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Research Article

Synthesis and Antimicrobial Activities of a Series of Disubstitutedarylazo-Barbituric- and Thiobarbituric Acid Derivatives

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Abstract

A simple, convenient and reproducible synthesis of several new disubstitutedarylazo-barbituric and thiobarbituric acids is described. The method involves condensation with urea and thiourea of 2,4-disubstituted phenylhydrazones formed from the reactions of the parent aniline diazonium salts with diethylmalonate. These new compounds were preliminary evaluated for their antimicrobial activities against five bacterial strains covering both (Gram positive and Gram negative bacterial strains) and two fungi. Three of the newly-synthesized compounds showed promising antimicrobial activities with no signs of human cells cytotoxicity.

Keywords: Arylazo-barbiturates, arylazo-thiobarbiturates, condensation reactions, cytotoxic activity, antimicrobial activity.

1. Introduction

Barbiturates and thiobarbiturates are a well-studied and important class of medicinal compounds. Barbituric acid or 2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione (**1**) is the lead compound of all barbiturates. The synthesis of simple barbituric or thiobarbituric acids involves the condensation of malonic acid esters with urea or thiourea, Despite their longevity in pharmaceutical use, these compounds continue to attract attention due to the wide range of biological activities that they exhibit. The use of this class of compounds include sedatives, hypnotics and antiepileptic drugs.[1] Furthermore, barbiturates and thiobarbiturates are reported to have antibacterial, antiurease and antioxidant activities.[2] In addition, some derivatives bearing the pyrimidine-2,4,6-trione unit are reported to have a potential therapeutic effect against diet-induced non-alcoholic fatty liver disease.[3] Also reported are some compounds which include a thiobarbiturate unit that are used as sirtuin inhibitors.[4] The substitution on the pyrimidine-2,4,6-trione moiety is the effective pharmacophore in many other compounds showing anti-invasive, antitumor and antiangiogenic effects,[5] and also as promising lead compounds for amyotrophic lateral sclerosis.[6] Recently, pyrimidine-2,4,6-triones attached to aryl hydrazones have been reported to be Ribosomal S6 Kinase 2 (RSK-2) inhibitors.[7]

We have been interested in the synthesis of analogues of these barbituric acid arylhydrazone compounds, namely **4a-d** and the thia analogues **5a-d**, as shown in **Scheme 1** in order to test for their potential biological properties. A search of the recent literature revealed that several published reports describe the syntheses of various substituted phenylhydrazones attached to the pyrimidine-2,4-6-trione nucleus. Xue *et al.* [7] and Shaaban *et al.* [8] reported the synthesis of other barbituric acid and thiobarbituric acid aryl hydrazone derivatives. Kandhavelu *et al.* reported the synthesis of such compounds by the azocoupling reaction of an aniline diazonium salt with barbituric acid under NaOH or NaOAc conditions.[9] Using similar methodology, Using a different approach, Jursic and

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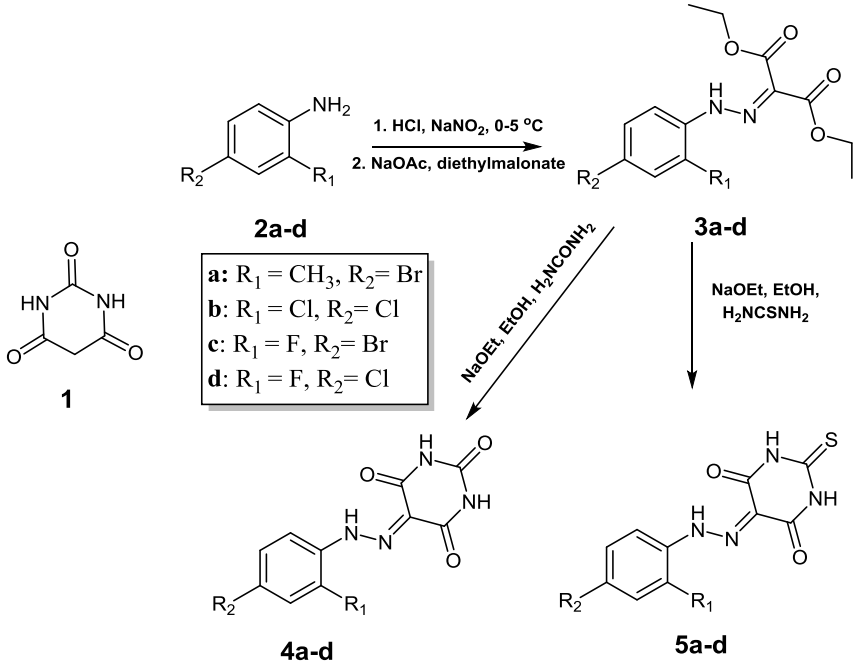
Neumann reported the synthesis of several nitrophenylhydrazones formed by the reaction of 5-formyl and 5-acetylbarbituric acids with 4-nitrophenylhydrazine.[10] To the best of our knowledge, only two of the compounds which we report herein namely, **4b** and **5b** have appeared in the literature, but neither their methods of preparation nor their physical properties were reported.[11] A very recent paper by Sahoo *et al* [12] which appeared after the present work was conducted, reported some other substituted aryl derivatives and their antimicrobial activities.

