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Optimizing the structure of (salicylideneamino)benzoic acids: Towards selective antifungal and anti-staphylococcal agents

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ABSTRACT

An increasing resistance of human pathogenic bacteria and fungi has become a global health problem. Based on previous reports of 4-(salicylideneamino)benzoic acids, we designed, synthesised and evaluated their me-too analogues as potential antimicrobial agents. Forty imines derived from substituted salicylaldehydes and aminobenzoic acids, 4-aminobenzoic acid esters and 4-amino-*N*-phenylbenzamide were designed using molecular hybridization and prodrug strategies. The target compounds were synthesized with high yields and characterized by spectral methods. They were investigated against a panel of Gram-positive and Gram-negative bacteria, mycobacteria, yeasts and moulds. The most active imines were tested to determine their cytotoxicity and selectivity in HepG2 cells. Dihalogenosalicylaldehydes-based derivatives showed potent broad-spectrum antimicrobial properties, particularly against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (minimum inhibitory concentrations, MIC, from 7.81 μM) and *Enterococcus faecalis* (MIC of ≥15.62 μM), yeasts (MIC from 7.81 μM) and *Trichophyton interdigitale* mould (MIC of ≥3.90 μM). Methyl 4-[(2-hydroxy-3,5-diiodobenzylidene)amino]benzoate **4h** exhibited excellent *in vitro* activity along with low toxicity to mammalian cells. This compound is selective for staphylococci, *Candida* spp. and *Trichophyton interdigitale*. In addition, this imine was evaluated as a potential inhibitor of Gram-positive biofilms. The successful approach used provided some promising derivatives with more advantageous properties than the parent 4-(salicylideneamino) benzoic acids.

1. Introduction

A loss of control of antibiotic- and antimycotic-resistant pathogens has emerged to become a global and constantly growing health problem issue due to serious and often untreatable infections. This state is strongly reflected with a complicated treatment, an increased length of hospital stays, health costs and also with a higher mortality (Thampi et al., 2015; Whitby et al., 2001). All these factors highlight the urgent need for strategies to combat infections. One of these strategies is based on discovery and development of new anti-infectives.

The increase in antibiotic resistance is most often mentioned in connection with bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, or multidrug-resistant Gram-negative bacilli (Cepas et al., 2019; Guo et al., 2020; (O'Driscoll and Crank, 2015). In addition, it should be pointed out that other infectious agents, such as yeasts or filamentous fungi, are equally affected by this phenomenon.

MRSA has become a serious threat to global health and is responsible for a wide range of infections, from skin and wound infections to life-threatening infections such as pneumonia or bloodstream infections (Zhen et al., 2020). This bacterial agent is for a long time included to the most clinically important nosocomial pathogens. Thus, its occurrence has been associated with health care settings, including hospitals and other health care environments (Kale and Dhawan, 2016). Moreover,

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this pathogen has been emerged as a major cause of community-associated infections, as well (Elston, 2007). MRSA strains are instantly classified as a multidrug resistant strains called „superbugs“. These strains have emerged with resistance to many commonly used antibiotics from different chemical groups, e.g., aminoglycosides, macrolides, fluoroquinolones, etc. (Kaur and Chate, 2015).

When compared to antibacterial agents, reports on resistance to antifungal agent is so far relatively rare (Arendrup and Patterson, 2017). However, with regard to the limited number of available antifungal agents for clinical use, a global multifaceted phenomenon of antimicrobial resistance spreading cannot be overlooked and it is desirable to invest effort to discovery and development of new compounds with antifungal activity, as well.

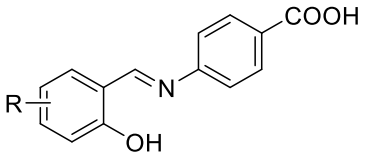
The yeast *Candida albicans* is the most common causative agent of fungal infections. As a commensal microorganism it causes usually relatively less severe infections, but under appropriate conditions (chemotherapy, haematological malignancies, gastrointestinal surgery, venous catheter usage, hospitalization in the intensive care units, etc.) is capable to break the immune barrier and cause life-threatening infections, which are associated with a markedly higher mortality (Pappas et al., 2018; Pfaller and Diekema, 2007).

Trichophyton interdigitale represents a pathogenic microorganism from the group of filamentous fungi. It is a strictly anthropophilic species causing generally cutaneous infections restricted to the non-living cornified layers of the human bodies (Zhang et al., 2019). Similarly to the above mentioned pathogens, also in the case of this infectious agent, the phenomenon of microorganisms adaptation to unfavourable conditions in the form of resistant strains selection and their spread has not been avoided. For example, there is a cumulative evidence indicating an increase in the number of cases caused by *T. interdigitale* strains resistant to terbinafine, which is the treatment of first-line choice (Saunte et al., 2019; Singh et al., 2018).

A systematic structural modification (so called me-too or follow-on approach) of a known lead bioactive molecule represents one of the most frequent and successful strategies to obtain new drugs, although it has some disadvantages and risks. A partial success has been also achieved with anti-infective agents whose development is challenging. It is largely believed that based on structural homology, an identification of novel promising agents will be enhanced and accelerated (Flick et al., 2019; Zhao and Guo, 2009).

Recently, we have published a systematic report (Krátký et al., 2020) of 4-aminobenzoic acid (PABA **1**)-salicylaldehydes mutual derivatives (Fig. 1) identified as potent antimicrobial agents supressing above all Gram-positive cocci including MRSA and fungal strains (*Candida albicans* and non-albicans *Candida* spp., *Trichophyton interdigitale*). In this study, 3,5-dihalogenosalicylidene pharmacophore was identified, especially with at least one heavier halogen (Fig. 1, substituents in bold). On the other hand, their antimicrobial activity was not separated from cytotoxic action (Krátký et al., 2020).

Based on here presented results, herein we extend the study to a systematic structure-activity relationship (SAR) investigation consisting in me-too approach for further development of selective antimicrobial drug candidates. The rational design of these analogues of PABA-derives imines **1a**-**1h** was performed in order to improve their antimicrobial activity against Gram-positive strains including MRSA and human pathogenic fungi, alleviate cytotoxicity and thus improve selectivity.



R = H, 5-F, 5-Cl, 5-Br, 5-I, 3-Cl, 4-Cl, 6-Cl, 5-NO_2, 5-CH_3, 5-*t*-Bu, 5-CH_3O, 5-OH, 3,5-Cl_2, **3-Br-5-Cl**, **3-I-5-Cl**, **3,5-I_2**

Fig. 1. Antimicrobial original 4-(salicylideneamino)benzoic acids (Krátký et al., 2020).

2. Material and Methods

2.1. Chemistry

2.1.1. General

All the reagents and solvents were purchased from Sigma-Aldrich (Darmstadt, Germany) or Penta Chemicals (Prague, Czech Republic) and they were used as received. The reactions and the purity of the products were monitored by thin-layer chromatography (TLC) using a mixture with a ratio of dichloromethane (DCM) to methanol (MeOH) of 4:1 (*v/v*) as the eluent. TLC plates were coated with 0.2 mm Merck 60 F254 silica gel (Merck Millipore, Darmstadt, Germany) with UV detection (254 nm). The melting points were determined on a Büchi Melting Point B-560 apparatus (BÜCHI, Flawil, Switzerland) using open capillaries. The reported values are uncorrected. Infrared spectra were recorded on a FT-IR spectrometer using ATR-Ge method (Nicolet 6700 FT-IR, Thermo Fisher Scientific, Waltham, MA, USA) in the range of 600–4000 cm^{-1}. The NMR spectra were measured in DMSO-d_6 and THF-d_8 at ambient temperature using a Varian V NMR S500 instrument (500 MHz for ^1H and 126 MHz for ^{13}C; Varian Comp. Palo Alto, CA, USA). The chemical shifts δ are given in ppm and were referred indirectly to tetramethylsilane *via* signals of DMSO-d_6 (2.49 for ^1H and 39.7 for ^{13}C spectra) and THF-d_8 (1.72 and 3.58 for ^1H). The coupling constants (*J*) are reported in Hz. Elemental analysis was performed on Vario MICRO Cube Element Analyzer (Elementar Analysensysteme, Hanau, Germany). Both calculated and found values are given as percentages.

Log *P* values, i.e., logarithm of partition coefficient (octan-1-ol/water) were calculated with the ChemOffice 2019 program (version 19.1, CambridgeSoft, Cambridge, MA, USA) using Crippen's fragmentation approach (Ghose and Crippen, 1987).

The identity of the known compounds was established using NMR (^1H and ^{13}C) and IR spectroscopy by the comparison with previously reported data. The purity was checked by melting points measurement and elemental analysis. The compounds were considered pure if they agree within ± 0.4% with theoretical values.

2.1.2. Synthesis of imines precursors

*Synthesis of esters **4** and **5**.* 4-Aminobenzoic acid **1** (0.02 mol; 2.743 g) was dissolved in 50 ml of appropriate alcohol (methanol for the synthesis of **4**, ethanol for **5**). The solution was cooled to 0°C and concentrated sulfuric acid was added dropwise (0.5 ml). The reaction mixture was refluxed for 4 hours, then let cool down to room temperature followed by addition of saturated sodium bicarbonate solution to adjust pH to a mildly basic (~8.5). After 2 hours at +4°C, the precipitate was filtered off, washed with distilled water and dried. Products were recrystallized from ethyl acetate.

*Synthesis of 4-amino-N-phenylbenzamide **6**.* 4-Nitrobenzoyl chloride (0.02 mol; 3.711 g) together with 1.5 of equivalents of triethylamine (Et_3N; 4.2 ml) were dissolved in dichloromethane (DCM; 35 ml), followed by a dropwise addition of freshly distilled aniline (1 equivalent, 1.8 ml) under vigorous stirring. After 2.5 hours stirring at room temperature, the reaction mixture was evaporated till dryness and using ethyl acetate and water, it was transferred into a separation funnel. The organic phase was washed twice with 0.1 M aqueous hydrochloric acid, 5% aqueous sodium bicarbonate, followed by saturated brine. The organic layer was dried over anhydrous sodium sulphate and filtered. The filtrate was concentrated under reduced pressure and *n*-hexane was added to start precipitation. After 24 hours at +4°C, the suspension was filtered off to give afforded 4-nitro-*N*-phenylbenzamide in almost quantitative yield. It was used for further reaction step without any

purification.

4-Nitro-*N*-phenylbenzamide was dissolved in a mixture of glacial acetic acid and ethyl acetate (30 ml; 1:1 *v/v*) and 10 equivalents of iron powder were added. The reaction mixture was refluxed for 1 hour and then stirred to cool down to room temperature. Then, insoluble portion was removed by filtration and the filtrate was evaporated till dryness. Using ethyl acetate and 5% aqueous sodium carbonate solution, the solid residue was transferred into a separation funnel. The organic phase was washed with 5% aqueous sodium carbonate and saturated brine. The solid insoluble material was washed thoroughly with hot ethyl acetate and acetic acid and this solution was treated analogously to the previous filtrate. The combined organic phases were dried over anhydrous sodium sulphate. The filtrate was concentrated under reduced pressure to initiate crystallization. Then, *n*-hexane was added to promote it. After 24 hours at +4°C, the suspension was filtered off to provide pure 4-amino-*N*-phenylbenzamide **6**.

Methyl 4-aminobenzoate **4**. Brownish solid; yield 71%; mp 111.5-112.5°C (111.4-112.4°C, Feng et al., 2014). IR (ATR): 3466, 3370 (N-H), 3251, 2952, 1677 (COO), 1625, 1595, 1568, 1435, 1285, 1181, 1168, 1122, 836, 770, 699 cm^{-1}. ^1H NMR (DMSO, 500 MHz): δ 7.65-7.61 (2H, m, H2, H6), 6.58-6.54 (2H, m, H3, H5), 5.95 (2H, s, NH_2), 3.72 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 166.51, 153.66, 131.23, 115.91, 112.82, 51.29. Anal. Calcd. for $C_8H_9NO_2$ (151.17): C, 63.56; H, 6.00; N, 9.27. Found: C, 63.71; H, 6.02; N, 8.97.

Ethyl 4-aminobenzoate **5**. Brownish solid; yield 69%; mp 87.6-89.5°C (88.5-89°C, Markiewicz et al., 2010). IR (ATR): 3423, 3344 (N-H), 3224, 2985, 1681 (COO), 1633, 1596, 1514, 1367, 1310, 1277, 1172, 1125, 1110, 1026, 847, 772, 700, 669 cm^{-1}. ^1H NMR (DMSO, 500 MHz): δ 7.68-7.64 (2H, m, H2, H6), 6.57-6.53 (2H, m, H3, H5), 5.94 (2H, s, NH_2), 4.23 (2H, q, $J = 7.0$ Hz, CH_2), 1.32 (3H, t, $J = 7.0$ Hz, Me). ^{13}C NMR (DMSO, 126 MHz): δ 166.30, 153.72, 131.48, 116.56, 113.10, 59.93, 14.71. Anal. Calcd. for $C_9H_{11}NO_2$ (165.19): C, 65.44; H, 6.71; N, 8.48. Found: C, 65.30; H, 6.61; N, 8.28.

4-Amino-*N*-phenylbenzamide **6**. Yellowish solid; overall yield 65 %; mp 138.5-139.4°C (138-140°C, Kakuta et al., 2008). IR (ATR): 3393 (N-H), 3351 (N-H), 1646 (CONH), 1598, 1525, 1509, 1440, 1321, 1260, 1182, 887, 847, 767, 751, 689, 671, 633 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 9.73 (1H, s, NH), 7.76-7.70 (4H, m, H2, H6, H2′, H6′), 7.32-7.27 (2H, m, H3′, H5′), 7.03 (1H, tt, $J = 7.4$, 1.2 Hz, H4′), 6.62-6.58 (2H, m, H3, H5), 5.74 (2H, s, NH_2). ^{13}C NMR (DMSO, 126 MHz): δ 165.47, 152.30, 139.95, 129.51, 128.61, 123.04, 121.30, 120.29, 112.72. Anal. Calcd. for $C_{13}H_{12}N_2O$ (212.25): C, 73.56; H, 5.70; N, 13.20. Found: C, 73.39; H, 5.88; N, 13.33.

2.1.3. Synthesis of imines ***2a-6h***

Imines **2a-2h, 3a-3h, 4a-4h, 5a-5h**, and **6a-6h** were synthesized according to Krátký et al. (2020). Briefly, 1 mmol of amino compound (**2, 4, 5, 6**) was dissolved in 7 ml of MeOH and then appropriate aldehyde (1.1 mmol) was added in one portion. In the case of imines **3a-3h**, 3,5-diaminobenzoic acid **3** (1 mmol) was dissolved in 10 ml of MeOH and 2.2 mmol of appropriate aldehyde was added. The reaction mixture was refluxed for 3 hours. After additional 12 hours of stirring at room temperature, the reaction mixture was stored at -20°C for 1 h. The precipitate formed was filtered off and washed successively by MeOH and diethyl ether thoroughly. If necessary, the products were crystallized from tetrahydrofuran/MeOH to obtain pure Schiff bases.

(*E*)-3-[(2-Hydroxybenzylidene)amino]benzoic acid **2a**. Yellow solid; yield 86%; mp 188.9-191.9°C (194°C, Grammaticakis, 1959). IR (ATR): 2823, 1678 (COO), 1621, 1573 (CH=N), 1464, 1450, 1420, 1307, 1281, 1194, 1153, 931, 812, 758, 746, 733, 679, 654 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 13.14 (1H, s, COOH), 12.85 (1H, s, OH), 9.02 (1H, s, CH=N), 7.92 (1H, s, H2), 7.88 (1H, d, $J = 7.6$ Hz, H6), 7.73-7.63 (2H, m, H4, H6′), 7.58 (1H, t, $J = 7.7$ Hz, H5), 7.44 (1H, t, $J = 7.9$ Hz, H4′), 7.01-6.96 (2H, m, H3′, H5′). ^{13}C NMR (DMSO, 126 MHz): δ 167.11, 164.58, 160.45, 148.67, 133.73, 132.86, 132.34, 129.97, 127.72, 126.22, 121.91, 119.49, 119.40, 116.81. Anal. Calcd. for $C_{14}H_{11}NO_3$ (241.25): C, 69.70; H, 4.60; N, 5.81. Found: C, 69.92; H, 4.55; N, 5.94.

