Substituted Salicylaldehydes as Potential Antimicrobial Drugs: Minimal Inhibitory and Microbicidal Concentrations

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Substituted salicylaldehydes are potent antibacterial and antifungal agents and may have chemotherapeutic potential. In the clinical setting, the minimal inhibitory concentration (MIC) as well as the minimal bactericidal and fungicidal concentrations (MBC and MFC, respectively) are of fundamental interest. Therefore, we have now, using a panel of five microbial species (*Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Staphylococcus aureus*), determined the MIC and MBC/MFC values of a total of 22 aromatic aldehydes, including 19 substituted salicylaldehydes and the unsubstituted parent compounds benzaldehyde and salicylaldehyde (2-hydroxybenzaldehyde). The results clearly indicate that both of the yeasts studied are remarkably sensitive to various salicylaldehydes and, especially, to halogenated ones. Some congeners clearly merit consideration as potential therapeutic agents for *Candida* infections. The MIC values of the most potent congeners are of roughly the same magnitude as that of amphotericin B, and the results of the MFC measurements indicate that the compounds are fungicidal. All of the bacteria studied are also sensitive to at least some of the compounds tested but, clearly, this class of antimicrobials has superior activity against yeasts. Structure-activity relationships are discussed for each microbial species and compared with each other. The comparison of the results of MIC and MBC/MFC measurements with those of agar diffusion tests revealed aspects that are of interest concerning the methodology of antimicrobial activity screening. Unexpectedly, it was found that some compounds that are completely devoid of activity in agar diffusion tests had potent activity in MIC tests, indicating that if only agar diffusion methodology is used in drug discovery, some highly active compounds may be missed.

Key words: 2-Hydroxybenzaldehydes, Minimal Inhibitory Concentration, Minimal Bactericidal Concentration, Minimal Fungicidal Concentration

Introduction

We have recently reported a systematic survey of the antimicrobial properties of substituted salicylaldehydes and some other aromatic aldehydes (Pelttari *et al.*, 2007a). All of the 18 differently substituted salicylaldehydes studied displayed distinct antimicrobial activity against one or more test organisms (*Aspergillus niger*, *Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, and *Staphylococcus epidermidis*), in contrast to the unsubstituted parent compounds salicylaldehyde (2-hydroxybenzaldehyde) and benzaldehyde that had minimal or no activity. Several aldehydes, most notably halogenated, nitro-substituted, and hydroxylated salicylaldehydes, had highly potent activity. A further characterization of the antimicrobial properties of substituted salicylaldehydes is thus of great interest.

Our previous studies were carried out employing the paper disc agar diffusion method. Concerning possible clinical applications of the compounds, it is important to verify that they are active against essentially all microbial cells of each susceptible strain. It would be expected that if a (small) proportion of the cells of a microbial strain/culture is resistant to the test compound, this may result in visible growth in a liquid culture but perhaps only individual (micro)-colonies on agar.

Antimicrobial susceptibility testing is important in diagnostic microbiology because the results are used by practising physicians to determine treatment regimens for patients (Peterson and Shanholtzer, 1992). In clinical microbiology, one important method of determining the antibiotic susceptibility of a microbial strain is the determination of the lowest concentration of a compound that inhibits the growth of the micro-

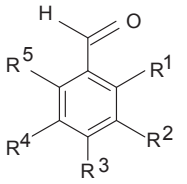
organism. This concentration is referred to as the minimal inhibitory concentration (MIC). Further important parameters in the clinical setting are the minimal bactericidal and fungicidal concentrations (MBC and MFC, respectively). Often, in clinical practice, the concepts of MBC and MFC are used so as to refer to the lowest concentration of a test compound that kills 99.9% of the bacteria or fungi (for details, see Anhalt *et al.*, 1980).

We have now, using a panel of five microbial species, studied the MIC values of the salicylaldehydes that were used in our previous agar diffusion studies, and have further determined the MBC and MFC values of the compounds. The results of those studies are reported here.

Experimental

Compounds tested

The structures of the compounds studied are shown in Fig. 1. A total of 19 substituted salicylaldehydes, plus benzaldehyde, unsubstituted salicylaldehyde and one congener (3,4-dihydroxybenzaldehyde; compound **6**) without the 2-hydroxy group characteristic of salicylaldehydes, were tested. They were obtained from E. Merck (Darmstadt, Germany), Aldrich-Chemie/Aldrich Chemical Company and EGA-Chemie (Steinheim, Germany), Fluka AG (Buchs, Switzerland), Kodak Eastman Fine Chemicals (Eastman Kodak Company, Rochester, NY, USA), and TCI (Tokyo, Japan). The compounds were dissolved in dimethyl sulfoxide (DMSO).



1 $R^1 = R^2 = R^3 = R^4 = R^5 = H$
2 $R^1 = OH, R^2 = R^3 = R^4 = R^5 = H$
3 $R^1 = R^2 = OH, R^3 = R^4 = R^5 = H$
4 $R^1 = OH, R^2 = H, R^3 = OH, R^4 = R^5 = H$
5 $R^1 = OH, R^2 = R^3 = H, R^4 = OH, R^5 = H$
6 $R^1 = H, R^2 = R^3 = OH, R^4 = R^5 = H$
7 $R^1 = R^2 = R^3 = OH, R^4 = R^5 = H$
8 $R^1 = OH, R^2 = H, R^3 = OH, R^4 = H, R^5 = OH$
9 $R^1 = OH, R^2 = H, R^3 = OCH_3, R^4 = H, R^5 = OCH_3$
10 $R^1 = OH, R^2 = R^3 = H, R^4 = OCF_3, R^5 = H$
11 $R^1 = OH, R^2 = F, R^3 = R^4 = R^5 = H$
12 $R^1 = OH, R^2 = R^3 = H, R^4 = Cl, R^5 = H$
13 $R^1 = OH, R^2 = R^3 = H, R^4 = Br, R^5 = H$
14 $R^1 = OH, R^2 = F, R^3 = H, R^4 = F, R^5 = H$
15 $R^1 = OH, R^2 = Cl, R^3 = H, R^4 = Cl, R^5 = H$
16 $R^1 = OH, R^2 = Br, R^3 = H, R^4 = Br, R^5 = H$
17 $R^1 = OH, R^2 = I, R^3 = H, R^4 = I, R^5 = H$
18 $R^1 = OH, R^2 = Cl, R^3 = H, R^4 = F, R^5 = H$
19 $R^1 = OH, R^2 = F, R^3 = H, R^4 = Br, R^5 = H$
20 $R^1 = OH, R^2 = R^3 = H, R^4 = NO2, R^5 = H$
21 $R^1 = OH, R^2 = NO_2, R^3 = H, R^4 = NO_2, R^5 = H$
22 $R^1 = OH, R^2 = R^3 = H, R^4 = CHO, R^5 = H$

