ORIGINAL SCIENTIFIC PAPER

CROATICA CHEMICA ACTA

Croat. Chem. Acta **2018**, *91*(1), 1–9
Published online: February 2, 2018
DOI: 10.5562/cca3225

HKD 1926

Antioxidant, Antimicrobial and Antiproliferative Activities of Synthesized 2,2,5,5-Tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione Derivatives

Selma Zukić,[1] Elma Veljović,[1] Selma Špirtović-Halilović,[1] Samija Muratović,[1] Amar Osmanović,[1] Snežana Trifunović,[2] Irena Novaković,[3] Davorka Završnik[1,*]

[1] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina

[2] IHTM, Center for Chemistry, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia

[3] Faculty of Chemistry, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

* Corresponding author's e-mail address: davorka.zavrsnik@ffsa.unsa.ba

RECEIVED: September 19, 2017 ✶ REVISED: December 1, 2017 ✶ ACCEPTED: December 11, 2017

Abstract: Ten biologically active 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione derivatives were synthesized and their structures were confirmed by IR, ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. Synthesized compounds were scanned for their antioxidant, antimicrobial and antiproliferative activity. Antibacterial activity was tested by the diffusion and dilution method against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, while antifungal activity was tested against *Candida albicans* and *Saccharomyces cerevisiae*. Antiproliferative activity was tested against HeLa (cervical carcinoma), SW620 (colorectal adenocarcinoma, metastatic), hepatocellular carcinoma (HEpG2), lung carcinoma cells (A549) and mouse embryo fibroblast cell line (3T3). The best antioxidant activity showed compound **2** with two hydroxy groups substituted on phenyl ring in positions 2' and 3'. The best antimicrobial activity of all synthesized compounds showed compound **8**, while the best antiproliferative activity showed compound **6**. Results signify the importance of xanthene-1,8-dione derivatives as potential antioxidant and antiproliferative agents.

Keywords: xanthene-1,8-diones, antimicrobial activity, antiproliferative activity, antioxidant potency.

INTRODUCTION

XANTHENES and its derivatives are known as an important class of heterocyclic compounds that have been widely used as leuco dyes, pH sensitive fluorescent materials for visualization of biomolecules and in laser technologies due to their spectroscopic properties.[1] Also, xanthenes constitute as an important class of biologically active compounds due to their broad spectrum of pharmacological activities such as antibacterial,[2] antiviral,[3] anti-inflammatory activities.[4] Natural and synthetic xanthene derivatives are also well-known for their ability to act as antioxidants and/or enzyme inhibitors.[5] Due to their wide range of applications, these compounds have received a great deal of attention regarding their synthesis. A wide variety of methods for the preparation of the xanthenes have been reported.[6–11]

Antioxidants are necessary to control degenerative reactions produced by reactive oxygen and nitrogen species. These species are involved in several ailments including cancer, heart diseases and Alzheimer's disease.[12]

With the development of new strains of bacteria resistant to many currently available antibiotic treatments, there is increasing interest in the discovery of new antibacterial agents. Antimicrobial resistance refers to microorganism that have developed the ability to inactivate, exclude or block the inhibition or lethal mechanism of the antimicrobial agents.[13]

In our previous work we prepared thirteen 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione derivatives using reliable one-pot synthesis followed by structure elucidating measurements, and performed *in vitro* antimicrobial potency evaluation against *Escherichia coli* and *Candida albicans* strains.[14] Antimicrobial

and antiproliferative studies on similar xanthenes were reported,[15–18] however none so far on these particular derivatives. Presented study therefore, was aimed to prepare new derivatives and evaluate their antimicrobial, antiproliferative and antioxidant activity.

EXPERIMENTAL

Instrumentation

Melting points of the compounds were determined with BŰCHI Melting Point B-545 and are presented uncorrected. Infrared (IR) spectra of synthesized compounds were recorded by *Shimadzu IR Prestige 21 ID* using KBr pellets. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 600 and 150 MHz, respectively, in $CDCl_3$ at 25 °C using NMR spectrometer *Bruker AV600*, with tetramethylsilane (TMS) as internal reference. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. Elemental analyses of synthesized compounds were recorded by Vario EL III C, H, N, S/O Elemental Analyzer, *Elementar Analysensysteme GmbH*, *Hanau-Germany*. Electrospray ionization mass spectrometry (ESI-MS) measurements were performed on a high performance liquid chromatography–mass spectrometry (HPLC-MS) triple quadrupole 6420 instrument equipped with an autosampler (Agilent Technologies, Palo Alto, CA, USA). The desolvation gas temperature was 300 °C with flow rate of 6.0 L min^{-1}. The fragmentor voltage was 135 V and capillary voltage was 4.0 kV. Mobile phase was 0.1 % formic acid in 50 % methanol and a flow rate of mobile phase was 0.2 mL min^{-1}.

General Procedure for Synthesis

A mixture of substituted benzaldehyde (1 mmol), 5,5-dimethylcyclohexane-1,3-dione (2 mmol) and DABCO (10 mmol %) in H_2O (20 mL) was refluxed for 30 min. The progress of the reaction was monitored by TLC by using silica gel 60G F254 plates and dichlormethane : hexane = 1 : 1 as the mobile phase. After completion of the reaction, the mixture was cooled to room temperature, and the solid was filtered off and washed with distilled water. The crude product was purified by recrystallization from 96 % ethanol.[19]

Analytical data for synthesized 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-diones are listed below.

