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ORIGINAL RESEARCH

X-ray structural analysis, antioxidant and cytotoxic activity of newly synthesized salicylic acid derivatives

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Abstract New salicylic (2-hydroxybenzoic) acid derivatives **1–6** were prepared by conventional heating or microwave irradiation of a mixture consisting of methyl salicylate and the corresponding amino alcohol (2,2′-dihydroxydiethylamine, 2,2′,2″-trihydroxytriethylamine or *N*-phenyl-2,2′-dihydroxydiethylamine) and metallic sodium as catalyst. For compounds **1**, **3**, and **5** X-ray structure analysis was performed, as well as molecular mechanics calculations (MMC), to define their conformation in terms of their energy minima. Comparison of crystal and MMC structures for these three compounds (**1**, **3**, and **5**) revealed that the intramolecular hydrogen bonds play an important role, stabilizing conformation of the most part of the molecule. The antioxidant activity and cytotoxicity of the synthesized derivatives were evaluated in a series of in vitro tests. The newly synthesized compounds exhibited strong activity against hydroxyl radical, as well as promising lipid peroxidation inhibition. The study showed that the electronic effects of the groups at the N atom are responsible for neutralization of the OH radical, i.e., antioxidant activity. Compounds **1–3** exhibited sub-micromolar cytotoxicity against HeLa S3, whereas compounds **1**, **3** and **5** efficiently inhibited the growth of PC3 cells.

Keywords Salicylic acid derivatives · Microwave assisted synthesis · Antioxidant and cytotoxic activities · Molecular mechanics calculations · X-ray structural analysis

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Introduction

Reactive products of oxygen are among the most potent and omnipresent threats for living organisms. Intracellular accumulation of reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxy radical, can arise from toxic insults or normal metabolic processes. These species may perturb the cell's natural antioxidant defense systems, resulting in damage to all of the major classes of biological macromolecules (e.g., lipids, nucleic acids, and proteins) [1]. Furthermore, radical reactions play a significant role in the development of life-limiting chronic diseases such as cancer, hypertension, arteriosclerosis, rheumatism, and/or neurodegenerative diseases [2–5]. For that reason the search for new molecules with antioxidant properties is a very active domain of research.

Many phenol substances of plant origin [6, 7] and synthetic products [8, 9] belong to powerful antioxidants. They have also been found to be effective at reducing the incidence of various types of carcinomas [6, 8, 10, 11].

On the other hand, some salicylic acid derivatives exhibit cytotoxicity against PC-3 (prostate cancer cells) [12], MCF-7 (human breast adenocarcinoma ER+ cells)

[13, 14], MDA-MB 231 (human breast adenocarcinoma ER− cells) [14], and other tumor cell lines.

In order to investigate the antioxidant and cytotoxic activities we applied conventional heating and microwave (MW) irradiation methods to synthesize mono-, bis- and tris-derivatives of salicylic acid **1**–**6**. Besides, we determined the X-ray crystal structure of compounds **1**, **3**, and **5**, discussed the hydrogen bonds involved and performed molecular mechanics calculations (MMC) for their crystalline states.

Experimental

Chemical synthesis

General

Melting points were determined using a Büchi SMP 20 apparatus and are uncorrected. Infrared spectra (wave numbers in cm^{-1}) were recorded in KBr pellets (for crystals) or as film (for oils) on a NEXUS 670 SP-IR spectrometer; UV/VIS–CECIL CE2021 spectrophotometer. NMR spectra were taken on a Bruker AC 250E spectrometer operating at 250 MHz (proton) and 62.5 MHz (carbon) with tetramethylsilane as the internal standard. Chemical shifts are given in ppm (δ-scale). Mass spectra were recorded on a Finnigan MAT 8230 instrument, using chemical ionization (isobutane) or electron impact (70 eV) technique; the first number denotes m/e value, and the ion abundances are given in parentheses. The microwave reactor was a monomode system (Microwave Synthesis System—Discover Bench Mate from CEM) with focused waves. All reagents used were of analytical grade.

*General procedure for preparation of compounds **1–6***

Conventional heating A mixture consisting of methyl 2-hydroxy benzoate (30 or 20 mmol), the corresponding aminoalcohol (2,2′-dihydroxydiethylamine, 2,2′,2″-trihydroxytriethylamine or *N*-phenyl-2,2′-dihydroxydiethylamine; 10 mmol) and sodium (1 mmol) was heated at 150 °C with continuous removal of methanol for 2.5 h. The reaction mixture was diluted with water (100 mL) and extracted with dichloromethane (2 × 50 mL) and ethyl acetate (2 × 50 mL). After drying over anhydrous Na_2SO_4 and removal of the solvent in vacuum, the result was an oily mixture of products. The pure compounds **1**–**6** were obtained after chromatography on a column of silica gel (100 g, toluene:ethyl acetate, 9:1).

Microwave irradiation A mixture consisting of methyl salicylate, the corresponding amino alcohol and metallic sodium in the mole ratios as in the above procedure was heated to 110 °C. When sodium reaction was completed the mixture was cooled to room temperature, and then irradiated using a MW source of 170 W at 150 °C for 10 min. Further treatment was the same as in the previous procedure. Chromatographic separation of crude product on silica gel column (100 g, hexane, then hexane:acetone, 9:1) afforded pure products **1** and **3**–**6**.

*N-(2-hydroxybenzoyl)-2,2′-bis(2-hydroxybenzoyloxy)diethylamine (**1**)*. Colorless crystals (10% by conventional heating, 2.5% by MW, mp 97–98 °C from acetone–hexane). IR (KBr): 3450–3150, 3140, 2960, 2880, 1675, 1620, 1590, 1490, 1460, 1390, 1370, 1260, 1220, 1170, 1100. ^{1}H NMR ($CDCl_3$): 3.99 (t, 4H, 2COOCH$_2$C**H$_2$**, J = 5.3 Hz); 4.59 (t, 4H, 2COOC**H$_2$**CH$_2$, J = 5.3 Hz); 6.77–7.70 (m, 12H, from H); 8.80 (bs, 1H, OH); 10.56 (s, 2H, 2OH from A,B rings). ^{13}C NMR ($CDCl_3$): 47.27 (2COOCH$_2$**C**H$_2$); 62.47 (2COO**C**H$_2$CH$_2$); 111.69 (2C-1 from A,B rings); 117.63 (2C-3 from A,B rings); 117.81 (C-3 from C ring); 118.45 (C-1 from C ring); 119.23 (C-5 from C ring); 119.29 (2C-5 from A,B rings); 127.75 (C-6 from C ring); 129.67 (2C-6 from A,B rings), 132.41 (C-4 from C ring), 136.09 (2C-4 from A,B rings), 156.94 (C-2 from C ring); 161.67 (2C-2 from A,B rings); 169.77 (2COO); 172.63 (N–C = O). MS (*m/z*, rel %): 465 (M^+, 4); 328 (46); 190 (52); 121 (100); 120 (31); 92 (27); 65 (21); 56(19). For $C_{25}H_{23}O_8N$ (465.4) calculated: 64.52% C, 4.95% H 3.01% N; found: 64.99% C, 5.10% H, 3.47% N.

*N-methyl-2,2′-bis(2-hydroxybenzoyloxy)diethylamine (**2**)*. Colorless oil (3.2% by conventional heating). IR (film): 3350, 3050, 2960, 2880, 1675, 1615, 1590, 1490, 1470, 1300, 1260, 1160, 1080. ^{1}H NMR ($CDCl_3$): 2.47 (s, 3H, CH_3); 2.92 (t, 4H, 2COOCH$_2$C**H$_2$**, J = 5.7 Hz); 4.47 (t, 4H, 2COOC**H$_2$**CH$_2$, J = 5.7 Hz); 6.84 (m, 2H, 2H-5 from A,B rings, $J_{(3,5)}$ = 1.1 Hz, $J_{(4,5)}$ = 7.1 Hz, $J_{(5,6)}$ = 8.1 Hz); 6.99 (m, 2H, 2H-3 from A,B rings, $J_{(3,4)}$ = 8.4 Hz, $J_{(3,5)}$ = 1.1 Hz); 7.44 (m, 2H, 2H-4, $J_{(3,4)}$ = 8.4 Hz, $J_{(4,5)}$ = 7.1 Hz, $J_{(4,6)}$ = 1.8 Hz); 7.75 (dd, 2H, 2H-6 from A,B rings, $J_{(4,6)}$ = 1.8 Hz, $J_{(5,6)}$ = 8.1 Hz); 10.65 (bs, 2H, 2OH from A,B rings). ^{13}C NMR ($CDCl_3$): 42.85 (CH_3); 55.81 (2COOCH$_2$**C**H$_2$); 62.94 (2COO**C**H$_2$CH$_2$); 112.36 (2C-1 from A,B rings); 117.53 (2C-3 from A,B rings); 119.12 (2C-5 from A,B rings); 129.83 (2C-6 from A,B rings); 135.66 (2C-4 from A,B rings); 161.50 (2C-2 from A,B rings); 169.89 (2COO). MS (*m/z*, rel %): 359 (M^+, 18); 221 (38); 208 (98); 165 (42); 121 (52); 88 (100); 93 (12); 83 (14); 70 (25); 65 (18); 58 (14); 44 (34).

