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Antimicrobial Activity of Mono- and Di-Methyl Substituted Benzhydrols and Benzophenones *In Vitro*

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Abstract: *Twelve compounds of mono methyl and symmetrical dimethyl substituted benzhydrol and benzophenone were synthesized using standard synthetic procedures and screened for possible antimicrobial activity against thirteen known Gram-positive and Gram-negative bacteria, as well as two yeasts. Most benzhydrol and benzophenone derivatives under investigation demonstrated some antimicrobial activity, with* ortho-*methylbenzophenone, dapsone,* meta-*dimethylbenzophenone, and* para-*dimethylbenzyhydrol showing the greatest inhibition. Only four compounds,* ortho-*methylbenzhydrol,* para-*methylbenzhydrol,* para-*methylbenzophenone, and* para-*diaminobenzophenone, completely lacked antimicrobial activity. An Analysis of Variance (ANOVA) showed a significant difference between both the microorganisms and the chemical compounds used, which may provide insight into novel compounds to combat infections in humans and animals.*

Key Words: *Antimicrobial, Bacteria, Benzhydrol, Benzophenone, Yeast*

Introduction

Bacteria and fungi continually evolve mechanisms to evade antimicrobial agents, causing drug resistance problems when treating human and animal infections. As natural or synthetic pharmaceutical drugs become less effective on microbes due to resistance mechanisms, new agents must be discovered or developed to combat this problem (Waugh and Long 2002). This process can encompass screening known natural and synthetic agents, or chemically synthesizing derivatives of known compounds (Ma *et al.* 1999, Setti and Micetich 2000).

Computational studies (Dais 1990, Gore *et al.* 1980, Trovato *et al.* 1973) and crystallographic studies (Kutzke *et al.* 1996) have been performed for benzophenone and benzhydrol derivatives to determine conformational preferences and minimum energy structures, but investigation of the use of these small molecules with unique conformations in medicine is minimal. Studies of benzophenone reduction by microorganisms (fungi and bacteria) conducted by Spassov *et al.* (1993) showed that benzophenone could be taken up by microbes and be used in their metabolic pathways. Therefore, the antimicrobial activity of benzophenone and benzhydrol (the reduced product of benzophenone, a metabolite in the process), which has not been previously investigated, warrants scientific investigation. This study compares potential antimicrobial activities of mono- and dimethyl-substituted benzhydrols and benzophenones on various microorganisms.

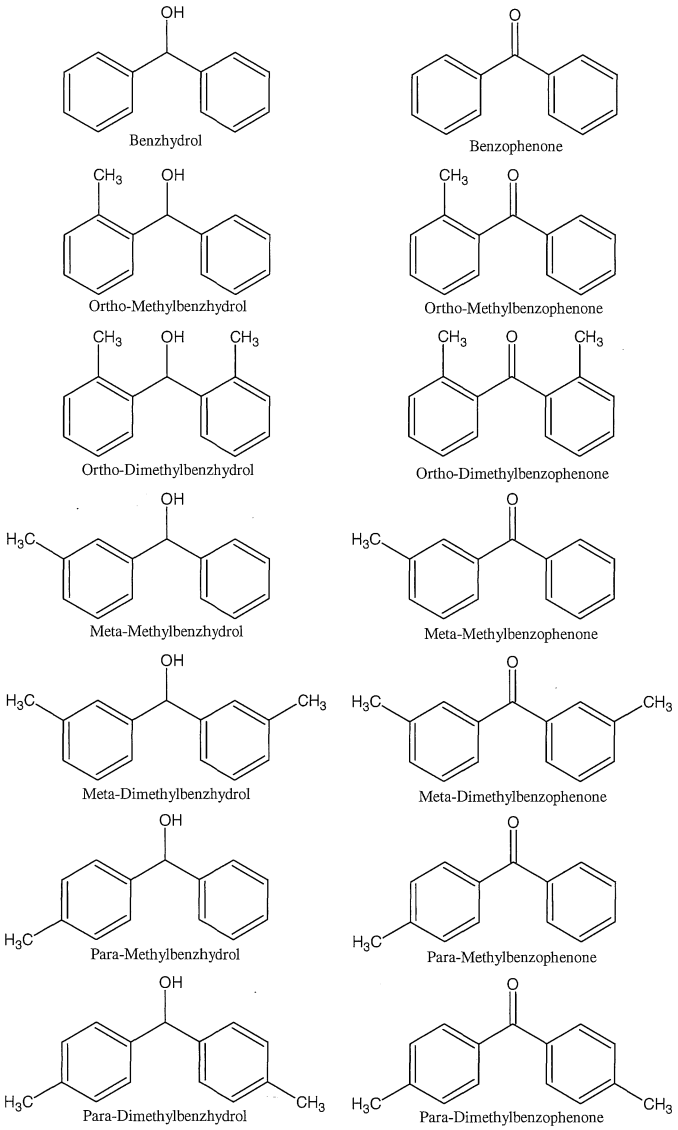
A series of methyl derivatives of benzophenone and benzhydrol were synthesized. Gram-positive and Gram-negative bacteria and yeast were subjected to the compounds to determine initial susceptibility since very little has been published thus far on the compounds. The substitution of methyl groups introduces lipophilicity to the compound without adding bulky side chains (Coleman *et al.* 1997).