(*E*)-3-[(3-Chloro-2-hydroxybenzylidene)amino]benzoic acid **2b**. Yellow solid; yield 86 %; mp 236.0-238.1°C. IR (ATR): 2996, 1683 (COO), 1616, 1600 (CH=N), 1581, 1449, 1439, 1417, 1294, 1274, 1182, 922, 855, 811, 783, 736, 682 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 14.10 (1H, s, OH), 13.19 (1H, s, COOH), 9.11 (1H, s, CH=N), 8.00 (1H, t, $J = 1.9$ Hz, H2), 7.91 (1H, dt, $J = 7.7$, 1.4 Hz, H6), 7.72 (1H, ddd, $J = 7.9$, 2.3, 1.2 Hz, H4), 7.67 (2H, dd, $J = 7.7$, 1.6 Hz, H6′), 7.63-7.58 (2H, m, H5, H4′), 7.02 (2H, t, $J = 7.8$ Hz, H5′). ^{13}C NMR (DMSO, 126 MHz): δ 167.02, 164.62, 156.60, 147.30, 133.52, 132.42, 132.16, 130.05, 128.26, 126.51, 121.93, 120.47, 120.27, 119.78. Anal. Calcd. for $C_{14}H_{10}ClNO_3$ (275.69): C, 60.99; H, 3.66; N, 5.08. Found: C, 60.84; H, 3.74; N, 5.03.

(*E*)-3-[(4-Chloro-2-hydroxybenzylidene)amino]benzoic acid **2c**. Yellow solid; yield 94%; mp 263.9-265.7°C. IR (ATR): 2873, 1676 (COO), 1621, 1601 (CH=N), 1561, 1495, 1461, 1321, 1260, 1193, 1077, 963, 905, 846, 813, 798, 779, 756, 677 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 13.25-13.11 (2H, m, COOH, OH), 9.03 (1H, s, CH=N), 7.92 (1H, t, $J = 1.9$ Hz, H2), 7.88 (1H, dt, $J = 7.6$, 1.8 Hz, H6), 7.73 (1H, d, $J = 8.0$ Hz, H6′), 7.66 (1H, ddd, $J = 7.9$, 2.3, 1.2 Hz, H4), 7.58 (1H, t, $J = 7.8$ Hz, H5), 7.07-7.03 (2H, m, H3′, H5′). ^{13}C NMR (DMSO, 126 MHz): δ 167.07, 163.43, 161.28, 148.33, 137.91, 134.00, 132.35, 129.98, 127.91, 126.30, 121.87, 119.69, 118.60, 116.70. Anal. Calcd. for $C_{14}H_{10}ClNO_3$ (275.69): C, 60.99; H, 3.66; N, 5.08. Found: C, 70.04; H, 3.60; N, 5.23.

(*E*)-3-[(5-Chloro-2-hydroxybenzylidene)amino]benzoic acid **2d**. Yellow solid; yield 90%; mp 261.2-261.9°C. IR (ATR): 2925, 2855, 1681 (COO), 1622, 1574 (CH=N), 1567, 1481, 1464, 1321, 1308, 1277, 1185, 934, 902, 823, 806, 780, 756, 685, 665, 649, 635 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 13.07 (1H, s, COOH), 12.72 (1H, s, OH), 8.96 (1H, s, CH=N), 7.88 (1H, t, $J = 2.0$ Hz, H2), 7.85 (1H, dt, $J = 7.4$, 1.5 Hz, H6), 7.75 (1H, d, $J = 2.8$ Hz, H6′), 7.61 (1H, dt, $J = 7.9$, 1.5 Hz, H4), 7.55 (1H, t, $J = 7.7$ Hz, H5), 7.41 (1H, dd, $J = 8.8$, 2.7 Hz, H4′), 6.97 (1H, d, $J = 8.9$ Hz, H3′). ^{13}C NMR (DMSO, 126 MHz): δ 167.42, 163.33, 159.41, 148.89, 133.51, 132.72, 131.58, 130.36, 128.35, 126.72, 123.19, 122.21, 121.18, 119.20. Anal. Calcd. for $C_{14}H_{10}ClNO_3$ (275.69): C, 60.99; H, 3.66; N, 5.08. Found: C, 61.12; H, 3.64; N, 5.00.

(*E*)-3-[(2-Hydroxy-5-iodobenzylidene)amino]benzoic acid **2e**. Yellow solid; yield 92%; mp 269.5-271.7°C. IR (ATR): 2838, 1678 (COO), 1621, 1578 (CH=N), 1558, 1463, 1356, 1318, 1274, 1188, 926, 902, 804, 778, 677, 663 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 13.11 (1H, s, COOH), 12.79 (1H, s, OH), 8.97 (1H, s, CH=N), 8.05 (1H, d, $J = 2.3$ Hz, H6′), 7.91 (1H, t, $J = 1.8$ Hz, H2), 7.88 (1H, dt, $J = 7.6$, 1.4 Hz, H6), 7.69 (1H, dd, $J = 8.7$, 2.3 Hz, H4′), 7.64 (1H, ddd, $J = 7.9$, 2.2 Hz, 1.2 Hz, H4), 7.58 (1H, t, $J = 7.7$ Hz, H5), 6.83 (1H, d, $J = 8.7$ Hz, H3′). ^{13}C NMR (DMSO, 126 MHz): δ 167.03, 162.89, 159.97, 148.50, 141.52, 140.21, 132.33, 129.98, 127.93, 126.32, 122.05, 121.87, 119.56, 80.91. Anal. Calcd. for $C_{14}H_{10}INO_3$ (367.14): C, 45.80; H, 2.75; N, 3.82. Found: C, 45.72; H, 2.77; N, 3.79.

(*E*)-3-[(3-Bromo-5-chloro-2-hydroxybenzylidene)amino]benzoic acid **2f**. Yellow-orange solid; yield 89 %; mp 235.8-237.5°C. IR (ATR): 3077, 2919, 2853, 1703 (COO), 1614, 1584 (CH=N), 1446, 1405, 1355, 1292, 1202, 1173, 892, 867, 809, 791, 738, 725, 679, 657 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 14.28 (1H, s, OH), 13.19 (1H, s, COOH), 9.07 (1H, s, CH=N), 8.01 (1H, t, $J = 1.9$ Hz, H2), 7.92 (1H, dt, $J = 7.8$, 1.4 Hz, H6), 7.84 (1H, d, $J = 2.5$ Hz, H4′), 7.80 (1H, d, $J = 2.5$ Hz, H6′), 7.72 (1H, ddd, $J = 7.9$, 2.3, 1.2 Hz, H4), 7.61 (1H, t, $J = 7.8$ Hz, H5). ^{13}C NMR (DMSO, 126 MHz): δ 166.94, 163.51, 156.79, 146.74, 135.33, 132.46, 131.75, 130.11, 128.57, 126.70, 122.78, 121.82, 120.47, 111.22. Anal. Calcd. for $C_{14}H_9BrClNO_3$ (354.58): C, 47.42; H, 2.56; N, 3.95. Found: C, 47.57; H, 2.45; N, 3.92.

(*E*)-3-[(5-Chloro-2-hydroxy-3-iodobenzylidene)amino]benzoic acid **2g**. Orange solid; yield 86%; mp 228.1-230.4°C. IR (ATR): 2970, 1688 (COO), 1617, 1582 (CH=N), 1431, 1295, 1225, 1201, 1161, 928, 871, 793, 759, 738, 717, 697, 680, 669, 658 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 14.40 (1H, s, OH), 13.19 (1H, s, COOH), 9.00 (1H, s,

CH=N), 8.01 (1H, t, $J = 1.9$ Hz, H2), 7.95 (1H, d, $J = 2.6$ Hz, H4′), 7.91 (1H, dt, $J = 7.7$, 1.3 Hz, H6), 7.81 (1H, d, $J = 2.6$ Hz, H6′), 7.72 (1H, ddd, $J = 8.0$, 2.3, 1.1 Hz, H4), 7.61 (1H, t, $J = 7.8$ Hz, H5). ^{13}C NMR (DMSO, 126 MHz): δ 166.95, 163.46, 159.24, 146.74, 140.95, 132.51, 132.46, 130.10, 128.52, 126.73, 123.26, 121.83, 119.34, 87.33. Anal. Calcd. for $C_{14}H_8ClINO_3$ (401.58): C, 41.87; H, 2.26; N, 3.49. Found: C, 42.04; H, 2.30; N, 3.55.

(*E*)-3-[(2-Hydroxy-3,5-diiodobenzylidene)amino]benzoic acid **2h**. Orange solid; yield 95%; mp 263.6-264.9°C. IR (ATR): 2999, 2819, 1687 (COO), 1615, 1580 (CH=N), 1439, 1296, 1272, 1156, 948, 866, 828, 791, 763, 738, 681, 663, 654 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.44 (1H, s, OH), 13.19 (1H, s, COOH), 8.99 (1H, s, CH=N), 8.16 (1H, d, $J = 2.1$ Hz, H4′), 8.04 (1H, d, $J = 2.2$ Hz, H6′), 8.00 (1H, t, $J = 1.9$ Hz, H2), 7.91 (1H, dt, $J = 7.7$, 1.3 Hz, H6), 7.72 (1H, ddd, $J = 8.0$, 2.3, 1.2 Hz, H4), 7.61 (1H, t, $J = 7.8$ Hz, H5). ^{13}C NMR (DMSO, 126 MHz): δ 166.95, 163.33, 160.09, 148.64, 146.71, 141.43, 132.45, 130.09, 128.46, 126.66, 121.87, 120.87, 88.35, 81.42. Anal. Calcd. for $C_{14}H_9I_2NO_3$ (493.04): C, 34.11; H, 1.84; N, 2.84. Found: C, 34.22; H, 1.90; N, 2.91.

3,5-Bis{[(*E*)-2-hydroxybenzylidene]amino}benzoic acid **3a**. Yellow solid; yield 92%; mp 258.5-261.0°C. IR (ATR): 2860, 1687 (COO), 1620, 1592 (CH=N), 1567, 1454, 1310, 1280, 1254, 1204, 1153, 1135, 983, 957, 904, 887, 752, 690, 673 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.31 (1H, s, COOH), 12.78 (2H, s, OH), 9.10 (2H, s, CH=N), 7.85 (2H, d, $J = 2.0$ Hz, H2, H6), 7.76 (1H, t, $J = 2.0$ Hz, H4), 7.71 (2H, dd, $J = 7.9$, 1.5 Hz, H6′), 7.44 (2H, td, $J = 7.6$, 1.7 Hz, H4′), 7.02-6.97 (4H, m, H3′, H5′). ^{13}C NMR (DMSO, 126 MHz): δ 166.78, 165.10, 160.50, 149.80, 133.88, 133.40, 132.91, 120.47, 119.46, 119.44, 118.77, 116.86. Anal. Calcd. for $C_{21}H_{16}N_2O_4$ (360.37): C, 69.99; H, 4.48; N, 7.77. Found: C, 70.11; H, 4.55; N, 7.69.

3,5-Bis{[(*E*)-3-chloro-2-hydroxybenzylidene]amino}benzoic acid **3b**. Yellow solid; yield 99%; mp 295.6-297.5°C. IR (ATR): 2864, 1712 (COO), 1616, 1588 (CH=N), 1564, 1445, 1416, 1303, 1234, 1186, 1142, 883, 833, 732, 704, 676 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.92 (2H, s, OH), 13.39 (1H, s, COOH), 9.19 (2H, s, CH=N), 7.96 (2H, d, $J = 2.0$ Hz, H2, H6), 7.91 (1H, t, $J = 2.0$ Hz, H4), 7.67 (2H, dd, $J = 7.8$, 1.6 Hz, H6′), 7.61 (2H, dd, $J = 8.0$, 1.6 Hz, H4′), 7.02 (2H, t, $J = 7.8$ Hz, H5′). ^{13}C NMR (DMSO, 126 MHz): δ 166.59, 165.20, 156.55, 148.44, 133.67, 133.57, 132.20, 120.92, 120.51, 120.22, 119.88, 119.52. Anal. Calcd. for $C_{21}H_{14}Cl_2N_2O_4$ (429.25): C, 58.76; H, 3.29; N, 6.53. Found: C, 58.97; H, 3.07; N, 6.43.

3,5-Bis{[(*E*)-4-chloro-2-hydroxybenzylidene]amino}benzoic acid **3c**. Yellowish solid; yield 91%; mp 291.5-292.6°C (decomp.). IR (ATR): 1716 (COO), 1614, 1583 (CH=N), 1556, 1489, 1412, 1367, 1299, 1259, 1242, 1209, 1143, 1078, 1005, 949, 930, 893, 856, 795, 770, 698, 681 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.11 (2H, s, OH), 9.09 (2H, s, CH=N), 7.84 (2H, d, $J = 2.0$ Hz, H2, H6), 7.74-7.71 (3H, m, H4, H6′), 7.07-7.03 (4H, m, H3′, H5′). ^{13}C NMR (DMSO, 126 MHz): δ 166.70, 163.91, 161.28, 149.48, 138.07, 134.01, 133.43, 120.53, 119.76, 119.01, 118.58, 116.74. Anal. Calcd. for $C_{21}H_{14}Cl_2N_2O_4$ (429.25): C, 58.76; H, 3.29; N, 6.53. Found: C, 58.79; H, 3.18; N, 6.70.

3,5-Bis{[(*E*)-5-chloro-2-hydroxybenzylidene]amino}benzoic acid **3d**. Yellowish solid; yield 90%; mp 321.7-323.8°C (decomp.). IR (ATR): 3071, 3976, 2885, 1703 (COO), 1627, 1595 (CH=N), 1562, 1477, 1355, 1308, 1290, 1276, 1198, 1147, 977, 867, 827, 769, 678, 649, 620 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.27 (1H, s, COOH), 12.60 (2H, s, OH), 9.03 (2H, s, CH=N), 7.82 (2H, d, $J = 2.0$ Hz, H2, H6), 7.75 (2H, d, $J = 2.8$ Hz, H6′), 7.68 (1H, t, $J = 2.0$ Hz, H4), 7.42 (2H, dd, $J = 8.8$, 2.7 Hz, H4′), 6.98 (2H, d, $J = 8.8$ Hz, H3′). ^{13}C NMR (DMSO, 126 MHz): δ 167.05, 163.80, 159.42, 150.09, 133.85, 133.68, 131.55, 123.26, 121.20, 120.86, 119.66, 119.26. Anal. Calcd. for $C_{21}H_{14}Cl_2N_2O_4$ (429.25): C, 58.76; H, 3.29; N, 6.53. Found: C, 58.65; H, 3.22; N, 6.47.

3,5-Bis{[(*E*)-2-hydroxy-5-iodobenzylidene]amino}benzoic acid **3e**. Yellow-orange solid; yield 97%; mp 301.5-303.8°C. IR (ATR): 2978, 1694 (COO), 1617, 1590 (CH=N), 1557, 1472, 1349, 1309, 1270, 1196, 1143, 906, 876, 815, 787, 764, 666 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.29 (1H, s, COOH), 12.68 (2H, s, OH), 9.04 (2H, s, CH=N), 8.05 (2H, d, $J = 2.3$ Hz, H6′), 7.85 (2H, d, $J = 2.1$ Hz, H2, H6), 7.73-7.68 (3H, m, H4, H4′), 6.84 (2H, d, $J = 8.7$ Hz, H3′). ^{13}C NMR (DMSO, 126 MHz): δ 166.68, 163.38, 159.99, 149.67, 141.67, 140.24, 133.44, 122.05, 120.54, 119.61, 119.18, 80.98. Anal. Calcd. for $C_{21}H_{14}I_2N_2O_4$ (612.16): C, 41.20; H, 2.31; N, 4.58. Found: C, 41.44; H, 2.23; N, 4.61.

