Synthesis and In vitro Analysis of Novel Dihydroxyacetophenone Derivatives with Antimicrobial and Antitumor Activities

ARTICLE *in* MEDICINAL CHEMISTRY (SHĀRIQAH (UNITED ARAB EMIRATES)) · NOVEMBER 2013

Impact Factor: 1.39 · DOI: 10.2174/15734064113096660070 · Source: PubMed

7 AUTHORS, INCLUDING:

Cosmin Teodor Mihai
Universitatea Alexandru Ioan Cuza
41 PUBLICATIONS **7** CITATIONS
SEE PROFILE

Ionel Mangalagiu
Universitatea Alexandru Ioan Cuza
113 PUBLICATIONS **376** CITATIONS
SEE PROFILE

Gheorghita Zbancioc
Universitatea Alexandru Ioan Cuza
21 PUBLICATIONS **78** CITATIONS
SEE PROFILE

Send Orders for Reprints to reprints@benthamscience.net

Medicinal Chemistry, **2014**, *10*, **000-000** **1**

Synthesis and *In vitro* Analysis of Novel Dihydroxyacetophenone Derivatives with Antimicrobial and Antitumor Activities

Ana-Maria Zbancioc[1], Anca Miron[1], Cristina Tuchilus[1], Pincu Rotinberg[2], Cosmin-Teodor Mihai[3], Ionel I. Mangalagiu[4] and Gheorghita Zbancioc[4]*

[1] University of Medicine and Pharmacy Iasi "GrigoreT. Popa", Str. Universitatii 16, 700115 Iasi, România; [2] National Institute of Research and Development for Biological Sciences, branch Institute of Biological Research Iasi, Str. Lascar Catargi 47, 700107, Iasi, România; [3] "Al. I. Cuza" University of Iasi, Department of Biology, Bd. Carol I 20A, 700505, Iasi, România; [4] "Al. I. Cuza" University of Iasi, Organic Chemistry Department, Bd. Carol 11, 700506 Iasi, Romania

Abstract: Herein we report a feasible study concerning the design, syntheses and *in vitro* antimicrobial and antitumoral activities of some novel compounds with dihydroxyacetophenone (**DA**) moiety. An efficient and general method for the preparation of diazine with dihydroxyacetophenone (**DDA**) skeleton under conventional thermal heating (TH), microwave (MW) and ultrasounds (US) irradiation is presented. Antimicrobial and antitumoral tests prove that some dihydroxyacetophenone compounds (the brominated derivatives **BrDA 3**) have a significant biological activity. It is also to be pointed out that, basically all the dihydroxyacetophenone derivatives proved to have a powerful antibacterial activity against drug resistant Gram-negative strain *Pseudomonas aeruginosa* ATCC 27853. Of particular interest could be the excellent antibacterial activity of our dihydroxyacetophenone compounds against drug resistant Gram-negative strain *Pseudomonas aeruginosa*.

Keywords: Antimicrobial, Antitumoral, Dihydroxyacetophenone, Microwaves, Phthalazine, Pyridazine, Ultrasounds.

INTRODUCTION

Infectious diseases caused by microorganisms (bacteria, fungus, Mycobacterium tuberculosis, etc.) have substantially increased over the last few years. This is largely due to the development of drug resistance, particularly multidrug resistance which remains a major concern in medicine, for hospitals especially [1, 3]. Despite the progress achieved by modern medicinal science in cancer therapy, neoplasm remains one of the most serious and merciless health problems of the mankind [2]. One of the most promising strategies in cancer therapy remains targeting DNA, designing and synthesizing DNA alkylators agents being one of the most attractive approach in the development of potent anticancer drugs [2, 3].

Pyridazine derivatives and its condensed systems phthalazines, are well known scaffolds in medicinal chemistry [3, 4], possessing a large variety of biological activities such as antihypertensive [5], diuretic [6], antiplatelet [7], cardiotonic [8], anticancer [9], anti-HIV [10], anti-inflammatory [11], antimicrobial (antibacterial, antifungal, anti-tuberculosis) [12, 13], so on. Synthesis of functionalized acetophenone derivatives is an important goal for organic synthesis, these derivatives and particulary dihydroxyacetophenone, exhibiting interesting biological properties, these including antimicrobial [14] and anticancer activities [15].

*Address correspondence to this author at the Al. I. Cuza" University of Iasi, Department of Organic and Biochemistry, 11 Carol I, Iasi-700506, Romania; Tel: +40 +232201278; Fax: +40+ 232 201313;
E-mail: zbanciocg@chem.uaic.ro

Recently published comprehensive books [16, 17] and papers [18, 19] indicate that MW and US irradiation is a new trend in organic chemistry, offering a versatile and facile pathway in a large variety of syntheses.

As part of our ongoing research in the field of biologically active 1,2-diazine [9,12,13,20,21], we decided to synthesize new dihydroxyacetophenone derivatives and diazine with dihydroxyacetophenone skeleton derivatives in order to combine their respective biological potentials, our attention being focused especially on antimicrobial and anticancer activities.

MATERIALS AND METHODS

Chemistry

General Information

All the reagents and solvents employed were of the best grade available and were used without further purification. Melting points were determined using an electrothermal apparatus (MELTEMP II) and are uncorrected. The IR spectra were recorded on an FTIR Shimadzu Prestige 8400s spectrophotometer. The NMR spectra were recorded on a Bruker Avance 400 DRX spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Chemical shifts were reported in ppm (δ-scale), coupling constants (J) in Hz. MS spectra were recorded on a Shimadzu GCMS-QP 2010. The instrument running in chemical ionization (CI)

1573-4064/14 $58.00+.00

mode. The system was equipped with a 25 m x 0.25 mm x 0.25 µm DB-5ms capillary column. The ion source, quadrupole and interface temperatures were 330 °C. Helium was used as carrier gas at constant flow (1.54 mL/min) with an initial pressure of 90.7 kPa, while methane was used as reagent gas in the mass spectrometer. The electron multiplier voltage was set at 1750 V. Two microliters of diluted solution was injected in cold pulsed splitless mode (initial injector temperature at 250 °C). The temperature of the DB-5ms column was programated from 50 °C, stay 2 min, then to 320 °C at a rate of 15 °C/min and finally stay 5 min to 320 °C.