2,2,5,5-TETRAMETHYL-9-(2',3'-DIHYDROXYPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (1)

77.25 %; m.p 214–217 °C; IR(KBr) ν_{max} / cm^{-1}: 3200–3400 (Ar–OH), 3000 (Ar–H), 1670 (C=O), 1300 (C–O), 1500 (C=O), 1200 (Ar–OH), 1152 (C–O–C); ^{1}H NMR ($CDCl_3$) δ / ppm: 1.01 (s, 3H, H-17), 1.04 (s, 3H, H-15), 1.08 (s, 6H, H-14 and H-16), 1.88–2.72 (m, 10H, H-3, H-5, H-9, H-11 and 2'-OH), 4.67 (s, 1H, H-13), 6.54 (dd, 1H, $J_{4'5'}$ = 7.6 Hz, $J_{4'6'}$ = 1.1 Hz, H-4'), 6.75 (dd, 1H, $J_{5'6'}$ = 8.1 Hz, $J_{4'6'}$ = 1.4 Hz, H-6'), 6.88 (t, 1H, $J_{5'6'}$ = 7.9 Hz, $J_{4'5'}$ = 7.8 Hz, H-5'); ^{13}C NMR ($CDCl_3$) δ / ppm: 26.42 (C-14), 27.13 (C-16), 27.68 (C-15), 29.15 (C-17), 29.81 (C-13), 30.93 (C-4), 32.31 (C-10), 41.36 (C-5), 43.14 (C-9), 49.91 (C-3), 50.62 (C-11), 111.50 (C-2), 114.35 (C-4'), 117.89 (C-12), 119.10 (C-5'), 124.63 (C-6'), 124.71 (C-1), 139.07 (C-7), 143.32 (C-1'), 167.99 (C-2'), 170.86 (C-3'), 196.86 (C-6), 200.93 (C-8); Anal. Calcd. mass fractions of elements, w / %, for $C_{23}H_{25}O_5$ (M_r = 382.45) are: C72.25, H6.54; found: C72.29, H6.32.

2,2,5,5-TETRAMETHYL-9-(3',4'-DIHYDROXYPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (2)

69 %; m.p 147–149 °C; IR(KBr) ν_{max} / cm^{-1}: 3500 (Ar–OH), 3000 (Ar–H), 1680 (C=O), 1320 (C–O), 1530 (C=O), 1200 (Ar–OH), 1154 (C–O–C); ^{13}C NMR ($CDCl_3$) δ / ppm: 27.47 (C-14 and C-16), 29.15 (C-15 and C-17), 29.49 (C-13), 31.46 (C-4), 32.25 (C-10), 46.46 (C-3 and C-11), 50.77 (C-5 and C-9), 114.41 (C-1 and C-7), 115.73 (C-2'), 118.61 (C-6'), 119.18 (C-5'), 130.93 (C-1'), 143.63 (C-6'); Anal. Calcd. mass fractions of elements, w / %, for $C_{23}H_{25}O_5$ (M_r = 382.45): C72.23, H6.85; found: C72.35, H6.72.

2,2,5,5-TETRAMETHYL-9-(2'-HYDROXY-3'-METHOXYPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (3)

93.4 %; m.p 229–231 °C; IR(KBr) ν_{max} / cm^{-1}: 3400 (Ar–OH), 3000 (Ar–H), 1670 (C=O), 1480 (O–CH_3), 1300 (C–O), 1500 (C=O), 1200 (OH), 1152 (C–O–C); ^{1}H NMR ($CDCl_3$) δ / ppm: 1.00 (s, 3H, H-17), 1.03 (s, 3H, H-15), 1.13 (s, 6H, H-14 and H-16), 1.88–2.75 (m, 9H, H-3, H-5, H-9, H-11 and 2'-OH), 3.89 (s, 3H, 3'-OCH_3), 4.70 (s, 1H, H-13), 6.59 (dd, 1H, $J_{4'5'}$ = 8.3 Hz, $J_{4'6'}$ = 0.9 Hz, H-4'), 6.76 (dd, 1H, $J_{5'6'}$ = 8.3 Hz, $J_{4'6'}$ = 0.9 Hz, H-6'), 6.95 (t, 1H, $J_{5'6'}$ = 8.3 Hz, $J_{4'5'}$ = 8.3 Hz, H-5'); ^{13}C NMR ($CDCl_3$) δ / ppm: 27.19 (C-14 and C-16), 27.80 (C-15 and C-17), 29.12 (C-13), 30.92 (C-4), 32.31 (C-10), 41.58 (C-3 and C-11), 49.96 (C-5 and C-9), 56.09 (C-7'), 110.45 (C-4'), 110.90 (C-1), 118.18 (C-7), 119.79 (C-5'), 124.23 (C-6' and C-1'), 125.21 (C-1'), 147.14 (C-2'), 147.25 (C-3'), 168.85 (C-2 and C-12), 200.96 (C-6 and C-8); MS m / z: 397.4 (M^+, 100 %); Anal. Calcd. mass fractions of elements, w / %, for $C_{24}H_{28}O_5$ (M_r = 396): C72.7, H7.07; found: C72.64, H7.24.

2,2,5,5-TETRAMETHYL-9-(2'-HYDROXY-5'-NITROPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (4)

96 %; m.p 205–207 °C; IR(KBr) ν_{max} / cm^{-1}: 3500 (Ar–OH), 3000 (Ar–H), 1750 (C=O), 1600 (C=C), 1500 (C=O), 1300 (C–O), 1200 (Ar–OH), 1152(C–O–C), 1250 (NO_2); ^{1}H NMR ($CDCl_3$) δ / ppm: 0.99 (s, 3H, H-15), 1.032 (s, 3H, H-17), 1.147 (s, 6H, H-14 and H-16), 1.87–2.73 (m, 9H, H-3, H-11, H-5, H-9 and 2'-OH), 4.70 (s, 1H, H-13), 7.11 (d, 1H, $J_{3'4'}$ = 8.9 Hz,

H-3′), 7.94 (d, 1H, $J_{4',6'}$ = 2.5 Hz, H-6′), 8.04 (dd, 1H, H-4′); ^{13}C NMR ($CDCl_3$) δ / ppm: 26.98 (C-14 and C-16), 27.82 (C-15 and C-17), 29.13 (C-13), 31.13 (C-4), 32.28 (C-10), 41.16 (C-3 and C-11), 49.87 (C-5 and C-9), 110.89 (C-1 and C-7), 116.37 (C-3′), 123.5 (C-1′), 123.7 (C-4′), 125.9 (C-6′), 144.25 (C-5′), 155.36 (C-2 and C-12), 167.78 (C-2′), 200.73 (C-6 and C-8); MS *m* / *z*: 412.4 (M^+, 100 %); Anal. Calcd. mass fractions of elements, *w* / %, for $C_{23}H_{25}O_6N$ (M_r = 411.45): C69.79, H6.32; found: C69.85, H6.38.