3,5-Bis{[(*E*)-3-bromo-5-chloro-2-hydroxybenzylidene]amino}benzoic acid **3f**. Pale orange solid; yield 91%; mp 329.4-332.0°C. IR (ATR): 3071, 1708 (COO), 1619, 1585 (CH=N), 1441, 1296, 1216, 1170, 1147, 886, 863, 773, 738, 716, 708 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.12 (2H, s, OH), 13.38 (1H, s, COOH), 9.13 (2H, s, CH=N), 7.97 (2H, d, $J = 2.0$ Hz, H2, H6), 7.88 (1H, t, $J = 2.0$ Hz, H4), 7.85 (2H, d, $J = 2.5$ Hz, H4′), 7.78 (2H, d, $J = 2.5$ Hz, H6′). ^{13}C NMR (DMSO, 126 MHz): δ 166.46, 164.10, 156.70, 147.87, 135.50, 133.68, 131.78, 122.92, 120.88, 120.44, 120.39, 111.27. Anal. Calcd. for $C_{21}H_{12}Br_2Cl_2N_2O_4$ (587.05): C, 42.97; H, 2.06; N, 4.77. Found: C, 43.03; H, 2.01; N, 4.90.

3,5-Bis{[(*E*)-5-chloro-2-hydroxy-3-iodobenzylidene]amino}benzoic acid **3g**. Orange solid; yield 92%; mp 305.2-307.1°C. IR (ATR): 3064, 1700 (COO), 1617, 1581 (CH=N), 1558, 1542, 1435, 1353, 1294, 1275, 1215, 1164, 1143, 876, 777, 739, 696, 676 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.28 (2H, s, OH), 13.38 (1H, s, COOH), 9.07 (2H, s, CH=N), 7.97 (2H, d, $J = 2.0$ Hz, H2, H6), 7.95 (2H, d, $J = 2.5$ Hz, H4′), 7.89 (1H, t, $J = 2.0$ Hz, H4), 7.78 (2H, d, $J = 2.5$ Hz, H6′). ^{13}C NMR (DMSO, 126 MHz): δ 166.77, 164.29, 159.47, 148.09, 141.41, 133.95, 132.78, 123.66, 121.16, 120.71, 119.57, 87.66. Anal. Calcd. for $C_{21}H_{12}Cl_2I_2N_2O_4$ (681.05): C, 37.04; H, 1.78; N, 4.11. Found: C, 36.95; H, 1.88; N, 4.00.

3,5-Bis{[(*E*)-2-hydroxy-3,5-diiodobenzylidene]amino}benzoic acid **3h**. Orange solid; yield 97%; mp 307.7-309.1°C. IR (ATR): 3058, 2975, 1695 (COO), 1614, 1575 (CH=N), 1541, 1463, 1434, 1414, 1291, 1278, 1157, 1144, 1003, 881, 863, 771, 740, 661 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.03 (2H, s, OH), 13.39 (1H, s, COOH), 9.05 (2H, s, CH=N), 8.16 (2H, d, $J = 2.1$ Hz, H4′), 8.01 (2H, d, $J = 2.1$ Hz, H6′), 7.97 (2H, d, $J = 2.1$ Hz, H2, H6), 7.90 (1H, t, $J = 2.0$ Hz, H4). ^{13}C NMR (DMSO, 126 MHz): δ 166.79, 164.20, 160.30, 149.11, 148.12, 141.78, 133.95, 121.22, 121.12, 120.51, 88.67, 81.89. Anal. Calcd. for $C_{21}H_{12}I_4N_2O_4$ (863.95): C, 29.19; H, 1.40; N, 3.24. Found: C, 28.98; H, 1.51; N, 3.40.

Methyl (*E*)-4-[(2-hydroxybenzylidene)amino]benzoate **4a**. Pale yellow solid; yield 90%; mp 144.8-145.1°C (145°C, Manchot and Furlong, 1909). IR (ATR): 2950, 1709 (COO), 1620, 1598, 1571 (CH=N), 1439, 1454, 1282, 1190, 1169, 1105, 1035, 961, 912, 864, 820, 783, 758, 766, 704, 682 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.64 (1H, s, OH), 8.99 (1H, s, CH=N), 8.06-8.00 (2H, m, H2, H6), 7.70 (1H, dd, $J = 7.6$, 1.8 Hz, H6′), 7.53-7.48 (2H, m, H3, H5), 7.45 (1H, td, $J = 7.8$, 1.8 Hz, H4′), 7.02-6.96 (2H, m, H3′, H5′), 3.86 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.95, 165.08, 160.46, 152.70, 134.06, 132.75, 130.75, 127.71, 121.89, 121.86, 119.49, 116.86, 52.31. Anal. Calcd. for $C_{15}H_{13}NO_3$ (255.27): C, 70.58; H, 5.13; N, 5.49. Found: C, 70.66; H, 5.04; N, 5.41.

Methyl (*E*)-4-[(3-chloro-2-hydroxybenzylidene)amino]benzoate **4b**. Yellow solid; yield 89%; mp 148.4-150.5°C. IR (ATR): 2949, 1711 (COO), 1592 (CH=N), 1570, 1450, 1435, 1307, 1279, 1208, 1183, 1162, 1146, 1114, 866, 852, 795, 778, 772, 738, 707, 690 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.92 (1H, s, OH), 9.07 (1H, s, CH=N), 8.06-8.01 (2H, m, H2, H6), 7.65 (1H, dd, $J = 7.8$, 1.6 Hz, H6′), 7.61 (1H, dd, $J = 7.9$, 1.6 Hz, H4′), 7.59-7.56 (2H, m, H3, H5), 7.01 (1H, t, $J = 7.8$ Hz, H5′), 3.86 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.85, 165.35, 156.65, 151.10, 133.81, 132.23, 130.76, 128.26, 122.03, 120.55, 120.17, 119.87, 52.35. Anal. Calcd. for $C_{15}H_{12}ClNO_3$ (289.72): C, 62.19; H, 4.18; N, 4.83. Found: C, 61.98; H, 4.09; N, 4.93.

Methyl (*E*)-4-[(4-chloro-2-hydroxybenzylidene)amino]benzoate **4c**. Yellow solid; yield 87%; mp 185.6-187.2°C. IR (ATR): 2963, 1718 (COO), 1621, 1597 (CH=N), 1561, 1434, 1389, 1357, 1283, 1188, 1169, 1104, 1077, 1015, 982, 933, 866, 827, 810, 771, 704, 607 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.97 (1H, s, OH), 9.00 (1H, s, CH=N),

8.05-8.01 (2H, m, H2, H6), 7.74 (1H, d, $J = 8.1$ Hz, H6′), 7.52-7.49 (2H, m, H3, H5), 7.08-7.04 (2H, m, H3′, H5′), 3.86 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.94, 163.85, 161.26, 152.46, 138.24, 133.84, 130.77, 127.87, 121.90, 119.82, 118.68, 116.76, 52.34. Anal. Calcd. for $C_{15}H_{12}ClNO_3$ (286.72): C, 62.19; H, 4.18; N, 4.83. Found: C, 62.30; H, 4.07; N, 4.91.

Methyl (*E*)-4-[(5-chloro-2-hydroxybenzylidene)amino]benzoate **4d**. Yellow solid; yield 83%; mp 190.9-192.0°C. IR (ATR): 3076, 2963, 1719 (COO), 1619, 1599 (CH=N), 1563, 1478, 1433, 1349, 1311, 1283, 1276, 1184, 1169, 1104, 1015, 953, 873, 831, 786, 769, 715, 702, 655 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.48 (1H, s, OH), 8.91 (1H, s, CH=N), 8.00 (2H, d, $J = 8.6$ Hz, H2, H6), 7.74 (1H, d, $J = 2.7$ Hz, H6′), 7.47-7.41 (3H, m, H3, H5, H4′), 6.98 (2H, d, $J = 8.7$ Hz, H3′), 3.83 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 166.31, 163.68, 159.40, 153.04, 133.85, 131.32, 131.15, 128.32, 123.32, 122.26, 121.28, 119.30, 52.70. Anal. Calcd. for $C_{15}H_{12}ClNO_3$ (286.72): C, 62.19; H, 4.18; N, 4.83. Found: C, 62.07; H, 4.29; N, 4.90.

Methyl (*E*)-4-[(2-hydroxy-5-iodobenzylidene)amino]benzoate **4e**. Yellow solid; yield 86%; mp 221.7-223.3°C. IR (ATR): 2949, 1708 (COO), 1616, 1596 (CH=N), 1557, 1470, 1433, 1347, 1310, 1280, 1181, 1169, 1103, 1015, 983, 954, 914, 868, 861, 845, 825, 784, 768, 703, 696 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.12 (1H, s, OH), 8.99 (1H, s, CH=N), 8.08-8.03 (3H, m, H2, H6, H6′), 7.67 (1H, dd, $J = 8.7$, 2.4 Hz, H4′), 7.58-7.54 (2H, m, H3, H5), 6.83 (1H, d, $J = 8.7$ Hz, H3′), 3.87 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 166.24, 163.58, 160.27, 152.97, 142.16, 140.27, 131.08, 128.20, 122.44, 122.20, 119.94, 81.37, 52.64. Anal. Calcd. for $C_{15}H_{12}INO_3$ (381.17): C, 47.27; H, 3.17; N, 3.67. Found: C, 46.99; H, 3.21; N, 3.81.

Methyl (*E*)-4-[(3-bromo-5-chloro-2-hydroxybenzylidene)amino]benzoate **4f**. Orange solid; yield 82%; mp 174.5-176.5°C. IR (ATR): 3069, 2957, 1703 (COO), 1596 (CH=N), 1561, 1452, 1433, 1414, 1363, 1294, 1206, 1191, 1166, 1121, 1016, 971, 873, 858, 772, 739, 726, 712, 694 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.10 (1H, s, OH), 9.03 (1H, s, CH=N), 8.07-8.04 (2H, m, H2, H6), 7.88 (1H, d, $J = 2.6$ Hz, H4′), 7.79 (1H, d, $J = 2.6$ Hz, H6′), 7.60-7.56 (2H, m, H3, H5), 3.87 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.82, 164.34, 156.86, 150.57, 135.67, 131.81, 130.83, 128.57, 122.89, 122.10, 120.41, 111.37, 52.41. Anal. Calcd. for $C_{15}H_{11}BrClNO_3$ (386.61): C, 48.88; H, 3.01; N, 3.80. Found: C, 48.74; H, 3.13; N, 3.77.

Methyl (*E*)-4-[(5-chloro-2-hydroxy-3-iodobenzylidene)amino]benzoate **4g**. Pale orange solid; yield 89%; mp 182.1-183.9°C. IR (ATR): 3071, 1713 (COO), 1592 (CH=N), 1551, 1436, 1359, 1290, 1277, 1202, 1163, 1102, 873, 773, 738, 719, 703, 691 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.20 (1H, s, OH), 8.95 (1H, s, CH=N), 8.06-8.03 (2H, m, H2, H6), 7.97 (1H, d, $J = 2.5$ Hz, H4′), 7.77 (1H, d, $J = 2.6$ Hz, H6′), 7.59-7.55 (2H, m, H3, H5), 3.86 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.81, 164.27, 159.31, 150.52, 141.29, 132.52, 130.81, 128.51, 123.35, 122.10, 119.22, 87.49, 52.40. Anal. Calcd. for $C_{15}H_{11}ClINO_3$ (415.61): C, 43.35; H, 2.67; N, 3.37. Found: C, 43.50; H, 2.58; N, 3.49.

Methyl (*E*)-4-[(2-hydroxy-3,5-diiodobenzylidene)amino]benzoate **4h**. Orange solid; yield 90%; mp 224.1-226.5°C. IR (ATR): 3061, 2952, 1703 (COO), 1585 (CH=N), 1542, 1437, 1353, 1311, 1292, 1275, 1205, 1158, 1117, 1103, 950, 869, 864, 776, 739, 695, 706, 695, 659 cm^{-1}. ^{1}H NMR (500 MHz, THF-d_8): δ 14.10 (1H, s, OH), 8.77 (1H, s, CH=N), 8.22-8.07 (3H, m, H2, H6, H4′), 7.92-7.89 (1H, m, H6′), 7.51-7.46 (2H, m, H3, H5), 3.89 (3H, s, Me). ^{13}C NMR (DMSO, 126 MHz): δ 166.38, 164.20, 161.36, 152.13, 150.45, 142.24, 131.71, 130.13, 122.31, 121.78, 87.56, 80.60, 52.18. Anal. Calcd. for $C_{15}H_{11}I_2NO_3$ (507.07): C, 35.53; H, 2.19; N, 2.76. Found: C, 35.70; H, 2.12; N, 2.83.

Ethyl (*E*)-4-[(2-hydroxybenzylidene)amino]benzoate **5a**. Yellow solid; yield 71%; mp 87.9-89.2°C (85-86.5°C, Kuder et al., 1975). IR (ATR): 2976, 1705 (COO), 1619, 1598 (CH=N), 1573, 1284, 1269, 1188, 1166, 1099, 1012, 912, 855, 840, 815, 787, 768, 751, 739, 699, 680 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.65 (1H, s, OH), 8.98 (1H, s, CH=N), 8.02 (2H, d, $J = 8.2$ Hz, H2, H6), 7.70 (1H, dd, $J = 7.7$, 1.8 Hz, H6′), 7.50 (2H, d, $J = 8.3$ Hz, H3, H5), 7.44 (1H, td, $J = 7.8$, 1.7 Hz, H4′), 7.02-6.97 (2H, m, H3′, H5′), 4.32 (2H, q, $J = 7.1$ Hz, CH_2), 1.33 (3H, t, $J = 7.1$ Hz, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.45, 165.03, 160.48, 152.64, 134.05, 132.75, 130.71, 128.00, 121.83, 121.78, 119.49, 116.87, 60.91, 14.34. Anal. Calcd. for $C_{16}H_{15}NO_3$ (269.30): C, 71.36; H, 5.61; N, 5.20. Found: C, 71.22; H, 5.72; N, 5.06.

Ethyl (*E*)-4-[(3-chloro-2-hydroxybenzylidene)amino]benzoate **5b**. Pale orange solid; yield 68%; mp 116.5-117.8°C. IR (ATR): 2983, 1705 (COO), 1618, 1594 (CH=N), 1565, 1446, 1366, 1307, 1273, 1238, 1206, 1183, 1168, 1141, 1125, 1105, 1025, 857, 835, 813, 736, 702, 691 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.94 (1H, s, OH), 9.07 (1H, s, CH=N), 8.07-8.02 (2H, m, H2, H6), 7.67-7.56 (4H, m, H3, H5, H4′, H6′), 7.01 (1H, t, $J = 7.8$ Hz, H5′), 4.32 (2H, q, $J = 7.1$ Hz, CH_2), 1.33 (3H, t, $J = 7.1$ Hz, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.36, 165.31, 156.68, 151.04, 133.82, 132.24, 130.74, 128.55, 122.01, 120.57, 120.18, 119.87, 60.99, 14.33. Anal. Calcd. for $C_{16}H_{14}ClNO_3$ (303.74): C, 63.27; H, 4.65; N, 4.61. Found: C, 63.14; H, 4.59; N, 4.88.

Ethyl (*E*)-4-[(4-chloro-2-hydroxybenzylidene)amino]benzoate **5c**. Yellow-orange solid; yield 76%; mp 131.2-132.3°C. IR (ATR): 3001, 1715 (COO), 1620, 1593 (CH=N), 1558, 1507, 1367, 1270, 1175, 1166, 1097, 1080, 1014, 933, 842, 858, 799, 771, 690 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.98 (1H, s, OH), 8.99 (1H, s, CH=N), 8.04-8.00 (2H, m, H2, H6), 7.73 (1H, d, $J = 8.0$ Hz, H6′), 7.52-7.48 (2H, m, H3, H5), 7.08-7.03 (2H, m, H3′, H5′), 4.32 (2H, q, $J = 7.1$ Hz, CH_2), 1.33 (3H, t, $J = 7.1$ Hz, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.43, 163.77, 161.27, 152.36, 138.23, 133.84, 130.72, 128.15, 121.85, 119.81, 118.66, 116.76, 60.94, 14.34. Anal. Calcd. for $C_{16}H_{14}ClNO_3$ (303.74): C, 63.27; H, 4.65; N, 4.61. Found: C, 63.36; H, 4.72; N, 4.49.