The microanalyses were in satisfactory agreement with the calculated values: C, ±0.15; H, ±0.10; N, ±0.30. For the MW irradiation we used a 800 W STAR SYSTEM-2 monomode reactor (CEM Corporation). The best results were obtained when we used 20% of the full power of the magnetron. US assisted reactions were carried out using a Bandelin Sonopuls GM 3200 reactor, with a nominal power of 200 W and frequency of 20 kHz. The optimal results were obtained when applying a pulse irradiation: 5 s pulse/ 5 s pause, 50% from the full power of the generator. Compounds **3a** and **3e** were initially investigated by us [22]; here we obtained these compounds using a new method, under MW irradiation.

General Procedure for Syntheses of Mono- and Bis-etherificated DA 2 a-e Under Conventional TH, MW and US Irradiation

To a mixture of dihydroyacetophenone derivatives (19 mmol, 2.88 g), potassium carbonate (38 mmol, 5.24 g) and potassium iodide (a spatula tip) in acetonitrile (100 mL under TH, 40 mL under MW and US irradiation), were added methylchloroacetate (38 mmol, 3.35 ml), at room temperature. Under conventional TH, the solution was then refluxed for 26 h on an oil bath. After completion of the reaction (TLC), the mixture was cooled to room temperature, water was added and then the compounds were extracted with chloroform (3 x 50 ml), washed with sodium hydroxide solution (10%), dried and evaporated to afford a precipitate. The crude product was purified by column chromatography on silica gel (eluted with a mixture of dichloromethane / methanol 99:1).

Under MW and US irradiation, the solution mixture of reagents was placed in the reaction vessel and exposed to irradiation (5 min for MW and 15 min for US). Once the heating cycle was completed, the reaction tube was cooled to ambient temperature, removed from the reactor, and processed as indicated for TH conditions.

Dimethyl 2,2'-(2-acetyl-1,3-phenylene)bis(oxy)diacetate (2c)

Yellow solid (4.391 g, 78% using TH; 0.169 g, 3% using MW; 0.169 g, 3% using US): R_f= 0.44 (CH_2Cl_2/CH_3OH, 9.9:0.1); mp: 80-81°C; ^{1}H NMR (400 MHz, DMSO): δ_{ppm}= 2.45 (s, 3H, CH_3 of acetyl group from 2 position), 3.69 (s, 6H, 2xCH_3 of methyl acetate groups from 1 and 3 positions), 4.85 (s, 4H, 2xCH_2 of methyl acetate groups from 1 and 3 positions), 6.65 (d, $J_{4,5} = J_{6,5}$ = 8.4 Hz, 2H, H_4, H_6), 7.25 (t, $J_{5,4} = J_{5,6}$ = 8.4 Hz, 1H, H_5); ^{13}C NMR (100 MHz, DMSO): δ_{ppm}= 31.95 (CH_3 of acetyl group from 2 position, COMe), 51.82 (2xCH_3 of methyl acetate groups from 1 and 3 positon, -CH_2-COOMe), 64.88 (CH_2 of methyl acetate groups from 1 and 3 position, -CH_2-COOMe), 105.63 (C_4, C_6), 120.78 (C_2), 130.36 (C_5), 154.33 (C_1, C_3), 168.97 (2xCO keto ester), 200.63 (CO keto); IR (KBr): ν/cm^{-1}= 3100, 3048, 3011, 2957, 2917, 1767, 1751, 1697, 1599, 1470, 1441, 1248, 1211, 1130, 1086. MS (CI) m/z(%)= 91 (7.6), 107 (5.7), 195 (5.7), 221 (9.4), 237 (34.0), 253 (20.8), 255 (15.1), 281 (Base peak, 100), 282 (15.1), 296 (M, 17.0), 297 (M+1, 11.3).

General Procedure for Syntheses of α-bromo-bis-etherificated BrDA 3a-e Under Conventional TH, MW and US Irradiation

To a suspension of copper (II) bromide (10 mmol, 2.24 g) in 20 mL mixture of chloroform/ethylacetate in a 1:2 ratio, **DA** derivatives **2a-e** (5 mmol, 1.48 g) in 10 mL mixture of chloroform/ethylacetate in a 1:2 ratio were added drop wise, in one hour, under stirring and refluxing. The stirring and refluxing were maintained for 300 minutes. The boiled solution was filtered (in order to remove the copper (I) bromide that formed), and the solvent was removed by reduced pressure distillation. The crude product was crystallization from toluene. The crude product was purified by column chromatography on silica gel (eluted with dichloromethane).

Under MW and US irradiation, the copper (II) bromide (10 mmol, 2.24 g), **DA** derivatives **2a-e** (5 mmol, 1.48 g), in 15 mL solvent (chloroform/ethylacetate, 1:2 ratio) were placed in the reaction vessel and exposed to irradiation (15 min for MW and 20 min for US). Once the heating cycle was completed, the reaction tube was cooled to ambient temperature, removed from the reactor, and processed as indicated for TH conditions.