Herein we report on our studies on the efficient synthesis of some previously-unreported 2,4-disubstituted phenyl pyrimidinone hydrazones as outlined in **Scheme 1**. The compounds reported herein were achieved in good isolated yields, using simple routes which did not require any need for further purification for the resulting products.

2. Materials and Methods

2.1 Experimental

Chemicals were purchased from Alfa-Aesar or Sigma-Aldrich and were used without further purification. Melting points were determined with a MPA 100 Optimelt automated melting point system and are uncorrected. ^{1}H NMR (300MHz) and ^{13}C NMR (75 MHz) spectra were recorded in DMSO-d_6 on a Bruker AVANCE III 300MHz. All chemical shifts are reported in δ (ppm) using TMS as an internal standard. APCI mass spectra (in positive mode) were recorded on LC/MSD (Trap) Agilent 1100 series SL. APPI HRMS (+mode) were recorded on a LC/MSD - TOF Agilent 6200 series instrument.



Scheme 1: Synthesis of arylazo- barbituric and thiobarbituric acid derivatives **4a-d** and **5a-d**.

General Procedure for the synthesis of Diethyl 2-(2-(2,4-disubstituted phenyl)hydra-zono-malonate (3a-d): A solution of the substituted aniline derivative **2a-d** (20 mmol) in aqueous concentrated HCl (6.0 ml) was cooled to 0-5 ℃, and an ice-cooled solution of sodium nitrite (2.0 g in 10 ml H_2O) was added dropwise to it over 20 min with vigorous stirring, to afford the diazonium salt of the aniline derivative .To an ice-cooled solution of the corresponding active methylene compound i.e. diethylmalonate (3.0 ml, 20 mmol) and sodium acetate (1.64 g, 20 mmol) in EtOH, the diazonium salt was added dropwise and the reaction mixture was stirred for 1 h. The formed solid was filtered, washed with 10 ml deionized water and crystallized from EtOH to afford the corresponding phenyl hydrazones **3a-d**.

Diethyl 2-(2-(4-bromo-2-methylphenyl)hydrazono-malonate (3a): Light yellow crystals; yield: 5.7g (80%); mp 90.2℃. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 12.36 (s, 1H), 7.53 – 7.37 (m, 3H), 4.30 (dq, *J* = 21.0, 7.1 Hz, 4H), 2.31 (s, 3H), 1.30 (td, *J* = 7.1, 3.8 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 162.34, 133.16, 130.00, 126.62, 121.79,

115.83, 115.69, 99.49, 61.37, 60.78, 15.95, 14.07, 13.85. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{14}H_{17}BrN_2O_4]$: 356.0372 ; found:356.0379.

Diethyl 2-(2-(2,4-dichlorophenyl)hydrazono-malonate (3b): Orange crystals; yield: 5.7g (85%); mp 96.4 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 12.59 (s, 1H), 7.71 (d, *J* = 2.2 Hz, 1H), 7.58 (d, *J* = 8.9 Hz, 1H), 7.49 (dd, J = 8.9, 2.3 Hz, 1H), 4.30 (dq, *J* = 19.2, 7.1 Hz, 4H), 1.29 (td, *J* = 7.1, 1.7 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ =162.02, 161.80, 137.09, 129.11, 128.85, 127.76, 123.75, 120.07, 116.49, 61.73, 61.09, 14.01, 13.80. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{13}H_{14}Cl_2N_2O_4]$: 332.0331 ; found:332.0338.