Ethyl (*E*)-4-[(5-chloro-2-hydroxybenzylidene)amino]benzoate **5d**. Orange solid; yield 82%; mp 144.8-146.1°C. IR (ATR): 2981, 1699 (COO), 1598 (CH=N), 1564, 1484, 1363, 1284, 1188, 1169, 1127, 1111, 1024, 871, 855, 773, 714, 696, 650, 635 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.49 (1H, s, OH), 8.91 (1H, s, CH=N), 8.01-7.98 (2H, m, H2, H6), 7.75 (1H, d, $J = 2.6$ Hz, H6′), 7.46-7.41 (3H, m, H3, H5, H4′), 6.98 (1H, d, $J = 8.9$ Hz, H3′), 4.28 (2H, q, $J = 7.1$ Hz, CH_2), 1.29 (3H, t, $J = 7.2$ Hz, Me). ^{13}C NMR (DMSO, 126 MHz): 165.80, 163.62, 159.40, 152.97, 133.83, 131.31, 131.10, 128.60, 123.32, 122.22, 121.27, 119.28, 61.31, 14.70. Anal. Calcd. for $C_{16}H_{14}ClNO_3$ (303.74): C, 63.27; H, 4.65; N, 4.61. Found: C, 63.11; H, 4.79; N, 4.70.

Ethyl (*E*)-4-[(2-hydroxy-5-iodobenzylidene)amino]benzoate **5e**. Orange solid; yield 79%; mp 154.2-156.1°C. IR (ATR): 3001, 1717 (COO), 1592 (CH=N), 1556, 1475, 1369, 1353, 1272, 1221, 1169, 1096, 1013, 873, 860, 811, 785, 771, 691 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.15 (1H, s, OH), 9.00 (1H, s, CH=N), 8.08-8.02 (3H, m, H2, H6, H6′), 7.66 (1H, dd, $J = 8.7$, 2.3 Hz, H4′), 7.59-7.54 (2H, m, H3, H5), 6.84 (1H, d, $J = 8.7$ Hz, H3′), 4.33 (2H, q, $J = 7.1$ Hz, CH_2), 1.33 (3H, t, $J = 7.1$ Hz, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.42, 163.22, 159.98, 152.59, 141.84, 139.98, 130.73, 128.18, 122.13, 121.86, 119.63, 81.07, 60.95, 14.34. Anal. Calcd. for $C_{16}H_{14}INO_3$ (395.20): C, 48.63; H, 3.57; N, 3.54. Found: C, 48.70; H, 3.44; N, 3.70.

Ethyl (*E*)-4-[(3-bromo-5-chloro-2-hydroxybenzylidene)amino]benzoate **5f**. Orange solid; yield 75%; mp 160.5-162.7°C. IR (ATR): 3067, 2988, 1698 (COO), 1595 (CH=N), 1557, 1449, 1413, 1362, 1250, 1199, 1166, 1123, 1106, 1023, 869, 857, 773, 741, 722, 697 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.09 (1H, s, OH), 9.03 (1H, s, CH=N), 8.08-8.03 (2H, m, H2, H6), 7.88 (1H, d, $J = 2.6$ Hz, H4′), 7.79 (1H, d, $J = 2.6$ Hz, H6′), 7.60-7.55 (2H, m, H3, H5), 4.33 (2H, q, $J = 7.2$ Hz, CH_2), 1.33 (3H, t, $J = 7.1$ Hz, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.32, 164.26, 156.91, 150.51, 135.66, 131.79, 130.78, 128.84, 122.85, 122.06, 120.41, 111.40, 61.04, 14.33. Anal. Calcd. for $C_{16}H_{13}BrClNO_3$ (382.64): C, 50.22; H, 3.42; N, 3.66. Found: C, 50.28; H, 3.59; N, 3.40.

Ethyl (*E*)-4-[(5-chloro-2-hydroxy-3-iodobenzylidene)amino]benzoate **5g**. Orange solid; yield 74%; mp 184-186.5°C. IR (ATR): 3064, 2981, 1698 (COO), 1590 (CH=N), 1550, 1429, 1367, 1358, 1300, 1199, 1162, 1099, 1016, 876, 854, 774, 740, 717, 694 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.23 (1H, s, OH), 8.96 (1H, s, CH=N), 8.07-8.03 (2H, m,

H2, H6), 7.98 (1H, d, J = 2.3 Hz, H4′), 7.79 (1H, d, J = 2.3 Hz, H6′), 7.60-7.55 (2H, m, H3, H5), 4.32 (2H, q, J = 7.0 Hz, CH_2), 1.33 (3H, t, J = 7.1 Hz, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.32, 164.23, 159.38, 150.50, 141.29, 132.50, 130.78, 128.79, 123.29, 122.08, 119.24, 87.57, 61.03, 14.33. Anal. Calcd. for $C_{16}H_{13}ClINO_3$ (429.64): C, 44.73; H, 3.05; N, 3.26. Found: C, 44.77; H, 2.96; N, 3.12.

Ethyl (*E*)-4-[(2-hydroxy-3,5-diiodobenzylidene)amino]benzoate **5h**. Orange solid; yield 76%; mp 211.5-213.8°C. IR (ATR): 3051, 2977, 1698 (COO), 1587 (CH=N), 1543, 1438, 1362, 1280, 1203, 1173, 1157, 1125, 1104, 1016, 855, 774, 740, 693, 679, 668, 660 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.08 (1H, s, OH), 8.95 (1H, s, CH=N), 8.17 (1H, d, J = 2.2 Hz, H4′), 8.08-8.02 (3H, m, H2, H6, H6′), 7.59-7.55 (2H, m, H3, H5), 4.33 (2H, q, J = 7.1 Hz, CH_2), 1.33 (3H, t, J = 7.1 Hz, Me). ^{13}C NMR (DMSO, 126 MHz): δ 165.34, 164.11, 160.29, 150.53, 148.97, 141.45, 130.78, 128.73, 122.07, 120.81, 88.74, 81.80, 61.02, 14.34. Anal. Calcd. for $C_{16}H_{13}I_2NO_3$ (521.09): C, 36.88; H, 2.51; N, 2.69. Found: C, 36.78; H, 2.51; N, 2.78.

(*E*)-4-[(2-Hydroxybenzylidene)amino]-*N*-phenylbenzamide **6a**. Pale yellow solid; yield 92%; mp 222-224°C (230-230.5°C, Levchenko, 1967). IR (ATR): 3359 (N-H), 3063, 1645 (C=O), 1597 (CH=N), 1564, 1526, 1504, 1443, 1323, 1282, 1264, 1194, 1177, 913, 852, 768, 749, 689, 644, 625, 609 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.83 (1H, s, OH), 10.26 (1H, s, NH), 9.03 (1H, s, CH=N), 8.09-8.06 (2H, m, H2, H6), 7.81-7.78 (2H, m, H2″, H6″), 7.71 (1H, dd, J = 7.6, 1.9 Hz, H6′), 7.57-7.53 (2H, m, H3, H5), 7.45 (1H, ddd, J = 8.2, 7.4, 1.8 Hz, H4′), 7.38-7.34 (2H, m, H3″, H5″), 7.10 (1H, tt, J = 7.3, 1.2 Hz, H4″), 7.03-6.97 (2H, m, H3′, H5′). ^{13}C NMR (DMSO, 126 MHz): δ 164.97, 164.71, 160.51, 151.14, 139.33, 133.92, 133.06, 132.81, 129.27, 128.78, 123.85, 121.50, 120.60, 119.51, 119.47, 116.87. Anal. Calcd. for $C_{20}H_{16}N_2O_2$ (316.36): C, 75.93; H, 5.10; N, 8.86. Found: C, 75.79; H, 5.03; N, 8.94.

(*E*)-4-[(3-Chloro-2-hydroxybenzylidene)amino]-*N*-phenylbenzamide **6b**. Orange solid; yield 90%; mp 188.1-189.4°C. IR (ATR): 3341 (N-H), 3040, 1658 (C=O), 1591 (CH=N), 1526, 1503, 1439, 1367, 1322, 1257, 1201, 1178, 1144, 857, 779, 765, 753, 735, 689, 630 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.28 (1H, s, NH), 9.12 (1H, s, CH=N), 8.11-8.08 (2H, m, H2, H6), 7.81-7.77 (2H, m, H2″, H6″), 7.76 (1H, dd, J = 7.8, 1.6 Hz, H6′), 7.64-7.60 (3H, m, H3, H5, H4′), 7.38-7.34 (2H, m, H3″, H5″), 7.11 (1H, tt, J = 7.4, 1.2 Hz, H4″), 7.02 (1H, t, J = 7.8 Hz, H5′). ^{13}C NMR (DMSO, 126 MHz): δ 164.87, 164.76, 156.77, 149.56, 139.28, 133.69, 133.62, 132.16, 129.31, 128.78, 123.88, 121.66, 120.59, 120.57, 120.21, 119.81. Anal. Calcd. for $C_{20}H_{15}ClN_2O_2$ (350.80): C, 68.48; H, 4.31; N, 7.99. Found: C, 68.39; H, 4.28; N, 7.91.

(*E*)-4-[(4-Chloro-2-hydroxybenzylidene)amino]-*N*-phenylbenzamide **6c**. Yellow solid; yield 94%; mp 271.5-273.4°C. IR (ATR): 3389 (N-H), 1655 (C=O), 1597 (CH=N), 1561, 1529, 1501, 1440, 1321, 1257, 1193, 1175, 1077, 930, 877, 849, 811, 766, 752, 697, 605 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.18 (1H, s, OH), 10.26 (1H, s, NH), 9.04 (1H, s, CH=N), 8.08-8.05 (2H, m, H2, H6), 7.80-7.77 (2H, m, H2″, H6″), 7.74 (1H, d, J = 8.1 Hz, H6′), 7.56-7.53 (2H, m, H3, H5), 7.38-7.34 (2H, m, H3″, H5″), 7.13-7.06 (3H, m, H3′, H5′, H4″). ^{13}C NMR (DMSO, 126 MHz): δ 164.93, 163.50, 161.35, 150.82, 139.31, 138.09, 133.93, 133.23, 129.28, 128.78, 123.87, 121.52, 120.59, 119.78, 118.65, 116.77. Anal. Calcd. for $C_{20}H_{15}ClN_2O_2$ (350.80): C, 68.48; H, 4.31; N, 7.99. Found: C, 68.32; H, 4.44; N, 8.04.

(*E*)-4-[(5-Chloro-2-hydroxybenzylidene)amino]-*N*-phenylbenzamide **6d**. Yellow-orange solid; yield 90%; mp 247.0-248.9°C. IR (ATR): 3379 (N-H), 1658 (C=O), 1598 (CH=N), 1558, 1525, 1502, 1480, 1438, 1319, 1277, 1259, 1173, 887, 864, 834, 783, 766, 717, 690, 646, 626 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.68 (1H, s, OH), 10.23 (1H, s, NH), 8.95 (1H, s, CH=N), 8.06-8.02 (2H, m, H2, H6), 7.78-7.74 (3H, m, H6′, H2″, H6″), 7.51-7.48 (2H, m, H3, H5), 7.43 (1H, dd, J = 8.9, 2.7 Hz, H4′), 7.35-7.33 (2H, m, H3″, H5″), 7.07 (1H, tt, J = 7.4, 1.2 Hz, H4″), 6.99 (1H, d, J = 9.0 Hz, H3′). ^{13}C NMR (DMSO, 126 MHz): δ 165.30, 163.39, 159.45, 151.42, 139.69, 133.69, 131.46, 129.66, 129.14, 124.23, 123.27, 121.89, 121.24, 120.96, 120.86, 119.27. Anal. Calcd. for $C_{20}H_{15}ClN_2O_2$ (350.80): C, 68.48; H, 4.31; N, 7.99. Found: C, 68.30; H, 4.25; N, 8.10.

(*E*)-4-[(2-Hydroxy-5-iodobenzylidene)amino]-*N*-phenylbenzamide **6e**. Yellow solid; yield 98%; mp 270.6-272.1°C. IR (ATR): 3299 (N-H), 1644 (C=O), 1596 (CH=N), 1539, 1501, 1471, 1441, 1327, 1278, 1172, 912, 890, 848, 823, 746, 689, 652, 612 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.75 (1H, s, OH), 10.26 (1H, s, NH), 8.97 (1H, s, CH=N), 8.09-8.05 (3H, m, H2, H6, H6′), 7.80-7.77 (2H, m, H2″, H6″), 7.71 (1H, dd, J = 8.7, 2.3 Hz, H4′), 7.54-7.51 (2H, m, H3, H5), 7.38-7.33 (2H, m, H3″, H5″), 7.10 (1H, tt, J = 7.3, 1.2 Hz, H4″), 6.85 (1H, d, J = 8.7 Hz, H3′). ^{13}C NMR (DMSO, 126 MHz): δ 164.93, 162.97, 160.03, 151.05, 141.71, 140.10, 139.31, 133.27, 129.28, 128.78, 123.86, 122.12, 121.53, 120.58, 119.64, 81.00. Anal. Calcd. for $C_{20}H_{15}IN_2O_2$ (442.26): C, 54.32; H, 3.42; N, 6.33. Found: C, 54.30; H, 3.50; N, 6.19.

(*E*)-4-[(3-Bromo-5-chloro-2-hydroxybenzylidene)amino]-*N*-phenylbenzamide **6f**. Orange solid; yield 96%; mp 212.9-213.9°C. IR (ATR): 3328 (N-H), 3080, 1656 (C=O), 1596 (CH=N), 1528, 1500, 1439, 1356, 1324, 1262, 1199, 1168, 890, 866, 855, 767, 750, 738, 707, 689 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.28 (1H, s, NH), 9.07 (1H, s, CH=N), 8.11-8.08 (2H, m, H2, H6), 7.88 (1H, d, J = 2.6 Hz, H4′), 7.81-7.77 (3H, m, H6′, H2″, H6″), 7.64-7.61 (2H, m, H3, H5), 7.37-7.33 (2H, m, H3″, H5″), 7.11 (1H, tt, J = 7.4, 1.2 Hz, H4″). ^{13}C NMR (DMSO, 126 MHz): δ 164.82, 163.71, 157.02, 148.98, 139.26, 135.54, 133.94, 131.75, 129.36, 128.79, 123.91, 122.80, 121.71, 120.58, 120.41, 111.40. Anal. Calcd. for $C_{20}H_{14}BrClN_2O_2$ (429.70): C, 55.90; H, 3.28; N, 6.52. Found: C, 55.81; H, 3.36; N, 6.73.

(*E*)-4-[(5-Chloro-2-hydroxy-3-iodobenzylidene)amino]-*N*-phenylbenzamide **6g**. Orange solid; yield 93 %; mp 170.0-172.3°C. IR (ATR): 3373 (N-H), 3062, 1660 (C=O), 1593 (CH=N), 1539, 1527, 1506, 1440, 1326, 1258, 1199, 1180, 1159, 888, 867, 852, 763, 719, 696, 626, 616 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.28 (1H, s, NH), 9.00 (1H, s, CH=N), 8.11-8.07 (2H, m, H2, H6), 7.98 (1H, d, J = 2.6 Hz, H4′), 7.81-7.77 (3H, m, H6′, H2″, H6″), 7.64-7.61 (2H, m, H3, H5), 7.38-7.33 (2H, m, H3″, H5″), 7.11 (1H, tt, J = 7.3, 1.2 Hz, H4″). ^{13}C NMR (DMSO, 126 MHz): δ 164.83, 163.66, 159.46, 148.97, 141.17, 139.27, 133.89, 132.46, 129.35, 128.79, 123.91, 123.27, 121.73, 120.58, 119.25, 87.55. Anal. Calcd. for $C_{20}H_{14}ClIN_2O_2$ (476.70): C, 50.39; H, 2.96; N, 5.88. Found: C, 50.49; H, 3.07; N, 5.96.