2,2,5,5-TETRAMETHYL-9-(2'-FLUOROPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (5)

83 %; m.p 171–174 °C; IR(KBr) ν_{max} / cm^{-1}: 3000 (Ar–H), 1680 (C=O), 1500 (C=O), 1470(C=C), 1300 (C–O), 1152 (C–O–C), 1100 (C–F); ^{1}H NMR ($CDCl_3$) δ / ppm: 0.99 (s, 6H, H-14 and H-16), 1.15 (s, 6H, H-15 and H-17), 2.25–2.45 (s, H-3, H-11, H-5 and H-9), 5.62 (s, 1H, H-13), 6.95 (m, 1H, H-5′), 6.99 (m, 1H, H-3′), 7.095 (m, 1H, H-6′), 7.27 (m, 1H, H-4′); ^{13}C NMR ($CDCl_3$) δ / ppm: 27.07 (C-14, C-16); 28.88 (C-15, C-17); 29.45 (C-13); 30.91 (C-4); 31.27 (C-10); 46.387 (C-3 and C-11), 76.97 (C-5 and C-9), 115.51 (C-1 and C-7), 118.57 (C-3′), 123.58 (C-1′), 125.5 (C-5′), 127.9 (C-4′), 129.17 (C-6′), 158.91 (C-2 and C-12), 162.84 (C-2′), 189.46 (C-6 and C-8); Anal. Calcd. mass fractions of elements, *w* / %, for $C_{23}H_{25}O_3F$ (M_r = 368.45): C74.98, H6.84; found: C75.01, H6.78.

2,2,5,5-TETRAMETHYL-9-(2'-BROMO-4'-METHYLPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (6)

89 %; m.p 213–214 °C; IR(KBr) ν_{max} / cm^{-1}: 3000 (Ar–H), 1750 (C=O), 1604, 1465 (C=C), 1300 (C–O), 1200 (C=O), 1152 (C–O–C), 750 (C–Br); ^{1}H NMR ($CDCl_3$) δ / ppm: 1.06 (s, 6H, H-14 and H-16), 1.12 (s, 6H, H-15 and H-17), 2.19–2.53 (m, 8H, H-3, H-11, H-5 and H-9), 2.27 (s, 3H, 4′-CH_3), 5.5 (s, 1H, H-13), 7.11 (s, 1H, H-3′), 7.15 (d, 1H, H-6′), 7.4 (d, 1H, H-5′); ^{13}C NMR ($CDCl_3$) δ / ppm: 20.38 (C-7′), 27.01 (C-14 and C-16), 28.52 (C-15 and C-17), 29.56 (C-13), 31.63 (C-4 and C-10), 42.78 (C-3 and C-11), 50.82 (C-5 and C-9), 115.80 (C-1 and C-7), 123.51 (C-2′), 127.83 (C-5′), 129.17 (C-6′), 134.13 (C-3′), 134.34 (C-4′), 134.94 (C-1′), 137.84 (C-2 and C-12), 189.69 (C-6 and C-8); MS *m* / *z*: 447.3 (M^+, 100 %); Anal. Calcd. mass fractions of elements, *w*/%, for $C_{24}H_{27}O_3Br$ (M_r = 446.37): C65.01, H6.14; found: C65.25, H6.07.

2,2,5,5-TETRAMETHYL-9-(4'-DIMETHYLAMINOPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (7)

73.6 %; m.p 199–201 °C; IR(KBr) ν_{max} / cm^{-1}: 3000 (Ar–H), 1680 (C=O), 1465 (C=C), 1400 (C=O), 1300 (C–O), 1158 (C–O–C), 980 (C–N); ^{1}H NMR ($CDCl_3$) δ / ppm: 1.11 (s, 6H, H-14 and H-16), 1.24 (s, 6H, H-15 and H-17), 2.22–2.42 (m, 8H, H-3, H-11, H-5 and H-9), 2.91 (s, 6H, 7′-$N(CH_3)_2$), 5.48 (s, 1H, H-13) 6.68 (d, 2H, $J_{2'3}$ = 8.8 Hz, H-2′ and H-6′), 6.95 (d, 2H, H-3′ and H-5′); ^{13}C NMR ($CDCl_3$) δ / ppm: 27.30 (C-14 and C-16), 29.76 (C-15 and C-17), 31.30 (C-4 and C-10), 32.78 (C-13), 40.75 (C-7′, $N(CH_3)_2$), 46.37 (C-3 and C-11), 46.99 (C-5 and C-9), 112.69 (C-3′ and C-5′), 115.87 (C-1 and C-7), 125.49 (C-1′), 127.44 (C-2′ and C-6′), 148.67 (C-4′), 189.21 (C-2 and C-12), 190.17 (C-6 and C-8); Anal. Calcd. mass fractions of elements, *w* / %, for $C_{25}H_{31}O_3N$ (M_r = 393.52): C76.30, H7.94; found: C76.34, H7.91.