Diethyl 2-(2-(4-bromo-2-fluorophenyl)hydrazono-malonate (3c): Black crystals; yield: 5.50g (76%); mp 70.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 12.23 (s, 1H), 7.74 – 7.63 (m, 1H), 7.56 – 7.41 (m, 2H), 4.30 (dq, *J* = 17.7, 7.1 Hz, 4H), 1.30 (td, *J* = 7.1, 1.8 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 162.02, 161.77, 152.03, 148.75, 129.62, 129.51, 128.56, 128.51, 123.77, 119.26, 118.98, 117.09, 114.83, 114.71, 61.61, 61.01, 14.01, 13.76. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{13}H_{14}BrFN_2O_4]$: 360.0121 ; found:360.013.

Diethyl 2-(2-(4-chloro-2-fluorophenyl)hydrazono-malonate (3d): Brown crystals; yield: 5.05g (80%); mp 73.4 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 12.22 (d, *J* = 2.2 Hz, 1H), 7.64 – 7.48 (m, 2H), 7.40 – 7.29 (m, 1H), 4.29 (dq, *J* = 18.1, 7.1 Hz, 4H), 1.29 (td, *J* = 7.1, 1.8 Hz, 6H). ^{13}C NMR (75 MHz, DMSO- d_6): δ = 162.03, 161.77, 151.96, 148.70, 129.25, 129.13, 127.43, 127.30, 125.71, 125.66, 123.71, 116.76, 116.62, 116.33, 61.61, 61.02, 14.00, 13.76. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{13}H_{14}ClFN_2O_4]$: 316.0626 ; found:316.0639.

General Procedure for the synthesis of 5-(2,4-disubstituted phenyl hydrazono-pyrimidine-2,4,6(1H,3H,5H)-trione (4a-d) / 5-(2-(2,4-disubstituted phenylhydrazono)-2-thioxodihydro-pyrimidine-4,6(1H,5H)-dione (5a-d): To an ethanolic 21% solution of sodium ethoxide (10.0 ml) in a round-bottom flask, under N_2 atmosphere, **3a (3b, 3c or 3d** , 5.0 mmol) in absolute ethanol (20 ml) was added, followed by a hot solution of urea or thiourea (5.0 mmol) in absolute ethanol (10.0 ml) and heated at reflux for ~ 2-4 h. The reaction was stopped and treated with hot water (40 ml). Aqueous concentrated HCl was added to the hot solution until the solution became acidic to litmus paper. The resulting solution was then cooled in a refrigerator for 2 h. The resulting solid product was filtered and washed with cold water to afford the corresponding compounds (**4a-d** and **5a-d**) without any further purification.

5-(2-(4-bromo-2-methylphenylhydrazono)-pyrimidine-2,4,6(1H,3H, 5H)-trione (4a): Brown solid; yield: 1.35 g (83 %); mp 314 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 14.38 (s, 1H), 11.63 (s, 1H), 11.36 (s, 1H), 7.63 – 7.45 (m, 3H), 2.34 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 162.73, 159.63, 149.69, 138.60, 133.39, 130.35, 127.95, 119.01, 117.73, 116.54, 15.91. MS (APCI+): *m/z* = 325.0 $[M+H]^+$. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{11}H_9BrN_4O_3]$: 323.9858; found: 323.9874.

5-(2-(2,4-dichlorophenylhydrazono)-pyrimidine-2,4,6(1H,3H,5H)-trione (4b): Light yellow solid; yield: 1.28 g (85 %); mp 340 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 14.44 (s, 1H), 11.72 (s, 1H), 11.45 (s, 1H), 7.82 – 7.74 (m, 2H), 7.57 (dd, *J* = 8.9, 2.3 Hz, 1H).^{13}C NMR (75 MHz, DMSO-d_6): δ = 162.50, 159.34, 149.67, 136.86, 129.46, 129.29, 129.10, 121.29, 120.69, 117.31. MS (APCI+): *m/z* = 301.0 $[M+H]^+$. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{10}H_6Cl_2N_4O_3]$:299.9817; found: 299.9822.

5-(2-(4-bromo-2-fluorophenylhydrazono)-pyrimidine-2,4,6(1H,3H,5H)-trione (4c): Dark yellow solid; yield: 1.20 g (73 %); mp 331 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 14.24 (s, 1H), 11.69 (s, 1H), 11.42 (s, 1H), 7.77 (dd, *J* = 10.9, 2.0 Hz, 1H), 7.65 (t, *J* = 8.5 Hz, 1H), 7.54 (ddd, *J* = 8.8, 2.1, 1.0 Hz, 1H).^{13}C NMR (75 MHz, DMSO-d_6): δ = 162.64, 159.38, 152.68, 149.64, 149.37, 129.08, 128.96, 128.91, 120.45, 119.55, 119.27, 117.73, 116.99, 116.87.. MS (APCI+): *m/z* = 329.0 $[M+H]^+$. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{10}H_6BrFN_4O_3]$: 327.9607 ; found:327.9608.

