chemistry proceedings

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Proceeding Paper

Effects of *N*-(Alkoxyphenyl)-1-hydroxynaphthalene-2-carboxamides on Intestinal Microbial Communities †

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† Presented at the 27th International Electronic Conference on Synthetic Organic Chemistry (ECSOC-27), 15–30 November 2023; Available online: https://ecsoc-27.sciforum.net/.

Abstract: The phylum Proteobacteria, more precisely, the family *Enterobacteriaceae*, has been shown to be a major cause of inflammation in the human microbiome. Their standard level in the human intestine is usually kept below 1% in a healthy person, and their overgrowth above this number leads to intestinal inflammation, which can cause the development of inflammatory bowel diseases, most often, Crohn's disease or ulcerative colitis. The minimum inhibitory concentrations (MICs) of a series of eighteen recently synthesized *N*-(alkoxyphenyl)-2-hydroxynaphthalene-1-carboxamides were determined against two representatives of the *Enterobacteriaceae* family–*Escherichia coli* CCM 3954 and *Salmonella typhimurium* LT 2-18. Although the tested compounds are cyclic analogues of salicylanilides known to have strong antimicrobial properties, the found MICs ranged between 50 μM and 1000 μM. However, it can be concluded that *S. typhimurium* was generally more sensitive to the tested antimicrobial agents than *E. coli*. *N*-[2-(But-2-yloxy)phenyl]-1-hydroxynaphthalene-2-carboxamide was the most active agent among the investigated compounds with an MIC of 100 μM against *E. coli* and an MIC of 50 μM against *S. typhimurium*.

Keywords: hydroxynaphthalenecarboxamides; synthesis; enterobacteriaceae; antibacterial activity

Citation: Nikolaeva, V.; Gonec, T.; Kushkevych, I.; Jampilek, J. Effects of *N*-(Alkoxyphenyl)-1-hydroxynaphthalene-2-carboxamides on Intestinal Microbial Communities. *Chem. Proc.* **2023**, *14*, 45. https://doi.org/10.3390/ecsoc-27-16142

Academic Editor: Julio A. Seijas

Published: 15 November 2023

1. Introduction

Enterobacteriaceae is a large family of Gram-negative, facultatively anaerobic rods, most of which live in the digestive tract as a natural part of the intestinal microflora. Most are non-pathogenic, but some are conditionally pathogenic and some species are dangerous agents of serious and fatal diseases (e.g., *Salmonella*, *Shigella*, *Yersinia pestis*, some strains of *Escherichia coli*) [1].

The presence of pathogenic enterobacteria in the gastrointestinal tract can greatly alter the diversity and abundance of beneficial bacteria. For example, it is well-known that adherent invasive strains of *E. coli* colonize the intestinal mucosa and prevent the proper function of intestinal immune cells, causing major damage to the epithelial barrier, which plays an important role in regulating interactions between the luminal contents of the gut and the rest of the body. Thus, pathogenic enterobacteria tend to be highly inflammatory and play a significant role in the development of inflammatory bowel diseases–chronic, immune-related diseases of the gastrointestinal tract with multifactorial and unclear pathogenesis [2–5].

Salicylanilides represented by, e.g., niclosamide [6,7], are simple, small and relatively old molecules, which, however, still surprise with a wide range of biological activities and

the ability to influence various cell structures [8–12]. Hydroxynaphthalenecarboxanilides can be considered as cyclic analogues of salicylanilides and have also been described mainly for their anti-invasive activity against both cancer lines and Gram-positive bacteria and mycobacteria [13–18].

Following the previous studies, a series of *N*-(alkoxyphenyl)-1-hydroxynaphthalene-2-carboxamides was tested for their ability to inhibit the growth of Gram-negative intestinal bacteria from the *Enterobacteriaceae* family represented by *Escherichia coli* and *Salmonella typhimurium* species, with the aim that if any of tested compounds achieves antimicrobial activity against the test species at a reasonable minimum inhibitory concentration (MIC), its possible potential for use in inflammatory bowel disease therapy will be explored.

2. Materials and Methods

2.1. Synthesis

All the discussed *N*-(alkoxyphenyl)-1-hydroxynaphthalene-2-carboxamides **1**–**18** were synthesized using microwave-assisted synthesis, as described recently by Gonec et al. [13,14].