(*E*)-4-[(2-Hydroxy-3,5-diiodobenzylidene)amino]-*N*-phenylbenzamide **6h**. Orange solid; yield 94%; mp 233.5-235.9°C. IR (ATR): 3361 (N-H), 3057, 1659 (C=O), 1590 (CH=N), 1542, 1527, 1504, 1439, 1325, 1260, 1205, 1152, 866, 764, 740, 697, 660, 630, 611 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.28 (1H, s, NH), 8.98 (1H, s, CH=N), 8.17 (1H, d, J = 2.1 Hz, H4′), 8.10-8.07 (2H, m, H2, H6), 8.03 (1H, d, J = 2.1 Hz, H6′), 7.81-7.77 (2H, m, H2″, H6″), 7.63-7.60 (2H, m, H3, H5), 7.38-7.33 (2H, m, H3″, H5″), 7.11 (1H, tt, J = 7.3, 1.2 Hz, H4″). ^{13}C NMR (DMSO, 126 MHz): δ 164.83, 163.50, 160.32, 148.92, 148.84, 141.42, 139.27, 133.83, 129.34, 128.79, 123.90, 121.70, 120.78, 120.56, 88.57, 81.42. Anal. Calcd. for $C_{20}H_{14}I_2N_2O_2$ (568.15): C, 42.28; H, 2.48; N, 4.93. Found: C, 42.01; H, 2.59; N, 5.05.

2.2. Biology

2.2.1. Antibacterial activity

Antibacterial activity was evaluated against four Gram-positive and four Gram-negative strains of a clinical importance, namely: *Staphylococcus aureus* ATCC (American Type Culture Collection) 29213, CCM (Czech Collection of Microorganisms) 4223, methicillin-resistant *Staphylococcus aureus* ATCC 43300, CCM 4750 (MRSA strain), *Staphylococcus epidermidis*, clinical isolate 143-2016, *Enterococcus faecalis* ATCC 29212, CCM 4224; *Escherichia coli* ATCC 25922, CCM 3954, *Klebsiella pneumoniae* ATCC 10031, CCM 4415, *Serratia marcescens*, clinical isolate 62-2016, and *Pseudomonas aeruginosa* ATCC 27853, CCM 3955. These strains were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic) or they are clinical isolates from the Department of Clinical Microbiology, University Hospital in Hradec

Králové, Czech Republic.

The microdilution broth method was performed according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing) instructions (EUCAST DISCUSSION DOCUMENT E.Dis 5.1 2003) with slight modifications starting from the concentration of 0.49 μM. Briefly, the cultivation was done in Cation-adjusted Mueller-Hinton broth (CAMHB, M-H 2 Broth, Sigma-Aldrich, St. Louis, MO, USA) at 35±2°C. Tested compounds were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) to produce stock solutions. The final concentration of DMSO in the testing medium did not exceed 1% (*v/v*) of the total solution composition and did not affect the growth of bacteria. Positive (microbe solely), negative (cultivation medium and DMSO) controls and internal quality standards were involved in each assay. Antibacterial activity is expressed as minimum inhibitory concentration (MIC, reported in μM) after 24 and 48 h of static incubation in dark and humidified atmosphere at 35±2°C. The experiments were performed in duplicates. For the results to be valid, the difference in MIC determined from two parallel measurements must not be greater than one step on the dilution scale. The results were analysed by visual inspection.

Parent amino compounds **2-6** together with topical antibiotics bacitracin (active especially against Gram-positive species including MRSA) and neomycin (an aminoglycoside active mainly against Gram-negative strains) were used as reference compounds. Standard antibiotics (ciprofloxacin, gentamicin; data not shown) and internal quality control/reference strains are routinely included in parallel testing-basic screening of antibacterial activity.

2.2.2. Anti-biofilm activity

Microtiter plate biofilm assay, determination of minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC) were done.

The assay was carried out using the biofilm microtiter plate method and two biofilm-forming strains, methicillin-resistant *Staphylococcus aureus* subsp. *aureus* (ATCC 43300, CCM 4223) and *Staphylococcus epidermidis* (ATCC 1228, CCM 784) were used for MBIC and MBEC evaluation.

Briefly, inocula were prepared from bacteria cultivated on Mueller-Hinton plates for 18 hours at 37°C in humidified atmosphere. The density of bacterial suspension was adjusted to 5×10^5 CFU/ml in tryptone soya broth (TSA, Himedia, India) and final volumes of 100 μl were seeded in flat-bottom, polystyrene, non-tissue-treated microtiter plates. The negative control (only TSA without bacterial agents) was included, as well. The plates were then incubated in a humidified incubator for 24 hours. After biofilm formation, the wells were washed three times with sterile 0.9% saline solution. The plates were left to air dry for 15 min.

The stock solution of the tested compound was prepared in DMSO. Subsequently, a serial twofold dilution of the antimicrobial agent in CAMHB was prepared (concentration range from 3.125 mg/ml to 0.0244 mg/ml, final concentration of DMSO did not exceed 1% *v/v* of the total solution composition). Ciprofloxacin (Sigma-Aldrich, St. Louis, USA) was used as a reference antibiotic in the assay. Stock solution of ciprofloxacin was prepared in water and solutions with concentration range from 0.0244 mg/ml to 0.000381 mg/ml were subsequently prepared by a serial twofold dilution.

Solutions with various compound concentrations were transferred to wells with preformed bacterial biofilms in triplicates, in total volume of 100 μl.

Biofilms were exposed to drugs for 24 hours at 37°C without shaking. After 24 hours of incubation, 90 μl of supernatant were transferred to the external plates for a purpose of MBIC value determination. Into each well of the external microtiter plates, 10 μl of metabolic indicator, Alamar Blue, was transferred and plates were incubated for 30 min at 37°C in the gentle shaking mode. After incubation, the visual inspection together with fluorescence measurement (λEx 530 nm and λEm 590 nm, multimode microplate reader Synergy HTX, BioTek Instruments, Inc., VT, USA) was done.

The MBIC value was defined as the minimum antimicrobial concentration at which there was no evidence of observable metabolic activity associated with bacterial growth in planktonic bacteria forming biofilm.

The wells of microtiter plate with preformed staphylococcal biofilm challenged by tested antimicrobial agent were washed four times with sterile 0.9% saline solution. After washing steps, 20 μl of CAMHB medium was transferred into each well of plate. Further, for a purpose of release of attached biofilm-forming bacteria into CAMHB medium, two sonication steps (for 5 minutes, vigorous shaking step between two sonication procedures) were performed.

For MBEC evaluation, the spot plate count method was used. Released biofilm-forming bacteria in total volume of 10 μl from each well were seeded on Baird-Parker, or Mueller-Hinton agar plates and cultivated for 24 hours at 37°C. The MBEC value was defined as the minimum antimicrobial concentration at which no observable bacterial growth or limited number of CFU (limit of 50 CFU per spot) was detected.

Each experiment was repeated twice in triplicates.

2.2.3. Antimycobacterial activity evaluation

The antimycobacterial activity was evaluated using a previously reported method (Krátký et al., 2013) against *Mycobacterium tuberculosis* 331/88 ($H_{37}Rv$), *Mycobacterium avium* 330/88 and a clinical isolate of *Mycobacterium kansasii* (6509/96). The investigated compounds were added as solutions in DMSO. The final volume contained 1.0 % DMSO (*v/v*). Isoniazid as a reference drug was dissolved in demineralised water. One single concentration of 32 μM was used. The activity was determined after incubation at 37°C for 14 and 21 days, for *M. kansasii* additionally for 7 days. First-line oral antitubercular drug isoniazid (INH) and parent amino compounds **2-6** were involved as reference compounds.

2.2.4. Antifungal activity

Antifungal activity was evaluated against four yeast strains, namely: *Candida albicans* ATCC 24443, CCM 8320, *Candida krusei* ATCC 6258, CCM 8271, *Candida parapsilosis* ATCC 22019, CCM 8260, *Candida tropicalis* ATCC 750, CCM 8264; and four strains of filamentous fungi, namely: *Aspergillus fumigatus* ATCC 204305, *Aspergillus flavus* CCM 8363, *Lichtheimia corymbifera* CCM 8077, and *Trichophyton interdigitale* ATCC 9533, CCM 8377. A microdilution broth method was performed according to EUCAST instructions (EUCAST 7.3.1. and 9.3.1, 2017) with slight modifications. Briefly, tested compounds were dissolved in DMSO and diluted in a twofold manner with RPMI-1640 medium with L-glutamine, supplemented with 2% glucose (*w/v*) and buffered to pH 7.0 with 3-(*N*-morpholino)propane-1-sulfonic acid (all components were purchased from Sigma-Aldrich, St. Louis, MO, USA). The final concentration of DMSO in the tested medium did not exceed 1% (*v/v*) of the total solution composition, and it was confirmed that this concentration did not inhibit the fungal growth. Static incubation was performed in dark and humidified atmosphere, at 35±2°C for 24 and 48 h (72 and 120 h for *Trichophyton interdigitale*). Positive controls consisted of test fungus solely, while negative controls consisted of medium and DMSO. Internal quality control was included too. Visual inspection was used for MIC endpoints evaluation. The experiments were conducted in duplicates. For the results to be valid, the difference in MIC determined from two parallel measurements must not be greater than one step on the dilution scale. MIC determination scale started from 0.49 μM.

Parent amino benzoic compounds **2-6** and triazole antimycotic drug fluconazole were involved as reference compounds for a comparison. MIC values of fluconazole mean MIC_{50} values, i.e., the lowest drug concentration giving growth inhibition of 50% of that of the drug-free control. Results were read after 24 h or 48 h microdilution plates cultivation without agitation at 35±2°C in humidified atmosphere. The results were read with a microdilution plate reader (SynergyTM HTX,

BioTek Instruments, Inc., VT, USA) at wavelength 530 nm.

2.2.5. Cytotoxicity determination

The human hepatocellular liver carcinoma cell line HepG2 purchased from Health Protection Agency Culture Collections (ECACC, Salisbury, UK; passage 15-16) was cultured in Minimum Essentials Eagle Medium supplemented with 10% foetal bovine serum, 1% L-glutamine solution and non-essential amino acid solution in a humidified atmosphere containing 5% CO_2 at 37°C. For subculturing, the cells were harvested after trypsin/EDTA treatment at 37°C. To determine cytotoxicity of the compounds, the cells treated with the tested substances were used as experimental groups whereas untreated HepG2 cells served as control groups. All the reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The cells were seeded in a density of 15 000 cells per well in a 96-well plate. The next day the cells were treated with each of the tested substances at a broad range of concentrations in triplicates. The compounds were dissolved in DMSO (maximal incubation concentration of DMSO was 1% *v/v*). The controls representing 100% cell viability, 0% cell viability (treated with 10% DMSO), no cell control and vehiculum control were incubated in parallel also as triplicates. After 24 hours of incubation in a humidified atmosphere containing 5% CO_2 at 37°C, the reagent from the kit CellTiter 96 AQueous One Solution Cell Proliferation Assay (CellTiter 96; PROMEGA, Fitchburg, WI, USA) was added. After 2 hours of incubation at 37°C, absorbance of samples was recorded at 490 nm (TECAN, Infinita M200, Grödig, Austria). A standard toxicological parameter IC_{50} was calculated by nonlinear regression from a semilogarithmic plot of incubation concentration versus percentage of absorbance relative to untreated controls using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results of the experiments are presented as inhibitory concentrations that reduce viability of the cell population to 50% from the maximal viability (IC_{50}). Parent amino compounds **2-6** and tamoxifen (as tamoxifen citrate) were involved as reference compounds.

3. Results and Discussion

3.1. Chemistry

3.1.1. Study design

The design of the targeted derivatives is depicted in Scheme 1. Following modifications were employed: 1) positional isomers (derivatives of 3-aminobenzoic acid, MABA, **2**); 2) duplication of imine bonds in the molecules (derivatives of 3,5-diaminobenzoic acid, DAB, **3**); 3) synthesis of more lipophilic analogues to enhance passive diffusion through cell membranes. The largest last group is comprised of imines derived from two PABA esters, methyl 4-aminobenzoate **4** and ethyl 4-aminobenzoate **5** (i.e., local anaesthetic drug benzocaine), and 4-amino-*N*-phenylbenzamide **6** that combines an enhanced lipophilicity and one more conjugated aromatic ring with a chance of hydrogen bond formation as parent derivatives **1a-1h**.

Promoting compounds **4a-6h**, the modification of carboxyl group to form ester and amide prodrugs of **1a-1h** belongs to traditional ways of achieving differentiation when compared to parent compounds (Zhao and Guo, 2009). The more lipophilic compounds than parent imines (**3a-6h**; Table 1) should cross biological membranes like bacterial cell wall by passive diffusion more easily than parent imines bearing free carboxyl moiety.

In addition, prodrugs are common powerful tool helping to overcome limiting drawbacks of new drugs including a low activity, bioavailability and cytotoxicity. Ester prodrugs have usually simple alcohols as they are expected to be cleaved by numerous either bacterial specific or nonspecific hydrolytic enzymes. However, it was demonstrated unequivocally that esterification can introduce original antimicrobial properties into previously inactive agents through changes of cellular permeability and/or solubility (Larsen and Johnson, 2019). These findings justify design of our amino carboxylic acids functional derivatives.

Supporting our design idea, imines of several of the parent amino compounds **2-6** have been reported as potential antimicrobial agents, illustratively those derived from MABA **2** (Qui et al., 2019a), methyl 4-aminobenzoate **4** (Qui et al., 2019b) and benzocaine **5** (Khair-ul-Bariyah et al., 2020). Moreover, the local anaesthetic drug benzocaine has exhibited mild intrinsic antimicrobial properties (Khair-ul-Bariyah et al., 2020).

Various substituents effective in the previous study of Krátký et al. (2020) such as 3,5-dihalogenosalicylaldehydes (3-Br-5-Cl **f**, 3-I-5-Cl **g**, 3, 5-I_2 **h**) were used together with isomeric chlorosalicylaldehydes (3-Cl **b**, 4-Cl **c**, 5-Cl **d**), 5-iodosalicylaldehyde (derivatives **e**) highlighting the role of iodine and unsubstituted salicylidene analogues **a** for comparison.

3.1.2. Synthesis

The targeted derivatives **2a-6h** were obtained by one step (derivatives of **2** and **3**), two steps (**4** and **5**) or three steps (**6**) procedures. Initially, we synthesized parent modified amino compounds, esters **4** and **5** and amide **6**. The synthesis pathways chosen are comparatively straightforward and quick to allow a synthesis of a large family of derivatives to determine SAR and to identify the most promising hits.

Amino acids **2** and **3** are commercially available, amino esters **4** and **5** were synthesized from PABA **1** *via* Fischer esterification using concentrated sulfuric acid as a catalyst (Scheme 2). The acid was dissolved in an excess of an appropriate alcohol and refluxed for 4 hours. After neutralization, the esters precipitated immediately in good yields (70 %).

4-Amino-*N*-phenylbenzamide **6** was synthesized in two steps (Scheme 3). At first, aniline reacted with 4-nitrobenzoyl chloride in the presence of a mild excess of triethylamine (Et_3N) to form 4-nitro-*N*-phenylbenzamide in quantitative yield. This intermediate was reduced to amino derivative **6** using iron as a source of electrons and acetic acid. The overall yield was 65%.

Imines **2a-2h, 3a-3h, 4a-4h, 5a-5h**, and **6a-6h** were synthesized according our previous publication (Krátký et al., 2020). Amino compounds were dissolved in MeOH and mixed with a mild excess of aldehydes (1.1 of equivalents) (Scheme 4). The mixture was heated to reflux for a period of 3 hours, then allowed to cool to room temperature over 12 hours. The imines precipitated from reaction mixture spontaneously and after filtration, they were purified by crystallization. In general, the yields ranged from 68 to 99%. Among the series, the highest yields were obtained for derivatives of 3,5-diaminobenzoic acid (**3a-3h**, 90-96%) and 4-amino-*N*-phenylbenzamide **6a-6h** (90-96%), followed by imines of MABA **3a-3h** (86-95%). The synthesis of less hydrophilic esters led to lower yields due to their better solubility in methanol. Methyl esters **4a-4h** were isolated in 82-90% yields, since for the ethyl esters **5a-5h**, there was a drop to only 68-82%.