Dimethyl 2,2'-(2-(2-bromoacetyl)-1,3-phenylene)bis(oxy) diacetate (3c)

Light brown solid (1.294 g, 69% using TH; 0.056 g, 3% using MW; 1.576 g, 84% using US): R_f= 0.43 (CH_2Cl_2/CH_3OH, 9.9:0.1); mp: 57-58°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ_{ppm}= 3.78 (s, 6H, 2xCH_3 of methyl acetate groups from 1 and 3 positions), 4.60 (s, 2H, CH_2 of bromoacetyl group from 2 position), 4.67 (s, 4H, 2xCH_2 of methyl acetate groups from 1 and 3 positions), 6.50 (d, $J_{4,5} = J_{6,5}$ = 8.4 Hz, 2H, H_4, H_6), 7.28 (t, $J_{5,4} = J_{5,6}$ = 8.4 Hz, 1H, H_5); ^{13}C NMR (100 MHz, $CDCl_3$): δ_{ppm}= 37.51 (CH_2 of bromoacetyl group from 2 position), 52.41 (2xCH_3 of methyl acetate groups from 1 and 3 positions, -CH_2-COOMe), 65.73 (2xCH_2 of methyl acetate groups from 1 and 3 positions, -CH_2-COOMe), 105.94 (C_4, C_6, C_2), 131.92 (C_5), 155.86 (C_1, C_3), 168.74 (2xCO keto ester), 193.42 (CO keto); IR (KBr): ν/cm^{-1}= 3093, 2994, 2947, 2911, 1749, 1676, 1595, 1470, 1435, 1377, 1220, 1182, 1145, 1124, 613; MS (CI) m/z(%)= 237 (7.6), 253 (17.0), 255 (11.3), 281 (Base peak, 100), 282 (18.9), 315 (24.5), 317 (22.6), 374 (M-1, 11.3), 376 (M+1, 9.4).

General Procedure for Syntheses of Cycloimmonium Salts 4a-e and 5a-e Under Conventional TH, MW and US Irradiation

BrDA derivatives **3a-e** (5 mmol, 1.88g) and diazine compound (5 mmol) were dissolved in dry acetone (40 mL under TH, 10 mL under MW and US irradiation). The resulting mixture was stirred at room temperature for 48 h. The

solvent was removed on a rotary evaporator and the obtained salts were washed twice with 10 mL ether. No further purification was required.

Under MW and US irradiation, the solution of reagents mixture was placed in the reaction vessel and exposed to irradiation (5 min for MW and 15 min for US). Once the heating cycle was completed, the reaction tube was cooled to ambient temperature, removed from the reactor, and processed as indicated for TH conditions (with the difference that dry acetone was used as washing solvent).

1-(2-(2,6-bis(2-methoxy-2-oxoethoxy)phenyl)-2-oxoethyl) pyridazin-1-ium bromide (4c)

Brown solid (1.343 g, 59% using TH; 1.457 g, 64% using MW; 1.571 g, 69% using US): R_f= 0.53 (CH_2Cl_2/CH_3OH, 3:1); mp: 127-128°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ_{ppm}= 3.73 (s, 6H, 2xCH_3 of methyl acetate groups from 10 and 14 positions), 4.99 (s, 4H, 2xCH_2 of methyl acetate groups from 10 and 14 positions), 6.39 (s, 2H, CH_2 from 7 position), 6.82 (t, overlapped peaks, 2H, H_{11}, H_{13}), 7.46 (t, $J_{12,11}$ = 8.0 Hz, $J_{12,13}$ =8.4 Hz, 1H, H_{12}), 8.77 (s, 1H, H_4), 8.89 (t, $J_{5,6}$ = 4.4 Hz, 1H, H_5), 9.76 (d, 1H, H_3), 9.94 (d, $J_{6,5}$ = 4.4 Hz, 1H, H_6); ^{13}C NMR (100 MHz, $CDCl_3$): δ_{ppm}= 52.07 (2xCH_3 of methyl acetate groups from 10 and 14 positions, -CH_2-COOMe), 65.40 (2xCH_2 of methyl acetate groups from 10 and 14 positions, -CH_2-COOMe), 72.86 (CH_2 from 7 position), 106.41 (C_{11}, C_{13}), 115.38 (C_9), 133.28 (C_{12}), 136.12 (C_5), 137.66 (C_4), 151.51 (C_6), 154.81 (C_{10}, C_{14}), 155.81 (C_3) 169.08 (CO keto ester from 10 and 14 positions), 192.46 (CO keto); IR (KBr): ν/cm^{-1}= 3095, 3032, 2969, 2949, 1753, 1690, 1597, 1470, 1437, 1423, 1253, 1219, 1130.

2-(2-(2,6-bis(2-methoxy-2-oxoethoxy)phenyl)-2-oxoethyl) phthalazin-2-ium bromide (5c)

Brown solid, (1.617 g, 64% using TH; 1.920 g, 76% using MW; 1.996 g, 79% using US): R_f= 0.67 (CH_2Cl_2/CH_3OH, 3:1); mp: 130-131°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ_{ppm}= 3.74 (s, 6H, 2xCH_3 of methyl acetate groups from 12 and 16 positions), 5.02 (s, 2H, CH_2 of methyl acetate groups from 12 and 16 positions), 6.37 (s, 2H, CH_2 from 9 position), 6.84 (d, $J_{13,14}$ = $J_{15,14}$ = 7.6 Hz overlapped peaks, 2H, H_{13}, H_{15}), 7.46 (t, $J_{14,13}$ = $J_{14,15}$ = 7.6 Hz, 1H, H_{14}), 8.52 (d, $J_{5,4}$ = 6.8 Hz, 1H, H_5), 8.62 (d, $J_{6,7}$ = 6.8 Hz, 1H, H_6), 8.68 (d, $J_{4,5}$ = 6.8 Hz, 1H, H_4), 8.81 (d, $J_{7,6}$ = 6.8 Hz, 1H, H_7), 10.23 (s, 1H, H_3), 10.80 (s, 1H, H_8); ^{13}C NMR (100 MHz, $CDCl_3$): δ_{ppm}= 52.00 (CH_3 of methyl acetate groups from 12 and 16 positions, -CH_2-COOMe), 65.34 (CH_2 of methyl acetate groups from 12 and 16 positions, -CH_2-COOMe), 71.60 (CH_2 from 9 position), 106.28 (C_{13}, C_{15}), 115.60 (C_{11}), 127.22 (C_4), 128.54 (C_7), 130.74 (C_{14}), 133.06 (C_{3a}), 136.05 (C_{7a}), 136.54 (C_5), 139.89 (C_6), 153.14 (C_8), 154.88 (C_3),155.72 (C_{12}, C_{16}) 169.04 (CO keto ester from 12 and 16 positions), 192.80 (CO keto); IR (KBr): ν/cm^{-1}: 3080, 3056, 2955, 2936, 1751, 1687, 1597, 1470, 1437, 1395, 1281, 1219, 1130, 1080.