*2,2′,2″-tris(2-hydroxybenzoyloxy)triethylamine (**3**)*. Colorless crystals (30% by conventional heating, 9% by MW, mp 89 °C from acetone–hexane). IR (KBr): 3450–3150, 3080, 2960, 2870, 1670, 1625, 1590, 1490, 1470, 1360; 1300, 1260, 1160, 1100. ^{1}H NMR ($CDCl_3$): 3.09 (t, 6H, 3COOCH$_2$C**H$_2$**, J = 5.7 Hz); 4.45 (t, 6H, 3COOC**H$_2$**CH$_2$,

$J = 5.7$ Hz); 6.79 (m, 3H, 3H-5 from A,B,C rings, $J_{(3,5)} = 1.1$ Hz, $J_{(4,5)} = 7.1$ Hz, $J_{(5,6)} = 8.1$ Hz); 6.92 (dd, 3H, 3H-3 from A,B,C rings, $J_{(3,4)} = 8.4$ Hz, $J_{(3,5)} = 1.1$ Hz); 7.41 (m, 3H, 3H-4 from A,B,C rings, $J_{(3,4)} = 8.4$ Hz, $J_{(4,5)} = 7.1$ Hz, $J_{(4,6)} = 1.8$ Hz); 7.75 (dd, 3H, 3H-6 from A,B,C rings, $J_{(5,6)} = 8.1$ Hz, $J_{(4,6)} = 1.8$ Hz); 10.69 (*s*, 3H, 3OH from A,B,C rings). ^{13}C NMR ($CDCl_3$)*:* 53,29 ($3COOCH_2\mathbf{C}H_2$); 63.19 ($3COO\mathbf{C}H_2CH_2$); 112.11 (3C-1 from A,B,C rings); 117.52 (3C-3 from A,B,C rings); 119.13 (3C-5 from A,B,C rings); 129.61 (3C-6 from A,B,C rings); 135.73 (3C-4 from A,B,C rings); 161.57 (3C-2 from A,B,C rings); 169.93 (3COO). MS (*m/z*, rel. %)*:* 508 (M^+-1, 9); 358 (70); 238 (40); 165 (60); 121 (100); 118 (57); 100 (18); 69 (14); 65 (22); 56 (20). For $C_{27}H_{27}O_9N$ (509.5) calculated: 63.65% C, 5.34% H, 2.75% N 2.75% found: 63.55% C, 5.53% H, 3.01% N.

*2-Hydroxy-2′,2″-bis(2-hydroxybenzoyloxy)triethylamine (**4**)*. Colorless oil (40.5% by conventional heating, 19% by MW). IR (film): 3450–3150, 2950, 2840, 1670, 1620, 1590, 1490, 1300, 1250, 1220, 1160, 1080. ^{1}H NMR ($CDCl_3$): 2.84 (t, 2H, $NC\mathbf{H_2}CH_2OH$, $J = 5.3$ Hz); 3.03 (t, 4H, 4H, $2COOCH_2C\mathbf{H_2}$, $J = 4.4$ Hz); 3.64 (t, 2H, $NCH_2C\mathbf{H_2}$ OH, $J = 5.3$ Hz); 3.80 (bs, 1H, OH), 4.43 (t, 4H, $2COOC\mathbf{H_2}CH_2$, $J = 4.4$ Hz); 6.80 (m, 2H, 2H-5 from A,B rings, $J_{(3,5)} = 1.1$ Hz, $J_{(4,5)} = 7.1$ Hz, $J_{(5,6)} = 8.1$ Hz); 6.91 (dd, 2H, 2H-3 from A,B rings, $J_{(3,4)} = 8.4$ Hz, $J_{(3,5)} = 1.1$ Hz); 7.41 (m, 2H, 2H-4, $J_{(3,4)} = 8.4$ Hz, $J_{(4,5)} = 7.1$ Hz, $J_{(4,6)} = 1.8$ Hz); 7.75 (dd, 2H, 2H-6 from A,B rings, $J_{(4,6)} = 1.8$ Hz, $J_{(5,6)} = 8.1$ Hz); 10.61 (bs, 2H, 2OH from A,B rings). ^{13}C NMR ($CDCl_3$): 52.86 ($2CO-OCH_2\mathbf{C}H_2$); 56.82 ($N\mathbf{C}H_2CH_2OH$); 58.84 ($NCH_2\mathbf{C}H_2OH$); 62.90 ($2COO\mathbf{C}H_2CH_2$); 111.91 (2C-1 from A,B rings); 117.45 (2C-3 from A,B rings); 119.17 (2C-5 from A,B rings); 129.50 (2C-6 from A,B rings); 135.74 (2C-4 from A,B rings); 161.50 (2C-2 from A,B rings); 169.94 (2COO). MS (*m/z*, rel. %): 389 (M^+, 16); 358 (52) 238 (72); 165 (54); 121 (72); 118 (100); 65 (12); 56 (19).

*N-phenyl-2,2′-bis(2-hydroxybenzoyloxy)diethylamine (**5**)*. Colorless crystals (49% by conventional heating, 8.5% by MW, mp 121–122 °C from acetone–hexane). IR (KBr): 3500–3100, 2960, 1670, 1600, 1460, 1400, 1210, 1160, 1060, 1090. ^{1}H NMR ($CDCl_3$): 3.87 (t, 4H, $2COOCH_2C\mathbf{H_2}$, $J = 6.1$ Hz); 4.57 (t, 4H, $2COOC\mathbf{H_2}CH_2$, $J = 6.1$ Hz); 6.76–6.95 (m, 5H, 2H-5 from A,B rings; H-2, H-6, and H-4 from Ph); 7.01 (dd, 2H, 2H-3 from A,B rings, $J_{(3,4)} = 8.4$ Hz, $J_{(3,5)} = 0.9$ Hz); 7.32 (dd, 2H, H-3 and H-5 from Ph, $J_{(2,3)} = 8.1$ Hz, $J_{(3,4)} = 7.3$ Hz); 7.48 (m, 2H, 2H-4 from A,B rings, $J_{(3,4)} = 8.4$ Hz, $J_{(4,6)} = 1.7$ Hz, $J_{(4,5)} = 7.8$ Hz); 7.81 (dd, 2H, 2H-6 from A,B rings, $J_{(4,6)} = 1.8$ Hz, $J_{(5,6)} = 8.0$ Hz); 10.74 (s, 2H, 2OH from A,B rings). ^{13}C NMR ($CDCl_3$): 49.94 ($2COOCH_2\mathbf{C}H_2$); 62.51 ($2COO\mathbf{C}H_2CH_2$); 112.17 (2C-1 from A,B rings); 112.64 (C-2 and C-6 from Ph); 117.75 (2C-3 from A,B rings and C-4 from Ph); 129.72 and 129.91 (C-3 and C-5 from Ph, 2C-6 from A,B rings); 136.02 (2C-4 from A,B rings); 147.11 (C-1 from Ph); 161.83 (2C-2 from A,B rings); 170.16 (2COO). MS (*m/z*, rel. %): 422 (M^++1, 100); 284 (5). For $C_{24}H_{23}O_6N$ (421.4) calculated: 68.40% C; 5.50% H; 3.30% N; found: 68.19% C; 5.52% H; 3.50% N.