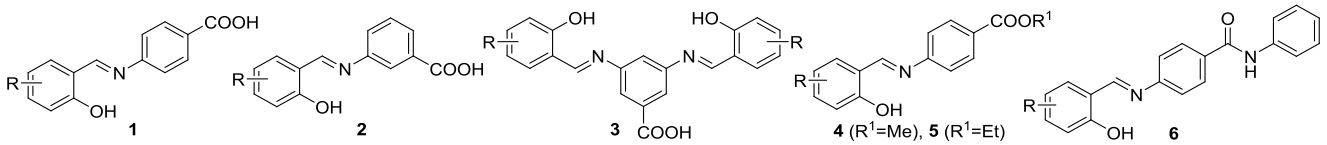
The identity of the known compounds was established using ^{1}H and ^{13}C NMR and IR spectroscopy by the comparison with previously reported data. The purity was checked by melting points measurement and elemental analysis. Identical methods were used for the characterization of novel compounds.

3.2. Biological activity

3.2.1. Antibacterial activity

Antimicrobial activity of the imines **2a-6h** was determined by the microdilution broth method according to the EUCAST guidelines (EUCAST DISCUSSION DOCUMENT E.Dis 5.1 2003) against four Gram-positive strains (*Staphylococcus aureus* ATCC 29213; methicillin-resistant *Staphylococcus aureus* ATCC 43300, MRSA; *Staphylococcus epidermidis*, clinical isolate 143-2016; *Enterococcus faecalis* ATCC 29212) and four Gram-negative strains (*Escherichia coli* ATCC 25922; *Klebsiella pneumoniae* ATCC 10031; *Serratia marcescens*, clinical

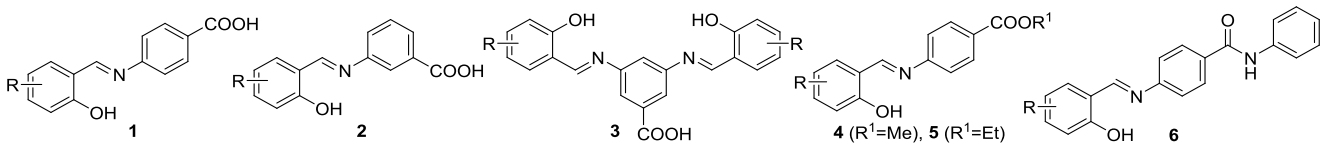
Table 1
Antibacterial activity of Schiff bases **1a-6h.**



Code	R	log *P*	MIC [μM]															
			SA		MRSA		SE		EF		EC		KP		SEMA		PA	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1a*	H	3.07	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1b*	3-Cl	3.63	>500	>500	>500	>500	500	500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1c*	4-Cl	3.63	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1d*	5-Cl	3.63	>500	>500	>500	>500	62.5	62.5	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1e*	5-I	4.43	>250	>250	250	250	62.5	62.5	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
1f*	3-Br-5-Cl	4.46	**15.62**	**15.62**	31.25	31.25	**15.62**	31.25	62.5	62.5	500	500	500	500	**500**	**500**	>500	>500
1g*	3-I-5-Cl	4.98	**15.62**	**15.62**	**15.62**	**15.62**	31.25	31.25	62.5	62.5	**62.5**	**62.5**	**125**	**125**	**500**	**500**	>500	>500
1h*	3,5-I_2	5.78	62.5	62.5	62.5	62.5	62.5	62.5	500	500	500	500	500	500	**500**	**500**	>500	>500
2a	H	3.07	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2b	3-Cl	3.63	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2c	4-Cl	3.63	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
2d	5-Cl	3.63	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
2e	5-I	4.43	500	500	500	500	500	500	500	500	500	500	500	500	>500	>500	>500	>500
2f	3-Br-5-Cl	4.46	125	125	250	250	250	250	500	500	500	500	>500	>500	>500	>500	>500	>500
2g	3-I-5-Cl	4.98	31.25	62.5	62.5	62.5	125	125	250	250	500	500	>500	>500	>500	>500	>500	>500
2h	3,5-I_2	5.78	**7.81**	**7.81**	**15.62**	**15.62**	31.25	62.5	**31.25**	62.5	500	500	500	500	>500	>500	>500	>500
3a	H	4.55	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
3b	3-Cl	5.66	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
3c	4-Cl	5.66	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
3d	5-Cl	5.66	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
3e	5-I	7.26	250	250	250	250	125	125	500	500	250	250	500	500	**500**	**500**	>500	>500
3f	3-Br-5-Cl	7.32	62.5	62.5	62.5	62.5	62.5	62.5	62.5	125	>125	>125	>125	>125	>125	>125	>125	>125
3g	3-I-5-Cl	8.38	**15.62**	**15.62**	**15.62**	31.25	**15.62**	31.25	62.5	125	250	500	500	>500	>500	>500	>500	>500
3h	3,5-I_2	9.98	**7.81**	**7.81**	**7.81**	**7.81**	**7.81**	**15.62**	**15.62**	**31.25**	500	500	>500	>500	>500	>500	>500	>500
4a	H	3.33	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
4b	3-Cl	3.89	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
4c	4-Cl	3.89	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
4d	5-Cl	3.89	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
4e	5-I	4.69	125	250	250	250	125	250	500	500	250	250	500	500	**500**	**500**	>500	>500
4f	3-Br-5-Cl	4.72	125	250	250	250	250	250	500	500	500	500	>500	>500	>500	>500	>500	>500
4g	3-I-5-Cl	5.25	31.25	62.5	62.5	62.5	125	250	500	500	500	500	>500	>500	>500	>500	>500	>500
4h	3,5-I_2	6.05	**7.81**	**7.81**	**7.81**	**15.62**	62.5	125	**31.25**	**31.25**	>125	>125	>125	>125	>125	>125	>125	>125
5a	H	3.67	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
5b	3-Cl	4.23	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
5c	4-Cl	4.23	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
5d	5-Cl	4.23	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
5e	5-I	5.03	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
5f	3-Br-5-Cl	5.06	125	125	250	250	250	500	500	500	500	500	>500	>500	>500	>500	>500	>500
5g	3-I-5-Cl	5.59	31.25	62.5	62.5	125	125	250	500	500	500	500	500	500	>500	>500	>500	>500
5h	3,5-I_2	6.39	**15.62**	**15.62**	**15.62**	**15.62**	**15.62**	**15.62**	125	125	>250	>250	>250	>250	>250	>250	>250	>250
6a	H	4.32	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
6b	3-Cl	4.88	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
6c	4-Cl	4.88	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
6d	5-Cl	4.88	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
6e	5-I	5.68	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250

(*continued on next page*)

Table 1 (*continued*)



Code	R	log *P*	MIC [μM]															
			SA		MRSA		SE		EF		EC		KP		SEMA		PA	
6f	3-Br-5-Cl	5.70	250	500	250	500	250	500	500	500	500	500	500	>500	>500	>500	>500	>500
6g	3-I-5-Cl	6.23	62.5	125	125	125	62.5	125	125	125	>125	>125	>125	>125	>125	>125	>125	>125
6h	3,5-I_2	7.03	31.25	31.25	31.25	62.5	31.25	31.25	62.5	125	250	250	500	>500	>500	>500	>500	>500
1*	-	-	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2	-	-	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
3	-	-	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
4	-	-	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
5	-	-	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
6		-	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
BAC	-	-	7.81	15.62	15.62	15.62	15.62	31.25	15.62	62.5	>500	>500	>500	>500	>500	>500	>500	>500
NEO	-	-	NT	NT	NT	NT	NT	NT	NT	NT	3.9	3.9	1.95	1.95	3.9	3.9	7.81	15.62

BAC = bacitracin; NEO = neomycin. SA: *Staphylococcus aureus* ATCC 29213; MRSA: methicillin-resistant *Staphylococcus aureus* ATCC 43300; SE: *Staphylococcus epidermidis*, clinical isolate 143-2016; EF: *Enterococcus faecalis* ATCC 29212; EC: *Escherichia coli* ATCC 25922; KP: *Klebsiella pneumoniae* ATCC 10031; SEMA: *Serratia marcescens*, clinical isolate 62-2016; PA: *Pseudomonas aeruginosa* ATCC 27853. One or two of the best MIC value(s) for each strain are shown in bold. NT: not tested. *: data from Krátký et al., 2020.

isolate 62-2016; *Pseudomonas aeruginosa* ATCC 27853). Bacitracin, neomycin and amino compounds **2-6** were used for comparison of MIC (Table 1).

Parent amino compounds **2-6** share no antibacterial properties (all MIC values above 500 μM). In general, Gram-positive bacteria were inhibited by novel imines **2a-6h** at lower concentrations (MIC from 7.81 μM) than Gram-negative species with MIC of ≥250 μM. *S. aureus* including MRSA strain were the most susceptible ones (MIC from 7.81 to 500 μM), followed by two remaining Gram-positive species (*S. epidermidis* and *E. faecalis*). Notably, there is no difference between methicillin-susceptible and resistant *S. aureus* which means that no cross-resistance of the imines **2a-6h** to beta-lactams was described, fortunately. On the other hand, the growth of *P. aeruginosa* was not affected by any of the imines. *E. coli* was the most susceptible Gram-negative pathogen, however, at comparatively higher concentrations (≥250 μM). Interestingly, the best activity was determined for 5-iodosalicylidene derivatives (**2e, 3e**, and **4e**) since additional iodine at the position 3 was found superfluous in this case.

Drawing a comparison to clinically used antibiotic, we chose bacitracin, a peptide drug used for local treatment of Gram-positive bacteria-caused infections, and neomycin, an aminoglycoside for therapy especially of topical Gram-negative infections. Due to lack of a significant activity against Gram-negative pathogens, none of the compounds outstripped neomycin. Contrarily, the most active new imines (**2h, 3g, 3h, 4h**, and **5h**) were not inferior, but fully comparable to bacitracin against three strains of the genus *Staphylococcus* and three of them (**2h, 3h**, and **4h**) also against *E. faecalis*.

SAR for the inhibition of Gram-positive bacteria are summarized in Fig. 2. Obviously, the highest suppression of the pathogens is related to the presence of 3,5-dihalogenosalicylic scaffold (derivatives **f, g**, and **h**) favouring diiodo compounds **h**, followed by 5-chloro-3-iodosalicylidenes **g**. The original series **1a-1h** derived from PABA exhibited partly different SAR with 3-I (or Br)-5-Cl-salicylaldehyde-based Schiff bases superiority. Among monohalogenated aldehydes, 5-iodine is preferred over chlorine. The presence of at least one heavier halogen is essential, favouring iodine atom(s) with the biggest atomic mass, lipophilicity and atomic radius. However, based on the data set, it is not possible to decide which parameter is a key one if any. Novel compounds derived from both all isomeric chlorosalicylaldehydes and unsubstituted 2-hydroxybenzaldehyde are virtually inactive. In fact, the substitution of the salicylidene scaffold is more important than the parent amino compound for antibacterial activity, although imines of methyl 4-aminobenzoate **4** and highly lipophilic "double" imines of 3,5-diaminobenzoic acid **3** tend to be more potent. Contrarily, Schiff bases obtained from 4-amino-*N*-phenylbenzamide **6** displayed increased MIC values and more lipophilic ethyl esters (benzocaine analogues **5a-5h**) did not provide any better

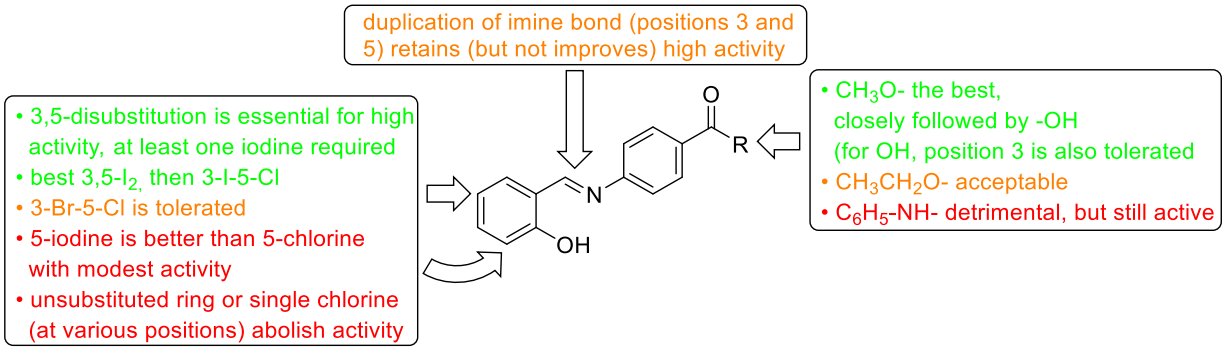


Fig. 2. Antibacterial structure-activity relationships of novel imines **2a-6h.**

activity than methyl esters **4a**-**4h**. In sum, the lipophilicity of parent amino compounds **2**-**6** is not the sole factor influencing the antibacterial activity, since order of descending lipophilicity of amino compounds and their imines is as follows: DAB **3** > 4-amino-*N*-phenylbenzamide **6** > ethyl 4-aminobenzoate **5** > methyl 4-aminobenzoate **4** > MABA **2** = PABA **1**.

According to our best knowledge, this study reports the antibacterial activity of the 3,5-diaminobenzoic acid **3** and also 4-amino-*N*-phenylbenzamide **6** derivatives at first time so far.

3.2.2. Anti-biofilm activity

Based on antibacterial activity of **4h**, we investigated also its ability to inhibit biofilm production and eradicate already formed biofilm. Biofilms are extracellular complex structures composed of microbes itself surrounded by polymeric matrix and attached to the surface. Formation of biofilm is a common feature of the bacterial physiology and it belongs to their key defensive strategies included also in adhesion to the surfaces and drug resistance. The resistance to antibacterial drugs can increase to 1,000 times that of planktonic phase (Guo et al., 2020). That is why development of drugs able to either disrupt a preformed biofilm or interfere with its *de novo* formation is required.

Microtiter plate biofilm assay was used for determination of minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC). MBIC is defined as the minimum concentration at which no activity associated with bacterial growth in planktonic bacteria forming biofilm was detected. MBEC means the minimum concentration at which no observable bacterial growth or limited number of colony-forming units (CFU) was found. Two biofilm-producing strains, methicillin-resistant *Staphylococcus aureus* subsp. *aureus* (ATCC 43300) and *Staphylococcus epidermidis* (ATCC 1228), were used for MBIC and MBEC evaluation. Fluoroquinolone drug ciprofloxacin highly active against staphylococci was employed as a reference antibiotic. Results are summarized in Table 2.

Methyl 4-[(2-hydroxy-3,5-diiodobenzylidene)amino]benzoate **4h** inhibited the formation of biofilm produced by MRSA strain at concentrations of two orders of magnitude higher than minimum inhibitory concentrations (1540 and 3081 μM, i.e., 99 and 197 times higher, respectively). When these MBIC concentrations were increased twofold, we obtained MBEC, where **4h** was able to eliminate preformed biofilm. Evaluating the activity against *S. epidermidis*, the imine **4h** was ineffective in first experiment within the range of the tested concentrations, while it exhibited a biofilm inhibiting activity in the repeated one. Its MBIC reached 1540 μM (i.e., 25 times higher value than MIC) but MBEC was not accomplished at the highest concentration investigated (6162.8 μM). This lower activity against *S. epidermidis* can be a consequence of increased MIC value (62.5 μM) when compared to *S. aureus*.

Ciprofloxacin, a potent antistaphylococcal drug, exhibited lower MIC values as expected and its ratios MBIC/MIC reached significantly lower values for both strains (up to three times), i.e., being a potent inhibitor of biofilm growth. On the other hand, rise in MBEC/MIC ratio of ciprofloxacin is noticeable and their values are at least comparable to **4h**.

In summary, although the suppression of biofilms produced by Gram-positive bacteria was not outstanding, the most selective imine **4h** exhibited a mild potency to affect them.

Table 2
Anti-biofilm activity of the imine **4h.**

Type of activity		Bacterial strain	
		MRSA	SSEE
Imine **4h**	MIC (μM)	7.81-15.625	62.5
	MBIC (μg/ml)	781.25-1562.5	781.25->1562.5
	MBIC (μM)	1540.07-3081.0	1540.07->3081.0
	Multiple of MIC value	98.6-197.2 ×	≥24.6 ×
	MBEC (μg/ml)	1562.5-3125.0	>1562.5
	MBEC (μM)	3081.0- 6162.8	>3081.0
	Multiple of MIC value	197.2-394.3 ×	>49.3 ×
CIP	MIC (μg/ml)	0.128	0.256
	MBIC (μg/ml)	0.381	0.381-0.7625
	Multiple of MIC value	3 ×	1.5-3 ×
	MBEC (μg/ml)	48.8	97.6-195.3
	Multiple of MIC value	381.3 ×	381.3-762.9 ×

MBIC: minimum biofilm inhibition concentration; MBEC: minimum biofilm eradication concentration. MRSA: methicillin-resistant *S. aureus* ATCC 43300; SE: *Staphylococcus epidermidis* ATCC 1228. CIP: ciprofloxacin.