Fig. 1. Chemical structures of the compounds studied.

Microbial strains and culture conditions

A total of five microbial strains were employed in this study (*Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Staphylococcus aureus*). The microbial strains, their origins, the media employed, and the growth temperatures used have been previously described (Pelttari *et al.*, 2002, 2007a, b; Elo, 2007; Elo *et al.*, 2007).

The antibiotics used as positive controls were: doxycycline (Doximycin; Orion, Espoo, Finland) for *B. cereus* and *S. aureus*, cefuroxime (Zinacef; Glaxo Operations UK Ltd, Barnard Castle, UK) for *E. coli* and *S. aureus*, and amphotericin B (Fungizone; Bristol-Myers Squibb, Bromma, Sweden) for the yeasts.

Micro-scale determination of MIC, MBC, and MFC

MIC, MBC, and MFC values were determined using novel micro-scale methods developed in our laboratory (Lehtinen *et al.*, 2011). The micro-scale MIC method is a modification of our previously published macro-scale MIC method (Elo *et al.*, 2007). In the micro-scale method, the measurements were carried out on 96-well deep-well plates made of non-transparent polypropylene, and detection of microbial growth was based on visual inspection of the wells of these non-transparent plates. For determining the MBC or MFC values, samples were taken from those wells of the MIC test plate, in which no growth could be visually observed (*i.e.*, from wells that on the basis of visual inspection were found to contain clear liquid). The samples were streaked on agar plates (as a single streak across the plate), the liquid was allowed to absorb into the plates, and finally the microbes were spread all over the plate with a bent rod. For details of the methods, the reader is referred to the paper of Lehtinen *et al.* (2011). After the incubation, colonies on the agar plates were counted and the MBC or MFC values were calculated according to common practise using the formula $N = n + 2\sqrt{n}$ for determination of the

cut-off number, where N is the corrected cut-off number and n is the (uncorrected) cut-off number (see Anhalt *et al.*, 1980).

Results

We performed MIC, MBC, and MFC measurements on a total of 22 compounds, including 19 substituted salicylaldehydes and, for comparison, one related dihydroxylated benzaldehyde in which the 2-position is unsubstituted (compound **6**) as well as the parent compounds benzaldehyde and salicylaldehyde (see Fig. 1 for details). All salicylaldehydes previously studied using the agar diffusion method (Pelttari *et al.*, 2007a) were included, except one that was no longer available for this study. In addition, two new congeners each containing two different halogen atoms were now included (compounds **18** and **19**). The results of the MIC measurements are shown in Table I and those of the MBC and MFC measurements in Table II. Some aspects of the results are also illustrated in Figs. 2–11. For comparison, we also studied the 'new' compounds using agar diffusion (Table III), and also performed agar diffusion studies on all of the present compounds using *S. aureus* as test organism (Table IV).

Because of the inherent problems of all MIC and, especially, MBC and MFC measurement methods, all results obtained by such methods must be considered as directional (see Lehtinen

Table I. Results of the MIC measurements.

Compound	MIC [μg/ml][a]									
	Bacillus cereus		*Candida albicans*		*Escherichia coli*		*Saccharomyces cerevisiae*		*Staphylococcus aureus*	
	24 h	44 h	24 h	44 h	24 h	44 h	24 h	44 h	24 h	44 h
1	> 1000	> 1000	94*	> 1000	> 1000	> 1000	500	> 1000	> 1000	> 1000
2	500	500	12*	63	375*	500	63	125	1000	1000
3	1000	750*	8	12*	> 1000	> 1000	31	63	> 1000	> 1000
4	1000	> 1000	16	63	1000	750*	250	500	> 1000	> 1000
5	1000	1000	63	250	1000	750*	125	375*	> 1000	> 1000
6	1000	1000	1000	> 1000	1000	> 1000	> 1000	> 1000	> 1000	> 1000
7	1000	> 1000	750*	150	> 1000	> 1000	1000	1000	> 1000	> 1000
8	> 1000	> 1000	63	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
9	> 1000	> 1000	16	94*	> 1000	> 1000	125	375*	> 1000	> 1000
10	375*	375*	63	94*	47*	250	16	94*	750*	750*
11	750*	750*	4	8	188*	375*	16	63	500	500
12	500	500	8	31	125	125	16	63	125	250
13	1000	1000	23*	31	63	63	16	31	375*	375*
14	500	375*	12*	63	125	125	31	94*	188*	125
15	125	125	8	47*	12*	47*	47*	63	94*	125
16	31	31	8	12*	47*	47*	63	125	375*	375*
17	23*	23*	8	12*	47*	47*	63	125	63	250
18	250	188*	8	31	94*	94*	31	63	> 1000	> 1000
19	188*	188*	16	23*	94*	94*	31	125	188*	188*
20[b]	250	125	31	31	94*	94*	94*	250	63	63
20[c]	250	188*	8	31	47*	47*	94*	188*	94*	125
21	> 1000	> 1000	1000	> 1000	500	500	1000	> 1000	> 1000	> 1000
22	375*	188*	31	47*	250	250	94*	250	188*	250

* Mean of two determinations (the results of the two determinations differed by one dilution).