*N-phenyl-2-hydroxy-2′-(2-hydroxybenzoyloxy)diethylamine (**6**)*. Colorless oil (22% by conventional heating, 27% by MW). IR (film): 3450–3100, 2890, 2940, 1675, 1600, 1470, 1390, 1300, 1210, 1160, 980. ^{1}H NMR ($CDCl_3$): 2.33 (bs, 1H, OH); 3.59 (t, 2H, $C\mathbf{H_2}OH$, $J = 5.8$ Hz); 3.76–3.86 (m, 4H, $C\mathbf{H_2}N(Ph)C\mathbf{H_2}$); 4.54 (t, 2H, $COOC\mathbf{H_2}$, $J = 6.1$ Hz); 6.75–6.94 (m, 4H, H-5, H-3 from A ring; H-2, H-4, H-6 from Ph); 7.00 (dd, 1H, H-3 from A ring, $J_{(3,4)} = 8.4$ Hz, $J_{(3,5)} = 0.9$ Hz); 7.28 (dd, 2H, H-3 and H-5 from Ph, $J_{(2,3)} = 8.1$ Hz, $J_{(3,4)} = 7.3$ Hz); 7.48 (m, 1H, H-4 from A ring, $J_{(4,5)} = 7.8$ Hz, $J_{(4,6)} = 1.7$ Hz); 7.75 (dd, 1H, H-6 from A ring, $J_{(5,6)} = 8.0$ Hz, $J_{(4,6)} = 1.7$ Hz); 10.76 (s, 1H, OH from A ring). ^{13}C NMR ($CDCl_3$): 50.21 ($COOCH_2\mathbf{C}H_2$); 53.61 ($\mathbf{C}H_2OH$); 59.75 ($N\mathbf{C}H_2CH_2OH$); 62.36 ($COO\mathbf{C}H_2CH_2$); 112.03 (C-1 from A ring); 112.95 (C-2 and C-6 from Ph); 117.46 (C-4 from Ph); 117.50 (C-3 from A ring); 119.16 (C-6 from A ring); 129.37 (C-3 and C-5 from Ph); 129.76 (C-6 from A ring); 135.79 (C-4 from A ring); 147.54 (C-1 from Ph); 161.53 (C-2 from A ring); 170.03 (COO). MS (*m/z*, rel. %): 302 (M^++1, 100); 182 (4); 164 (9); 137 (4).

Crystal structure determination

The diffraction data for compounds **1** and **5** were collected at room temperature on an Oxford Diffraction Gemini S diffractometer with graphite-monochromated MoKα radiation ($\lambda = 0.7107$ Å) and for compound **3** also at room temperature using Enraf–Nonius CAD-4 diffractometer with CuKα radiation ($\lambda = 1.5418$ Å). The data reduction for compounds **1** and **5** were performed with program package CrysAlis RED [15], and for compound **3** with program package XCAD4 [16]. The space group determinations were based on an analysis of the Laue class and the systematically absent reflections. The structures **1** and **5** were solved by direct methods using SIR92 [17], and the structure **3** was solved using SHELXS97 [18]. All structures were refined using full-matrix least-squares. For all three compounds non-hydrogen atoms were refined anisotropically, the C–H hydrogen atoms were included on calculated positions riding on their attached atoms with fixed distances 0.93 (CH) or 0.97 Å (CH_2) and all O–H hydrogen atoms were identified on difference electron density maps and isotropically refined. All calculations were performed using SHELXL97 [18], PARST [19], and PLATON [20], as implemented in the WINGX [21] system

Table 1 The crystal data and refinement parameters

	(**1**)	(**3**)	(**5**)
Chemical formula	$C_{25}H_{23}NO_8$	$C_{27}H_{27}NO_9$	$C_{24}H_{23}NO_6$
M_r	465.44	509.50	421.43
Cell setting, space group	Monoclinic, $P2_1/c$	Monoclinic, $C2/c$	Monoclinic, $P2_1/c$
Temperature (K)	297 (2)	293 (2)	297 (2)
a (Å); b (Å); c (Å)	15.9876 (7); 14.0448 (6); 10.8187 (4)	37.5130 (10); 10.3100 (10); 12.813 (2)	7.8021 (4); 30.9049 (17); 8.9008 (4)
β (°)	105.559 (5)	90.900 (10)	106.112 (5)
V (Å^3)	2340.24 (17)	4954.9 (9)	2061.89 (18)
Z	4	8	4
D_x (mg m^{-3})	1.321	1.366	1.358
Radiation type	Mo$K\alpha$	Cu$K\alpha$	Mo$K\alpha$
μ (mm^{-1})	0.10	0.86	0.10
Crystal form, color	Prism, colorless	Prism, colorless	Prism, colorless
Crystal size (mm)	0.44 × 0.20 × 0.18	0.53 × 0.35 × 0.20	0.50 × 0.25 × 0.21
Diffractometer	Gemini S; Oxford Diffraction	Enraf–Nonius CAD4	Gemini S; Oxford Diffraction
Data collection method	ω–θ-scans	ω–2θ	ω–θ-scans
Absorption correction	None	ψ scan	Multi-scan
T_{min}	–	0.727	0.953
T_{max}	–	0.841	0.987
No. of measured, independent and observed reflections	14193, 5334, 2460	10514, 5099, 4244	12011, 4738, 2752
Criterion for observed reflections	$I > 2\sigma(I)$	$I > 2\sigma(I)$	$I > 2\sigma(I)$
R_{int}	0.023	0.010	0.025
θ_{max} (°)	29.5	75.0	29.5
Refinement on	F^2	F^2	F^2
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.039, 0.099, 0.86	0.045, 0.145, 1.09	0.058, 0.136, 1.04
No. of relections	5334 reflections	5099 reflections	4738 reflections
No. of parameters	319	347	288
H-atom treatment	Mixture of independent and constrained refinement	Mixture of independent and constrained refinement	Mixture of independent and constrained refinement
Weighting scheme	Calculated $w = 1/[\sigma^2(F_o^2) + (0.0515P)^2]$ where $P = (F_o^2 + 2F_c^2)/3$	Calculated $w = 1/[\sigma^2(F_o^2) + (0.0691P)^2 + 1.8743P]$ where $P = (F_o^2 + 2F_c^2)/3$	Calculated $w = 1/[\sigma^2(F_o^2) + (0.0457P)^2 + 0.6147P]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max}$	<0.0001	0.004	<0.0001
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.13, −0.12	0.27, −0.15	0.13, −0.16

of programs. The crystal data and refinement parameters are summarized in Table 1.

Biological methods

Free radical scavenging assays

Free radical scavenging activity of examined compounds was evaluated by measuring their ability to neutralize DPPH- and OH-radicals.

DPPH assay The DPPH-assay was performed as described before [22], with small modifications. The different aliquots (0.10–2.00 mL) of 0.01 M sample solution in methanol were added to 1.00 mL of 90 μmol/L DPPH$^{\bullet}$ in methanol (Sigma; St. Louis, MO) and filled up with 95% (v/v) methanol to a final volume of 4.00 mL. The same reaction mixture without tested compound was used as the control. Absorbances of the reaction mixtures and control were recorded at 515 nm (CECIL CE2021 spectrophotometer) after 1 h. Commercial synthetic antioxidants, 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) (Aldrich;

Taufkirchen, Germany) and 3-*tert*-butyl-4-hydroxyanisole (BHA) (Fluka; Taufkirchen, Germany) were used as positive controls. For each sample, three replicates were recorded.

DPPH scavenging activity was expressed as radical scavenging activity (DPPH RSC). Percentage of DPPH RSC was calculated using the following equation:

$$\mathrm{RSC}\,(\%) = 100 \times (A_{\mathrm{control}} - A_{\mathrm{sample}}/A_{\mathrm{control}}).$$

IC_{50} values (the concentration of the tested compound in the reaction mixture which causes 50% of RSC) were determined by linear regression analysis from the obtained RSC values.

Hydroxyl-radical scavenging assay Hydroxyl-radicals scavenging capacity ($HO^{\bullet}$ RSC) of the tested compounds was evaluated by measuring the degradation of 2-deoxy-D-ribose (Aldrich; Taufkirchen, Germany) in the reaction with OH radicals, generated in situ in Fenton's reaction [23].