Materials and Methods

Compounds

ortho, *meta*, and *para* mono-methyl substituted benzhydrols (Fig. 1) were prepared using benzaldehyde and a corresponding bromotoluene isomer utilizing a standard Grignard Reaction (Zanger and McKee 1995). Symmetrical di-methyl substituted benzhydrols (Fig. 1) were prepared using substituted bromotoluenes and respective tolualdehydes. All starting materials to prepare the compounds were purchased from Sigma-Aldrich (St. Louis, MO). All benzhydrol derivatives and benzophenone derivatives are known in the literature, but only

Figure 1. Benzhydrol and benzophenone compounds used in this study.



a few were commercially available during our studies. All starting materials and subsequent products were characterized by proton Nuclear Magnetic Resonance (NMR) spectroscopy, C-13 NMR with a 270 MHz Jeol spectrometer, and melting point determination for solids. Spectral data for all compounds were matched with the literature values.

Synthesis of Benzophenones

Mono- and di-methyl substituted benzophenones (Fig. 1) were synthesized from mono- and di-methyl substituted benzhydrols via a silica gel-supported Jones Oxidation. Ten grams of silica gel was added to a 250 mL Erlenmeyer flask. Jones Reagent (Ali and Wiggin 2001) (2.7 M, 8.8 mL) was added drop-wise to the silica gel with stirring until an orange "slurry" was formed. An additional 30 mL of dichloromethane was added to the reaction mixture after the slurry was formed with the substituted benzhydrols (250 mg, mono = 1.267 mmol, dimethyl = 1.183 mmol). The reaction mixtures were allowed to stir vigorously at room temperature overnight. Upon completion, the mixture was filtered, washed with three 10 mL portions of dichloromethane, the solvent was removed on a rotary evaporator, and then dried on a vacuum pump. Depending upon the states, products were recrystallized using hexane, and dried under vacuum before the antimicrobial study was conducted. Tables 1 and 2 show the states of benzhydrol and benzophenone, and the percent yields. The commercially available *para*-diaminobenzophenone and *para*-dihydroxybenzophenone structures are depicted in Figure 2.

Bacteria and growth

Fifteen different microorganisms including Gram-positive, Gram-negative, and yeasts were used in this study. Cultures were purchased from Hardy Diagnostics (Santa Maria, CA) or Wards Scientific (Rochester, NY) and subjected to biochemical testing before experimentation to ensure bacterial purity and identity. Cultures included *Bacillus cereus* ATCC 14579, *Candida albicans*, *Citrobacter freundii* ATCC 8090, *Enterobacter*

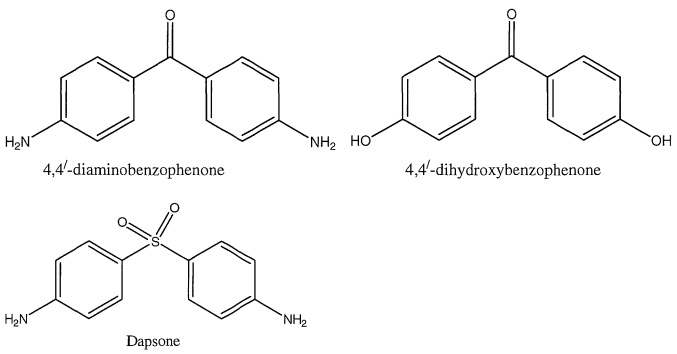
Table 1. Compound characteristics of the benzhydrol derivatives.