Biological Activity

The synthesized compounds were tested for their *in vitro* antimicrobial activity against six different strains Gram-positive (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Bacillus cereus* ATCC 14579, *Bacillus subtilis*) and Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) bacteria, and one yeast strain (*Candida albicans* ATCC 10231).

The *in vitro* anticancer activity of the synthesized compounds was screened against human cervix epithelial carcinoma cell line (HeLa) [24, 25].

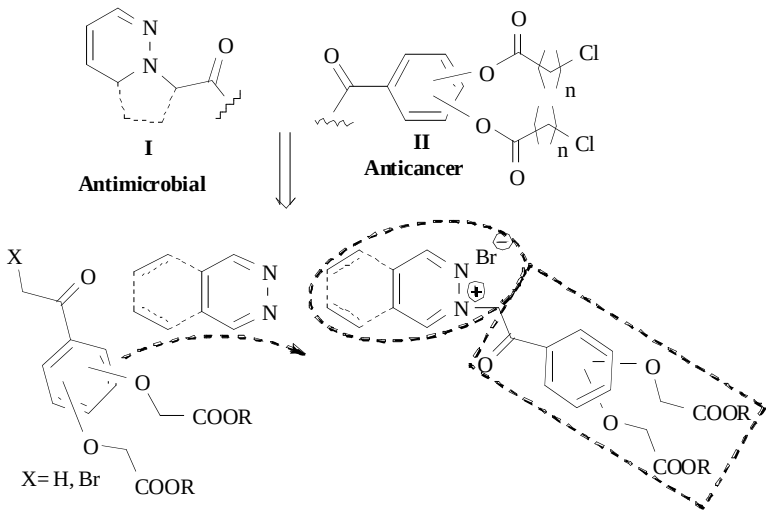
Broth Micro Dilution Assay

A broth microdilution assay was used to determine the minimum inhibitory concentrations (MIC) and the minimum bactericidal/fungicidal concentrations (MBC/MFC) [27-29]. The compounds were dissolved in dimethyl sulfoxide at a concentration of 10 mg/mL. 50 µL of each compound solution were mixed with 50 µL of Mueller Hinton broth for antibacterial assays and 50 µL of Sabouraud broth for antifungal assays and subjected to further serial two-fold dilutions in 96-well plates. An aliquot of 2 µL of microbial suspension (0.5 McFarland) was dispersed in each well. The plates were incubated at 37 °C for antibacterial tests and 24 °C for antifungal tests, for 24 h. The MIC value represents the lowest concentration of compound inhibiting the visible growth of microorganisms. The MBC/MFC values represent the lowest concentration of the compound killing completely the microorganisms to be tested, were determined by transferring 10 µL of samples showing inhibition of visible growth on the surface of an agar plate [28]. The subcultures were incubated at 37 °C for antibacterial tests and 24 °C for antifungal tests for 24 h. There were also evaluated the MIC and MBC/MFC values of ampicillin/nystatin towards bacteria/yeast strains.

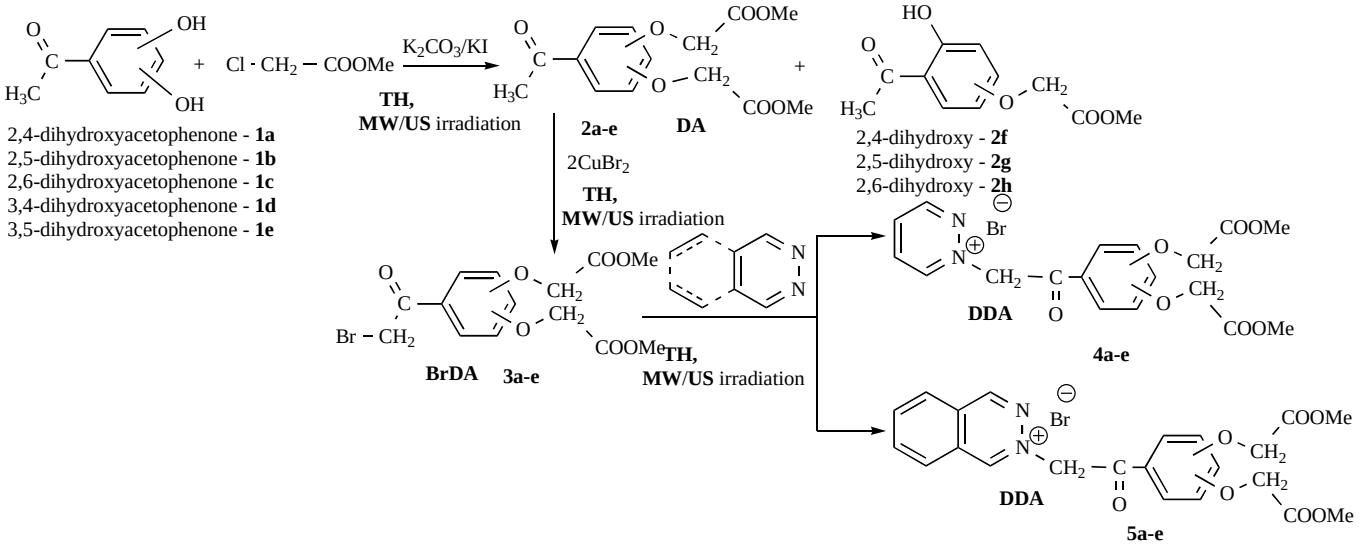
Cytotoxic Assay

The HeLa cells were cultured in DMEM medium (Dulbeco's Modified Essential Medium, Biochrom AG, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany), 100 µg/mL streptomycin (Biochrom AG, Germany), 100 IU/mL penicillin (Biochrom AG, Germany) and 50 µg/mL amphotericin B (Biochrom AG, Germany), at a density of 5x10^5 cells, in a humidified 5% CO_2 atmosphere at 37°C in a Binder CB 150 incubator (Tuttlingen, Germany).