2,2,5,5-TETRAMETHYL-9-(2',3'-DIMETHOXY-5'-BROMOPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (8)

69 %; m.p 185–187 °C; IR(KBr) ν_{max} / cm^{-1}: 3500 (Ar–OH), 3000 (Ar–O), 1300 (C–O), 1152 (C–O–C), 750 (C–Br); ^{1}H NMR ($CDCl_3$) δ / ppm: 1.02 (s, 6H, H-14 and H-16), 1.08 (s, 6H, H-15 and H-17), 1.88–2.65 (m, 8H, H-3, H-11, H-5 and H-9), 3.66 (s, 3H, 2′-OCH_3), 3.79 (s, 3H, 3′-OCH_3), 3.99 (s, 1H, H-13), 4.98 (s, 1H, H-4′), 5.58 (s, 1H, H-6′); ^{13}C NMR ($CDCl_3$) δ / ppm: 27.40 (C-14 and C-16), 28.23 (C-15 and C-17), 31.63 (C-4 and C-10), 32.80 (C-13), 46.38 (C-3 and C-11), 49.34 (C-5 and C-9), 55.80 (C-7′ and 2′-OCH_3), 60.58 (C-8′ and 3′-OCH_3), 114.66 (C-1 and C-7), 115.94 (C-4′), 123.62 (C-5′), 124.85 (C-1′), 133.97 (C-6′), 138.46 (C-2′), 153.21 (C-3′), 189.19 (C-2 and C-12), 196.24 (C-6 and C-8); MS *m* / *z*: 389.3 (M^+, 100 %); Anal. Calcd. mass fractions of elements, *w* / %, for $C_{25}H_{29}O_5Br$ (M_r = 488.4): C61.35, H5.97; found: C61.32, H 6.01.

2,2,5,5-TETRAMETHYL-9-(2',4',6'-TRIMETHOXYPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (9)

81.5 %; m.p 184–185 °C; IR(KBr) ν_{max} / cm^{-1}: 3065 (Ar–H), 1700 (C=O), 1600, 1585 (C=C), 1437 (O–CH_3), 1300 (C–O), 1400 (C=O), 1158 (C–O–C); ^{1}H NMR ($CDCl_3$) δ / ppm: 1.02 (s, 6H, H-14 and H-16), 1.09 (s, 6H, H-15 and H-17), 1.8–2.9 (m, 9H, H-3, H-11, H-5 and H-9), 3.69–3.76 (sss, 9H, 2′, 4′ and 6′-OCH_3), 5.01 (s, 1H, H-13), 6.2 (m, 2H, H-3′ and H-5′); ^{13}C NMR ($CDCl_3$) δ / ppm: 26.87 (C-14 and C-16), 28.07 (C-15 and C-17), 28.69 (C-13), 31.88 (C-4), 33.14 (C-10), 42.92 (C-3 and C-11), 48.38 (C-5 and C-9), 55.19 (4′-OCH_3), 56.00 (2′ and 6′-OCH_3), 92.26 (C-3′ and C-5′), 100.01 (C-1′), 112.00 (C-1 and C-7), 156.87 (C-4′), 159.47 (C-2 and C-12), 163.90 (C-2′ and C-6′), 196.32 (C-6 and C-8); Anal. Calcd. mass fractions of elements, *w* / %, for $C_{26}H_{32}O_6$ (M_r = 440.5): C70.89, H7.32; found: C70.93, H7.24.

2,2,5,5-TETRAMETHYL-9-(2',5'-DIMETHOXYPHENYL)-3,4,5,6,7,9-HEXAHYDRO-1*H*-XANTHENE-1,8(2*H*)-DIONE (10)

92 %; m.p 181–182 °C; IR(KBr) ν_{max} / cm^{-1}: 3500 (Ar–OH), 3000 (Ar–H), 1680 (C=O), 1604, 1465 (C=C), 1440 (O–CH_3), 1400 (C=O), 1300 (C–O), 1158 (C–O–C), 860 (trisubstituted benzene); ^{1}H NMR ($CDCl_3$) δ / ppm: 1.11 (s, 12H, H-14, H-16, H-15 and H-17), 1.8–2.45 (m, 8H, H-3, H-11, H-5 and H-9), 3.67, 3.74 (2s, 6H, 2′ and 5′-OCH_3), 5.58 (s, 1H, H-13),

6.65 (s, 1H, H-6′), 6.94 (d, 2H, H-3′ and H-4′); ^{13}C NMR ($CDCl_3$) δ / ppm: 26.36 (C-14 and C-16), 27.18 (C-15 and C-17), 29.40 (C-13), 31.22 (C-4 and C-10), 42.79 (C-3 and C-11), 48.84 (C-5 and C-9), 55.47 (5′-OCH_3), 55.91 (2′-OCH_3), 111.16 (C-4′), 112.21 (C-1 and C-7), 116.32 (C-3′), 128.01 (C-1′), 151.42 (C-2′), 153.03 (C-5′), 189.12 (C-6 and C-8); MS Ž *m* / *z*: 412.4 (M^+, 100 %); Anal. Calcd. mass fractions of elements, *w* / %, for $C_{25}H_{30}O_5$ (M_r = 411.5): C73.15, H7.37; found: C73.28, H7.19.