Compound	State	Percent Yield
Benzhydrol		
Ortho-Dimethylbenzhdryol	Liquid	82
Ortho-Methylbenzhydrol	Liquid	81
Meta-Dimethylbenzhydrol	Solid (mp118°C)	82
Meta-Methylbenzhydrol	Solid (mp 95°C)	69
Para-Dimethylbenzhydrol	Liquid	81
Para-Methylbenzhydrol	Liquid	59

Table 2. Compound characteristics of the benzophenone derivatives.

Compound	State	Percent Yield
Benzophenone	Solid	Commercially available
Ortho-Dimethylbenzophenone	Liquid	Quantitative yield ranging form 95–98%
Ortho-Methylbenzophenone	Liquid	
Meta-Dimethylbenzophenone	Solid (mp 72.5°C)	
Meta-Methylbenzophenone	Liquid	
Para-Dimethylbenzophenone	Solid (mp 95°C)	
Para-Methylbenzophenone	Solid (59°C)	

Figure 2. Diamino- and dihydroxy-benzophenones and dapsone used in this study.



cloacae ATCC 13047, *Escherichia coli* ATCC 14948, *Micrococcus luteus* ATCC 9341, *Micrococcus roseus*, *Mycobacterium phlei*, *Providencia stuartii* ATCC 33672 49809, *Pseudomonas aeruginosa* ATCC 27853, *Saccharomyces cerevisiae* ATCC 2601, *Serratia marcescens* ATCC 14756, *Shigella flexneri* ATCC 9199, and *Staphylococcus aureus* ATCC 6538. *Bacillus cereus* produces toxins, endospores, causes food poisoning, and was chosen for its similarity to *Bacillus anthracis*, the gram positive causative agent of anthrax. Since we wanted to compare two yeasts, *Candida albicans*, a dimorphic yeast known to cause reproductive tract infections, thrush, and nosocomial deaths, and *Saccharomyces cerevisiae*, used in fermentation and usually considered non-pathogenic, were chosen for this study. *Enterobacter cloacae, E. coli, Providencia stuartii, Serratia marsescens*, and *Shigella flexneri* belong to the Gram-negative Enterobacteriaceae family known to inhabit the intestines of humans and animals. They all cause urinary tract infections, food-borne infections, are opportunistic pathogens, and can be drug resistant, leading to death. *Micrococcus luteus* and *M. roseus* are Gram-positive bacteria commonly found in the environment that do not produce endospores or toxins, and are rarely pathogenic. *Pseudomonas aeruginosa* is a gram negative, multi-drug resistant bacterium that is pathogenic causing skin, ear, lung, and urinary tract infections, with only a few drugs available for treatment. *Mycobacterium phlei* contains mycolic acid as part of it's cell wall, which allows it to be drug resistant, and is considered to be an acid-fast bacterium. We chose this microbe because we could not work with the more pathogenic *Mycobacterium tuberculosis* or *M. leprae*, which cause tuberculosis and leprosy respectively, but we could determine if a drug could potentially penetrate the mycolic acid layer. Finally, *Staphylococcus aureus* is a Gram-positive skin bacterium that produces many toxins, is a leading cause of nosocomial and food-borne infections, and can be drug resistant.

Bacteria were initially grown on Tryptic Soy Agar (TSA) or Nutrient Agar (NA), yeasts were incubated on Sabouraud (Sab) Agar, and all cultures were subsequently transferred to broth. *Mycobacterium phlei* was grown on TSA supplemented with sterile glycerol. All cultures were incubated for 24–48 h at their optimal temperature (25°C, 30°C, or 37°C) to achieve log phase growth.