The cells monolayer was further removed with 0.25% trypsin and 0.02% EDTA (ethylenediaminetetraacetic acid, Biochrom AG, Germany) and centrifuged in a Sigma - Sartorius (Gottingen, Germany) centrifuge at 1800 rpm for 2 min. The sediment of the cells was then suspended in the normal medium (DMEM medium). Volumes of 2 mL of suspension (1x10^5 cells) were inoculated in the experimental tubes which were kept in the same conditions mentioned above. After 24 h the medium was discarded and replaced either with a normal one (control cultures) or with one containing the synthesized compounds at different concentrations. At least four concentrations were used for each compound: 200, 300, 400 and 500 µg/mL (the brominated compounds were also tested in lower concentrations at 25, 50 and 100 µg/mL). After 48 h the medium was discarded from the test tubes; the cells layer was washed with PBS (phosphate buffer saline) and then subjected to Lowry method modified by Oyama in order to evaluate the total protein content [30]. The test tubes containing HeLa cells were treated with 2% sodium carbonate, 0.1 N sodium hydroxide, 0.02% potassium tartrate and



Scheme 1. Design in the class of diazine with dihydroxyacetophenone skeleton derivatives.



Scheme 2. Reaction pathway for preparation of **DA**, **BrDA** and **DDA**.

0.5% copper sulphate. After mixing, the absorbance of each test tube was measured at λ= 660 nm. The results were expressed as IC_{50} values representing the concentration of the drug inducing a 50% inhibition of cell growth. Each experiment was performed five times. *5-Fluoro-uracil, etoposide* were used as the references drug.

RESULTS AND DISCUSSION

Recently, we reported successful results on the identification of new antimicrobial (type **I**) [12-14] and anticancer (type **II**) [9,21] compounds, which contain a diazine or an acetophenone skeleton as pharmacophoric moiety. As a consequence, in an attempt to increase the potential of pharmacophoric models, we decided to combine their respective biological potentials. In this respect a *bis*-etherificated **DA** unit was grafted onto a diazine heterocycles, (Scheme **1**).

The *bis*-etherificated **DA** would probably act as *bis*-acylation units, analogous to *bis*-alkylating units from nitrogen mustards anticancer derivatives. As a whole, the entire molecule of **DDA** could exert both antimicrobial and anticancer activity.

In according with our goal, we have synthesized three new types of dihydroxyacetophenone: bis-etherificated dihydroxyacetophenone (**DA**, **2a-e**), α-brominated dihydroxyacetophenone (**BrDA**, **3a-e**) and diazine with dihydroxyacetophenone skeleton derivatives (**DDA**, **4a-e** and **5a-e**). The strategies adopted for the construction of our dihydroxyacetophenone derivatives are depicted in (Scheme **2**).

In the first step, using conventional TH, we obtained the expected 2,4-; 2,5-; 2,6-; 3,4- and 3,5- *bis*-etherificated **DA**, **2a-e**, in good yields (between 78-87%, Table **1**). Unexpectedly, in the case of dihydroxyacetophenone bearing a hydroxyl group in the 2nd the position (2,4-; 2,5-; 2,6- **DA**), we also obtained traces of *mono*-etherificated **DA**, **2f-h**, regardless of the conditions that were employed (Table **1**).

As we may notice from (Table **1**), under conventional TH the reaction pathways had some major disadvantages, including moderate selectivity, long reaction time (1560 min), etc. Having in view the above considerations, we decided to perform these reactions using US and MW irradiation. For the MW irradiation we used a 800 W STAR SYSTEM-2 monomode reactor (CEM Corporation).

Table 1. Synthesis of DA Derivatives Under Conventional TH, MW and US Irradiation

Comp.	MW		US		Conventional TH	
	Reaction Time (min)	Yield (%)	Reaction Time (min)	Yield (%)	Reaction Time (min)	Yield (%)
2a	5	traces	15	traces	1560	83
2b	5	traces	15	traces	1560	83
2c	5	traces	15	traces	1560	78
2d	5	92	15	93	1560	84
2e	5	95	15	96	1560	87
2f	5	90	15	90	1560	traces
2g	5	87	15	89	1560	traces
2h	5	50	15	87	1560	traces

The US assisted reactions were carried out using a Sonoplus GM 3200 reactor (Bandelin) with a frequency of 20 KHz and a nominal power of 200 W. As indicated in (Table **1**), under MW and US irradiation, the reactions occured differently in accordance with the relative position of the two hydroxyl groups on the acetophenone ring. When the hydroxyl groups were in 3,4- and 3,5- positions, the reactions occured selective, the *bis*-etherificated **DA 2d**,**e**, being obtained with better yields compared with TH. When the hydroxyl groups were in 2,4-, 2,5-, and 2,6- positions, the *mono*-etherificated compounds (**DA 2f-h**) were obtained as major products (in good to moderate yields) while the *bis*-etherificated compounds (**DA 2a-c**) were obtained only in traces. We may also notice from (Table **1**) that, in all cases, under MW and US irradiation, the reaction time decreased considerably from hours to minutes.