These radicals attack the sugar 2-deoxy-D-ribose and degrade it into a series of fragments, some or all of which react on heating with 2-thiobarbituric acid (TBA) (Sigma; St. Louis, MO) at low pH to give a pink chromogen, which can be determined spectrophotometrically at 532 nm [12]. Different aliquots (0.005–0.5 mL) of sample solution in methanol were added to test tubes (final concentration ranged from 0.01 to 8 mmol/L), each containing 0.1 ml of 5 mmol/L H_2O_2, 0.1 mL of 10 mmol/L $FeSO_4$, and 0.1 mL of 0.05 mol/L 2-deoxy-D-ribose and 0.067 mol/L KH_2PO_4-K_2HPO_4 buffer pH 7.4 to a final volume of 3.00 mL. The same reaction mixture without sample was used as the control. After an incubation period of 1 h at 37 °C, 2 mL of TBA reagent (10.4 mL of 60% (v/v) $HClO_4$, 3 g TBA and 120 g of trichloroacetic acid (Sigma; St. Louis, MO)), and 0.2 mL of 0.1 mol/L EDTA (Sigma; St. Louis, MO) were added to the reaction mixture, and the tubes were heated at 100 °C for 20 min. After cooling, absorbances of the reaction mixtures and of the control were recorded at 532 nm.

Percentage of $HO^{\bullet}$ RSC was calculated using the following equation:

$$\mathrm{RSC}\,(\%) = 100 \times (A_{\mathrm{control}} - A_{\mathrm{sample}}/A_{\mathrm{control}}).$$

Three replicates were recorded for each sample; BHT and BHA were used as reference compounds.

Lipid peroxidation (LP) assay The extent of LP was determined by measuring the absorbance of adduct produced in the reaction between TBA and malondialdehyde as an oxidation product in the peroxidation of phospholipids from liposomes, by the TBA assay [23].

The commercial preparation of liposomes "PRO-LIPO S" (Lucas-Meyer, Hamburg, Germany), pH 5–7, was used as a model system of biological membranes. The liposomes, 225–250 nm in diameter, were obtained by dispersing the commercial preparation in demineralized water (1:10) in an ultrasonic bath.

In the Fe^{2+}/ascorbate-induced lipids peroxidation, a 60 μL suspension of liposomes was incubated with 20 μL of 0.01 mol/L $FeSO_4$, 20 μL of 0.01 mol/L ascorbic acid, and 10 μL of tested compound and filled with 0.05 mol/L KH_2PO_4–K_2HPO_4 buffer, pH 7.4 to a final volume of 3.00 mL. Samples were incubated at 37 °C for 1 h. LP was terminated by adding 2 mL of TBA reagent, and 0.2 mL of EDTA, and heating the test tubes at 100 °C for 20 min. After centrifugation (4000 rpm for 10 min), the content of the MDA (TBARS) was determined by measuring the absorbance of the product at 532 nm.

Analyses were compared with the commercial synthetic antioxidants BHT and BHA as a positive control. All reactions were carried out in triplicate.

The percentage of LP inhibition was calculated by the following equation:

$$I(\%) = (A_o\ --A_1)/A_o \times 1000$$

where A_0 was the absorbance of the control reaction (without the test compound) and A_1 was the absorbance in the presence of the inhibitor.

Cytotoxic activity

Cell lines

Six human tumor cell lines and one human non-tumor cell line were used in the study: human breast adenocarcinoma ER+, MCF-7, human breast adenocarcinoma ER−, MDA-MB-231, prostate cancer PC3, cervix epithelioid carcinoma, HeLa S3, human melanoma, Hs 294T, chronic myelogenous leukemia, K562, and normal fetal lung fibroblasts, MRC-5.

The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose (MCF7; MDA-MB-231; PC3; MRC-5) or in RPMI 1640 (K562). Media were supplemented with 10% of fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU/mL of penicillin and 100 μg/mL of streptomycin (ICN Galenika). All cell lines were cultured in flasks (Costar, 25 cm^2) at 37 °C in the 100% humidity atmosphere and 5% of CO_2. Only viable cells were used in the assay. Viability was determined by dye exclusion assay with trypan blue.

SRB assay

Cytotoxicity was evaluated by colorimetric sulforhodamine B (SRB) assay [24]. Briefly, single cell suspension was plated into 96-well microtiter plates (Costar, flat bottom): 5×10^3 cells (MCF7; MDA-MB-231; PC3; MRC-5) or 10^4 (K562) cells per 180 mL of medium. Plates were

pre-incubated 24 h at 37 °C, 5% CO_2. Tested substances at concentrations ranging from 10^{-8} to 10^{-4} mol/L were added to all wells except for the control ones. After incubation period (48 h/37 °C/5% CO_2) SRB assay was carried out as follows: 50 μL of 80% trichloroacetic acid (TCA) was added to all wells; an hour later the plates were washed with distilled water, and 75 μL of 0.4% SRB was added to all wells; half an hour later the plates were washed with citric acid (1%) and dried at room temperature. Finally, 200 μL of 10 mmol Tris (pH = 10.5) basis was added to all wells. Absorbance (*A*) was measured on the microplate reader (Multiscan MCC340, Labsystems) at 540/690 nm. The wells without cells, containing complete medium only, acted as blank.

Cytotoxicity was calculated according to the formula:

$$(1 - A_{\text{test}}/A_{\text{control}})100.$$

and expressed as a percent of cytotoxicity (CI%).

Two independent experiments were set out in quadruplicate for each concentration of the compound. IC_{50} value defines the dose of compound that inhibits cell growth by 50%. The IC_{50} of compounds was determined by median effect analysis.

Results and discussion

Chemistry

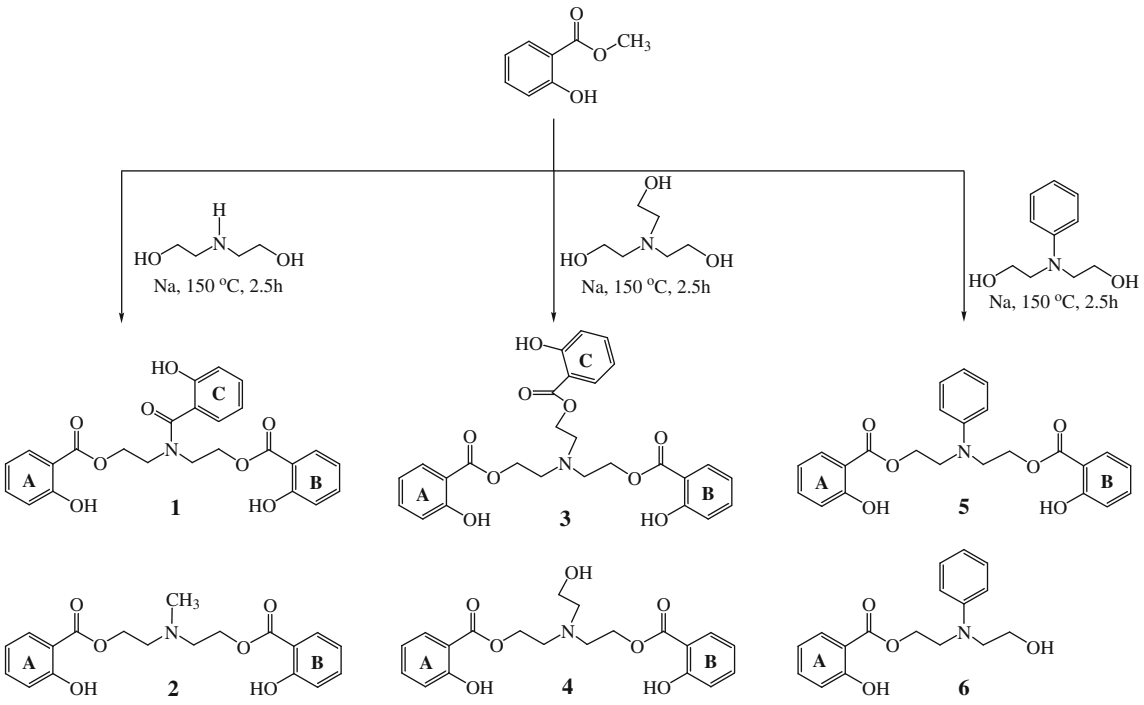
Compounds **1–6** were prepared by transesterification of methyl salicylate (methyl 2-hydroxybenzoate) with selected amino alcohols: 2,2′-dihydroxydiethylamine, 2,2′,2″-trihydroxy triethylamine and *N*-phenyl-2,2′-dihydroxydiethylamine. The reactions were carried out at 150 °C for 2.5 h in the presence of sodium as catalyst (Scheme 1).