3.2.3. Antimycobacterial activity

Antimycobacterial activity of **2a-6h** against three mycobacterial strains (*Mycobacterium tuberculosis* $H_{37}Rv$, *Mycobacterium avium, Mycobacterium kansasii*) was screened initially at a single concentration of 32 μM. However, none of the imines produced any growth inhibition of the tested strains. These findings are consistent with the previous study (Krátký et al., 2020) concerning imines of PABA (**1a**-**1h**) with only a mild-to-moderate activity (MIC ≥62.5 μM). Parent aromatic amino compounds **2**-**6** avoided any significant antimycobacterial properties too.

3.2.4. Antifungal activity

Parent amino compounds **2**-**6** together with their imines **2a**-**6h** were evaluated against eight human pathogenic fungi according to the EUCAST standards for yeasts (EUCAST 7.3.1., 2017) and moulds (EUCAST 9.3.1., 2017). The yeasts within this study were *Candida albicans* ATCC 24443, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 750. *Aspergillus fumigatus* ATCC 204305, *Aspergillus flavus* CCM 8363, *Lichtheimia* (*Absidia*) *corymbifera* CCM 8077, and *Trichophyton interdigitale* ATCC 9533 were used as representatives of filamentous fungi. Fungistatic triazole drug fluconazole was involved for comparison of MIC values (Table 3). In other words, activity data of fluconazole mean IC_{50} values determined spectrophotometrically; on the other hand, MIC of the investigated compounds report concentrations inhibiting the growth of pathogens completely (visual inspection). The parent structures **2**-**6** lack any antifungal action (MIC >500 μM).

Generally, *L. corymbifera* was identified as the most resistant strain (MIC of ≥31.25 μM, but for majority of the active compounds of ≥250 μM and many derivatives are inactive), followed by both strains of *Aspergillus* (MIC values from 15.62 μM). By contrast, the growth of yeasts (especially *C. albicans*) and *T. interdigitale* was supressed from the concentration of 3.90 μM. The last strain mentioned was the most susceptible to the largest number of derivatives. Non-albicans *Candida* species showed a uniform susceptibility predominantly.

As shown in Table 3, most of the modifications produced *in vitro* antifungal efficacy against at least one strain excepting the majority of non-halogenated salicylidene (**2a, 3a, 4a**, and **6a**), 4-chlorosalicylidene (**2c, 4c**, and **5c**) and **6e** compounds. The remaining derivatives can be divided into two groups. First, Schiff bases with a moderate activity (here defined as MIC of ≥62.5 μM) consisted of remaining 4-chlorosalicylidene (**3c, 6c**) and with its isomeric 3- and 5-chlorosalicylidene (**2b, 2d, 3b, 3d, 4b, 4d, 5b, 5d, 6b**, and **6d**), majority of 5-iodosalicylidene (**2e, 3e**, and **5e**) and 3-bromo-5-chlorosalicylidene (**2f, 3f, 4f**) analogues. Interestingly, several members of this group exhibited a broad spectrum of activity with an inhibition of all fungal strains (5-I: **2e, 3e**; 3-Cl: **5b**; 3-Br-5-Cl: **2f**). Focusing on isomeric chlorosalicylaldehydes, 3-chloro one is optimal. The second group of highly active analogues (MIC from 3.90 μM) covers condensates of 3,5-diiodo- and 5-chloro-3-iodosalicylaldehydes **h** and **g**, respectively, providing balanced activity cross-sectionally as well as **5f**. Ten molecules were able to abolish the growth of all fungal strains (3-Cl: **5b**; 5-I: **2e, 3e**; 3-Br-5-Cl: **2f**; 3-I-5-Cl: **2g, 3g, 6g**; 3,5-I_2: **2h, 3h, 6h**). Thus, from the point of view of a wide spectrum of antifungal activity, derivatives of MABA **2** and DAB **3** proved to be superior to others. The esters **5a**-**5h** share a limited inhibition of both *Aspergillus* species and *L. corymbifera*.

It is worth drawing a comparison between fluconazole and imines. The best imines were able to abolish the growth of *C. albicans* at

Table 3
Antifungal activity of Schiff bases **1a-6h.**

Code	R	MIC [μM]															
		CA		CK		CP		CT		AF		AFl		LC		TI	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	72 h	120 h
1a*	H	>500	>500	>500	>500	NT	NT	>500	>500	>500	>500	NT	NT	>500	>500	>500	>500
1b*	3-Cl	31.25	31.25	250	250	NT	NT	62.5	62.5	250	250	NT	NT	500	500	125	125
1c*	4-Cl	250	>500	500	>500	NT	NT	500	>500	250	>500	NT	NT	>500	>500	500	>500
1d*	5-Cl	>125	>125	125	125	NT	NT	125	125	>125	>125	NT	NT	>125	>125	125	125
1e*	5-I	62.5	62.5	62.5	62.5	NT	NT	125	125	125	125	NT	NT	>125	>125	31.25	31.25
1f*	3-Br-5-Cl	31.25	31.25	62.5	62.5	NT	NT	62.5	62.5	250	250	NT	NT	500	500	62.5	62.5
1g*	3-I-5-Cl	**7.81**	**7.81**	**15.62**	**15.62**	NT	NT	**31.25**	**31.25**	125	125	NT	NT	**250**	**250**	**7.81**	**7.81**
1h*	3,5-I_2	**7.81**	**7.81**	**31.25**	**31.25**	NT	NT	**15.62**	**15.62**	125	125	NT	NT	**250**	**250**	**7.81**	**7.81**
2a	H	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2b	3-Cl	125	250	500	500	250	250	250	250	250	250	500	500	500	500	125	125
2c	4-Cl	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
2d	5-Cl	125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	125	125
2e	5-I	125	250	125	125	125	125	250	250	250	250	250	250	500	500	62.5	62.5
2f	3-Br-5-Cl	62.5	62.5	62.5	62.5	125	250	250	250	250	500	250	500	500	500	125	125
2g	3-I-5-Cl	**15.62**	**15.62**	**15.62**	**31.25**	**15.62**	**31.25**	**15.62**	**15.62**	**15.62**	**31.25**	**15.62**	**31.25**	500	500	**3.90**	**7.81**
2h	3,5-I_2	**7.81**	**7.81**	**15.62**	**15.62**	**15.62**	**15.62**	**31.25**	62.5	62.5	62.5	125	250	250	500	**3.90**	**7.81**
3a	H	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
3b	3-Cl	125	125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	62.5	62.5
3c	4-Cl	125	>125	>125	>125	>125	>125	>125	>125	62.5	125	>125	>125	>125	>125	125	125
3d	5-Cl	125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	125	>125
3e	5-I	62.5	62.5	62.5	62.5	125	125	125	125	62.5	125	250	250	250	250	125	250
3f	3-Br-5-Cl	62.5	62.5	62.5	62.5	125	125	>125	>125	>125	>125	>125	>125	>125	>125	62.5	62.5
3g	3-I-5-Cl	**15.62**	**15.62**	**15.62**	**31.25**	**15.62**	**31.25**	**31.25**	62.5	62.5	125	125	125	500	500	**3.90**	**7.81**
3h	3,5-I_2	**7.81**	**7.81**	**15.62**	**15.62**	**15.62**	**15.62**	**31.25**	125	125	250	125	250	250	500	**3.90**	**7.81**
4a	H	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
4b	3-Cl	250	250	500	500	250	500	500	500	250	500	>500	>500	500	>500	125	250
4c	4-Cl	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
4d	5-Cl	125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	125	125
4e	5-I	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4f	3-Br-5-Cl	62.5	62.5	62.5	125	125	125	62.5	62.5	500	>500	500	>500	>500	>500	62.5	125
4g	3-I-5-Cl	**15.62**	**15.62**	**31.25**	**31.25**	**31.25**	**31.25**	**31.25**	62.5	125	250	250	500	>500	>500	15.62	31.25
4h	3,5-I_2	**7.81**	**7.81**	**31.25**	62.5	**15.62**	**31.25**	62.5	62.5	62.5	125	62.5	125	>500	>500	**3.90**	**7.81**
5a	H	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	250	250
5b	3-Cl	125	250	500	500	250	250	250	250	250	250	250	250	500	500	125	125
5c	4-Cl	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
5d	5-Cl	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	125	>125
5e	5-I	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	250	250
5f	3-Br-5-Cl	125	125	125	125	125	250	250	250	>500	>500	>500	>500	>500	>500	31.25	31.25
5g	3-I-5-Cl	31.25	31.25	**31.25**	**31.25**	**31.25**	**62.5**	62.5	250	>500	>500	>500	>500	>500	>500	**3.90**	**3.90**
5h	3,5-I_2	**7.81**	**7.81**	62.5	125	**15.62**	**62.5**	125	125	125	125	125	250	>250	>250	**3.90**	**3.90**
6a	H	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
6b	3-Cl	250	500	500	>500	500	500	250	500	500	>500	500	500	500	500	500	500
6c	4-Cl	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	125	>125
6d	5-Cl	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	125	>125
6e	5-I	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
6f	3-Br-5-Cl	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	>125	>125	125	>125	>125	>125	>125	>125
6g	3-I-5-Cl	31.25	31.25	**31.25**	**31.25**	**31.25**	**31.25**	**31.25**	**31.25**	**31.25**	62.5	**31.25**	**31.25**	**62.5**	125	31.25	31.25
6h	3,5-I_2	**7.81**	**7.81**	**31.25**	**31.25**	**31.25**	**31.25**	62.5	62.5	62.5	62.5	**15.62**	**31.25**	**31.25**	**31.25**	31.25	31.25
1*	-	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2		>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
3		>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
4		>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
5		>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
6		>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
FLU	-	6.5	6.5	>104.5	>104.5	3.3	3.3	6.5	6.5	>104.5	>104.5	>104.5	>104.5	>104.5	>104.5	52.2	52.2

FLU = fluconazole. CA: *Candida albicans* ATCC 24433; CK: *Candida krusei* ATCC 6258; CP: *Candida parapsilosis* ATCC 22019; CT: *Candida tropicalis* ATCC 750; AF: *Aspergillus fumigatus* ATCC 204305; AFl: *Aspergillus flavus* CCM 8363; LC: *Lichtheimia corymbifera* CCM 8077; TI: *Trichophyton interdigitale* ATCC 9533. One or two of the best MIC value(s) for each strain are shown in bold. ND: not determined due to solubility problems. NT: not tested. *: data from Krátký et al., 2020.

concentrations identical to IC_{50} of fluconazole (**2h, 3h, 4h, 5h**, and **6h**). For other strains, MIC of the active imines were usually superior or fully comparable to IC_{50} values of this widely clinically used fungistatic drug, *C. tropicalis* and *C. parapsilosis* being partial exceptions. Some of the new modifications provide equal or superior antimycotic activity compared to the PABA-derived hit compounds **1a**-**1h**.

3,5-Dihalogenosalicylaldehyde derivatives containing at least one iodine are optimal choice to combat fungi, favouring especially 3-[(5-chloro-2-hydroxy-3-iodobenzylidene)amino]benzoic acid **2g**. There is no direct relationship between lipophilicity of parent amino compounds **2**-**6** and MIC values again. In summary, these SAR follow identical trends as antibacterial properties.

To the best of our knowledge, the activity of imines of 3,5-diaminobenzoic acid **3** and 4-amino-*N*-phenylbenzamide **6** against human pathogenic fungi is described herein for the first time.

3.2.5. Cytotoxicity and selectivity

The most active analogues, i.e., 5-chloro-3-iodosalicylidene **g** and 3,5-diiodosalicylidene **h** imines, and the parent amino derivatives **2**-**6** were evaluated further for their *in vitro* cytotoxicity on standard hepatic cell line HepG2. The used CellTiter 96 assay is based on the reduction of tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; MTS) in living cells to formazan, which is then determined colorimetrically. The reduction of the reagent is related to availability of NADH or NADPH. The decline in levels of these metabolically important compounds in the cell causes reduced production of formazan dye.

The parameter IC_{50} was used as a measure of cytotoxicity, which allows the quantitative comparison of the toxicity among the tested compounds. IC_{50} parameters were determined for the most substances on the base of concentration range of 0-500 μM, but the employed concentrations had to be limited in several agents due to a lower solubility (**3h** and **5h**). However, despite this obstacle we were able to determine the exact IC_{50} of all Schiff bases (Table 4). The parent amino compounds **2**-**6** with a comparatively better aqueous solubility were evaluated up to the concentration of 1000 μM and they were found non-toxic at this concentration. Cytotoxic drug tamoxifen was used as a comparator.

Based on the results, the derivatives **g** and **h** can be divided into three groups. First, compounds with an escalated cytotoxicity comparable to tamoxifen, i.e., with IC_{50} values lower than 20 μM (**2h, 3g, 5h**). Second, the largest group comprised of imines with moderate IC_{50} ranging from 53.3 to 87.1 μM (**1g, 1h, 2g, 3h, 4g, 5g, 6g**, and **6h**). A little cytotoxic methyl ester **4h** is the only one member of the last group (IC_{50} = 215.1 μM). The concentration-cytotoxicity response curves for the most (**2h**) and least toxic (**4h**) compounds are illustrated in Fig. 3.

The introduction of salicylidene scaffold led to an increased cytotoxicity when compared to parent amino derivatives **2**-**6**. Focusing on original PABA derivatives **1g** and **1h** (Krátký et al., 2020), their modification to the compounds **2**-**6** has an unclear effect. Among 5-chloro-3-iodosalicylidene imines **g**, three molecules (**2g, 4g**, and **6g**) were less toxic than **1g**, one comparable (**5g**) and remaining one showed an enhanced toxicity (**3g**). Drawing a comparison between 3,5-diiodo derivatives **h**, two of them (**2h, 5h**) showed lower IC_{50} values than **1h**, two compounds (**4h** and **6h**) share higher IC_{50}. From structure-activity relationships point of view, especially the esterification of PABA by methanol provided fewer toxic derivatives **4g** and **4h**. The formation of *N*-phenylamides proves a benefit too, but in a lesser extent. Both ethyl esters **5** and derivatives with duplicated imine bond **3** are rather disadvantageous, since 3-(salicylideneamino)benzoic acids **2** are ambiguous. The contradictory data did not enable to draw a distinct conclusion, which halogen (if iodine or chlorine) at the position 5 is the best choice.

We utilized IC_{50} for calculation of selectivity indexes (SI). Analogously to therapeutic index, unitless SI is defined as a ratio of the IC_{50} and MIC values. Its value above a threshold of 10 indicates a desired dissociation of toxicity and targeted biological action. We determined SI for the most susceptible pathogens: *S. aureus* including the MRSA strain, *Candida albicans* and *Trichophyton interdigitale*. The methyl ester **4h** shares satisfactory high values for all the strains, *S. aureus* including MRSA, *C. albicans* and *T. interdigitale* (27.5, 27.5, and 55.2, respectively). For mould *T. interdigitale*, three other compounds were selective, namely 3,5-diiodo derivative **3h** (13.7) and two 5-chloro-3-iodosalicylidene analogues **2g** and **5g** (22.3 and 14.5, respectively). Thus, this strain is inhibited superiorly in term of the selectivity. When compared to parent PABA imines **1g** and **1h**, several of the new modification offer an improved selectivity for both methicillin-susceptible and resistant *S. aureus* (**1h** vs. **2h, 3h, 6h** and above all **4h**), *C. albicans* (**1h** vs. **4h** and **6h**) and also *T. interdigitale* (**1g** vs. **2g** and **5g, 1h** vs. **3h** and **4h**), even up to 15.3 times. Thus, design of simple structural analogues and/or prodrugs can provide derivatives with better *in vitro* toxicity and selectivity properties. In this case, type of amino compound scaffold used modulates toxicity and selectivity considerably.

