RESEARCH ARTICLE

Inhibition of bacteria and white-rot-fungi by newly synthesized furfural derivatives

Jian-Lan Wang[1,*], **Wen Gu**[2], **Shi-Fa Wang**[2]
[1] School of Conservation, Shanghai Institute of Visual Arts, Shanghai 201620, P.R. China
[2] College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, P.R. China

Abstract: New *N*-alkylfurfurylacetamides were synthesized and characterized from abundant biomass derived furfural by a simple and straightforward procedure with overall yields of 51 to 92%. The antimicrobial activity of the synthesized compounds were then investigated and it was found that *N*-benzyl-*N*-furfurylacetamide and *N*-cyclohexyl-*N*-furfurylacetamide showed promising activity against bacteria, particularly white-rot-fungi, suggesting a feasible new type of anti-fungal agent in applications to protection and conservation of cultural heritage.
Keywords: furfural, *N*-alkyl furfurylamines, *N*-alkyl-*N*-furfurylacetamide, antibiotic activity, white-rot-fungi

[*] **Correspondence to:** Jian-Lan Wang, Shanghai Institute of Visual Arts, Shanghai 201620, China; E-mail: wjlwjl07@163.com

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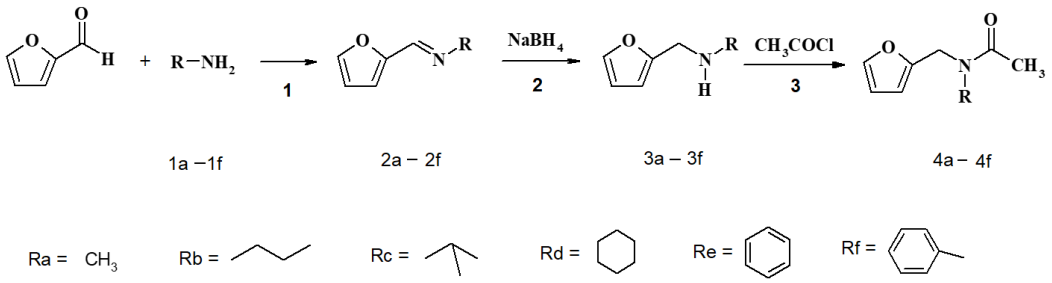
1 Introduction

Fossil fuel resource as the primary raw materials of pharmaceuticals and chemicals is becoming more and more restricted and regulated, this is due to their non-sustainability and undesirable environmental impact during mining and extraction, which has resulted in pollution and global climate change related environmental issues. Biomass feedstock is an attractive alternative and plays an increasing role in the chemical and pharmaceutical industry. It provides economical, environmental, and social sustainable options for renewable fuels and energy strategies (Bridgwater, 2003; Kamm, 2004; Yaman, 2004; Cherubini, 2010).

Furfural is one of the widely used, high value-added chemicals produced from biomass materials. It has been extensively used in many areas including food industry for synthesis of additives such as flavours and preservatives, textile industry for producing dyestuff, coating materials, synthetic fibres; and in manufacture of plastics, resins, semiconducting materials, pesticides, chemicals, biopolymers and increasingly in medicine research and development (Anthonia and Philip, 2015). Furfural and furfural-derived compounds are extensively used in anti-cancer and anti-inflammatory therapy and antimicrobials, as intermediate in the synthesis of pharmaceuticals (Anto et al., 1998). Furfural stands out in the natural products research and in sustainable resources for it is readily availability from low-cost and abundant agricultural waste materials including sasanqua hulls, corn cobs, bagasse, cotton seed hulls, and logging residues *etc*. Furfural can be obtained with simple chemical treatment, and become one of the most important and good starting materials for making furan-containing derivatives of enormous diversity (Weilmuenster and Jordan, 1945; Galletti et al., 2009).

In the recent developments of the pharmaceutical industry, although contribution and total volume of natural products show a decrease, natural products lead to a continuous new discovery of drugs for antibacterial activity with biologically and chemically novelty and potential applications. Due to long evolution, natural products often have more complex structure than synthetic molecules with multiple contacts that contribute to irreversible inhibition. This allows inhibition of challenging targets with complex or poorly defined active sites, inherent molecule stability, *etc.*, that can be exploited to a success of antibiotic discovery (Leeds et al., 2006). Furfural and its diversity of derived compounds therefore are considered as the meaningful candidates for antibacterial drug screening. In contrast to synthetic methods, antimicrobials extracted from essential oils are usually regarded as non-toxic to humans, but their application is often limited due to a lack of supply and their high cost of processing (Leeds et al., 2006; Schelz et al., 2006). Therefore, utilization of the readily available biomass furfural as a starting point to synthesize a series of furfural derivatives based on structure-activity relationships as the compound-design idea can be cost effective (Kabara and Conley, 1972; Atherton et al., 1986; Tang et al., 2013). In particular considering furan or furfural cooperated with amide structure, it is likely



Scheme 1. The synthetic route to *N*-alkyl-*N*-furfurylacetamide

to possess antibiotic activity (Sun, 2001; Patch and Barron, 2003; Fu et al., 2010). Therefore, the underlying antimicrobial potency might be presented in the novel synthetic compounds.