2.2. In Vitro Antibacterial Susceptibility Testing

In vitro antibacterial activity of the synthesized compounds was evaluated against representatives of two strains of intestinal bacteria (*Escherichia coli* CCM 3954 and *Salmonella typhimurium* LT 2-18), which are included into the *Enterobacteriaceae* family. The bacterial cultures were cultivated at 37 °C in standard liquid medium (nutrient broth, code: CM0001). A 1% solution of bacteria and nutrient broth was prepared before each experiment and subsequently incubated for approx. 24 h. After this incubation, the bacterial culture was diluted 10 more times (with pure nutrient broth) in order to achieve a suitable concentration that will provide an easily discernible growth curve. The optimal pH for the growth of both *E. coli* CCM 3954 and *S. typhimurium* LT 2-18 is in the range of 6.5 to 7.5. The pH of the nutrient broth used in each experiment was adjusted to fall between these values using hydrochloric acid to reduce the pH and sodium hydroxide to increase it. Each of the tested agents was dissolved using dimethyl sulfoxide [19]. Due to the difference in mass and molecular weight, the above calculation was repeated for each compound and for each concentration tested.

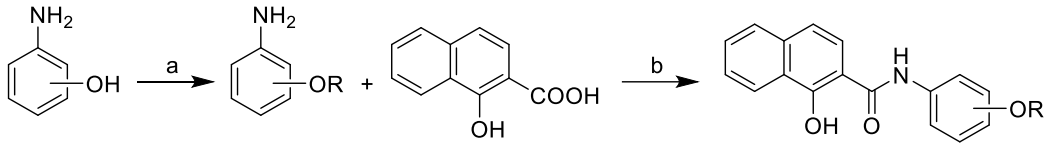
The equipment used for the majority of the experiment was a Bioscreen C (Bioscreen, Helsinki, Finland), an automated machine suitable for various microbiological experiments [20]. It monitors the growth of microorganisms by measuring the turbidity of liquid growth medium kinetically using the principle of vertical photometry, and then recording it as optical density measurements. A light beam penetrates though the bottom of the plate well, through the sample suspension, and to the detector. The growth of the bacteria in a Bioscreen C becomes visible twice faster than in a Petri dish, and four times faster if the shaking feature is used (it was not used in this experiment). A controlled environment is maintained by software throughout the whole process [20]. The incubation temperature, time, measuring intervals and wavelength can all be manually set. The wavelength used in this experiment was 600 nm, measuring intervals were set at 1 h. A total of 48 measurements were collected over the course of 48 h, 37 °C temperature was maintained during this time. The maximum capacity of the machine is two honeycomb plates, which altogether contain 200 wells; up to 31 concentrations were measured each time (9 wells had to be reserved for controls: three for negative control, three for positive control for *S. typhimurium* LT 2-18, and three for positive control for *E. coli* CCM 3954).

The second stage of the experiment used Bioscreen C and successfully generated a growth curve and determined a minimum inhibitory concentration for most of the antimicrobial agents by measuring their optical density (O.D.) at 1 h intervals over the course of 48 h. For this experiment, the incubation and subsequent dilution of the bacterial cultures before each experiment followed the same steps as the process. A honeycomb plate with 100 wells was used for the Bioscreen C, each well had a capacity of 250 µL. Six wells were used in total for each concentration tested, three of which contained bacteria and nutrient broth

solution + compound, and three more wells after them containing only plain nutrient broth and compound (no bacteria). This was conducted to eliminate the possibility of the growth curve being influenced by any colour changes of the compounds, as most of them were not colourless. Three of each was the amount chosen as 3 is the minimum amount needed to obtain a statistically valid result. The values received from the 3 wells without compound were averaged, and then subtracted from the values received from the 3 wells with compound (also averaged beforehand). The final value obtained formed 1 of the 48 points in each growth curve. Pure nutrient broth was used as a negative control, and nutrient broth + bacterial solution was used as a positive control. Each experiment had two positive controls–one for *S. typhimurium* LT 2-18, and one for *E. coli* CCM 3954, therefore 9 wells were reserved for controls every time.