In vitro antimicrobial assessment

Mueller Hinton plates (pH 7.3) were inoculated with a microbial lawn of growth from standardized broth cultures, which were measured by spectrophotometry at 625 nm. Powdered solids were aseptically weighed and 10 mg was added to the surface of the agar. Liquids (7 µl) were added directly to blank disks measuring six mm in diameter, and plates were incubated at the optimal temperature for 24–48 h. Final concentrations of the liquids were 0.007567 g for monobenzhydrol, and 0.007469 g for dimethylbenzophenone. Zones of inhibition were measured and recorded. The Clinical and Laboratory Standards Institute guidelines (2007) mix insoluble compounds with dimethyl sulfoxide (DMSO) to determine the minimum inhibitory concentration, but no consideration is given to compound structure or functionality when it is mixed with the DMSO in a research setting. Thus, for solids, zones were measured in two perpendicular locations since no work has been performed on insoluble solids to achieve standardization (Gould 2000). Inhibition ratios were determined by dividing the zone of inhibition area by the compound area, thus allowing for comparisons between liquids and solids. A ratio of one represents no compound activity.

Results

Antimicrobial Effectiveness

The null hypothesis was that no difference existed for the inhibition ratios between compounds and between organisms. An analysis of variance (ANOVA) for the fourteen compounds revealed a P value of 0.026, thus demonstrating significance ($P \leq 0.05$) between the compounds, and testing of each organism revealed a value $P < 0.0001$, demonstrating that there was also a difference in the ratios seen between the microbes.

Six compounds, *ortho*-methylbenzhydrol, *ortho*-dimethylbenzhydrol *para*-methylbenzhydrol, benzophenone, *para*-methylbenzophenone, and *para*-diaminobenzophenone completely lacked antimicrobial activity by demonstrating no zones of inhibition. Of the six, only *para*-methylbenzophenone is a solid, with the rest in a liquid state. Minimal inhibitory activity (a ratio <3.5) was seen by *ortho*-dimethylbenzophenone and *meta*-methylbenzophenone against all microbes tested.

A ratio of five was used to qualify a microbe as inhibited, since the inhibition zone was considered large enough to avoid any technical error. A ratio of five meant that for discs, the zone of inhibition was at least 30 mm, and for solids, the zone was at least 30 mm and could potentially be even larger, depending on the surface area of the solid. According to the Clinical and

Laboratory Standards Institute guidelines (2007) for reference microbes, zones from 18–21 mm and greater were classified as sensitive. Thus, we can be confident that those microbes were indeed inhibited by the compound with a ratio of five. The other nine compounds demonstrated antimicrobial activity with at least one microbe having a zone ratio greater than five. Based upon the sizes of the inhibition zones, dapsone, benzhydrol, monomethylbenzhydrol, *ortho*-methylbenzophenone and *meta*-dimethylbenzhydrol showed the greatest inhibition, exhibiting ratios above 25, which is shown in Table 3. Two compounds, *para*-dimethylbenzyhydrol and *ortho*-dimethylbenzophenone, each inhibited eight microbes, thus demonstrating broad spectrum activity. *meta*- dimethylbenzhydrol, *meta*-dimethylbenzophenone, and *para*-dihydroxybenzophenone each inhibited five microbes. Dapsone and *meta*-methylbenzhydrol each inhibited two bacteria, whereas benzhydrol and *para*-diaminobenzophenone each inhibited one bacterium.

Bacterial responses to the compounds are also shown in Table 3. *Citrobacter freundii* was resistant to all the compounds tested, whereas *M. phlei* and *M. roseus* was susceptible to six of the compounds. *Micrococcus luteus* showed susceptibility to five compounds. *Pseudomonas aeruginosa* was the only microbe susceptible to *para*-dimethylbenzophenone, but it was also inhibited by *ortho*-methylbenzophenone and *meta*-dimethylbenzhydrol. The two yeasts were both susceptible to *ortho*-methylbenzophenone and *meta*-dimethylbenzophenone. All other bacteria were inhibited by at least one compound.