In the next step we have synthesized the α-bromo-*bis*-etherificated **BrDA 3a-e**, (Scheme **2**). As bromination method we chose a procedure previously reported by us [22], the bromination in heterogeneous catalysis, taking into consideration the advantages of better selectivity, better yields and less polluted setup procedure. Using this method, the desired α-bromo-*bis*-etherificated **BrDA 3a-e** were synthesized by bromination of **DA** derivatives **2a-e** with copper (II) bromide. In the final stage, by quaternization of 1,2-diazine heterocycles (pyridazine and phthalazine) with the α-brominated **BrDA 3a-e**, we obtained the corresponding diazine with dihydroxyacetophenone skeleton derivatives, **4a-e** and **5a-e**, (Scheme **2**). In the (Table **2**) are listed the optimized conditions used for bromination and quaternization, under MW, US and conventional TH.

As indicated in (Table **2**), under conventional TH, the bromination occured highly selective (no nuclear bromination was observed) but the yields were moderate. In the case of US irradiation, the yields were higher, by an average of 10-15% and the reaction time decreases from hours to minutes. Unexpectedly, under MW irradiation, regardless of the conditions we employed, there were obtained only traces of the desired brominated compounds **3a-e**, and the unreacted starting materials remained. In the quaternization case the data presented in (Table **2**), leads to the conclusion that MW and US irradiation induced again a remarkable acceleration of the reactions and, in some cases, the yields were higher.

A preliminary antimicrobial screening was performed by agar diffusion assay [23] using nutrient agar medium (Mueller Hinton agar for antibacterial tests and Sabouraud agar for antifungal tests). The most active compounds were subjected to a broth micro dilution assay in order to determinate the minimum inhibitory concentrations (MIC) and the minimum bactericidal/fungicidal concentrations (MBC/MFC) [27-29] (Table **3**).

Compound **3e** showed the highest effects against all tested bacteria and yeast strains. It exhibited bactericidal effects against *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Bacillus subtilis* with MIC and MBC values of 0.625 mg/mL. The other Gram-positive bacteria strains (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Bacillus cereus* ATCC 14579) were more susceptible to compound **3e**, with MIC values of 0.31 mg/mL.

It is worthy to note that all the brominated compounds showed good antibacterial effects. If we compare the effects of non-brominated derivatives **2a**, **2c** and **2g**, with those of the brominated analogues **3a**, **3c** and **3b**, we may notice a considerable increase in antibacterial activity for the brominated compounds, the influence of bromine atom being certain.

In the case of diazine with dihydroxyacetophenone skeleton derivatives, an unexpected behaviour occurred. Literature data mentions that diazine salts with acetophenone skeleton possess a very good antimicrobial activity [10-12]. As far, for our derivatives, the antibacterial and antifungal effects were lower in comparison to those of the brominated analogues.

All tested compounds showed a good antifungal activity against *Candida albicans* ATCC 10231.

Table 2. Synthesis of α-bromo-bis-etherificated BrDA (3a-e) and DDA (4a-e and 5a-e), Under Conventional TH, MW and US Irradiation

Comp.	MW		US		Conventional TH	
	Reaction Time (min)	Yield (%)	Reaction Time (min)	Yield (%)	Reaction Time (min)	Yield (%)
3a	15	traces	20	86	300	71
3b	15	traces	20	88	300	75
3c	15	traces	20	84	300	69
3d	15	traces	20	83	300	76
3e	15	traces	20	80	300	65
4a	5	81	15	79	2880	69
4b	5	71	15	70	2880	61
4c	5	64	15	69	2880	59
4d	5	73	15	73	2880	66
4e	5	79	15	82	2880	64
5a	5	85	15	86	2880	76
5b	5	77	15	74	2880	65
5c	5	76	15	79	2880	64
5d	5	85	15	87	2880	77
5e	5	85	15	89	2880	79

Table 3. Antibacterial and Antifungal Activities of the Tested Compounds 2-5

Strain Comp.	*S. Aureus* ATCC 25923		*S. Lutea* ATCC 9341		*B. Cereus* ATCC 14579		*B. Subtilis*		*E. Coli* ATCC 25922		*P. Aeruginosa* ATCC 27853		*C. Albicans* ATCC 10231	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
2a[a]	2.5	5	0.62	1.25	1.25	5	1.25	2.5	1.25	1.25	1.25	2.5	1.25	2.5
2c[a]	1.25	2.5	0.62	1.25	2.5	5	10	10	1.25	5	2.5	5	1.25	5
2g[a]	1.25	2.5	0.62	1.25	1.25	2.5	1.25	2.5	1.25	2.5	2.5	10	1.25	2.5
3a[a]	0.62	2.5	0.62	2.5	1.25	2.5	1.25	2.5	1.25	2.5	2.5	5	1.25	2.5
3b[a]	1.25	2.5	0.62	2.5	0.62	1.25	1.25	2.5	1.25	2.5	2.5	5	1.25	2.5
3c[a]	1.25	2.5	0.62	1.25	2.5	5	1.25	2.5	1.25	2.5	2.5	5	2.5	5
3d[a]	1.25	2.5	0.62	1.25	0.62	1.25	0.62	1.25	1.25	2.5	2.5	5	1.25	2.5
3e[a]	0.31	0.62	0.31	0.62	0.31	0.62	0.62	0.62	0.62	0.62	0.62	0.62	1.25	2.5
4e[a]	1.25	2.5	0.62	1.25	1.25	2.5	10	10	2.5	5	2.5	5	2.5	5
5c[a]	2.5	5	0.62	1.25	1.25	5	2.5	5	2.5	5	2.5	10	1.25	5
Ampicillin[b]	0.25	0.5	0.15	0.31	-[c]	-[c]	0.15	0.62	0.31	0.62	-[c]	-[c]	-	-
Nystatin[b]	-	-	-	-	-	-	-	-	-	-	-	-	1	2

[a]concentration expressed in mg/mL
[b]concentration expressed in μg/mL
[c]no zone inhibition

The *in vitro* anticancer activity of the synthesized compounds was evaluated and screened against human cervix epithelial carcinoma cell line (HeLa) [24, 25]. At least four concentrations were tested for each compound: 200, 300, 400 and 500 µg/mL. In order to calculate IC_{50} values, lower concentrations of brominated compounds were tested. The results were expressed as IC_{50}, which is the concentration of the drugs inducing a 50% inhibition of cell growth. Each experiment was performed five times. *5-Fluorouracil* and *Etoposide* were used as the references drugs. (Table **4**) summarizes the antitumoral activity of the compounds expressed as IC_{50}.