When methyl 2-hydroxybenzoate and the corresponding aminoalcohol 2,2′-dihydroxydiethylamine were taken in a molar ratio 3:1, the tris-derivative of 2-hydroxybenzoic acid *N*-(2-hydroxybenzoyl)-2,2′-bis(2-hydroxybenzoyloxy) diethylamine (**1**), and bis-derivative *N*-methyl-2,2′-bis (2-hydroxybenzoyloxy)diethylamine (**2**) were obtained in the yields of 10 and 3.2%, respectively.

Using 2,2′,2″-trihydroxytriethylamine under the same reaction conditions, the tris-derivative 2,2′,2″-tris(2-hydroxybenzoyloxy)triethylamine (**3**) (30%) and bis-derivative 2-hydroxy-2′,2″-bis(2-hydroxybenzoyloxy)triethylamine (**4**) (40.5%) were obtained.

The transesterification using methyl 2-hydroxybenzoate and *N*-phenyl-2,2′-dihydroxydiethylamine in a molar ratio 2:1, yielded the bis-derivative *N*-phenyl-2,2′-bis(2-hydroxybenzoyloxy)diethylamine (**5**) (49%) and mono derivative *N*-phenyl-2-hydroxy-2′-(2-hydroxybenzoyloxy) diethylamine (**6**) (22%).

The preparation of compound **2** can be explained in terms of the known but still unusual disruption of the alkyl-oxygen bond in the methyl ester of 2-hydroxybenzoic acid, whereby the formed methyl cation binds to the nitrogen atom of the amino alcohol, yielding either *N*-alkylated amino alcohol or the subsequently *N*-alkylated bis derivative **2** (CH_3O-$COC_6H_4OH \rightarrow CH_3^+ + {}^-OCOC_6H_4OH$). The *N*-methyl



Scheme 1 Synthesis of salicylic acid derivatives **1–6**: **a** Na, 150 °C, 2.5 h; **b** Na, 110 °C, 5 min, than MW, 170 W, 150 °C, 10 min

cation formed as an intermediate can be stabilized by deprotonation only in the case of 2,2′-dihydroxydiethylamine.

Compounds **1** and **3–6** were also obtained by applying MW irradiation (170 W) of the mixture of methyl 2-hydroxybenzoate, the corresponding amino alcohol and metallic sodium as catalyst at 150 °C for 10 min. Under these conditions, compound **2** could not be obtained, compounds **1**, **3**, **4**, and **5** were obtained in a lower yield (2.5–19%), whereas compound **6** was obtained in a somewhat higher yield (27%) than by conventional method (22%).

Crystal structures analyses and molecular mechanics calculations

The perspective views of molecules **1**, **3**, and **5** are shown in Fig. 1 [25]. Selected bond lengths, bond angles, and torsion angles are given in Table 2.

Since we wanted to investigate the antioxidant behavior and cytotoxicity of compounds **1**, **3**, and **5**, we were interested in the conformation of molecules released from the influence of crystalline field. The next step, after determining the three-dimensional structures of these compounds in crystalline state, was to define the conformation of the molecules **1**, **3**, and **5** in terms of energy minima. To do that, we performed the MMC using PCMODEL [26].

The conformation of molecules remains stable in their energy minima in spite of the significant number of interatomic single bonds. This is confirmed by comparing selected torsion angles of molecules, obtained in crystal structure analyzes and after MMC (Table 2).

Figure 2 illustrates the very good overlapping of the molecular structures in crystalline state and after MMC for all three molecules (**1**, **3**, and **5**). It seems that the presence of intramolecular hydrogen bonds in all three molecules stabilizes their conformations in spite of the possible influence of crystal packing (Fig. 1; Table 3).

Comparing the structures of three molecules (**1**, **3**, and **5**) in crystalline state, we noticed the difference in N–C1c bond length. Molecules **1** and **5** have shorter N–C1c distance than molecule **3**. This is due to the resonance effect of the free (lone) electron pair on the N atom and π-electrons of the C=O bond in **1** and benzene ring in **5**, which results in partly double bond character of the N–C1c. This effect does not appear in compound **3**. In other words, hybridization on the N atom in molecules **1** and **5** is partly sp^2 and in molecule **3** is sp^3.

The best evidence of planarity around an atom is to consider the sum of the three bond angles because a perfectly planar system would have a sum equal to 360°. The sums of bond angles, which N atom forms with C1a, C1b, and C1c atoms in compounds **1** and **5** are 359.99 in both cases (Table 2). This is also the confirmation of partial sp^2 hybridization on the N atom in molecules **1** and **5**. On the other hand, the sum of the same bond angles for compound **3** is 342.68° (Table 2), confirming the sp^3 hybridization on N atom.

After the MMC for molecule **3**, the values of the same bond angles decreased (Table 2) and improved the trigonal configuration around N atom.

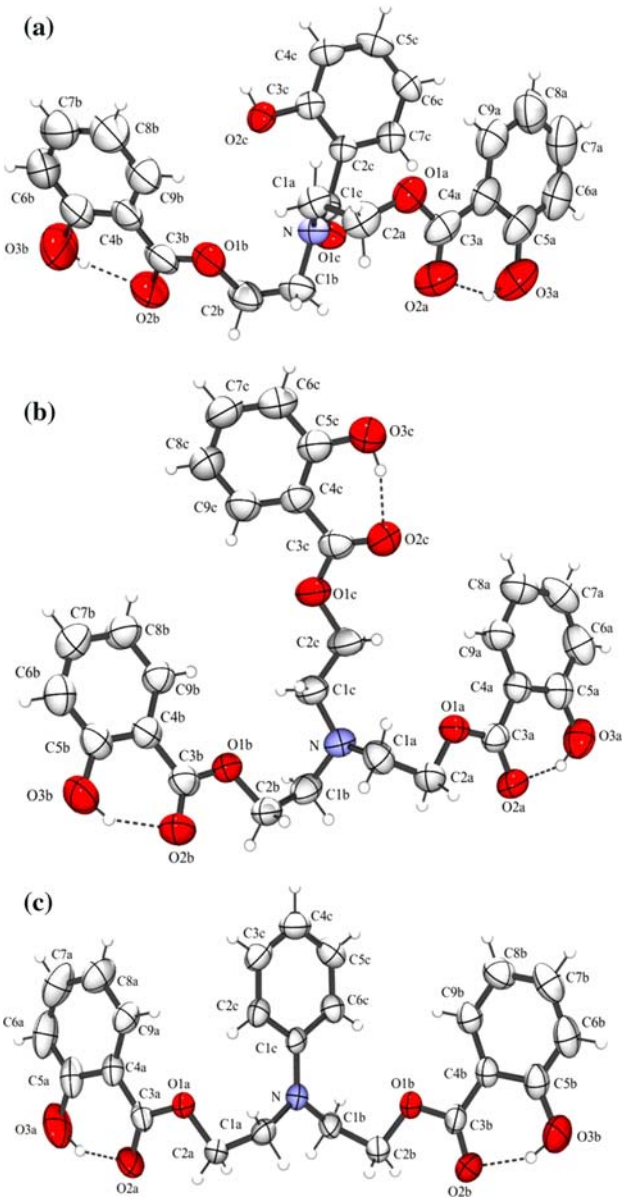


Fig. 1 ORTEP drawings of molecular structures of compound **1** (**a**), **3** (**b**), and **5** (**c**) with the labeling of non-H atoms. Displacement ellipsoids are shown at the 50% probability level and H atoms are drawn as spheres of arbitrary radii. Intramolecular hydrogen bonds are shown as *dashed lines*

Table 2 Selected bond lengths (Å), angles (°), and torsion angles (°)