Discussion

The most broad spectrum compounds were *ortho*-methylbenzophenone and *para*-dimethylbenzhydrol. The yeasts *Candida albicans* and *Saccharomyces cerevisiae* both showed similar inhibition zones to *ortho*-methylbenzophenone and *meta*-dimethylbenzophenone, although *C. albicans* was also susceptible to *para*-dimethylbenzhydrol. Benzhydrol and *para*-dimethylbenzophenone appeared to have narrow spectrum activity to one bacterium, *M. phlei* and *P. aeruginosa*, respectively. Besides *M. phlei*, *M. luteus* and *M. roseus* were the most susceptible to the compounds, which is expected of environmental microbes due to lack of antibiotic selective pressures.

Although the cut-off for the significant values were based upon the zone of inhibition ratios so that solid and liquid compounds could be compared, one must keep in mind that organisms demonstrate variability in sensitivities of up to 2 mm (Gould 2000). Media pH can affect the zone sizes seen on the media. Zones can be too small if the pH of the media is too low, or vice versa. Our media pH was 7.3, and inoculation and incubation conditions were all within guidelines (Clinical Laboratory Standards Institute 2007). Since the compounds have a high pK_a, the pH of the media was not changed and did not affect zone sizes.

The numbers bold-faced in Table 3 indicate the microbes and compounds who exhibited five mm zones and greater. Although some microbes were not highly inhibited, we are still able to report that these compounds do inhibit microbes to some extent, and can possibly be developed as alternative antimicrobial agents to existing, sometimes ineffective, drugs. Each compound's solubility was tested in deionized water and found to be poorly soluble. Benzyhydrol is slightly soluble in water, but benzophenone is reported as insoluble (www.chemcialland21.com), which correlates with the lack of antimicrobial activity seen by the benzophenone. However, the addition of methyl groups appears to allow some of the derivatives to become slightly soluble and exhibit antimicrobial activity. Since the disk diffusion method is based upon agents diffusing into the agar media in order to control microbial growth, this makes the results of the inhibition zones even more profound. The compounds which demonstrated an inhibition zone below five may even be more effective *in vivo* than our results suggest. Further, the results of the small zones must be carefully evaluated since increasing the agents' bioavailability may also increase their effectiveness. Industrial applications such as antimicrobial agents in cosmetics and fuel lines of naval ships may exist.

Mycobacterium phlei was greatly inhibited by six compounds. Dapsone was used as a control for *Mycobacterium* (Levy 1976) as it is commercially available and standardized inhibition zones are known with this compound. Other antibacterial studies performed in the laboratory on the same strain of *Mycobacterium* (data not shown) showed standard resistance patterns to most antibiotics, so the strain was not considered unusually susceptible to compounds. Drug resistance is often due to the impervious mycolic acid layer on the outside of the cells of *Mycobacterium leprae* and *M. tuberculosis*, so these results show potential new drugs to treat these disfiguring and deadly diseases.

Another major finding was that *para*-dimethylbenzophenone only inhibited the highly resistant bacterium, *P. aeruginosa*. *Pseudomonas* is well documented to use alternative metabolic pathways like the Entner Duoderoff pathway, so this could explain why no other microbes were inhibited. *ortho*-methylbenzophenone and *meta*-dimethylbenzhydrol also showed activity against this pathogen. *Pseudomonas* frequently grows in disinfectants and causes swimmer's ear, wound infections, and is often a cause of death in burn patients.

Staphylococcus aureus, a major cause of nosocomial and wound infections, was inhibited by *para*-dimethylbenzhydrol. Due to increasing drug resistance, overall cost to society in billions of dollars per year (Rubin *et al.* 1999), and human deaths attributed to this bacterium, any new potential drug should be evaluated.

Candida albicans, a major cause of reproductive tract yeast infections in women and thrush in immunocompromised individuals, was inhibited by *ortho*-methylbenzophenone and *para*-dimethylbenzhydrol. Since yeasts are eukaryotic, further studies would need to be performed to determine potentially harmful effects on human cells.

