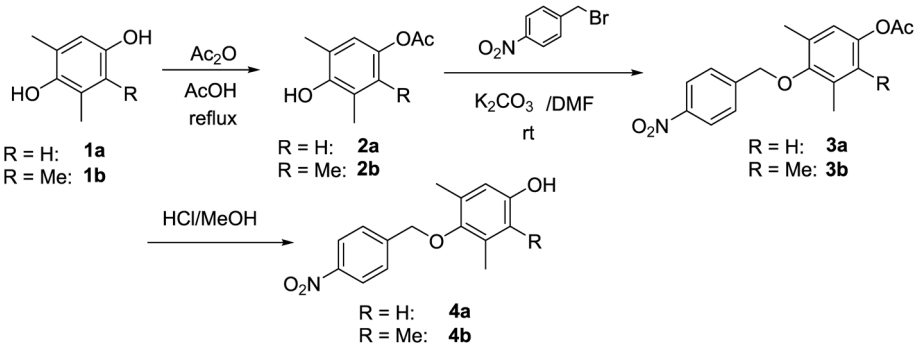


Chart 1. Synthesis of 3,5-Dimethyl-4-((4-nitrobenzyl)oxy)phenol (**4a**) and 2,3,5-Trimethyl-4-((4-nitrobenzyl)oxy)phenol (**4b**)

Table 1. Antibacterial Activity of Test Compounds and Reference Antibiotics

Bacterial strains	4a		4b		Monobenzone		Ciprofloxacin		Gentamycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
M. catarrhalis^{T}	91	365	11	22	250	499	9	19	0.21	0.41
M. catarrhalis$^{Cl\,1}$	91	365	11	22	250	499	9	19	0.41	0.82
M. catarrhalis$^{Cl\,2}$	91	365	11	22	250	499	9	19	0.41	0.82
M. catarrhalis$^{Cl\,3}$	91	365	11	22	250	499	9	19	0.41	0.82
M. catarrhalis$^{Cl\,4}$	91	365	11	22	250	499	9	19	0.21	0.41
M. catarrhalis$^{Cl\,5}$	91	365	11	22	250	499	9	19	0.21	0.41

T, type strain; Cl, clinical strain; MIC (μM), minimum inhibitory concentration, *i.e.*, the lowest concentration of the compound to inhibit the growth of bacteria completely; MBC (μM), minimum bactericidal concentration, *i.e.*, the lowest concentration of the compound for killing the bacteria completely.

to 52 μM for gentamycin and 9 to 1200 μM for ciprofloxacin and tested samples were prepared in tubes including broth and DMSO 10% (v/v). A 100 μL suspension of test microorganism was added to individual tubes and incubated at 37°C for 24 h.

RESULTS AND DISCUSSION

There has been growing interest in the development of new compounds to deal with resistant bacteria. *De novo* drug discovery is still one of the most important areas of antibacterial research. However, identification of new indications for known drugs (drug repositioning) has been gaining popularity as an alternative approach for drug discovery. Drug repositioning offers opportunities for faster development times and reduced risk.[25] The two approaches of drug discovery were performed in this study, the synthetic approach afford two new compounds while drug repositioning afford one compound active against both clinical isolates and type strain of *M. catarrhalis*. However, all tested compounds showed weak activity against other tested microorganisms.

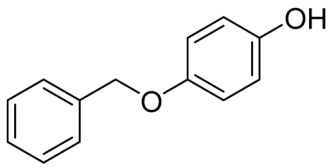
Selective protection of the less hindered hydroxyl groups of 2,6-dimethylbenzene-1,4-diol and 2,3,5-trimethylbenzene-1,4-diol was achieved by mono-acetylation with acetic anhydride in acetic acid at reflux and afforded compounds **2a** and **b** in excellent yields (Chart 1). Introduction of the nitrobenzyl group into the free hydroxyl groups of **2a**, **b** was achieved by addition of 4-nitrobenzyl bromide in the presence of K_3CO_3/DMF to generate compounds **3a** and **b**. After deprotection of the acetyl groups of **3a** and **b** by treatment with HCl/methanol the 3,5-dimethyl-4-((4-nitrobenzyl)oxy)phenol (**4a**) and 2,3,5-trimethyl-4-((4-nitrobenzyl)oxy)phenol (**4b**) were obtained.

The antimicrobial activity of **4a** was found to be reasonable, with MIC and MBC of 91 and 365 μM respectively (Table 1). There are many phenolic compounds with similar MICs.[26–31] However, the introduction of a methyl group in the 2-position of the phenol ring resulted in the very active compound **4b** that had the highest antibacterial activity, which equivalent to that of ciprofloxacin, with MIC and MBC values of 11 and 22 μM respectively (Table 1). The zone of inhibition on agar plates for **4a**, **b** and ciprofloxacin against *M. catarrhalis* type strain were found to be 13, 15 and 20 mm, respectively.

It has been found previously that the free phenolic hydroxyl group is responsible for the antimicrobial[32] and antioxidant activities of phenols.[14–16,33] We also found that the free phenolic hydroxyl group to be essential for the antibacterial activity of the test compounds. Acetylated compounds **3a**, **b** were inactive against the test microorganisms.

Drug repositioning is an accelerated route for drug discovery and has resulted in new opportunities for the use of old drugs. In the United States of America for example, drug repositioning accounts for approximately 30% of new Food and Drug Administration (FDA) approved drugs and vaccines.[34]

Drug repositioning approach for drug discovery was also performed in this study by observing the structural similarity between **4a**, **b** and monobenzone (Fig. 1). Monobenzone is a melanin synthesis inhibitor with bleaching properties which used for the treatment of pigmentary skin disorders.[35] There has been no previous report regarding the antibacterial effect of monobenzone, but we found monobenzone to be modestly active against *Moraxella catarrhalis* and its activity was comparable to that of **4a**, with MIC and MBC values of 250 and 499 μM respectively (Table 1). Based on this result, the antibacterial activity might not be attributed to the nitro group of compound **4a**. On the other hand, introduction of methyl group at 2-position of the phenol ring significantly increased



monobenzone

Fig. 1. Structure of Monobenzone

its antibacterial activity without affecting its activity spectrum.

CONCLUSION

Two new phenolic compounds were synthesised using a successful method under mild conditions from economical starting materials and tested for their antibacterial activity. The MICs and MBCs of the tested compounds were determined. Compound **4a** and monobenzone showed equivalent modest antibacterial activity, however, compound **4b** showed strong antibacterial activity comparable to the reference antibiotic ciprofloxacin. Thus, *M. catarrhalis* might serve as a model organism for the discovery of new drug target, or studying the mechanism of some side effects of these compounds which might be regarded as good starting points to design and synthesise optimised broad spectrum antibacterial compounds.

Conflict of Interest The authors declare no conflict of interest.

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