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Halogenated Cinnamanilides and Their Activity Against Selected Gram-Negative Bacteria †

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Abstract

Recently published halogenated anilides of chlorinated and trifluorinated cinnamic acids, such as (2*E*)-*N*-[3,5-bis(trifluoromethyl)phenyl]-3-(3,4-dichlorophenyl)prop-2-enamide, (2*E*)-*N*-(3,5-dichlorophenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-enamide or (2*E*)-*N*-[3,5-bis(trifluoromethyl)phenyl]-3-[4-(trifluoromethyl)phenyl]prop-2-enamide, showed excellent antibacterial activities in vitro against Gram-positive bacteria, especially against reference and quality control strains *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, as well as against representatives of multidrug-resistant bacteria and clinical isolates of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecalis* (VRE) with minimum inhibitory concentrations (MICs) against staphylococci <0.2 μg/mL and against enterococci < 4 μg/mL. It should be noted that all these compounds are rather lipophilic (software predicted log *p* values close to 5) and carry electron-withdrawing substituents that allow them to be classified as so-called Michael acceptors. All these facts inspired further investigation of the spectrum of effectiveness against other bacteria, and the most effective agents with various substitutions in both the anilide part and on the phenyl ring of the parent cinnamic acid were chosen and tested against selected pathogenic Gram-negative bacteria, such as reference and quality control strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27859 and clinical isolate of *Klebsiella pneumoniae* 797. Unfortunately, it was found that none of the selected halogenated anilide derivatives with such high potency against Gram-positive bacteria demonstrated better efficacy against the tested Gram-negative bacteria than MICs 256 μg/mL.

Keywords: halogenated cinnamanilides; synthesis; Gram-negative bacteria; antibacterial activity

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1. Introduction

Antimicrobial resistance (AMR) is a serious problem that threatens the effectiveness of treatments for infections caused by bacteria, viruses, fungi and parasites. The greatest

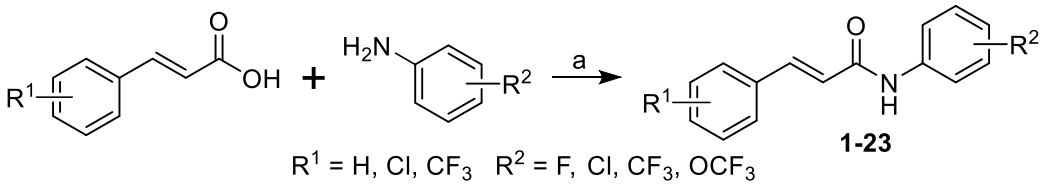
burden and cost in healthcare is associated with bacterial resistance to antibiotics [1–3]. According to estimates from 2021, approximately 4.71 million deaths were associated with AMR, of which 1.14 million were directly caused by bacteria [4]. Moreover, AMR also poses a critical threat to the global economy and could cost up to $100 trillion by 2050 [5].

The World Health Organization (WHO) responded to this problem in 2015 with the Global Action Plan on Antimicrobial Resistance [6]. In 2017, WHO published the Bacterial Pathogens Priority List (BPPL), which classified 25 antibiotic-resistant pathogens into three priority levels: critical, high, and medium [7]. The list was designed to focus research and development of new antibacterial agents and to streamline the monitoring of AMR [3,5,8,9]. In 2024, the WHO updated the list, but the leading places are still occupied by resistant strains of bacteria such as *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Enterococcus* sp., *Salmonella* sp., *Shigella* sp. and others [10]. Despite increasing research efforts in the field of antimicrobial resistance, progress in the development of new drugs is stagnant [11–15].

To overcome antimicrobial resistance, the development of new agents with innovative mechanisms of action is essential [16–18]. One promising strategy is to take inspiration from natural compounds [19–21] and modify them into so-called multi-target agents. These agents, in contrast to single-target agents, are more effective in combating the development of resistance [22–24]. Cinnamic acid, which has a long history of human use, has been used as a scaffold for new compounds [25–27]. From it, novel anilides, specifically diverse series of halogenated cinnamanilides, have been designed. These compounds were prepared, and tested for their efficacy against a variety of pathogens, including bacteria, mycobacteria, and protozoa [28–34]. Some of these compounds have been shown to be highly effective against Gram-positive bacteria [28,30,31], and therefore their research has been extended to Gram-negative bacteria. This article summarizes the findings on the effect of halogenated cinnamanilides against selected Gram-negative bacteria.