2 Materials and Methods

2.1 Synthesis

Twelve compounds of two categories with furfural as the starting material were syntheized: *N*-alkylfurfurylamines and *N*-alkyl-*N*-furfurylacetamides, under general synthetic pathway shown in Scheme 1 involving reductive amination ($NaBH_4$ as the Schiff base) followed by acetylation (Mamedov et al., 1994; Ganushchak et al., 2001). Furfural was supplied by Zouping Furfural Company in Shandong province; all the rest chemicals were provided by Sigma Aldrich. For the reaction solvent in the synthesis, cyclohexane, ethyl acetate, diethyl ether and dichloromethane were examined; of the bases in Step 3 of the synthetic path, pyridine, KOH and $KHCO_3$ were investigated in the experiments.

In order to minimize the side-products in the acetylation step, drops of chloroacetyl chloride was slowly added. This reduced the potential for furfural to react as a free radical in acid medium. Commonly, the reaction was completed within 2 h of adding chloroacetyl chloride. The work-up temperature was modified from room temperature to -20°C, generally, if the temperature was held under 5°C, the yield of reaction was acceptable and the formation of side products was significantly decreased. The heat generated by the acetylation was thought to initiate the polymerization of the furan and this significantly increased when the reaction temperature was higher than 15°C.

2.2 Antimicrobial testing

The antimicrobial testing involved seven strains of bacteria and fungi. All strains were provided by Microbiological Lab in College of Chemical Engineering in Nanjing Forestry University, Nanjing, China. The antibacterial activity of all compounds was tested on four different strains of bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, and *Escherichia coli*. The antifungal activity of all compounds was tested on three different strains of fungi: *Candida albicans*, *Phanerochaete chrysosporium*, and *Coriolus versicolor*.

The culture of bacteria and fungi: bacteria were grown in broth (NB) medium, the NB medium was composed of 3 g/L beef extract, 5 g/L polypeptone, 1 g/L yeast extract, and 10 g/L sucrose, and the pH was adjusted to 7.0 with 1 M sodium hydroxide (Liang et al., 2016) and then sterilized by autoclaving at 121°C for 20 min. Fungi were grown in potato dextrose agar (PDA) medium (Atlas, 1998) and sterilized by autoclaving at 121°C for 20 min.

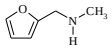
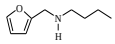
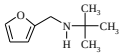
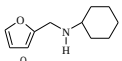
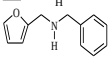
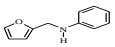
Bacterial and fungal suspension were then prepared with a population of ca. 10^6-10^7 CFU/mL separately (Moreno et al., 2017; Higginbotham et al., 2014), after incubation cultures at 37°C with for 24 h (for strains of baeteria); at 28°C with for 72 h (for strains of fungi). The seven strains of bacterial and fungal suspensions were then separately added into a 96-well microplate (200 μL each well, and three wells for each replicate). The minimal inhibitory concentration (MIC) tests of twelve furfural derivatives (3a-3f, 4a-4f) were determined according to Andrew's methods (Andrews, 2001), in a dilution series from 0.095 μg/mL to 200 μg/mL. The lowest concentration showing a clear zone of inhibition was considered as the MIC. Each treatment was represented by three replicates, and this experiment was repeated three times. For the positive control of the antibiotic tests of obtained compounds, kanamycin was used in bacterial test, and ketoconazole was used in fungal test.

3 Results and Discussion

The synthetic furfural derivatives in Tables 1 and 2 were obtained following synthetic route in Scheme 1, with comparative high yields varying from 76%∼92%, apart from *N*-methyl-*N*-furfurylacetamide and *N*-t-butyl-*N*-furfurylacetamide with yield around 50%. In solvent selection of the experiments, compared with diethyl ether and dichloromethane, the yield of products obtained in either cyclohexane or ethyl acetate was considerably higher. Of these, ethyl acetate was preferable as an environmentally-friendly solvent which was finally selected (Capello et al., 2007). Of the bases examined in Step 3 of the synthetic route in Scheme 1, pyridine was proven non-productive, whilst it provided a homogeneous reaction system, which

reached to equilibrium with incomplete reaction. In contrast, when either KOH or $KHCO_3$ was employed as the base, the formation of insoluble potassium salts as products shifted the equilibrium to favour product formation. Overall, $KHCO_3$ was proven to be better than KOH as the latter generated viscous oil, which caused difficulty in the purification process. In conclusion, ethyl acetate as the solvent, combined with $KHCO_3$ as the base, and a reaction temperature under 0°C for 2 h were experimental-optimised condition in the series of synthetic experiments.