Table 4. ***In vitro*** **Cytotoxicity of the Synthesized Compounds Against HeLa Cancer Cell Lines**

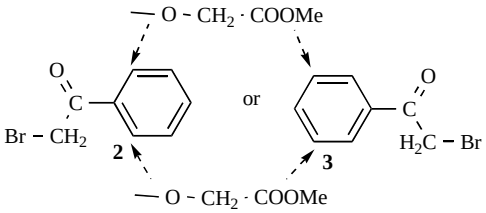
Compound	Cytotoxicity (IC_{50}[a], µg/mL)
2a	364.3±17.5
2b	380.8±16.7
2c	442.4±27.7
2d	380.6±15.3
2e	486.7±18.0
2f	412.8±15.2
2g	462.6±25.5
2h	221.7±48.8
3a	**41.5±1.3**
3b	**101.8±9.1**
3c	**30.9±7.23**
3d	**64.6±8.0**
3e	**203.4±11.6**
4a	>500[b]
4b	>500[b]
4c	>500[b]
4d	>500[b]
4e	>500[b]
5a	459.3±5.5
5b	>500[b]
5c	343.1±10.2
5d	432.0±7.3
5e	468.9±13.7
5-Fluorouracil	17.2±3.87
Etoposide	19.3±2.1

[a]IC_{50} value: the average of three IC_{50} values ± SD
[b]Compounds with IC_{50} > 500 µg/mL were considered to be inactive.

The *in vitro* evaluation revealed that some of the tested compounds showed high or moderate antitumoral activity. The brominated compounds **3a-e** were proved to be the most active against the tested HeLa cells, while the *non*-bromurated **DA 2** and the salts **4** and **5** do not have or have only a moderate cytotoxic activity (IC_{50} between >500 to roughly 300 µg/mL). As for the antimicrobial assay, these results suggest a certain influence of bromine atom concerning anticancer activity.

We may notice that the brominated series the antitumoral activity decreases in order **3c** (IC_{50} 30.9 µg/mL, 2,6-disubstituted **BrDA**) > **3a** (IC_{50} 41.5 µg/mL, 2,4-disubstituted **BrDA**) > **3e** (IC_{50} 64.6 µg/mL, 3,5-disubstituted **BrDA**) > **3b** (IC_{50} 101.8 µg/mL, 2,5-disubstituted **BrDA**) > **3d** (IC_{50} 203.4 µg/mL, 3,4-disubstituted **BrDA**).

These results suggest that the positions of O-methyl acetate substituents onto phenyl ring have also importance, a location into 2 or 3 positions onto phenyl ring being favourable for activity, (Scheme **3**).



Scheme 3. Brominated **DA**: positions onto phenyl ring favourable for activity.

CONCLUSION

In conclusion, we reported herein an efficient and general method for preparation of new diazine with dihydroxyacetophenone skeleton under conventional TH, MW and US irradiation. MW and US irradiation induce a remarkable acceleration for reactions and, in some cases, the yields were higher. The *in vitro* antimicrobial and anticancer activities of the **DA**, **BrDA** and **DDA** compounds were evaluated. The tested compounds have a significant antibacterial activity against Gram-positive strain *Bacillus cereus* ATCC 14579 and, more important, against drug resistant Gram-negative strain *Pseudomonas aeruginosa* ATCC 27853. The brominated derivatives **BrDA** have the most powerful antibacterial activity, the influence of bromine atom being certain. All the tested compounds have a moderate antifungal activity. The *in vitro* antitumoral evaluation revealed that some of the tested compounds show high or moderate anticancer activity. The brominated derivatives **BrDA** proved to be the most active against tested HeLa cells, suggesting again (as for the antimicrobial assay) a certain influence of bromine atom concerning anticancer. In the brominated **BrDA** series, the position of substituents onto phenyl ring seems to be important concerning anticancer activity.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

To CNCS Bucharest, Romania, for financial support, project PN-II-DE-PCE-2011-3-0038, no. 268/05.10.2011.

SUPPORTING INFORMATION

Details of the NMR spectra (^{1}H NMR and ^{13}C NMR for compounds **2c**, **3c**, **4c** and **5c**) and the preliminary biological screening which has been done in order to evaluate the biological activity of the synthesized compounds can be found in the Supporting Information.

Supplementary material is available on the publisher's web site along with the published article.

REFERENCES

[1] Grahman, P.L. *An introduction to medicinal chemistry*, 2nd ed.; Oxford University Press, **2001**.

[2] Avendaño, C.; Menéndez, J.C. *Medicinal chemistry of anticancer drugs*, Elsevier: Amsterdam, **2008**.

[3] Mangalagiu, I.I. Recent achievements in the chemistry of 1,2-diazines. *Curr. Org. Chem.*, **2011**, *15*, 730-752.

[4] Wermuth, C.G. Are pyridazines privileged structures? *Med. Chem. Commun.*, **2011**, *2*, 935-941.

[5] Demirayak, S.; Karaburun, A.C.; Beis, R. Some pyrrole substituted aryl pyridazinone and phthalazinone derivatives and their antihypertensive activities. *Eur. J. Med. Chem.*, **2004**, *39*, 1089-1095.

[6] Akahane, A.; Katayama, H.; Mitsunaga, T. Discovery of 6-oxo-3-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl)-1(6*H*)-pyridazinebutanoic acid (FK 838): a novel non-xanthine adenosine A_1 receptor antagonist with potent diuretic activity. *J. Med. Chem.*, **1999**, *42*, 779-783.