[a] The MIC value of doxycycline for *S. aureus* was (0.4 ± 0.1) μg/ml at 24 h and (0.5 ± 0) μg/ml at 44 h (n = 18, *i.e.* 9 determinations in duplicate at both 24 h and 44 h), while the MIC value of cefuroxime was (2 ± 1) μg/ml at 24 h and (1 ± 0.3) μg/ml at 44 h (n = 18). The MIC value of cefuroxime for *E. coli* was (3 ± 1) μg/ml at 24 h and at 44 h (n = 4). The MIC value of amphotericin B for *C. albicans* was (0.9 ± 0.5) μg/ml at 24 h and (2 ± 1.5) μg/ml at 44 h (n = 20), and that for *S. cerevisiae* was (8 ± 4) μg/ml at 24 h and (11 ± 5) μg/ml at 44 h (n = 16). In the case of *B. cereus*, the MIC value of doxycycline was variable, the average value ± standard deviation being (0.5 ± 0.6) μg/ml at 24 h and (0.7 ± 0.6) μg/ml at 44 h (n = 18).

[b] Product of Aldrich Chemical Company.

[c] Product of TCI.

Table II. Results of the MBC and MFC studies.

Compound	MBC or MFC [μg/ml][a]				
	B. cereus	*C. albicans*	*E. coli*	*S. cerevisiae*	*S. aureus*
1	> 1000	> 1000	> 1000	> 1000	> 1000
2	> 1000	375*	1000	1000	> 1000
3	> 1000	375*	> 1000	250	> 1000
4	> 1000	500	> 1000	> 1000	> 1000
5	> 1000	> 1000	> 1000	> 1000	> 1000
6	> 1000	> 1000	> 1000	> 1000	> 1000
7	> 1000	> 1000	> 1000	> 1000	> 1000
8	> 1000	> 1000	1000	> 1000	1000
9	> 1000	> 1000	> 1000	> 1000	> 1000
10	500	250	750*	250	1000
11	> 1000	250	750*	> 1000	> 1000
12	> 1000	125	250	250	125
13	> 1000	125	375*	31	> 1000
14	> 1000	125	375*	1000	750*
15	> 1000	125	188*	188*	125
16	47*	63	63	125	1000
17	47*	12*	1000	750*	250
18	1000	94*	250	750*	> 1000
19	750*	94*	125	500	> 1000
20[b]	500	63	500	> 1000	1000
20[c]	> 1000	188*	1000	> 1000	500
21	> 1000	> 1000	500	> 1000	1000
22	750*	188*	750*	> 1000	1000

* Mean of two determinations (the results of the two determinations differed by one dilution).

[a] The MBC value of cefuroxime for *E. coli* was 6 μg/ml (mean of two determinations) and that for *S. aureus* was (4 ± 2) μg/ml (n = 8). The MFC value of amphotericin B for *C. albicans* was (3 ± 2) μg/ml (n = 6) and that for *S. cerevisiae* was (37 ± 32) μg/ml (n = 8; most of the individual MFC values being between 13 and 50). The MBC value of doxycycline for *S. aureus* was variable [(60 ± 40) μg/ml (n = 8), range 31–125 μg/ml, most values being 31 μg/ml], and that for *B. cereus* was even more variable [(160 ± 190) μg/ml (n = 6), range 31–500 μg/ml, most values being either 31 or 63 μg/ml].

[b] Product of Aldrich Chemical Company.

[c] Product of TCI.

Table III. Results of agar diffusion tests on compounds **14**, **18**, and **19** against *B. cereus*, *E. coli*, *C. albicans*, and *S. cerevisiae*. The diameter of the paper disc was 6 mm.

Compound	Concentration [mg/ml]	Diameter of inhibitory zone ± S.D. [mm]*			
		B. cereus	*E. coli*	*C. albicans*	*S. cerevisiae*
14[a]	40	12 ± 1	32 ± 1	> 45	29 ± 3
	20	11 ± 1	6 ± 0	> 40	23 ± 2
	10	7 ± 1	6 ± 0	21 ± 3	7 ± 1
	5	6 ± 0	6 ± 0	11 ± 1	6 ± 0
14[b]	40	23	21	7	23
	20	15	11	6	7
	10	12	7	6	7
	5	11	6	6	7
18	40	26 ± 1	45 ± 1	> 45	35 ± 1
	20	18 ± 1	27 ± 1	> 40	34 ± 1
	10	10 ± 0	15 ± 1	20 ± 1	32 ± 1
	5	7 ± 1	6 ± 0	16 ± 1	18 ± 0
19	40	26 ± 1	27 ± 1	> 40	38 ± 1
	20	21 ± 2	15 ± 1	24 ± 1	33 ± 1
	10	18 ± 2	8 ± 1	22 ± 1	29 ± 2
	5	7 ± 0	7 ± 1	14 ± 1	18 ± 1
Doxycycline	20	39 ± 1**			
Cefuroxime	5		29 ± 1**		
Amphotericin B	8			16 ± 1**	11 ± 1**

* Four determinations in each case, except for the control antibiotics. All measurements were carried out according to Method II of Elo (2007).

** Twenty determinations in each case.

[a] Product of Sigma-Aldrich. The results of the MIC tests were compared with these agar diffusion data, since this lot was used also in the MIC tests and MBC/MFC tests.

[b] Synthesized in our laboratory as described in Pelttari *et al.* (2007a). These test results are shown for comparison purposes and are taken from Pelttari *et al.* (2007a). This lot was not available for the present MIC and MBC/MFC studies.

et al., 2011, for details), and this applies also to the present results. Small differences between MIC, MBC, and MFC values must thus be treated cautiously.