Table 1. Synthesized *N*-alkyl furfurylamines

Entry	Compound	Structure	MW	Yield (%)
3a	*N*-methyl furfurylamine		111	76
3b	*N*-*n*-butyl furfurylamine		153	83
3c	*N*-*t*-butyl furfurylamine		153	77
3d	*N*-cyclohexyl furfurylamine		179	79
3e	*N*-phenyl furfurylamine		173	81
3f	*N*-benzyl furfurylamine		187	90

The structures of the synthesised compounds were confirmed by ^{1}H-NMR, GC-MS, and IR spectroscopy. The IR spectral data of compounds 3a to 3f and 4a to 4f showed characteristic bands of NH at 3114 cm^{-1} to 3412 cm^{-1}. The stretching vibration bands of the furan ring skeleton appeared at 1505 cm^{-1}, 1457 cm^{-1}, 1377 cm^{-1}, 1334 cm^{-1}; C-H stretching vibration band of furan ring appeared at 3116 cm^{-1}, respectively. In particular, characteristic absorption peak of amide structure $\nu_{C=O}$ of 4a to 4f vibrated at 1647-1652 cm^{-1}; $\delta_{N-C=O}$ at 599-600 cm^{-1}. C-H stretching vibration of benzene ring was also remarkable in the IR absorption bands around 3067 cm^{-1} -3085cm^{-1}. In the ^{1}H-NMR spectra of the target compounds, taking *N*-phenyl furfuryl amine ($CDCl_3$, 400MHz) as an example: δ 3.089 (1H, s, N-H); δ 3.397 (2H, s, CH_2); δ 5.313 (1H, d, J=3.2, CH); δ 5.401 (1H, dd, J1=3.2, J2=1.6, CH) and δ 6.445 (1H, d, J=0.8, CH) were signals of three protons of the furan ring; Notably, the phenyl protons of compound 3e present at 5.769-5.747 (2H, m, CH); 5.824 (1H, t, J=7.6, CH); 6.327-6.249 (2H, m, CH). In the Mass spectrum of target compounds, typical ion/group fragment peaks generated at: 138(50) CH_3, 69(10) furyl, 96(58) amidoxime, 81(100) sulfhydryl, 57(12) tert-butyl.

It is clearly seen in the Table 4 that the growth of *Phanerochaete chrysosporium* was inhibited by *N*-cyclohexyl-*N*-furfurylacetamide (4d, MIC=50) and *N*-benzyl-*N*-furfurylacetamide (4f, MIC=12.5). *N*-cyclohexyl-*N*-furfurylacetamide also showed noble antifungal activity in inhibiting the growth of *Coriolus versicolor* (4d, MIC=25). The results also indicated that the antibacterial and antifungal effect was not observed with other common bacteria and fungi such as *E.coli, S.aureus, C. albicans etc.* (All MIC $\geq$ 200 μg/mL).

Table 2. Synthesized *N*-alkyl-*N*-furfurylacetamides

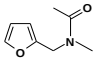
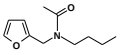
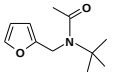
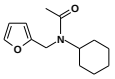
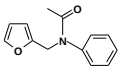
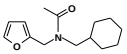
Entry	Compound	Structure	MW	Yield (%)
4a	*N*-methyl-*N*-furfurylacetamide		153	51
4b	*N*-*n*-butyl-*N*-furfurylacetamide		195	90
4c	*N*-*t*-butyl-*N*-furfurylacetamide		195	52
4d	*N*-cyclohexyl-*N*-furfurylacetamide		223	88
4e	*N*-phenyl-*N*-furfurylacetamide		215	91
4f	*N*-benzyl-*N*-furfurylacetamide		229	92

Table 3. The minimal inhibitory concentration (MIC) results of *N*-alkyl furfurylamines series

Test microorganisms	MIC (μg/mL)						
	3a	3b	3c	3d	3e	3f	PC[a]
Bacillus subtilis	>200	>200	>200	>200	>200	>200	0.78
Staphylococcus aureus	>200	200	>200	>200	>200	>200	3.12
Pseudomonas	>200	>200	>200	>200	>200	>200	3.12
Escherichia coli	>200	>200	>200	>200	>200	>200	0.78
Candida albicans	>200	>200	>200	>200	>200	>200	1.56
Phanerochaete	200	>200	200	200	100	200	1.56
Coriolus versicolor	>200	>200	>200	>200	>200	>200	0.78