Evaluation of Antioxidant Activity by DPPH Method

The free radical scavenging activity of synthesized 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-diones was evaluated using a stable radical, 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) in methanol solution described in literature.[19]

When determining the antioxidant activity by using DPPH method, the absorbance of the 0.2 mM methanol solution of the test substance was mixed with the same ratio of 0.2 mM DPPH solution, representing the absorbance of the sample (A_A (t)). The control solution is a mixture of methanol and DPPH. The measurement was done 30 minutes after the addition of DPPH at 517 nm. In order to determine the concentration of a compound that inhibits DPPH activity by 50 % (EC_{50}), a dilution series was made for each sample individually, and from the calibrated curves the concentration ratio and percent inhibition were determined by EC_{50}. The inhibition percentage (%) of radical scavenging activity was calculated by using the equation 1:

$$\%\ \text{inhibition} = \frac{A_c(0) - A_A(t)}{A_c(0)} \times 100 \qquad (1)$$

where A_c (0) is the absorbance of control at t = 0 and A_A (t) is the absorbance of antioxidant at t = 30 min. All measurements were done in triplicate.[20,21]

Antimicrobial Activity

Antibacterial activity was tested by the diffusion method against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 9027 and *Pseudomonas aeruginosa* ATCC 15442, while antifungal activity was tested against *Candida albicans* ATCC 1023 and *Saccharomyces cerevisiae* ATCC 9763. Test samples were dissolved in 99.5% dimethyl sulfoxide (DMSO) to obtain a 1 mg/mL stock solutions which were then applied to Müller-Hinton and Sabouraud nutritious bases. The inhibition zones for bacteria were measured in millimetres at the end of an incubation period of 18 h at 37 °C, and for fungal strains after 48 h at 25 °C. Compounds that showed good antimicrobial activity tested by the diffusion method were further tested by dilution method. For this, Casein soya bean digest broth (Triptic soya bujon) was used. As referent compounds chloramphenicol and fluconazole are used in concentration of 500 μg mL^{-1}. Test solution of the compound was prepared, followed by formation of a series of 12 dilutions with liquid nutritious base. After the incubation for 24 h, the last tube with no growth of microorganisms was taken to represent minimum inhibitory concentration (MIC) expressed in mg mL^{-1}. The concentrations of the prepared solutions were in range 0.5–0.00024 mg mL^{-1}.

Cell Culturing for Antiproliferative Evaluation

The cell lines HeLa (cervical carcinoma), SW620 (colorectal adenocarcinoma, metastatic), hepatocellular carcinoma (HEpG2), lung carcinoma cells (A549) and mouse embryo fibroblast cell line (3T3), were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM, Lonza, Austria) supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a humidified atmosphere with 5 % CO_2 at 37 °C. Trypan blue solution was used to determine cell viability using an automatic cell counter (Countess, Invitrogen, USA). Morphology was determined under light microscope (Axio Vision-Zeiss, Germany).

Proliferation Assays

A panel of adherent tumour cell lines were inoculated onto a series of standard 96-well microtiter plates on day 0, at 5000 cells per well. Test agents were then added in five, 10-fold dilutions (0.01 μM to 100 μM) and incubated for further 72 h. Working dilutions were freshly prepared on the day of testing in the growth medium. The solvent (DMSO) was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in the working concentrations (DMSO concentration never exceeded 0.1 %). After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay. End-point absorbance was measured at 570 nm. Each test point was performed in quadruplicate in three individual experiments. Experimentally determined absorbance values were transformed into a cell percentage growth (PG) using the formulas proposed by NIH and described previously.[22] This method directly relies on control cells at the day of assay because it compares the growth of treated cells with the growth of untreated cells in control wells on the same plate. The results are therefore a percentile difference from the calculated expected value. The IC_{50} and LC_{50} values for each compound were calculated from dose-response curves using linear regression analysis by fitting the mean test concentrations that give PG values above and below the reference value. If, however, all of the tested concentrations produce PGs exceeding the respective reference level of effect (*e.g.* PG

value of 50) for a given cell line, the highest tested concentration is assigned as the default value (in the screening data report that default value is preceded by a ">" sign).

RESULTS AND DISCUSSION

Synthesis

The synthesis of 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-diones (**1–10**) was assumed to proceed via formation of a Knoevenagel product (**A**), which by addition of second molecule gave the Michael adduct intermediate (**B**) and was followed by cyclization (Figure 1). An α,α'-bis(arylidene)cycloalkanone (**A**) was firstly condensed with dimedone to afford the intermediate (**B**) by addition of second molecule of dimedone where this step can be regarded as a Michael addition. Then the intermediate **B** was cyclized by nucleophilic attack of the hydroxy group on the C=C moiety and 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-diones (**1**-**10**) were afforded.[19]

According to described procedure we synthesized ten new 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione derivatives (**1–10**), that are structural analogues with different substituents at aryl ring (Table 1).

Structural Characterisation of Compounds 1–10

In all IR spectra of synthesized 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9 -hexahydro-1*H*-xanthene-1,8(2*H*)-dione derivatives there are absorption bands characteristic for xanthene structure; absorption bands at 1600-1680 cm^{-1} come from the C=O group and 1152 cm^{-1} band from C–O–C vibration. Also, in all IR spectra of synthesized xanthene compounds,

Table 1. Substituents at phenyl ring of synthesized compounds.

Compound	R_1	R_2	R_3	R_4	R_5
1	OH	OH	H	H	H
2	H	OH	OH	H	H
3	OH	OCH_3	H	H	H
4	OH	H	H	NO_2	H
5	F	H	H	H	H
6	Br	H	CH_3	H	H
7	H	H	$N(CH3)_2$	H	H
8	OCH_3	OCH_3	H	Br	H
9	OCH_3	H	OCH_3	H	OCH_3
10	OCH_3	H	H	OCH_3	H