Studies on E. coli

In the case of *E. coli*, the results of the MIC tests are very clear-cut, indicating that, generally, halogenated and nitro-substituted salicylaldehydes are active, while di- and trihydroxylated benzaldehydes (that contain one or more 'extra' hydroxy substituents in addition to the 2-hydroxy group of salicylaldehyde) are essentially inactive.

All 3,5-dihalogenated salicylaldehydes had potent growth-inhibitory activity against *E. coli*, displaying MIC values that ranged between 12 and 125 μg/ml (24 h). The activity of these compounds in both the MIC and MBC tests as a function of the position of the halogen atom(s) in the periodic table is shown in Fig. 2. The most potent compound in the MIC test was 3,5-dichlorosalicylaldehyde (**15**), the least active one being its difluoro analogue **14**. All dihalogenated compounds retained their activity even upon longer

Table IV. Results of agar diffusion tests against *S. aureus*. The diameter of the paper disc was 6 mm.

Compound	Diameter of inhibitory zone ± S.D. [mm]*			
	40 mg/ml	20 mg/ml	10 mg/ml	5 mg/ml
1	6 ± 0	6 ± 0	6 ± 0	6 ± 0
2	7 ± 1	6 ± 0	6 ± 0	6 ± 0
3	10 ± 1	7 ± 1	7 ± 1	6 ± 0
4	8 ± 1	7 ± 1	6 ± 0	6 ± 0
5	17 ± 1	13 ± 1	8 ± 1	6 ± 1
6	6 ± 0	6 ± 0	6 ± 0	6 ± 0
7	28 ± 1	24 ± 1	19 ± 1	6 ± 1
8	15 ± 0	12 ± 1	12 ± 1	10 ± 1
9	8 ± 1	7 ± 0	6 ± 0	6 ± 0
10	6 ± 0	6 ± 0	6 ± 0	6 ± 0
11	22 ± 2	13 ± 2	7 ± 0	6 ± 0
12	8 ± 2	6 ± 0	6 ± 0	6 ± 0
13	20 ± 2	17 ± 1	6 ± 0	6 ± 0
14	45 ± 1	26 ± 1	16 ± 1	8 ± 1
15	22 ± 1	15 ± 1	12 ± 1	6 ± 1
16	24 ± 2	23 ± 2	19 ± 1	18 ± 2
17	24 ± 1	22 ± 1	20 ± 2	19 ± 1
18	22 ± 1	20 ± 1	6 ± 0	6 ± 0
19	26 ± 1	15 ± 1	9 ± 1	7 ± 1
20[a]	33 ± 1	27 ± 2	24 ± 1	24 ± 1
20[b]	26 ± 1	23 ± 1	10 ± 1	12 ± 2
21	8 ± 0	7 ± 1	7 ± 1	6 ± 0
22	25 ± 0	12 ± 1	8 ± 1	6 ± 0
Cefuroxime				30 ± 1**
Doxycycline		34 ± 2***		

* Four determinations in each case, except for the control antibiotics.
** Forty determinations.
*** Ten determinations.
[a] Product of Aldrich Chemical Company.
[b] Product of TCI.

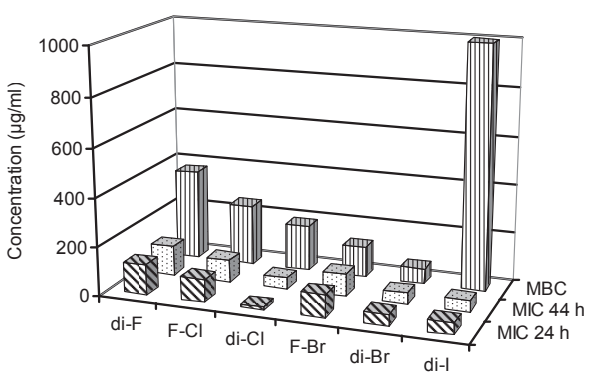


Fig. 2. MIC and MBC values of dihalogenated salicylaldehydes for *Escherichia coli*, shown as a function of the position of the halogen atom(s) in the periodic system of the elements.

cultivation (44 h), although the dichloro congener displayed a somewhat higher MIC value than it did at 24 h.

The MBC values of the dihalogenated congeners appeared to markedly decrease on going from period 2 to period 4, even the results obtained on congeners that carry two different halogen substituents being well in line with this behaviour, and then to rise suddenly and abruptly on going from the dibromo compound to the diiodo one, possibly because of the increasing size of the halogen atoms.

Interestingly, the MBC values of the different 3,5-dihalogenated salicylaldehydes were markedly different, ranging between 63 and 1000 μg/ml. The MBC value decreased steadily on going from the difluoro congener to the ones containing heavier halogen atoms (Cl, Br), but after that it suddenly rose markedly. Also the results obtained on 'mixed-halogen' congeners were in line with this behaviour. Thus, these compounds had bactericidal activity against *E. coli*, albeit in the case of 3,5-diiodosalicylaldehyde (**17**) only at very high concentrations.

Analogously with the dihalogenated compounds, also the three monohalogenated salicylaldehydes tested showed activity in the MIC test and in the MBC test. Yet, the MBC value of 3-fluorosalicylaldehyde (**11**) was quite high. Also 5-trifluoromethoxysalicylaldehyde (**10**) was tested and displayed marked activity in the MIC test at 24 h but its activity decreased on longer culturing, and it had a quite high MBC value.

5-Nitrosalicylaldehyde (**20**) from two different suppliers was tested, and both substances had marked activity in the MIC test, with 24-h MIC values of 47 and 94 μg/ml. The MBC values of the two lots of this compound (1000 and 500 μg/ml) indicated minimal activity. The 3,5-dinitro congener **21** in turn had quite low activity in both the MIC test and the MBC test. 5-Formylsalicylaldehyde (**22**) had some activity in the MIC test.