[7] Saracoglu, M.; Kandemirli, F. The structure-AChE inhibitory activity relationships study in a series of pyridazine analogues. *Med. Chem.*, **2009**, *5*(4), 325-335.

[8] Asif, M.; Singh, A.; Siddiqui, A.A. The effect of pyridazine compounds on the cardiovascular system. *Med. Chem. Res.*, **2012**, *21*, 3336-3346.

[9] Zbancioc, A.M.; Zbancioc, G.; Tanase, C.; Miron, A.; Ursu, C.; Mangalagiu, I.I. Design, synthesis and *in vitro* anticancer activity of a new class of dual DNA intercalators. *Lett. Drug Des. Discov.*, **2010**, *7*, 644-649.

[10] De Clercq, E. New approaches towards anti-HIV chemotherapy. *J. Med. Chem.*, **2005**, *48*, 1297-1313.

[11] Refaat, H.M.; Khalil, O.M.; Kadry, H.H. Synthesis and anti-inflammatory activity of certain piperazinylthienylpyridazine derivatives. *Arch. Pharm. Res.*, **2007**, *30*, 803-811.

[12] Butnariu, R.; Mangalagiu, I.I. New pyridazine derivatives: synthesis, chemistry and biological activity. *Bioorg. Med. Chem.*, **2009**, *17*, 2823-2829.

[13] Mantu, D.; Luca, M.C.; Moldoveanu, C.; Zbancioc, G.; Mangalagiu, I.I. Synthesis and antituberculosis activity of some new pyridazine derivatives. Part II. *Eur. J. Med. Chem.*, **2010**, *45*, 5164-5168.

[14] Balan, A.M.; Florea, O.; Moldoveanu, C.; Zbancioc, G.; Iurea, D.; Mangalagiu, I.I. Diazinium salts with dihydroxyacetophenone skeleton: Syntheses and antimicrobial activity. *Eur. J. Med. Chem.*, **2009**, *44*, 2275-2279.

[15] Basabe, P.; Román, M.; Marcos, I.S.; Diez, D.; Blanco, A.; Bodero, O.; Mollinedo, F.; Sierra, B.G.; Uronesa, J.G. Prenylflavonoids and prenyl/alkyl-phloroacetophenones: synthesis and antitumour biological evaluation. *Eur. J. Med. Chem.*, **2010**, *45*, 4258-2269.

[16] Loupy, A. *Microwaves in organic synthesis*, 2nd ed.;Wiley-VCH: Weinheim, **2006**.

[17] Mason, T.J.; Peters, D. *Practical sonochemistry*, 2nd ed.; Ellis Horwood: London, **2002**.

[18] Perreux, L.; Loupy, A. A tentative realization of microwave effects in organic synthesis according to the reaction medium, and mechanistic consideration. *Tetrahedron*, **2001**, *57*, 9199-9223.

[19] Mason, T.J. Sonochemistry and the environment-providing a "green" link between chemistry, physics and engineering. *Ultrason. Sonochem.*, **2007**, *14*, 476-483.

[20] Caprosu, M.; Butnariu, R.; Mangalagiu, I.I. Synthesis and antimicrobial activity of some new pyridazine derivatives. *Heterocycles*, **2005**, *65*, 1871-1879.

[21] Luca, M.C.; Tura, V.; Mangalagiu, I.I. Considerations concerning design and mechanism of action of a new class of dual DNA intercalators. *Med. Hyp.*, **2010**, *75*, 627-629.

[22] Zbancioc, G.; Zbancioc, A.M.; Mantu, D.; Miron, A.; Tanase, C.; Mangalagiu, I.I. Ultrasounds assisted synthesis of highly functionalized acetophenone. *Rev. Roum. Chim.* **2010**, *55*, 983-987.

[23] Brown, D.F.J.; Blowers, R. *Disc methods of sensitivity testing and other semiquantitative methods*. In: Reeves, D.S.; Phillips, I.; Williams, J.D.; Wise, R. eds. *Laboratory methods in antimicrobial chemotherapy*; Churchill Livingstone: Edinburgh, **1978**, pp. 8-23.

[24] Doyle, A.; Griffiths, J.B. *Cell and tissue culture: laboratory procedures in biotechnology*, John & Sons Ltd: West Sussex, **1998**.

[25] Leiter, J.; Abott, D.J.; Schepartz, S.A. Screening data from the Cancer Chemotherapy National Service Center screening laboratories. XXVI. *Cancer Res.*, **1965**, *25*, 1253-1431.

[26] De Leon, G.P.; Elowe, N.H.; Koteva, K.P.; Valvano, M.A.; Wright, G.D. An *in vitro* screen of bacterial lipopolysaccharide biosynthetic enzymes identifies an inhibitor of ADP-heptose biosynthesis. *Chem. Biol.*, **2006**, *13*, 437-441.

[27] Mighri, H.; Hajlaoui, H.; Akrout, A.; Najjaa, H.; Neffati, M. Antimicrobial and antioxidant activities of *Artemisia herba-alba* essential oil cultivated in Tunisian arid zone. *C. R. Chim.*, **2010**, *13*, 380-386.

[28] Apetrei, C.L.; Spac, A.; Brebu, M.; Tuchilus, C.; Miron, A. Composition, and antioxidant and antimicrobial activities of the essential oils of a full-grown *Pinus cembra* L. tree from the Calimani Mountains (Romania). *J. Serb. Chem. Soc.*, **2013**, *78* (1), 27-37.

[29] Wayne, P.A. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard*, 9th ed., CLSI publication M07-A9, **2012**.

[30] Oyama, V.; Eagle, H. Measurement of cell growth in tissue culture with a phenol reagent (folin-ciocalteau). *Proc. Soc. Exp. Biol. Med.*, **1956**, *91*, 305-307.

Received: April 19, 2013 Revised: October 03, 2013 Accepted: October 26, 2013