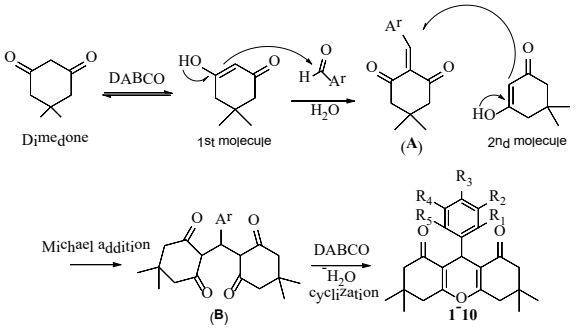

Figure 1. Mechanism of synthesis of 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-diones.[19]

there are bands at 1300 cm^{-1}, characteristic for C–O stretching, and deformation vibrations of the OH group at 1200 cm^{-1}. The IR spectra of the synthesized xanthene compounds differ in absorption bands that come from the corresponding substituent attached to the phenyl ring. For compounds with trisubstituted benzene ring in the structure there are visible characteristic bands at 860 cm^{-1} (compounds **8** and **9**). For compound with bromine in the structure there is visible characteristic band at 750 cm^{-1} from C–Br stretching, while for methoxy substituted derivatives (compounds **3**, **8**, **9** and **10**), IR spectra contain bands between 1200 and 1450 cm^{-1} characteristic for stretching of O–CH_3. Compound with dimethylamino group (compound **7**) shows band at 1440 cm^{-1} which originates from N–CH_3 stretching. Synthesized compounds with hydroxy groups as substituents (compounds **1**, **2**, **3**, **4**) on IR spectra showed bands at 2500–3400 cm^{-1}. In IR spectra of compound with fluor as substituent (compound **5**) there is visible band at 1100 cm^{-1} from C–F streching.

The ^{1}H NMR spectra of synthesized 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione derivatives contain signals characteristic for 2,2,5,5-tetramethyl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione structure. Thus, in all spectra are visible singlets in the range 1.0–1.25 ppm from H-14, H-15, H-16 and H-17 atoms. Also, all spectra possess multiplets (m) in the range 1.88–2.75 ppm from H-3, H-5, H-9 and H-11 atoms, and in the range 4.7–6.04 ppm singlet originating from H-13 atom. Compounds with methoxy group as substituent on the benzene ring possess singlet at 3.7–3.9 ppm which is characteristic for protons from the methoxy group (compounds **3**, **8**, **9**). Compound with dimethylamino group substituted on the benzene ring (compound **7**) on ^{1}H NMR spectra has a singlet at 2.91-3.10 ppm, derived from the protons of the dimethylamino group. Compound **6,** with methyl group in the structure, reveals a singlet at 2.27 ppm which comes from the protons of the methyl group.

^{13}C NMR spectra of synthesized 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione

derivatives show characteristic. All spectra possess signals in the range of 26.4–29.9 ppm from the carbon atoms of the methyl groups (C-14, C-15, C-16, C-17), also at 30.1–32.02 ppm signals from C-4 and C-10 atoms and at 32.72 ppm signals of the C-13 atom.

Antioxidant Activity

For synthesized 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione antioxidant acivity was determinated by DPPH method. For this test, 0.2 mM methanolic solutions for each of the compounds were used. As a standard in the determination of antioxidant activity trolox was used which at the same concentration as the synthesized derivatives showed an inhibition of 84.54 %.

In order to determine the exact concentration in which the activity of the DPPH reagent was inhibited by 50 % (EC_{50}), dilution series were individually made and for each dilution the absorbances were measured at 517 nm. The results of antioxidant activity are shown in Table 2.

According to the obtained results compound **2** (with two hydroxy groups substituted on phenyl ring in positions 3' and 4') has shown the best antioxidant effect with EC_{50} of 0.017 mM. The higher scavenging activity of compound **2** could be attributed to the formation of two radicals on hydroxy groups both of which are stabilized by resonance. Similarly compound **1** also has two hydroxy groups on phenyl ring, but in positions 2' and 3' and its EC_{50} of 0.075 mM indicates antioxidant potency lower than of the compound **2**, so we can conclude that position of hydroxy groups has significant effect on the activity. This difference in potency could be due to the difference in the stability of the radicals. Also, in literature good antioxidant activity is described for dibenzoxanthene and xanthene-11-one derivatives against hydroxy radicals which correlates well with our results.[23,24]

Antimicrobial Activity

Microbiological activity was determinated by diffusion and dilution method on four strains of bacteria and two strains of fungi. The tests were carried out on gram negative bacteria *Escherichia coli* ATCC 9027 and *Pseudomonas aeruginosa* ATCC 15442, and gram positive bacteria *Staphylococcus aureus* ATCC 6538P and *Bacillus subtilis* ATCC 6051. The antifungal activity of synthetized xanthene compounds was tested against fungi *Candida albicans* ATCC

Table 2. Absorbances, percentage of inhibition and EC_{50} of 0.2 mM solution of synthesized compounds.

Compound	Absorbance (t = 30 min)	% Inhibition	EC_{50} / mM
1	0.429	68.74	0.075
2	0.178	87.03	0.017
3	0.510	62.83	0.13
4	0.595	58.86	0.16
5	0.527	61.59	0.135
6	0.528	61.52	0.130
7	0.321	76.60	0.03
8	0.503	63.34	0.129
9	0.383	72.08	0.04
10	0.440	67.93	0.095
Control	1.372; t = 0		
Trolox	0.212	84.54	0.018

Table 3. Results of antibacterial activity by diffusion method.

Compound	Zone of inhibition / mm			
	Escherichia coli (ATCC 9027)	*Pseudomonas aeruginosa* (ATCC 15442)	*Staphylococcus aureus* (ATCC 6538P)	*Bacillus subtilis* (ATCC 6051)
1	9	10	16.5	14
2	11	12	14.5	12
3	8	11	11	13
4	11	14	13	16
5	11	15	11	18.5
6	12	16	12.5	19.5
7	10.5	14	16	20
8	20	13.5	19	13
9	11	15	14	19
10	12	14	15	18
Chloramphenicol	23.5	20	24	22.5

1023 and *Saccharomyces cerevisiae* ATCC 9763 As standard in the study, chloramphenicol (for antibacterial testing) and fluconazole (for the examination of antifungal activity) were used.

The results of antimicrobial acivity of synthesized compounds by diffusion method are shown in Table 3 and Table 4.