	(**1**)		(**3**)		(**5**)	
	Crystal structure	MMC structure	Crystal structure	MMC structure	Crystal structure	MMC structure
Bond lengths						
N–C1a	1.464 (2)	1.5	1.458 (2)	1.5	1.449 (2)	1.5
N–C1b	1.466 (2)	1.5	1.452 (2)	1.5	1.456 (2)	1.5
N–C1c	1.3431 (17)	1.3	1.451 (2)	1.4	1.384 (2)	1.4
Bond angles						
C1a–N–C1b	117.23 (11)	117.6	113.05 (13)	112.8	115.16 (16)	116.4
C1c–N–C1a	124.96 (12)	123.4	115.65 (14)	112.9	122.23 (16)	121.7
C1c–N–C1b	117.80 (11)	119.1	113.98 (14)	112.8	122.61 (16)	121.9
N–C1a–C2a	113.38 (11)	113.3	111.78 (14)	111.0	115.17 (19)	114.4
N–C1b–C2b	113.20 (14)	112.8	118.06 (14)	117.8	114.48 (17)	114.3
Torsion angles						
C1c–N–C1a–C2a	−103.87 (16)	−103.8	−134.79 (15)	−141.7	−99.4 (2)	−101.4
C1c–N–C1b–C2b	−72.34 (16)	−70.2	−67.8 (2)	−60.6	−100.5 (2)	−100.9
C1a–N–C1b–C2b	108.52 (15)	111.3	66.95 (19)	68.8	79.3 (2)	79.1
C1b–N–C1a–C2a	75.21 (17)	74.7	91.25 (17)	89.0	80.8 (2)	78.6
N–C1a–C2a–O1a	72.59 (16)	68.0	71.59 (18)	68.2	66.3 (2)	59.3
N–C1b–C2b–O1b	−52.17 (17)	−53.0	67.07 (19)	69.7	67.6 (2)	59.0
C1a–C2a–O1a–C3a	−104.73 (15)	−97.9	−172.41 (14)	175.8	−178.37 (18)	−177.5
C1b–C2b–O1b–C3b	176.42 (14)	176.0	175.18 (13)	−173.9	−177.42 (17)	−178.9
C2a–O1a–C3a–C4a	175.50 (12)	−179.1	−177.68 (13)	−178.2	174.78 (17)	−179.1
C2b–O1b–C3b–C4b	−171.17 (13)	−177.0	176.76 (13)	176.7	175.70 (17)	−179.7
C2a–O1a–C3a–O2a	−4.2 (2)	1.2	0.9 (2)	1.8	−5.0 (3)	0.7
C2b–O1b–C3b–O2b	6.6 (3)	2.8	−3.0 (2)	−2.8	−4.2 (3)	0.3
O1a–C3a–C4a–C5a	−175.94 (14)	−169.4	176.24 (14)	−167.0	−176.31 (18)	−178.0
O1b–C3b–C4b–C5b	−179.01 (17)	−173.0	−178.37 (14)	171.3	−179.18 (18)	−175.2

The differences between the conformations (in the region of atoms labeled with A and B, where the structural formulas are the same for all three compounds) of molecules **3** and **5** on one side and molecule **1** on the other, are shown by the values of selected torsion angles N–C1b–C2b–O1b [−52.17 (17)° in 1, 67.07 (19)° in 3, and 67.6 (2)° in 5] and C1a–C2a–O1a–C3a [−104.73 (15)° in 1, −172.41 (14)° in 3, and −178.37 (18)° in 5] (Table 2). These differences in conformations originate from the interaction of free electron pair of the N atom with O1c atom from the C=O group in molecule **1** [O1c···N = 2.2313 (15) Å]. Moreover, the environment around the N and O1c atoms in compound **1** is characterized by the steric overcrowding.

Hydrogen bonding analyses

The information on intramolecular hydrogen bonding, in particular, is very useful to understand various molecular properties (the molecular geometries, the stability of a certain predominant conformation, and, consequently, biological activity). As can be seen from Fig. 2, all three compounds (**1**, **3**, and **5**) contain intramolecular O–H···O hydrogen bonds. The hydrogen bond parameters are given in Table 3.

Interaction of free electron pair of the N atom with the C=O group in molecule **1** and the steric overcrowding around the O1c atom in molecule **1** results in the position of O1c atom that is not energy predominant for construction of expected hydrogen bond with O2c atom. Instead of intra-, the O1c and O2c atoms form strong inter-molecular hydrogen bond O2c–H2C···O1C [D···A = 2.674 (1) Å; D–H···A = 179 (2) o; symmetry code: x, 1/2 − y, −1/2 + z], which is illustrated in Fig. 3. Crystal packings of all three compounds (**1**, **3**, and **5**) are illustrated in Fig. 3.

As illustrated in Fig. 3, the intramolecular O–H···O hydrogen bonds in all three compounds (**1**, **3**, and **5**) are also preserved in the crystalline state after the MMC. This is the confirmation that intramolecular hydrogen bonds in all three compounds stabilize molecular conformation.

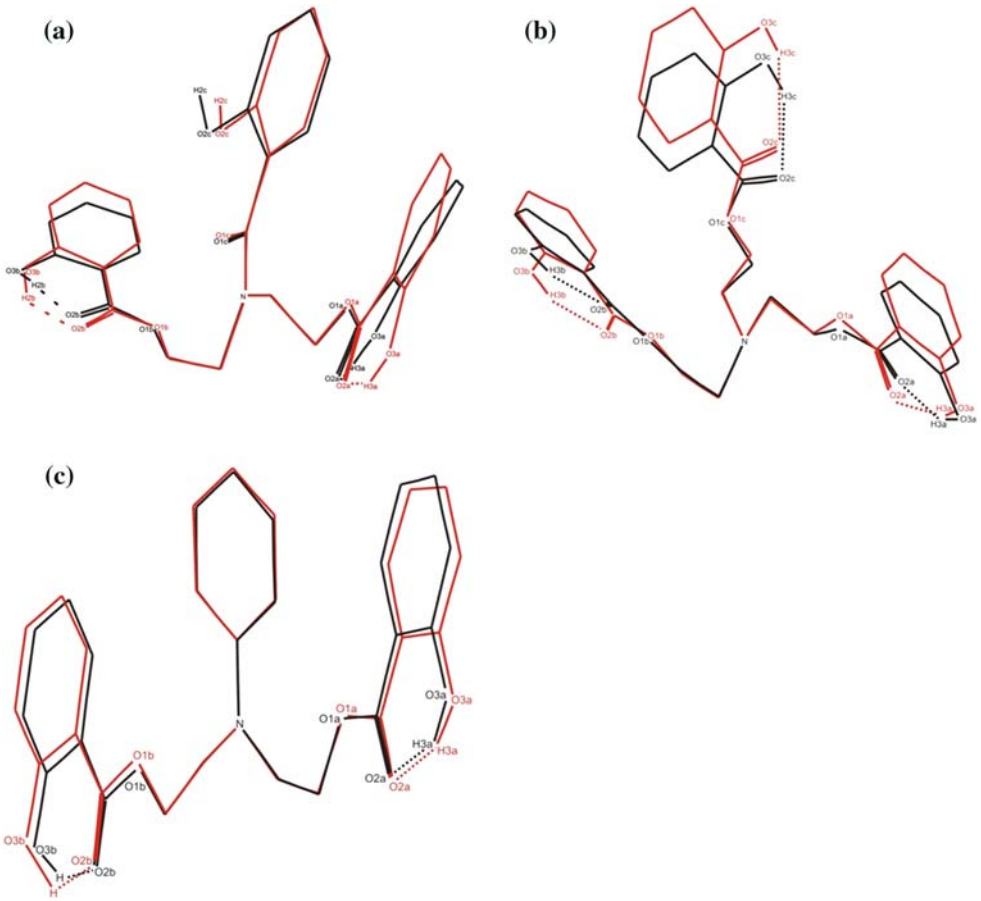


Fig. 2 Superimposed fit of the molecules after MMC (*lighter line*) and the molecule in the crystalline state (*darker line*): (**a**) compound **1**, (**b**) compound **3**, and (**c**) compound **5**

Table 3 Intramolecular O–H···O hydrogen-bond parameters (Å, °)

D–H···A	D–H	H···A	D···A	D–H···A
(**1**)				
O3A–H3A···O2A	0.96 (2)	1.76 (2)	2.598 (2)	144 (2)
O3B–H3B···O2B	0.82 (2)	1.89 (3)	2.589 (3)	143 (3)
(**3**)				
O3A–H3A···O2A	0.95 (3)	1.74 (3)	2.616 (2)	150 (3)
O3B–H3B···O2B	0.88 (3)	1.82 (3)	2.605 (2)	148 (3)
O3C–H3C···O2C	0.90 (3)	1.74 (3)	2.607 (2)	161 (2)
(**5**)				
O3A–H3A···O2A	1.00 (3)	1.66 (4)	2.577 (3)	151 (3)
O3B–H3B···O2B	0.91 (4)	1.78 (4)	2.615 (3)	151 (3)

Antioxidant activity

The antioxidant activities of the synthesized derivatives of salicylic acid were evaluated in a series of in vitro tests.