None of the numerous di- and trihydroxylated benzaldehydes tested displayed noteworthy activity in the MIC and MBC tests, most congeners being totally inactive in the concentration range tested and the rest having minimal activity. Benzaldehyde was likewise totally inactive, while salicylaldehyde displayed slight activity in the MIC test.

Studies on B. cereus

The results obtained on *B. cereus* are largely similar to those on *E. coli*. Thus, di- and trihydroxylated congeners were either totally inactive or showed minimal activity. Among the 3,5-dihalogenated salicylaldehydes, the MIC value decreased systematically when going from fluorine to iodine in the periodic table, the antibacterial activity of the aldehyde thus increasing on going to larger and less electronegative halogen atoms (see Fig. 3). The MIC value of compound **18**, *i.e.*, the aldehyde that contains one fluorine and one chlorine atom, is well in line with this conclusion, and that of the other aldehyde containing two different halogens (compound **19** that contains one fluorine and one bromine atom) is also roughly in line with the conclusion. The high activity of the dibromo and diiodo congeners **16** and **17** is noteworthy. These two compounds not only had low MIC values but also were effective bactericidal agents, the MBC value of both ones being 47 μg/ml. In contrast to the dihalogenated congeners, the monohalogenated ones tested had weak activity in the MIC test, the 5-bromo congener **13** being essentially inactive, and were completely inactive in the MBC test. 5-Nitrosalicyladehyde (**20**) was moderately active in the MIC test but had essentially no activity in the MBC test, and the 3,5-dinitro congener **21** was completely inactive in both tests.

Studies on S. aureus

In the case of *S. aureus*, the results obtained in the MIC and MBC tests are largely similar to those on *E. coli* and *B. cereus*. Thus, benzaldehyde and all of the di- and trihydroxylated aldehydes tested as well as 2,4-dimethoxybenzaldehyde (**9**) were totally inactive in both the MIC and the MBC tests. Unsubstituted salicylaldehyde had minimal activity in the MIC test and was inactive in the MBC test.

Most other congeners tested were completely inactive in the MBC test, indicating that they are not bactericidal against *S. aureus*, or had a very high MBC value. 5-Chlorosalicylaldehyde (**12**) and its 3,5-dichloro analogue **15** (MBC of both ones 125 μg/ml) and 3,5-diiodosalicylaldehyde (**17**) (MBC, 250 μg/ml) constituted the only noteworthy exceptions (Fig. 4). All halogenated compounds except 3-chloro-5-fluorosalicylaldehyde (**18**) had some degree of activity in the MIC test, as did also 5-nitro- (**20**) and 5-formylsalicylaldehyde (**22**), but also the latter were essentially inactive in the MBC test. The 3,5-dinitro congener **21** was completely inactive in all tests.

Agar diffusion tests of the present compounds against *S. aureus* have not been reported previously. Therefore, we performed those measurements in connection with the present study (see Table IV for results). Most of the halogenated congeners, 5-nitrosalicylaldehyde (**20**), both trihydroxysalicylaldehydes **7** and **8**, 2,5-dihydroxybenzaldehyde (**5**), and 5-formylsalicylaldehyde (**22**) displayed noteworthy activity in these tests, in spite of the fact that most compounds had no activity in the MBC test and few of them had potent activity in the MIC test.

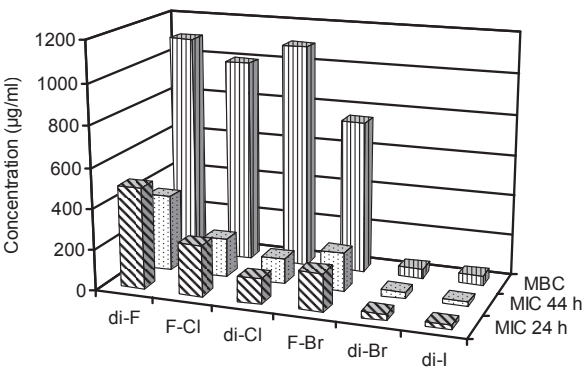


Fig. 3. MIC and MBC values of dihalogenated salicylaldehydes for *Bacillus cereus*, shown as a function of the position of the halogen atom(s) in the periodic system of the elements.

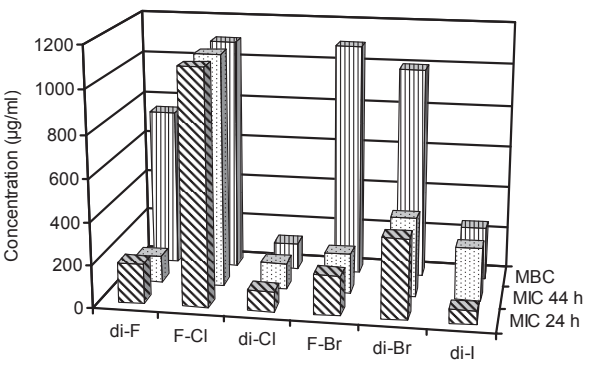


Fig. 4. MIC and MBC values of dihalogenated salicylaldehydes for *Staphylococcus aureus*, shown as a function of the position of the halogen atom(s) in the periodic system of the elements.

Studies on C. albicans

The results obtained on the pathogenic yeast *C. albicans* differ drastically from those obtained in the case of the bacteria studied. Thus, all salicylaldehydes except the 3,5-dinitro congener **21** had at least some activity in the MIC test. In most cases, clearly higher activity was displayed than in the case of the bacteria studied.

3-Fluorosalicylaldehyde (**11**) had the lowest 24-h MIC value, only 4 μg/ml, its activity being thus comparable with that of the clinically important antifungal drug amphotericin B, whose MIC value at 24 h was (0.9 ± 0.5) μg/ml. Also at 2 days, 3-fluorosalicylaldehyde had a MIC value (8 μg/ml) comparable with that of amphotericin B [(2 ± 1.5) μg/ml]. The MFC value of 3-fluorosalicylaldehyde was, however, quite high. Another compound with prominent fungicidal activity was 3,5-diiodosalicylaldehyde (**17**) (Fig. 5), whose MFC value was as low as 12 µg/ml, that of amphotericin B varying between (3 ± 2) μg/ml. Also several other salicylaldehydes had high activity against *C. albicans* in the MIC test but the MFC values were not equally low. Even unsubstituted salicylaldehyde itself had fairly high activity in the MIC test and displayed some activity in the MFC test.