Results showed that the best antibacterial activity against *Escherichia coli* ATCC 9027 and *Staphylococcus aureus* ATCC 6538P possesses compound **8.** The best activity against *Pseudomonas aeruginosa* ATCC 15442 showed compound **6** and against *Bacillus subtilis* ATCC 6051 compounds **6** and **7**. Also, compound **6** showed the best antifungal activity with zone of inhibition of 18.5 mm against *Candida albicans* ATCC 1023 and 18 mm against *Saccharomyces cerevisiae* ATCC 9763.

Described *in vitro* antibacterial activity of tetrahydrobenzoxanthene-11-one derivative against *Pseudomonas* strains suggests that antibacterial activity increased with increasing number of hydroxy groups in molecule.[25] In our research the best activity against *Pseudomonas* strain showed compound with bromine in structure, while compounds with hydroxy groups possess weak antibacterial activity against same bacterium. The results of antimicrobial activity by dilution method are shown in Table 5 and Table 6.

Table 4. Results of antifungal activity by diffusion method.

Compound	Zone of inhibition / mm	
	Candida albicans (ATCC 1023)	*Saccharomyces cerevisiae* (ATCC 9763)
1	13.5	14
2	11	16.5
3	12.5	16
4	12	12
5	14.5	14.5
6	18.5	18
7	18	16.5
8	15	16
9	16	14.5
10	16	14
Fluconazole	20.5	21

Table 6. Results of antifungal activity by dilution method.

Compound	MIC / mg mL^{-1}	
	Candida albicans (ATCC 1023)	*Saccharomyces cerevisiae* (ATCC 9763)
1	2.50	1.25
2	2.50	1.25
3	2.50	1.25
4	2.50	1.25
5	2.50	1.25
6	2.50	1.25
7	2.50	1.25
8	2.50	1.25
9	2.50	0.625
10	2.50	1.25
Fluconazole	0.313	0.313

Table 5. Results of antibacterial activity by dilution method.

Compound	MIC / mg mL^{-1}			
	Escherichia coli (ATCC 9027)	*Pseudomonas aeruginosa* (ATCC 15442)	*Staphylococcus aureus* (ATCC 6538P)	*Bacillus subtilis* (ATCC 6051)
1	2.50	2.50	1.25	2.50
2	1.25	2.50	1.25	1.25
3	5.00	2.50	2.50	2.50
4	1.25	1.25	1.25	1.25
5	1.25	1.25	1.25	1.25
6	1.25	1.25	1.25	1.25
7	2.50	1.25	1.25	2.50
8	0.625	1.25	0.625	0.625
9	2.50	1.25	1.25	1.25
10	2.50	1.25	1.25	1.25
Chloramphenicol	0.078	0.313	0.01	0.01

By dilution method, the best antibacterial activity against *Escherichia coli* ATCC 9027, *Staphylococcus aureus* ATCC 6538P and *Bacillus subtilis* ATCC 6051 showed compound **8** with Minimum Inhibitory Concentration (MIC) of 0.625 mg mL^{-1}.

All synthesized compounds showed weak antifungal activity by dilution method against tested fungal strains; the best activity was of compound **9** against *Saccharomyces cerevisiae* ATCC 9763 with MIC of 0.625 mg mL^{-1}.

Antiproliferative Activity

Antiproliferative activity of synthesized compounds were tested by MTT test and results are showed in Table 7.

Antitumor effect of synthetized 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-diones showed that only some of the synthesized compounds affected tested tumor cells. According to the metastatic carcinoma of colon (SW620) compounds **2**, **6**, **8**, **9** and **10** showed activity where compound **6** with IC_{50} of 0.87 μM was the best one. Other synthesized 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione derivatives in the tested concentrations have not shown antiproliferative activity against SW620 cell lines. Against the cells of hepatocellular carcinoma (HEpG2), active were compounds **1**, **2**, **6**, **8**, **9** and **10**, where the best activity again showed compound **6** with IC_{50} of 6.0 μM. Compounds **2**, **3**, **6**, **8**, **9** and **10** showed activity against the cervical adenocarcinoma (HeLa); compound **6** with IC_{50} of 5.6 μM was again the most effective at inhibiting the tumor cells growth. High activity of xanthene compounds against tumor cells has been described in literature.[26,27] Comparing antiproliferative activity of xanthene derivatives with other research[28] where similar 9-aryl-3,4,6,7,9,10-hexahydroacridine-1,8-diones, with nitrogen instead of oxygen in the main moiety, were tested and showed ineffective against tested cells, we can conclude that xanthene ring is important for antiproliferative activity. The worst antiproliferative effect of the synthesized compounds was reported against lung carcinoma cells (A549), where only compounds **2** and **9** showed antiproliferative effect in the tested concentrations (compound **9** being better with IC_{50} of 57.0 μM).

Table 7. Results of antiproliferative activity presented as IC_{50}.

Compound	Cell line				
	SW620	HEpG2	3T3	HeLa	A549
1	> 100	88.1	10.1	> 100	> 100
2	43.9	89.3	9.3	87.9	95.45
3	> 100	> 100	7.4	97.97	> 100
4	> 100	> 100	0.01	> 100	> 100
5	> 100	> 100	16.2	> 100	> 100
6	0.87	6.0	< 0.01	5.6	> 100
7	> 100	> 100	< 0.01	> 100	> 100
8	58.2	33.0	< 0.01	24.4	> 100
9	65.7	34.5	39.1	25.2	57.0
10	62.4	50.1	62.3	26.9	> 100