The hydroxyl radical scavenging activity of the examined compounds was measured by the deoxyribose assay [23]. The protective effects of the tested compounds on 2-deoxy-D-ribose were assessed as their ability to remove hydroxyl radicals (formed in the Fenton reaction) from the test solution and prevent the sugar degradation. Generally, all of the examined compounds inhibited the degradation of deoxyribose more than BHT and BHA. The highest activity exhibited compounds **3** and **5**, with respective IC_{50} values being about 60- and 70-fold lower than those observed for the BHT and BHA. Of bis-derivatives, significantly lower activity showed compound **4**, while compound **2** was the least active. It can be presumed that electronic effects of the groups at the N atom are responsible for the neutralization of OH radical by examined bis- and tris-derivatives. Namely, compound **5** contains phenyl group at the nitrogen atom, compound **4** has 2-hydroxyethyl group, and compound **2** bears methyl group. That is the order of decreasing of their scavenging activities $\mathbf{5} \sim \mathbf{3} > \mathbf{4} > \mathbf{6} \sim \mathbf{1} > \mathbf{2}$. The tris-derivative **3**, with the 2-salicyloyloxyethyl group at the N atom expressed stronger activity than tris-derivative **1** with salicyloyl group. It can also be presumed that the differences between conformations of molecules **3** and **5** on one side and molecule **1** on the other, shown by X-ray structure analyses, lead to the differences in their antioxidant activity. Besides, it can be presumed that the different conformation in the region of the N atom in molecule **1**, comparing with the conformations of the same region in the molecules **3** and **5**, changes the positions and distances

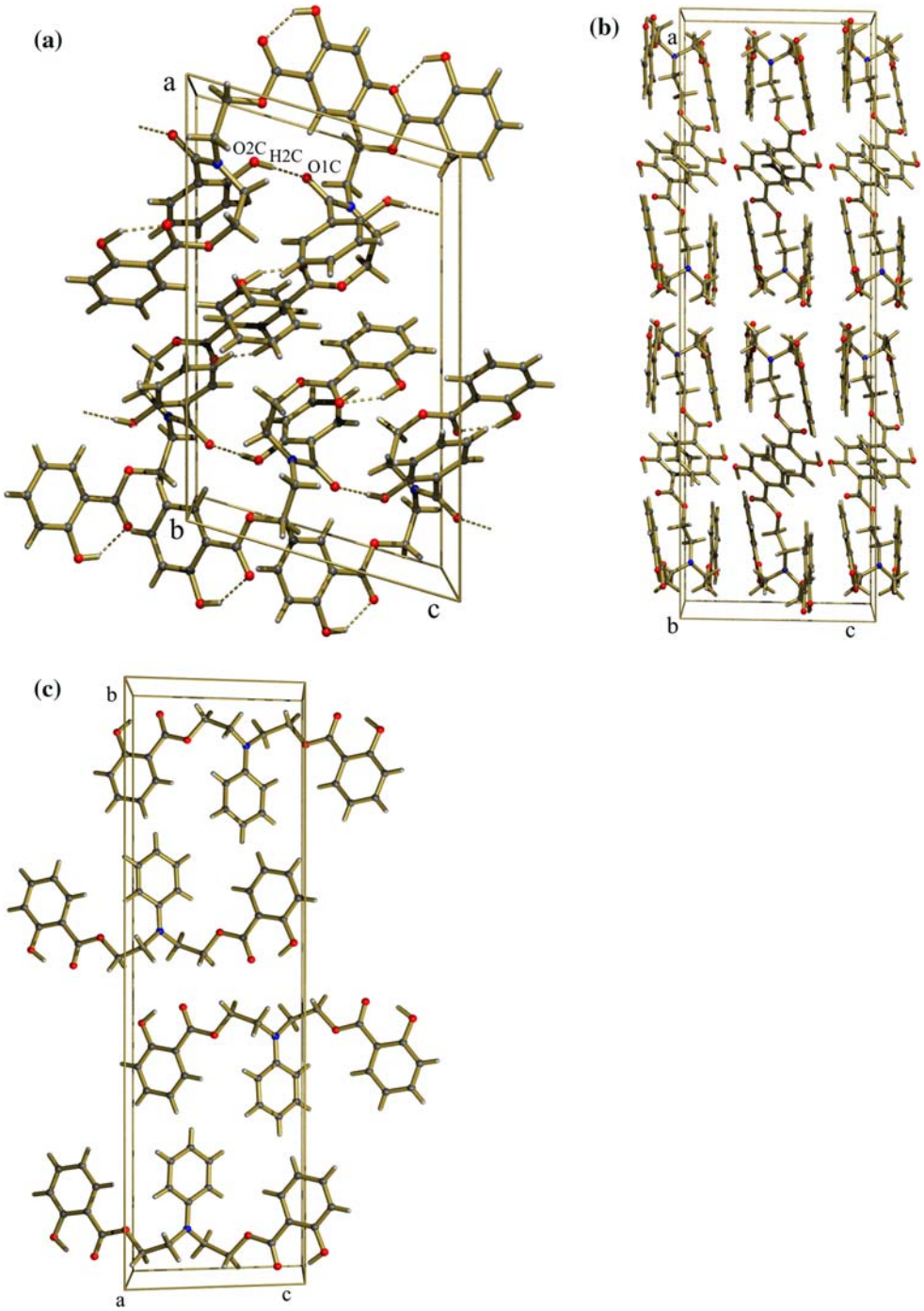


Fig. 3 PLATON drawings showing the crystal packing of: **a** compound **1**; Intermolecular hydrogen bond O2C–H2C···O1C in compound **1** is shown as *dashed line*, **b** compound **3**, and **c** compound **5**

among the active centers in the molecules (compound **1** has 10 times higher IC_{50} value than compounds **3** and **5** (Table 4)).

Furthermore, in the DPPH assay, the ability of tested compounds to act as donors of hydrogen atoms or electrons in transforming $DPPH^{\bullet}$ into its reduced form, DPPH-H, was measured by spectrophotometric method [22]. Most of tested compounds were able to reduce the stable, purple-colored radical $DPPH^{\bullet}$ into yellow-colored DPPH-H form. The strongest scavenging activity of DPPH radicals was expressed by mono derivative **6** (Table 4). Among bis-derivatives, the highest DPPH–RSC exhibited compound **2**, somewhat lower derivative **4**, whereas compound **5** exhibited no activity at all. Of tris-derivatives, only compound **1** exhibited scavenging activity, while derivative **3** appeared to be inactive. Commercial synthetic antioxidants

Table 4 Radical scavenging activities of the tested salicylic acid derivatives and commercial antioxidants (BHA, BHT)

Compound	IC_{50} (mmol/L)		
	$HO^{\bullet}$	DPPH	LP
1	0.376	7.1	0.52
2	0.705	6.5	1.76
3	0.032	–	0.51
4	0.198	8.0	0.38
5	0.028	–	0.22
6	0.338	6.2	0.82
BHT	1.940	0.040	0.210
BHA	2.130	0.012	0.048

BHT and BHA exhibited higher DPPH scavenging properties than those observed for the examined compounds.