Studies on S. cerevisiae

Also in the case of the other yeast studied, *S. cerevisiae*, most compounds displayed considerable activity in the MIC test, with the exception of 3,5-dinitrosalicylaldehyde (**21**), the two trihydroxylated benzaldehydes **7** and **8**, and 3,4-dihydroxybenzaldehyde (**6**).

The most potent congener was 5-bromosalicylaldehyde (**13**), whose 24-h MIC (16 μg/ml), 44-h MIC (31 μg/ml), and MFC (31 μg/ml) values are comparable with those of amphotericin B [24-h MIC, (8 ± 4) μg/ml, and 44-h MIC, (11 ± 5) μg/ml (n = 16); MFC, (37 ± 32) μg/ml (n = 8)]. All other halogenated compounds also displayed remarkable activity in the MIC test and most of them were also fungicidal (Fig. 6). 3-Fluorosalicylaldehyde (**11**) was, however, without fungicidal activity in the concentration range tested, and the 3,5-difluoro congener **14** had fungicidal activity only at the highest concentration tested.

Even unsubstituted salicylaldehyde had noteworthy antifungal activity (24-h MIC, 63 μg/ml), but its MFC value was high (500 μg/ml), and even benzaldehyde displayed slight activity at 24 h but not at 44 h. 2,3-Dihydroxybenzaldehyde (**3**) displayed marked activity (24-h MIC, 31 μg/ml; MFC, 250 μg/ml). Other dihydroxylated congeners and the dimethoxylated compound **9** as well as 5-formylsalicylaldehyde (**22**) had weaker activity in the MIC test and were inactive in the MFC test.

Interestingly, 3,4-dihydroxybenzaldehyde (**6**) that is devoid of the 2-hydroxy substituent characteristic of salicylaldehydes was completely inactive or essentially inactive in all MIC, MBC, and MFC tests performed, irrespective of the microbe in question.

Discussion. Comparison of Different Methods

When the results of the MIC and MBC/MFC tests are compared with previously published

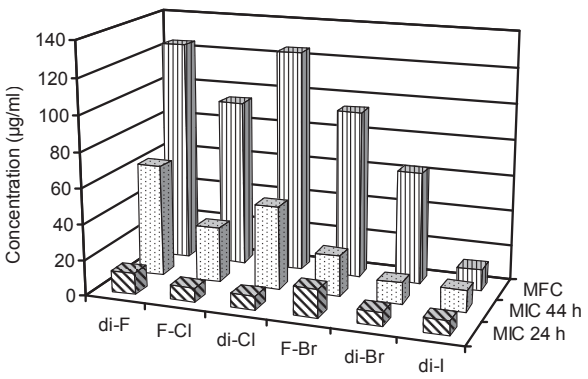


Fig. 5. MIC and MFC values of dihalogenated salicylaldehydes for *Candida albicans*, shown as a function of the position of the halogen atom(s) in the periodic system of the elements.

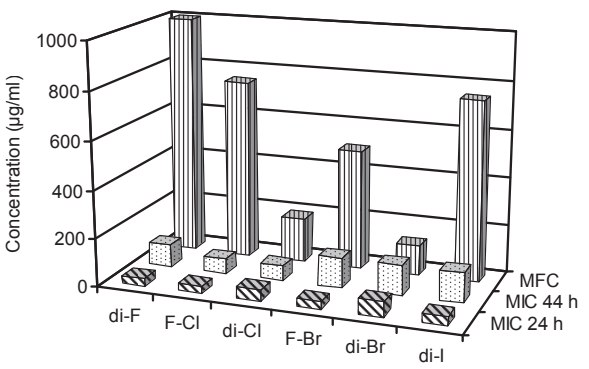


Fig. 6. MIC and MFC values of dihalogenated salicylaldehydes for *Saccharomyces cerevisiae*, shown as a function of the position of the halogen atom(s) in the periodic system of the elements.

results of inhibitory zone studies (Pelttari *et al.*, 2007a), some similarities but also some prominent differences are immediately evident. Thus, in the MIC as well as MBC/MFC tests, the compounds tested displayed most potent activity against the yeasts *S. cerevisiae* and, especially, *C. albicans*. Only two of the 22 compounds tested were inactive or essentially inactive against *C. albicans*, and 18 congeners displayed lower MIC values against it than against any other one of the microbes tested, the remaining two congeners being most active against *S. cerevisiae* but displaying nearly equal activity also against *C. albicans*. This superior activity against the two yeasts, especially *Candida*, was not seen in the previously reported agar diffusion studies (Pelttari *et al.*, 2007a).

In the case of *C. albicans*, a comparison of 24-h MIC values and inhibitory zones (Fig. 7) indicates that all compounds that displayed distinct activity in the agar diffusion test had high activity in the MIC test, while a few compounds that were inactive in the agar diffusion test displayed high activity in the MIC test and some others were essentially inactive also in the latter. Thus, on one hand the agar diffusion test did not reveal all active compounds but, on the other hand, all compounds that were active against *C. albicans* in it were active also in the MIC test. Essentially similar conclusions can be drawn in the case of MIC values measured at 44 h. Also in the case of *S. cerevisiae*, essentially similar conclusions can be drawn (Fig. 8), especially if results of diffusion tests with the lowest concentration used (5 mg/ml) are considered.

Some compounds (*e.g.* unsubstituted salicylaldehyde) that displayed minimal or low activity against *C. albicans* or *S. cerevisiae* in agar diffusion tests had, however, fairly low MFC values, being thus able to kill the yeasts in fairly low concentrations, which is potentially important concerning possible practical applications.