From an alternative point of view, the most cytotoxic derivatives exhibited potency comparable (**3g**) or even superior (**2h, 5h**) to tamoxifen, an established anticancer agent useful for the treatment of not only oestrogen-dependent but also other types of cancer (Farrar and Jacobs, 2020). That is why our three derivatives can be considered as potential anti-cancer compounds with adjunctive antimicrobial properties. This feature has been described and applied partly in the practice. Clioquinol and fluoroquinolones are examples of established antimicrobial agents with anticancer action. On the other hand, anthracyclines largely used in cancer treatment share antibacterial properties as well (Alibek et al., 2012).

Table 4
Toxicity and selectivity results of the most active derivatives.

Code	IC_{50} [μM]	Range of concentrations tested	SI for SA and MRSA	SI for CA	SI for TI
1g*	53.4	1-500	3.4	6.8	6.8
1h*	57.8	1-500	0.9	7.4	7.4
2g	87.1	1-500	1.4-2.8	5.6	**11.2-22.3**
2h	13.0	1-500	0.8-1.7	1.7	1.7-3.3
3g	19.6	1-500	0.6-1.3	1.3	2.5-5.0
3h	53.3	1-250	6.8	6.8	6.8-**13.7**
4g	78.1	1-500	1.2-2.5	5	2.5-5.0
4h	215.1	1-1000	**13.8-27.5**	**27.5**	**27.5-55.2**
5g	56.4	1-500	0.5-1.8	1.8	**14.5**
5h	14.8	1-250	0.9	1.9	3.8
6g	64.7	1-500	0.5-1	2.1	2.1
6h	74.4	1-500	1.2-2.4	9.5	2.4
1*	>1500	1-1500	ND	ND	ND
2	>1000	1-1000	ND	ND	ND
3	>1000	1-1000	ND	ND	ND
4	>1000	1-1000	ND	ND	ND
5	>1000	1-1000	ND	ND	ND
6	>1000	1-1000	ND	ND	ND
Tamoxifen	19.6	1-500	-	-	-

ND: not determined due to low activity; SI: selectivity index. SA: *Staphylococcus aureus* ATCC 292138; MRSA: methicillin-resistant *Staphylococcus aureus* ATCC 43300; CA: *Candida albicans* ATCC 24433; TI: *Trichophyton interdigitale* ATCC 9533. *: data from Krátký et al., 2020.

3.2.6. Key SAR findings

The spectrum of the biological tests realized enabled us to explore SAR for particular pharmacological activities that are summarized in Table 5.

Obviously, 3,5-diiodo- and 3-iodo-5-chlorosalicylidene motifs were found as a general pharmacophore for antimicrobial action (Gram-positive and Gram-negative bacteria, fungi). In contrast, a substitution with at least one halogen atom at position 3 or 5 is necessary for any

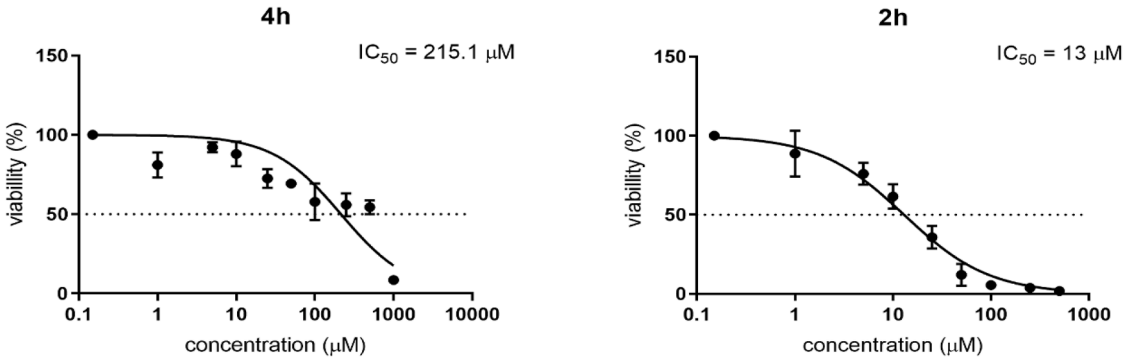
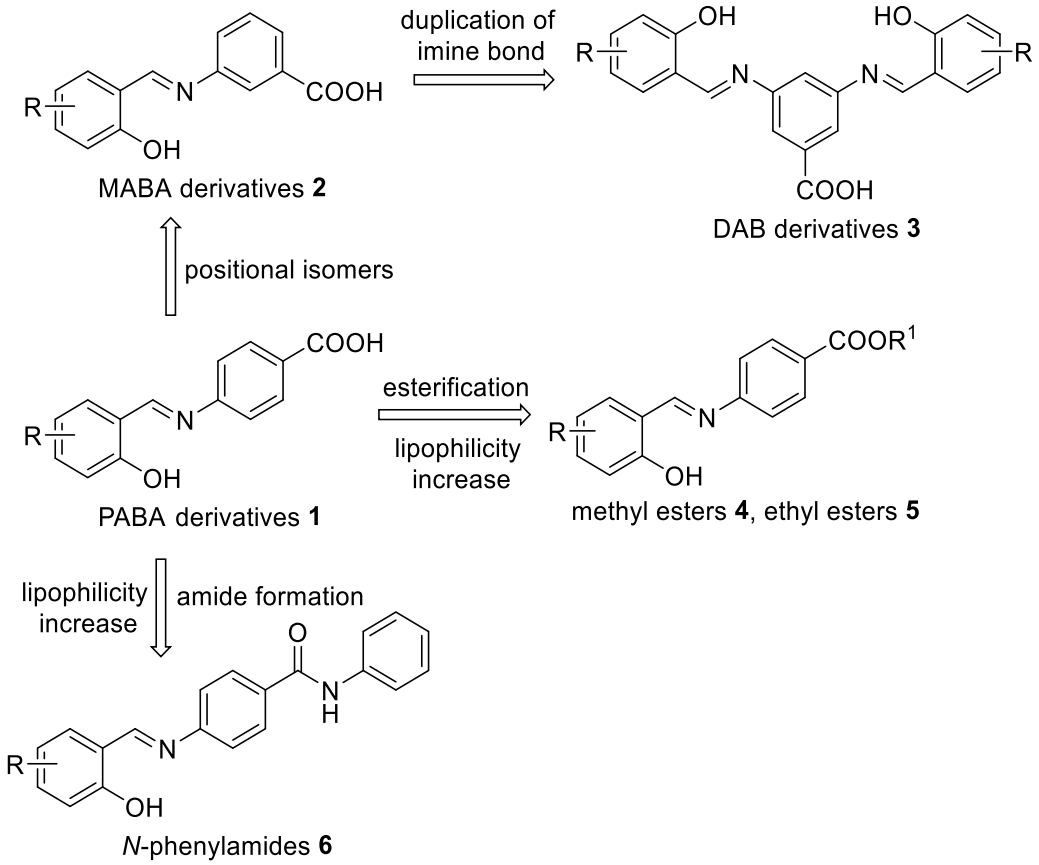
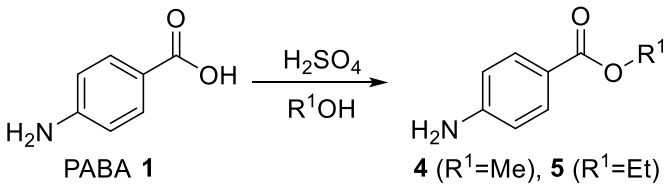


Fig. 3. Cytotoxic effects of different incubation concentrations of the most (**2h**) and the least (**4h**) toxic compounds on HepG2 cells.



Scheme 1. Design of analogues of 4-aminobenzoic acid (PABA)-based imines **1a-1h** (MABA: 3-aminobenzoic acid; DAB: 3,5-diaminobenzoic acid).

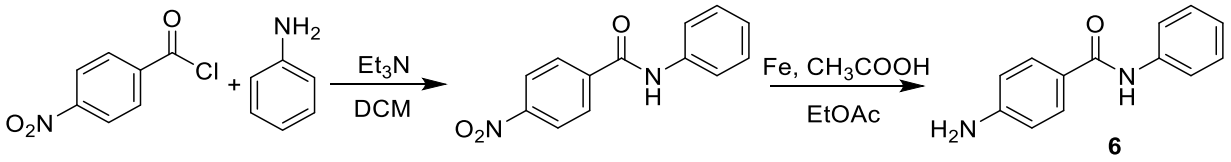


Scheme 2. Synthesis of precursor PABA-esters **4** and **5**.

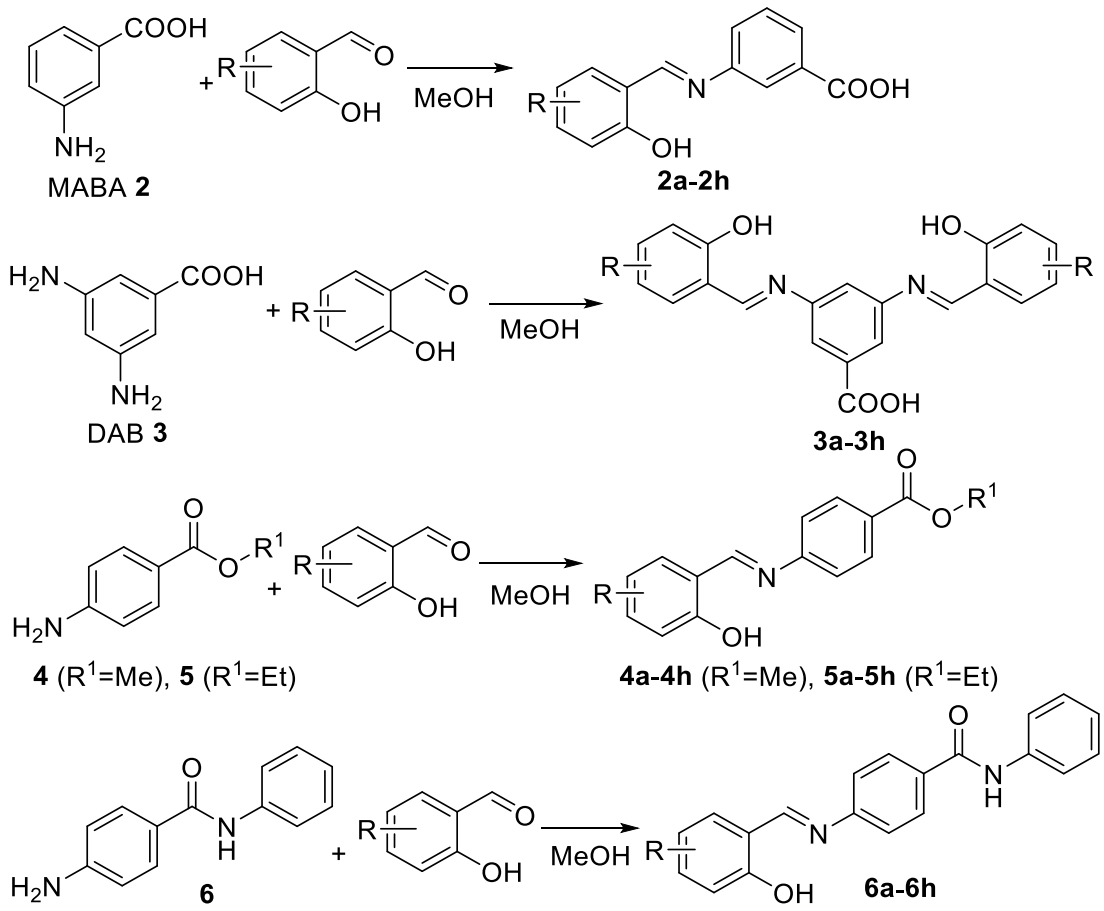
antimicrobial activity. Heavier halogens are generally favoured. Focusing on "aminobenzoic" part of the molecules, the optimal activity against both Gram-positive and negative bacteria is associated with methyl ester (fortunately together with low cytotoxicity) and free carboxyl. The presence of 3-/4-COOH group is also translated into potent antifungal properties. The derivatization of 3,5-diaminobenzoic acid led to both high inhibition of Gram-negative bacteria, fungi and opposing escalated toxicity. On the other hand, comparably toxic ethyl esters and anilides of 4-aminobenzoic acid provide only moderate overall antimicrobial activity.

4. Conclusions

We designed and synthesized forty analogues of 4-(salicylideneamino)benzoic acids previously reported as antimicrobial and cytotoxic agents (Krátký et al., 2020). The design consisted in positional isomers, doubling of imine bond and increasing lipophilicity *via* prodrug approach (esters, amides) of original Schiff bases. Aminobenzoic acid derivatives and halogenated salicylaldehydes were used for the synthesis of target hybrid compounds. These novel imines were prepared in



Scheme 3. Synthesis of 4-amino-*N*-phenylbenzamide **6** precursor (DCM: dichloromethane; EtOAc: ethyl acetate).



Scheme 4. Synthesis of Schiff bases **2a-6h** (R = H **a**, 3-Cl **b**, 4-Cl **c**, 5-Cl **d**, 5-I **e**, 3-Br-5-Cl **f**, 3-I-5-Cl **g**, 3,5-I_2 **h**; MABA: 3-aminobenzoic acid, DAB: 3,5-diaminobenzoic acid).

Table 5
SAR findings for all bioactivities.

Activity	SAR for salicylidene moiety	SAR for aminobenzoic part
Gram-positive bacteria	best: 3,5-I_2, 3-I-5-Cl detrimental: 3-/4-/5-Cl, H	best: 4-CH_3OCO-, 3-/4-COOH detrimental: PhNHCO-
Gram-negative bacteria	best: 5-I, 3-Br-5-Cl, 3-I-5-Cl, 3,5-I_2	best: 3,5-NH_2, 4-COOH, 4-CH_3OCO-
antifungal	best: 3,5-I_2, 3-I-5-Cl detrimental: 4-Cl, H	best: 3-/4-COOH, 3,5-NH_2
cytotoxicity	only 3,5-I_2 and 3-I-5-Cl tested; heterogeneous SAR, higher toxicity than parent amino compounds	lower: 4-CH_3OCO- higher: 3,5-NH_2, 4-CH_3CH_2OCO-

order to improve antimicrobial properties and reduce cytotoxicity for mammalian cells, and they were obtained in very good yields.

The novel compounds avoided any significant inhibition of both tuberculous and atypical mycobacteria as well as Gram-negative bacteria, but they were highly efficient *in vitro* against Gram-positive cocci including methicillin-resistant *Staphylococcus aureus* (MIC of ≥7.81 μM) and *Enterococcus faecalis*. Also, their broad-spectrum of antimycotic activity against yeasts and moulds was notable (MIC values from 3.90 μM) and improved when compared to parent original imines. The most important issue is the drop of cytotoxicity for HepG2 cells. Several imines provided a satisfactory selectivity for staphylococci, *Candida* spp. and *Trichophyton interdigitale*. Structure-activity relationships indicated the importance of 3,5-dihalogenosalicylidene fragment for the bioactivity, favouring compounds containing at least one iodine atom. The type of amino compound used do not modify antibacterial activity crucially, but it is important for antifungal and cytotoxic action. Methyl 4-[(2-hydroxy-3,5-diiodobenzylidene)amino]benzoate **2h** was identified as the most convenient molecule with high antimicrobial activity and substantially lower toxicity, but its potential to inhibit formation or eradicate biofilm produced by Gram-positive bacteria is only limited.

In summary, we demonstrated unequivocally that it is possible to tune up properties of parent PABA-based imines to escalate antimicrobial properties and modulate cytotoxicity. Thus, me-too analogues concept is a viable way to improve the activity profile. Several promising novel hits were identified.

CRediT authorship contribution statement

Martin Krátký: Conceptualization, Methodology, Investigation, Writing - original draft, Supervision. **Klára Konečná:** Methodology, Investigation, Writing - original draft, Supervision. **Kateřina Brokešová:** Investigation. **Jana Maixnerová:** Methodology, Investigation, Writing - review & editing. **František Trejtnar:** Methodology, Writing - review & editing. **Jarmila Vinšová:** Writing - review & editing, Supervision.

Declarations of Competing Interest

None.

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