2. Results and Discussion

The discussed anilides were synthesized using microwave-assisted synthesis as shown in Scheme 1 and described by Kos and Strharsky [28–32]. Reaction of ring-substituted cinnamic acids with appropriately substituted anilines using phosphorus trichloride in chlorobenzene afforded anilides **1**–**23**, the structures of which are listed in Table 1.



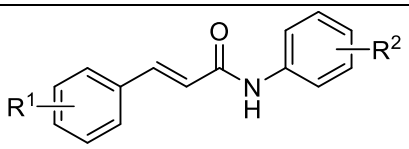
Scheme 1. Synthesis of ring-substituted cinnamanilides **1**–**23**. *Reagents and conditions*: (a) PCl_3, chlorobenzene, MW.

All compounds presented in this contribution have been recently tested in vitro against Gram-positive bacteria and mycobacteria. First, universally susceptible collection strains *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were selected. The second aspect of strain selection was the current status of strains with epidemiologically relevant resistance patterns, represented by clinical isolates, e.g., methicillin-resistant *S. aureus* (MRSA) isolates and vancomycin-resistant *E. faecalis* (VRE) isolates. In addition, all compounds were tested in vitro against fast and slow growing mycobacterial strains. In general, the minimum inhibitory concentrations (MICs) against *Staphylococcus*

strains were <0.2 μg/mL and against facultatively anaerobic enterococci the MICs were <4 μg/mL [28–32]. It is important to note that all of these effective agents were bactericidal.

Due to the problematic search for active agents against Gram-negative bacteria, and the excellent results of these compounds on Gram-positive bacteria, it was decided to test the most effective derivatives also on selected Gram-negative pathogens. The selection of bacteria was carried out in the context of BPPL [10] and the reference strains (from American Type Culture Collection (ATCC) [35]) *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27859 and the clinical isolate *Klebsiella pneumoniae* 797 (from the collection of the Department of Infectious Diseases and Microbiology, Faculty of Veterinary Medicine, Veterinary University Brno, Czech Republic) [36] were selected for primary screening. The results of the investigation are presented in Table 1, the activities are expressed as MICs.

Table 1. Structures of ring-substituted cinnamanilides **1–23** and antibacterial activities against selected Gram-negative bacteria.



No.	R¹	R²	MIC [μg/mL]		
			E. coli ATCC 25922	*P. aeruginosa* ATCC 27859	*K. pneumoniae* 797
1	H	3-CF_3	>256	>256	>256
2	H	3,4-Cl	>256	>256	>256
3	H	3,5-Cl	>256	>256	>256
4	H	3,5-CF_3	>256	>256	>256
5	H	3-F-4-CF_3	>256	>256	>256
6	4-Cl	3-CF_3	>256	>256	>256
7	4-Cl	4-CF_3	>256	>256	>256
8	4-Cl	3,5-Cl	>256	>256	>256
9	4-Cl	3,5-CF_3	>256	>256	>256
10	3,4-Cl	3,5-CF_3	>256	>256	>256
11	2-CF_3	3-CF_3	>256	>256	>256
12	2-CF_3	4-CF_3	>256	>256	>256
13	2-CF_3	3,5-Cl	>256	>256	>256
14	2-CF_3	3,5-CF_3	>256	>256	>256
15	2-CF_3	4-OCF_3	>256	>256	>256
16	3-CF_3	3-CF_3	>256	>256	>256
17	3-CF_3	4-CF_3	>256	>256	>256
18	3-CF_3	3,5-Cl	>256	>256	>256
19	3-CF_3	3,5-CF_3	>256	>256	>256
20	4-CF_3	3-CF_3	>256	>256	>256
21	4-CF_3	4-CF_3	>256	>256	>256
22	4-CF_3	3,5-Cl	>256	>256	>256
23	4-CF_3	3,5-CF_3	>256	>256	>256
ciprofloxacin			0.125	0.125	1.00

It is evident that the compounds in Table 1 did not show activity against Gram-negative bacteria, as their MIC values were 256 μg/mL or higher. However, the effect of cinnamic acid derivatives on Gram-negative bacteria has been reported in the literature. Depending on the used test method, the bacteria (*E. coli*, *P. aeruginosa*, *K. pneumoniae*) and evaluated compounds (ring-substituted acid, ester or amide), the activity varies in a wide range of MIC values from 1 to >1000 μg/mL [37–39].