CONCLUSION

Ten new 2,2,5,5-tetramethyl-9-aryl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione derivatives were synthesized and tested for antioxidant, antimicrobial and antiproliferative activities. The best antioxidant compound was 2,2,5,5-tetramethyl-9-(3',4'-dihydroxyphenyl)-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione (**2**) with two hydroxy groups substituted on phenyl ring in positions 3' and 4' indicating the importance of the substitutents position since the isomere, compound **1**, also bearing 2 hydroxy groups in positions 2' and 3' had almost 20 % lower inhibition effect. The best antimicrobial activity of all synthesized compounds showed compounds with bromine, **6** and **8** (which also possesses two methoxy groups in positions 2' and 3') indicating that bromine in the molecule might play important role in inhibiting growth factors of these microorganisms. On the other side, similar molecules with methoxy group (**9** and **10**), but without the bromine, showed somewhat lesser activity. The best antiproliferative activity against three tumour cell lines (HeLa, SW620 and HEpG2) showed again compound with bromine, 2,2,5,5-tetramethyl-9-(2'-bromo-4'-methylphenyl)-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione (**6**) indicating that bromine was responsible for inhibiting the proliferation of the tumor cells. The obtained results indicate the need for further synthesis of compounds of similar structures, as well as the need to test this group of compounds for other pharmacological activities, for which xanthenes are known to possess. This would give a more complete picture of the pharmacological effects of these agents.

REFERENCES

[1] A. M. El-Brashy, M. E. Metwally, F. A. El-Sepai, *Il Farmaco* **2004**, *59*, 809.

[2] H. Marona, H. Szkaradek, E. Karczewska, D. Trojanowska, A. Budak, P. Bober, W. Przepiórka, M. Cegla, E. Szneler, *Arch. Pharm.* **2009**, *342*, 9.

[3] K. R. M. Naidu, B. S. Krishna, M. A. Kumar, P. Arulselvan, S. I. Khalivulla, O. Lasekan, *Molecules* **2012**, *17*, 7543.

[4] H. N. Hafez, M. I. Hegab, I. S. Ahmed-Farag, A. B. A. El-Gazzar, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4538.

[5] A. M. Seca, S. B. Leal, D. C. Pinto, M. C. Barreto, A. Silva, *Molecules* **2014**, *19*, 8317.
[6] M. Seyyedhamzeh, P. Mirzaei, A. Bazgir, *Dyes Pigm.* **2008**, *76*, 836.
[7] J. Q. Wang, R. G. Harvey, *Tetrahedron* **2002**, *58*, 5927.
[8] G. Casiraghi, G. Casnati, M. Catellani, M. Corina, *Synthesis* **1974**, *8*, 564.
[9] S. Gupta, P. Gupta, A. Sachar, R. L. Sharma, *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.* **2010**, *49*, 1243.
[10] M. M. Heravi, H. Alinejhad, K. Bakhtiari, M. Saeedi, H. A. Oskooie, F. F. Bamoharram, *Bull. Chem. Soc. Ethiop.* **2011**, *25*, 399.
[11] M. Kaya, *Chin. J. Chem.* **2011**, *29*, 2355.
[12] M. Pohanka, *Folia Microbiol.* **2013**, *58*, 503.
[13] F. C. Tenover, *Am. J. Med.* **2006**, *119*, S3-10. 34:64-73.
[14] E. Veljović, S. Špirtović-Halilović, S. Muratović, A. Osmanović, A. Badnjević, L. Gurbeta, B. Tatlić, Z. Zorlak, S. Imamović, Đ. Husić, D. Završnik, *CMBEBIH 2017*, IFMBE Proceedings 62, Springer, Singapore, **2017**, p. 617.
[15] P. Iniyavan, S. Sarveswari, V. Vijayakumar, *Tetrahedron Lett.* **2015**, *56*, 1401.
[16] G. S. Kumar, A. A. Prabhu, P. G. Seethalashmi, N. Bhuvanesh, S. Kumaresan, *J. Mol. Struct.* **2014**, *1059*, 51.
[17] N. Mulakayala, P. V. Murthy, D. Rambabu, M. Aeluri, R. Adepu, G. R. Krishna, C. M. Reddy, K. R. Prasad, M. Chaitanya, C. S. Kumar, M. B. Rao, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 2186.
[18] M. Nisar, I. Ali, M. R. Shah, A. Badshah, M. Qayum, H. Khan, I. Khan, S. Ali, *RSC Adv.* **2013**, *3*, 21753.
[19] P. Paliwal, S. R. Jetti, A. Bhatewara, T. Kadre, S. Jain, *ISRN Org. Chem.* **2013**, Article ID 526173, 6 pages.
[20] W. Brand-Williams, M. E. Cuvelier, C. L. Berset, *LWT--Food Sci. Technol.* **1995**, *28*, 25.
[21] S. Burda, W. Oleszek, *J Agric Food Chem.* **2001**, *49*, 2774.
[22] T. Gazivoda, S. Raić-Malić, S. Krištafor, D. Makuc, J. Plavec, S. Bratulić, S. Kraljević Pavelić, K. Pavelić, L. Naesens, G. Andrei, R. Snoeck, J. Balzarini, M. Mintas, *Bioorg. Med. Chem.* **2008**, *16*, 5624.
[23] X. Z. Wang, B. Y. Yang, G. J. Lin, Y. Y. Xie, H. L. Huang, Y. J. Liu, *DNA Cell Biol.* **2012**, *31*, 1468.
[24] J. M. Khurana, A. Lumb, A. Chaudhary, B. Nand, *RSC Adv.* **2013**, *3*, 1844.
[25] A. Akbari, A. Hosseini-Nia, *J. Saudi Chem. Soc.* **2017**, *21*, S7–S11.
[26] A. Singha, N. Kaurb, S. Sharmaa, P. M. S. Bedi. *J. Chem. Pharm. Res.* **2016**, *8*, 75.
[27] R. Giri, J. R. Goodell, C. Xing, A. Benoit, H. Kaur, H. Hiasa, D. M. Ferguson, *Bioorg. Med. Chem.* **2010**, *18*, 1456.
[28] F. M. Wang, L. Zhou, J. F. Li, D. Bao, L. Z. Chen, *J. Heterocycl. Chem.* **2017**.