[a]Positive control: bacterial test used kanamycin, fungal test used ketoconazole

In general, *N*-alkyl-*N*-furfurylacetamide derivatives (4a-4f) exhibited better antibacterial and anti-microbial activities, particularly to the growth of wood rot fungi, which were superior to *N*-alkyl furfurylamine derivatives (3a-3f); indicating that *N*-alkyl furfurylamine derivatives with the acetamide group exhibit particularly high activity in the antibacterial and antifungal assay. Moreover, if the group of nitrogen of the investigated synthetic compounds linked to an aryl group, than to an aliphatic hydrocarbon, the final

Table 4. The minimal inhibitory concentration (MIC) results of *N*-alkyl-*N*-furfurylacetamide series

Test microorganisms	MIC (μg/mL)						
	4a	4b	4c	4d	4e	4f	PC[a]
Bacillus subtilis	>200	>200	>200	>200	>200	200	0.78
Staphylococcus aureus	>200	>200	>200	>200	>200	200	3.12
Pseudomonas fluorescens	>200	>200	>200	>200	>200	>200	3.12
Escherichia coli	>200	>200	>200	>200	>200	>200	0.78
Candida albicans	>200	>200	>200	>200	>200	>200	1.56
Phanerochaete chrysosporium	200	200	100	50	200	12.5	1.56
Coriolus versicolor	200	>200	>200	25	100	100	0.78

[a]Positive control: bacterial test used kanamycin, fungal test used ketoconazole

effect did not show any improvement.

Both *Phanerochaete chrysosporium* and *Coriolus versicolor* are taxonomically belong to white-rot-fungi, which degrade hemicelluloses and lignin of wood (Millati et al., 2011). The existence of wood-decay fungus is detrimental to the wood industry and also a major threat to the world cultural heritage, principally if considering the conservation of ancient wooden constructions and architecture all over the world and wooden artifacts in and outside museums, including manuscript (paper) collections in libraries and archives. In the conservation of cultural heritage, the growth of wood-decay fungi will not only occur and influence wooden structure, causing insect infestation which certainly accelerates the degradation of wood. It also causes discoloration, which affects other type of heritage including textiles as well (Keharia and Madamwar, 2002; Shah and Nerud, 2002; Itoh and Yatome, 2004). Environmental control in temperature and relative humidity (Gu, 2003) is regarded as the best way to prevent the growth of wood-decay fungi in the conservation of cultural heritage. This might be accessible in well-funded museums, but, for many private or local museums and historical sites, due to financial consideration and/or environmental limits, it is impossible to sustain the *in situ* balance of the conservation environment. Therefore, degradation or decolorization caused by wood-decay fungi would require responsible choices of chemical treatment if possible, with bearing the essentials of conservation principles, *i.e.*, applying lower toxic and heritage-friendly antifungal drug in the heritage environment, particularly the treatment will be reversible, with minimal intervention, and for long term conservation purpose.

Further study is needed to investigate this type of compounds and their anti-fungal activities compared to other types of wood-decay fungi such as brown-rot fungi and their interaction with local environment (Green and Highley, 1997; Hyde and Wood, 1997; Alper et al., 2009; Mir-Tutusaus et al., 2014). Toxicity tests will also be performed to screen those synthetic products from natural sources at suitable ecosystem of relevant applications (Gu, 2003). The future research is also enlightened by the mechanism of metabolism of white-rot-fungi, including producing various enzymes in furfural derivatives related environment (Cabana et al., 2007; Schulz and Dickschat, 2010).

Furfural used to be labeled as waste or by-product in the past. It is obtained from lignocellulosic materials with treatment in acidic water at high temperature on an industrially basis or gained in the liquid fraction from pyrolysis of wood and cellulose. Natural products, like wood, usually have certain antimicrobials that developed through evolution, thus the industrialized by-product of natural products from modern process may be worth reconsideration, exploring and exploiting its hidden value.

4 Conclusions

Our results show *N*-alkylfurfurylacetamide series of compounds exhibit noble anti-white-rot-fungi potency, superior to *N*-alkyl furfurylamines. Synthetic pathway and methods are simple and straightforward to obtain high yield target compounds, with convenient post processing and treatment. The approachable biomass refined furfural provides a promising green route for sustainable and economical starting materials in synthesizing high value-added microbial inhibitors. *N*-cyclohexyl-*N*-furfurylacetamide and *N*-benzyl-*N*-furfurylacetamide are structurally novel antimicrobial compounds, indicating that natural products are informative for pharmaceutical research in structure–activity relationship and target-oriented synthesis.

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