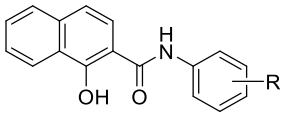
3. Results and Discussion

All eighteen studied compounds were prepared by condensation of 1-hydroxynaphthalene-2-carboxylic acid with appropriately alkoxy-substituted anilines in dry chlorobenzene using phosphorous chloride and microwave radiation in a microwave reactor as shown in Scheme 1. Alkoxy-substituted anilines occur either commercially and were purchased (anisidines) or were prepared from aminophenols and alkyl bromide according to De Marco et al. [21]. All the discussed compounds were described in detail in Gonec et al. [13,14] and are listed in Table 1.



Scheme 1. Synthesis of *N*-(alkoxyphenyl)-1-hydroxynaphthalene-2-carboxamides. *Reagents and conditions*: (**a**) R-Br, NaH, acetonitrile, room temperature, 24 h [21]; (**b**) PCl_3, chlorobenzene, MW, 15 min. [13,14].

Table 1. Structures of discussed ring-substituted *N*-(alkoxyphenyl)-1-hydroxynaphthalene-2-carboxamides **1**–**18** and in vitro activity (MIC) against *Escherichia coli* CCM 3954 and *Salmonella typhimurium* LT 2-18.



No.	R	MIC (μM)	
		E. coli	*S. typhimurium*
1	H	>1000	>1000
2	2-OCH_3	900	1000
3	3-OCH_3	200	200
4	2-OC_2H_5	300	300
5	3-OC_2H_5	400	200
6	4-OC_2H_5	600	600
7	2-OC_3H_7	500	200
8	3-OC_3H_7	300	200
9	4-OC_3H_7	500	200
10	2-OC_4H_9	400	200
11	3-OC_4H_9	300	200
12	4-OC_4H_9	400	300
13	2-$OCH(CH_3)_2$	400	200
14	3-$OCH(CH_3)_2$	300	200
15	4-$OCH(CH_3)_2$	400	300
16	2-$OCH(CH_3)CH_2CH_3$	100	50
17	3-$OCH(CH_3)CH_2CH_3$	400	300
18	4-$OCH(CH_3)CH_2CH_3$	200	200

All compounds were evaluated for their ability to inhibit in vitro growth of Gram-negative bacteria *Escherichia coli* CCM 3954 and *Salmonella typhimurium* LT 2-18, members

of the *Enterobacteriacea* family. The efficacy of the individual compounds was expressed as minimal inhibitory concentrations (MICs), i.e., 90% or more (IC_{90}) reduction of bacterial growth compared to the control [22]. The results are shown in Table 1, and, according to the MIC values, it can be seen that the compounds showed insignificant activity against the mentioned types of bacteria, which ranged from 50 to 1000 μM.

N-[2-(But-2-yloxy)phenyl]-1-hydroxynaphthalene-2-carboxamide (**16**) was the most effective against both tested bacterial strains with an MIC of 100 μM against *E. coli* and an MIC of 50 μM against *S. typhimurium*. 1-Hydroxy-*N*-(3-methoxyphenyl)naphthalene-2-carboxamide (**3**) and *N*-[4-(but-2-yloxy)phenyl]-1-hydroxynaphthalene-2-carboxamide (**18**) were other derivatives, which showed some effect in inhibiting the growth of both bacteria (all MICs = 200 μM). As evident from the results mentioned in Table 1, the compounds were slightly more active against *S. typhimurium*. Unsubstituted 1-hydroxy-*N*-phenylnaphthalene-2-carboxamide (**1**) was completely inactive (MICs >1000 μM).

4. Conclusions

Thus, although hydroxynaphthalenecarboxamides have shown strong antibacterial and antimycobacterial effects even when substituted with methyl and methoxy groups, 1-hydroxynaphthalene-2-carboxamides substituted in this way with longer alkoxy tails are not suitable as agents for the inhibition of intestinal microorganisms potentially involved in the development and progression of inflammatory bowel diseases.

Author Contributions: Conceptualization, J.J.; methodology, T.G. and I.K.; investigation, V.N., T.G. and I.K.; writing—original draft preparation, T.G., I.K. and J.J.; funding acquisition, T.G., I.K. and J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Grant Agency of Masaryk University (MUNI/A/1280/2022 and MUNI/A/1210/2022), by VEGA 1/0116/22 and by APVV-22-0133.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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