The inhibition of LP was determined by measuring the formation of malondialdehyde, using liposomes as an oxidizable substrate [22]. All examined compounds showed notable inhibition of the Fe2+/ascorbate induced LP in liposomes, compound **5** expressing the highest inhibition. The bis- and tris-derivatives showed similar order of decreasing activities as in the case of the neutralization of OH radicals **5** > **4** > **3** ~ **1** > **6** > **2**. Comparison of the IC_{50} values for tested compounds with the IC_{50} values for BHT and BHA shows that compounds **4** and **5** could be classified as promising inhibitors of LP.

Cytotoxicity

The newly synthesized compounds **1**–**6** were evaluated for their in vitro cytotoxicity against MCF7, human breast adenocarcinoma ER+, MDA-MB-231, human breast adenocarcinoma ER−, PC3, prostate cancer, HeLa S3, cervix epithelioid carcinoma, Hs 294T, human melanoma, K562, chronic myelogenous leukemia, as well as MRC-5, normal fetal lung fibroblasts. Cytotoxic activity was determined by using the standard SRB assay, after exposure of cells to the tested compounds for 48 h [24]. The results are presented in Table 5.

Compound **4** was active against four out of six cell lines, but its activity significantly depended on the cell line. HeLa S3 and PC3 cells were the most sensitive cell line-pronounced cytotoxicity was achieved by all tested compounds. Four (**1**–**3** and **6**) out of six compounds were active at nanomolar concentrations against HeLa S3 cells. PC3 cells were highly sensitive to compounds **1**, **3,** and **5**. Estrogen receptor negative MDA-MB-231 cells were more sensitive than MCF7 cells.

Both of tris-derivatives (**1** and **3**) and only one bis-derivative (**2**) exhibited potent cytotoxicity against the HeLa S3 cells. The other two derivatives (**4** and **5**) exhibited a lower, but still potent, activity against the same cell lines. Both of tris-derivatives (**1** and **3**) and only one bis-derivative (**5**) exhibited strong cytotoxycity against the PC3 cells. Among four compounds that exhibited activity against the MDA-MB-231 cells, the most potent was tris-derivative **3**, but only compound **4** showed activity against the MCF7 cells. Only compound **2** showed a moderate cytotoxicity against the normal fetal lung fibroblasts MRC-5.

The bis derivative **5**, which showed strong activity as a scavenger of OH radicals and inhibitor of LP, exhibited also a potent cytotoxic activity against the PC3 cells. The tris-derivative **3**, which exhibited a similar antioxidative activity, apart from showing potent activity against the PC3 cells, showed also a strong cytotoxic activity against the HeLa S3 line.

If compared with doxorubicin (DOX), BHT and BHA, compounds **1**–**6** showed a stronger cytotoxicity against PC3 cell lines, e.g., the activity of compound **5** was 340 times higher than that of DOX. Compounds **1**–**3** and **6** exhibited stronger cytotoxicity against HeLa S3 compared to DOX; compound **1** was 30 times more active than DOX. Compound **4** exhibited cytotoxicity against MCF7 cells, in contrast to BHT and BHA, which were not toxic to these cells. Compounds **1**, **3**, **4**, and **5** were toxic to MDA-MB-231 cells,

Table 5 In vitro cytotoxicity of the tested compounds

Compound	IC_{50} (μmol/L)						
	MCF-7	MDA-MB-231	PC-3	HeLa S3	Hs 294T	K562	MRC-5
1	>100	41.81	0.59	0.04	>100	>100	>100
2	>100	>100	24.58	0.05	>100	45.16	32.77
3	>100	12.08	0.58	0.06	>100	>100	>100
4	11.68	61.07	23.33	4.67	>100	>100	>100
5	>100	26.84	0.28	9.87	54.90	134.04	>100
6	>100	>100	37.82	0.21	>100	>100	>100
DOX	0.75	0.12	95.61	1.17	15.39	0.36	0.12
BHT	>100	>100	138.21	–	–	–	>100
BHA	>100	>100	212.89	–	–	–	>100

while BHT and BHA were not. Tested compounds did not exhibit cytotoxicity toward Hs 294T and K562. It should be pointed out that all compounds but **2** were not toxic to healthy MRC-5 cells; compound **2** was significantly less toxic compared to DOX.

Conclusions

Comparison of crystal and MMC structures for newly synthesized compounds **1**, **3**, and **5** revealed that the intramolecular hydrogen bonds play an important role, stabilizing conformation of the most part of molecule. The influence of crystal packing on molecular conformation, including the strong O2C–H2C···O1C intermolecular hydrogen bond in compound **1**, is not predominant.

The study showed that the electronic effects of the groups at the N atom are responsible for neutralization of OH radical, i.e., antioxidant activity. This electronic effect in molecule of compound **1** between O1c atom from the C=O group and N atom resulted in conformational changes in the region of atom N (comparing with the conformations of the same region in the molecules **3** and **5**). It seems that it is the reason for reduced antioxidant activity of compound **1**. The results of studying the biological effects show that the investigated compounds **1**–**6**, possessing salicyloyl group, exhibited strong activities as scavengers of OH radicals and inhibitors of LP, as well as a strong cytotoxic activity against the HeLa S3 and PC-3 cells.

Supplementary data

CCDC 637570-637572 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44(0)1223-336033; email: deposit@ccdc.cam.ac.uk].

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References

1. Dröge W (2002) Physiol Rev 82:47–95
2. Soler-Rivas C, Espin JC, Wichers H (2000) Phytochem Anal 11:330–338
3. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J (2004) Mol Cell Biochem 266:37–56
4. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006) Chem Biol Interact 160:1–14
5. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J (2007) Int J Biochem Cell Biol 39:44–84
6. Al-Dabbas MM, Suganuma T, Kitahara K, Hou D, Fuujii M (2006) J Ethnopharmacol 108:287–293
7. Ljubincic P, Azaizeh H, Portnaya I, Cogan U, Said O, Abu Saleh K, Bomzon AJ (2005) J Ethnopharmacol 99:43–47
8. Fujisawa S, Atsumi T, Kadoma Y, Sakagami H (2002) Toxicology 177:39–54
9. Sestili P, Diamantini G, Bedini A, Cerioni L, Tommasini I, Tarzia G, Cantoni O (2002) Biochem J 364:121–128
10. Simpson J, Forrester R, Tisdale MJ, Billington DC, Rathbone DL (2003) Bioorg Med Chem Lett 13:2435–2439
11. Zheng LF, Wei QY, Cai YJ, Fang JG, Zhou B, Yang L, Liu ZL (2006) Free Radic Biol Med 41:1807–1816
12. Rotem R, Tzivony Y, Flescher E (2000) Prostate 42:172–180
13. Lograd LPL, Silveira D, Romeiro LAS, de Moraes MO, Cavalcanti BC, Costa-Lotufo LV, do Ó Pessoa C, dos Santos MLJ (2005) Braz Chem Soc 16:1217–1225
14. Ott I, Schmidt K, Kircher B, Schumacher P, Wiglenda T, Gust RJ (2005) Med Chem 48:622–629
15. Oxford Diffraction CrysAlis CCD and CrysAlis RED. Versions 1.171. (2008) Oxford Diffraction Ltd, Abingdon, Oxfordshire, England
16. Harms K (1996) XCAD4, data reduction program for CAD4 diffractometers. Philipps-Universitat Marburg, Germany
17. Altomare A, Cascarano G, Giacovazzo C, Guagliardi A (1993) J Appl Cryst 26:343–350
18. Sheldrick GM (1997) SHELX97, programs for crystal structure analysis. University of Göttingrn, Germany
19. Nardelli MJ (1995) Appl Cryst 28:659–662
20. Spek AL (1998) PLATON. A multipurpose crystallographic tool. University of Utrecht, The Netherlands
21. Farrugia LJ (1999) J Appl Cryst 32:876–881
22. Mimica-Dukić N, Božin B, Sokolović M, Simin N (2004) J Agric Food Chem 52:2485–2489
23. Halliwell B (1990) Free Radical Res Commun 9:1–32
24. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren TJ, Bokesch H, Kenney S, Boyd RM (1990) J Natl Cancer Inst 82:1107–1112
25. Farrugia LJ (1997) J Appl Cryst 30:565
26. Serena Software (1989) PCMODEL, version 4.0. Serena Software Bloomington, Indiana, USA