In the case of the bacteria tested, the situation is more complex. In the case of *E. coli*, all compounds that had marked or moderate activity (zone diameter ≥ 9 mm) at the lowest concentration (5 mg/ml) in the agar diffusion test were active also in the MIC test (Fig. 9), just as in the case of the yeasts, but when higher concentrations (10–40 mg/ml) of the test substances were used in the diffusion test, the correlation between the results of the two tests was not equally straightforward, and several compounds with inhibitory zone diameters up to 23 mm were essentially inactive in the MIC test.

In the case of *S. aureus*, the comparison of the results of the MIC determinations and those of the agar diffusion tests carried out with the lowest concentration of the test substances (5 mg/ml) led to largely the same conclusions as in the case of *E. coli* (Fig. 10). Thus, all compounds except one that was active at the lowest concentration in the agar diffusion test had distinct activity in the MIC test (MIC usually below 200 and in all cases below 400 μg/ml), and several compounds that were completely inactive in the agar diffusion test displayed distinct and some of them even high activity in the MIC test. When higher concentrations were used in the agar diffusion test, the correlation between MIC values and inhibitory zones

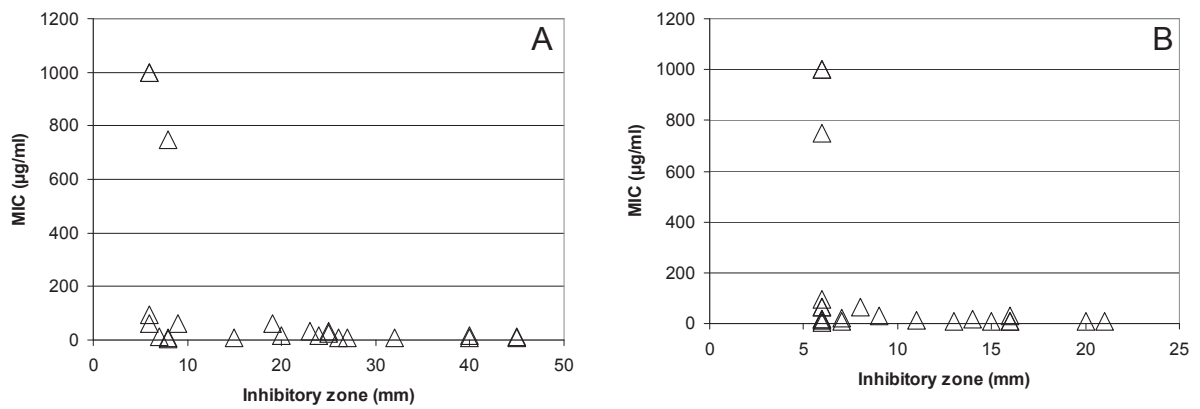


Fig. 7. Correlation between 24-h MIC and agar diffusion test results for *Candida albicans* at (A) 40 mg/ml and (B) 5 mg/ml of the test substances in the agar diffusion tests.

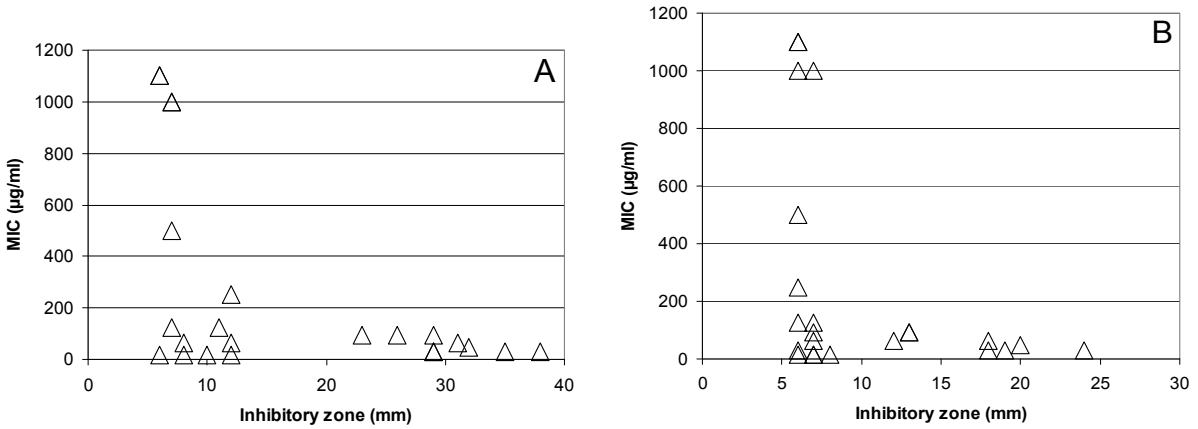


Fig. 8. Correlation between 24-h MIC and agar diffusion test results for *Saccharomyces cerevisiae* at (A) 40 mg/ml and (B) 5 mg/ml of the test substances in the agar diffusion tests. One compound had a MIC value > 1000 µg/ml (*i.e.,* above the measurement range), and this value is displayed in the figures as if it was 1100 µg/ml.