Ferulic and sinapic acids at 1000 μg/mL inhibited quorum sensing (QS), significantly impaired biofilm formation, and reduced virulence of *P. aeruginosa* [40]. Rajkumari et al. also confirmed that cinnamic acid alone at a sublethal concentration of 250 μg/mL effectively inhibited both the production of QS-dependent virulence factors and biofilm formation in P. *aeruginosa* without affecting bacterial viability [41]. Cinnamoyl hydroxamates have been reported as potential inhibitors of QS and biofilm formation of *P. aeruginosa* at concentrations of approximately 300 μg/mL [42]. It is true that testing any compounds at such high concentrations can be controversial. On the other hand, this approach allows us to determine whether the compounds show at least some activity against Gram-negative bacteria. Although it is clear that no substances with clinically relevant efficacy have been found, testing on such a large scale can provide valuable insights, e.g., on the threshold of action or the nature of resistance. The results obtained can thus serve as a starting point for further research.

In this study, derivatives were evaluated up to a meaningful concentration of 256 μg/mL. This low potency compared to the high activity against Gram-positive bacteria raises the question of whether these compounds lack intrinsic antibacterial activity or whether there are other factors that prevent their action, such as (*i*) limited permeability into the cell of Gram-negative bacteria [43] or (*ii*) becoming a substrate for efflux transporters [44]. Cinnamic acid derivatives have been described as inhibitors of efflux pumps in Gram-positive bacteria [45], but are substrates for efflux pumps in Gram-negative bacteria [46]. Similarly, the complexity of the cell wall of Gram-negative bacteria is known to provide an effective barrier against good permeability [43] of antibiotics. Therefore it is possible that the tested cinnamanilides are effective (have intrinsic activity) but cannot reach their target or effective concentration inside the Gram-negative bacterial cell. Further tests, for example using efflux pump inhibitors or membrane permeability assays, will be necessary to confirm this hypothesis.

3. Experimental Section

3.1. Chemistry

All discussed ring-substituted (2*E*)-*N*-aryl-3-phenylprop-2-enamides **1–5** [27,28], (2*E*)-3-(4-chlorophenyl)-*N*-arylprop-2-enanilides and (2*E*)-3-(3,4-dichlorophenyl)-*N*-arylprop-2-enanilides **6–10** [29], trifluoromethylcinnamanilide **11–23** [30,31] were previously prepared and characterized.

3.2. In Vitro Antibacterial Evaluation

In vitro antibacterial activity of the synthesized compounds was evaluated against representatives of Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27859 [35] and clinical isolate of human origin *Klebsiella pneumoniae* 797 (from the collection of the Department of Infectious Diseases and Microbiology, Faculty of Veterinary Medicine, Veterinary University Brno, Czech Republic) [36]. The minimum inhibitory concentrations (MICs) were evaluated by the microtitration broth method according to the Clinical and Laboratory Standards Institute (CLSI) [47,48] with some modifications, as detailed in Markuliak et al. [49]. The results are summarized in Table 1.

4. Conclusions

Twenty-three differently halogenated cinnamic acid anilides, which were highly active against Gram-positive bacteria, were evaluated by the microtiter broth method against the reference strains *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27859 and the clinical isolate *K. pneumoniae* 797. Their MIC values were >256 μg/mL, so they did not cause any real observable growth/viability inhibition of these Gram-negative bacteria. Additional

experiments are needed to gain a deeper understanding of the expected activity activities of these agents, such as inhibition of quorum sensing and virulence, or to verify their intrinsic antibacterial activity (e.g., synergism with efflux pump inhibitors or with compounds affecting membrane permeability).

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