The promising results we obtained against *Mycobacterium, Pseudomonas, Staphylococcus*, and *Candida* are even more

Table 3. Ratios of antimicrobial activities as determined by dividing the compound area by the inhibition zone. Highlighted squares indicate zones of inhibition of ≥25 mm and bolded numbers represent zones ≥5 mm. Abbreviations: Daps = dapsone, BH = benzhydrol, OMB = *ortho*-methylbenzhydrol, ODMB = *ortho*-dimethylbenzhydrol, MMB = *meta*-methylbenzhydrol, MDMB = *meta*-dimethylbenzhydrol, PMB = *para*-methylbenzhydrol, PDMB = *para*-dimethylbenzhydrol, BP = benzophenone, OMBP = *ortho*-methylbenzophenone, ODMBP = *ortho*-dimethylbenzophenone MMBP = *meta*-methylbenzophenone, MDMBP = *meta*-dimethylbenzophenone, PMBP = *para*-methylbenzophenone, PDMBP = *para*-dimethylbenzophenone, PDHBP = *para*-dihydroxybenzophenone, PDABP = *para*-diaminobenzophenone.

	Daps	BH	OMB	ODMB	MMB	MDMB	PMB	PDMB	BP	OMBP	ODMBP	MMBP	MDMBP	PMBP	PDMBP	PDHBP	PDABP
B. cereus	3.75	3.667	1	1	2.04	3.714	1	**8.571**	1	2.286	1	1	3.184	1	1.6	3.75	1
C. albicans	1	2.27	1	1	3.673	2.918	1	**13.796**	1	**21.778**	1	1	**6.245**	1	3.3	1	1
C. freundii	3.517	1	1	1	2.245	1	1	1.102	1	1.8	1	1	2.02	1	1	1.5	1
E. cloacae	1.067	1.92	1	1	1.796	**5.816**	1	**7.714**	1	4.767	1.203	2.02	3.429	1	1	**6.38**	1
E. coli	1	2.763	1	1	1	1	1	1.306	1	1	1	1	1	1	1	**8**	1
K. pneumoniae	1	1.65	1	1	2.245	1	1	1	1	**12.5**	1	1	1	1	1	1.944	1
M. luteus	1	2.222	1	1	4.571	**5.143**	1	**18.367**	1	**7.875**	1.481	1	**8.449**	1	1.867	**10.357**	1
M. roseus	1	2.743	1	1	10	**5.551**	1	**18.163**	1	**11.333**	1.3	1	**18.367**	1	2.24	**7.14**	1
M. phlei	**100**	**27.188**	1	1	**25.306**	3.429	1	**6.429**	1	**36.417**	1	1.469	**48.98**	1	1	2.75	1
P. stuartii	1	1.778	1	1	1.653	1	1	4.163	1	3.575	1.143	1	3.061	1	1	**5.25**	1
P. aeruginosa	1.429	1.2	1	1	3.449	**15.429**	1	1	1	**28.125**	1.212	3.184	1	1	**14.667**	1	1
S. cerevisiae	1	1.909	1	1	3.98	1	1	1	1	**19.5**	1.731	1.837	**6.612**	1	4.667	1	1
S. marcescens	**8**	1.328	1	1	1	**11.102**	1	4.571	1	1	1.821	1	1	1	1	2.4	1
S. flexneri	1	1	1	1	1	4.408	1	**8.143**	1	**7.4**	1.658	1.561	1	1	1	3.6	1
S. aureus	1	1.95	1	1	2.245	4.286	1	**7.367**	1	4.375	1	1	2.245	1	2.4	3.091	1

significant since these microbes often exhibit antimicrobial resistance problems in clinical settings, and can cause death in humans and animals (Poikonen *et al.* 2003, Rubin *et al.* 1999, Van Delden and Iglewski 1998). Any new drugs to combat these microorganisms could help alleviate some chronic infections, to which there currently is no treatment.

The location of the methyl groups does appear to have an effect on the compounds ability to serve as an antimicrobial. Benzophenone lacked activity, but the *ortho*-methylbenzophenone exhibited broad spectrum activity against a large number of microbes, and *para*-dimethylbenzophenone only inhibited *Pseudomonas*, thus demonstrating narrow spectrum activity. Although we do not know the mechanisms of antimicrobial activity at this time, placement of the methyl groups, as well as dimethyl composition, appears to show differences in activity among the various microbes.

Due to the lack of activity by racemic monomethylbenzhydrols, we did not pursue synthesis of enantiomerically pure monosubstituted benzhydrols (Ohkuma *et al.* 2000), although this could be a potential new avenue to pursue in antimicrobial agent development.

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