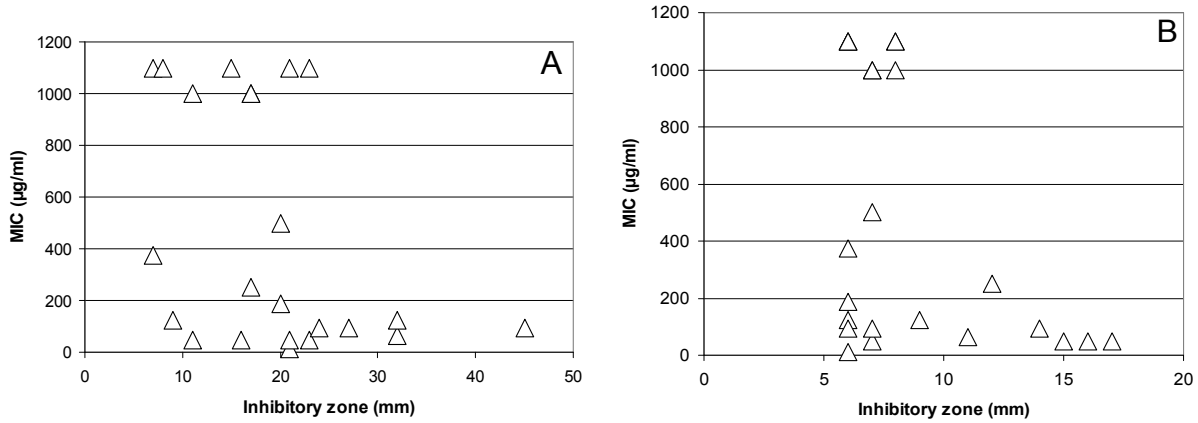


Fig. 9. Correlation between 24-h MIC and agar diffusion test results for *Escherichia coli* at (A) 40 mg/ml and (B) 5 mg/ml of the test substances in the agar diffusion tests. MIC values > 1000 µg/ml (*i.e.*, above the measurement range) are displayed in the figures as if they were 1100 µg/ml.

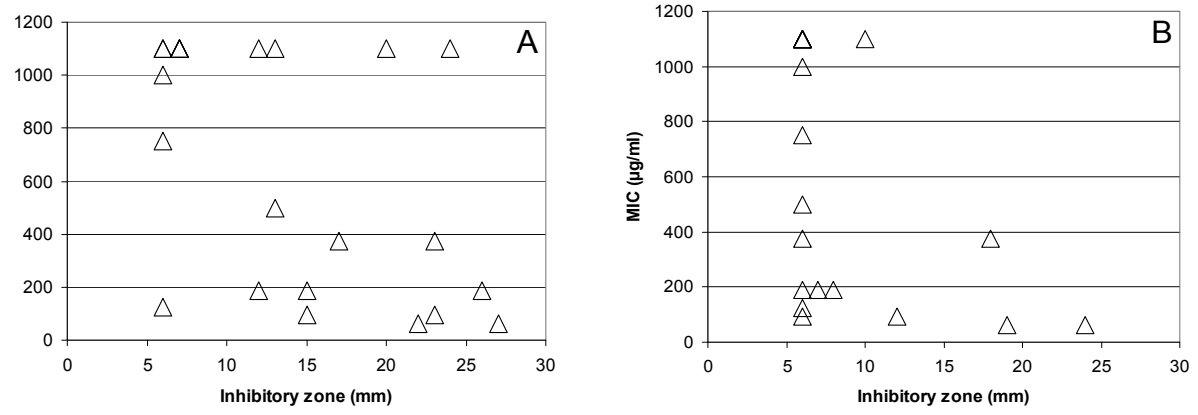


Fig. 10. Correlation between 24-h MIC and agar diffusion test results for *Staphylococcus aureus* at (A) 20 mg/ml and (B) 5 mg/ml of the test substances in the agar diffusion tests. MIC values > 1000 µg/ml (*i.e.*, above the measurement range) are displayed in the figures as if they were 1100 µg/ml.

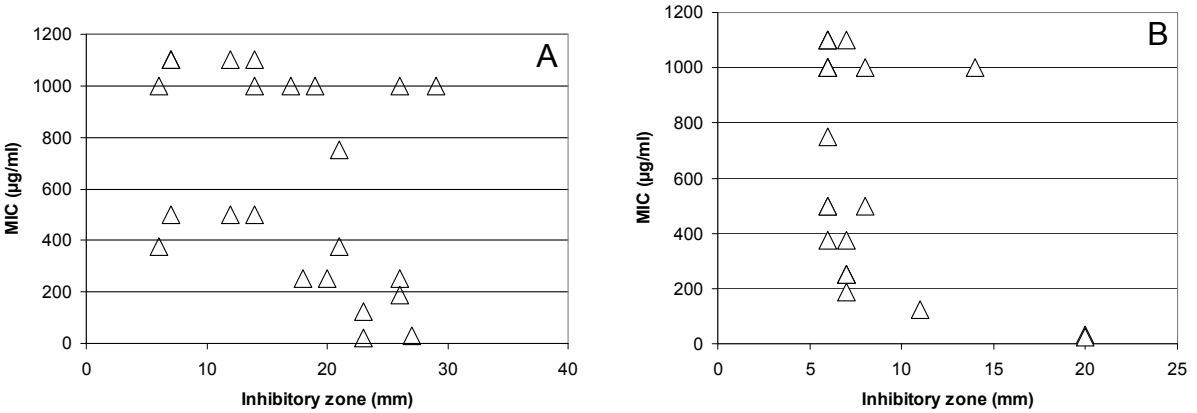


Fig. 11. Correlation between 24-h MIC and agar diffusion test results for *Bacillus cereus* at (A) 40 mg/ml and (B) 5 mg/ml of the test substances in the agar diffusion tests. MIC values > 1000 µg/ml (*i.e.*, above the measurement range) are displayed in the figures as if they were 1100 µg/ml.

was, however, lost. Already when the concentration of the test substances in the diffusion test was 20 mg/ml, some compounds with remarkably large inhibitory zones were completely inactive in the MIC test, while some others displayed varying degrees of activity in the latter test. In the case of *B. cereus*, the comparison of the results of MIC and agar diffusion studies (Fig. 11) leads to essentially the same conclusions as in the case of *S. aureus*.

It should be borne in mind that the above conclusions concerning the correlation (and lack of correlation in some cases) between agar diffusion test results and MIC values cannot necessarily be generalized and may not be valid for other types of compounds. Further studies on this topic are of interest for the development of the methodology of antimicrobial drug discovery and development.

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