5-(2-(4-chloro-2-fluorophenylhydrazono)-pyrimidine-2,4,6(1H,3H, 5H)-trione (4d):
Green solid; yield: 1.10 g (77 %); mp 329 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 14.25 (s, 1H), 11.69 (s, 1H), 11.42 (s, 1H), 7.76 – 7.61 (m, 2H), 7.42 (ddd, *J* = 8.8, 2.3, 1.2 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 162.62, 159.36, 152.63, 149.63, 149.34, 129.41, 129.28, 128.70, 128.58, 126.08, 126.04, 120.38, 117.40, 116.87, 116.59.

MS (APCI+): *m/z* = 285.0 $[M+H]^+$. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{10}H_6ClFN_4O_3]$: 284.0112; found: 284.011.

5-(2-(4-bromo-2-methylphenylhydrazono)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (5a): Orange solid; yield: 1.40 g (82 %); mp 317 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 14.42 (s, 1H), 12.56 (s, 2H), 7.68 – 7.43 (m, 3H), 2.36 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) :δ = 177.46, 160.52 , 157.85 , 138.56 , 133.50 , 130.47 , 128.50 , 119.93 , 118.34 , 116.85 , 15.93 . MS (APCI+): *m/z* = 341.0 $[M+H]^+$. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{11}H_9BrN_4O_2S]$: 339.963; found: 339.9632.

5-(2-(2,4-dichlorophenylhydrazono)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (5b):
Dark yellow solid; yield: 1.34 g (85 %); mp 325 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 14.51 (s, 1H), 12.77 (s, 1H), 12.57 (s, 1H), 7.81 (d, *J* = 2.2 Hz, 1H), 7.77 (d, *J* = 8.9 Hz, 1H), 7.58 (dd, *J* = 8.9, 2.3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 177.64, 160.41, 157.81, 136.68, 129.94, 129.40, 129.21, 121.68, 121.46, 117.60.MS (APCI+): *m/z* = 317.0 $[M+H]^+$. HRMS (+APPI): *m/z* $[M+ H]^+$ calcd for $[C_{10}H_6Cl_2N_4O_2S]$: 315.9589; found: 315.961.

5-(2-(4-bromo-2-fluorophenylhydrazono)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (5c): Orange solid; yield: 1.14 g (66 %); mp 313 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 14.34 (s, 1H), 12.76 (s, 1H), 12.56 (s, 1H), 7.81 (dd, *J* = 10.8, 2.0 Hz, 1H), 7.68 (t, *J* = 8.5 Hz, 1H), 7.57 (ddd, *J* = 8.8, 2.1, 0.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 177.59, 160.53, 157.83, 149.57, 129.07, 121.23, 119.66, 119.39, 117.99, 117.59, 117.48. MS (APCI+): *m/z* = 345.0 $[M+H]^+$. HRMS (+APPI): *m/z* $[M+H]^+$ calcd for $[C_{10}H_6BrFN_4O_2S]$: 343.9379; found: 343.9399.

5-(2-(4-chloro-2-fluorophenylhydrazono)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (5d): Orange solid; yield: 1.20 g (80 %); mp 315 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 14.35 (s, 1H), 12.76 (s, 1H), 12.56 (s, 1H), 7.76 (d, *J* = 8.7 Hz, 1H), 7.73 – 7.67 (m, 1H), 7.45 (ddd, *J* = 8.8, 2.2, 1.2 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 177.58 , 160.51 , 157.81 , 152.84 , 149.54 , 129.95 , 129.82 , 128.55 , 128.43 , 126.19 , 121.15 , 117.68 , 116.97 , 116.68. MS (APCI+): *m/z* = 301.0 $[M+H]^+$. HRMS (+APPI): *m/z* $[M + H]^+$ calcd for $[C_{10}H_6ClFN_4O_2S]$: 299.9884; found: 299.9901.

2.2 Primary antimicrobial screening

Primary antimicrobial screening was conducted by whole cell growth inhibition assays, of each sample tested at a single concentration, in duplicate (n=2). The inhibition of growth was measured against 5 bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (MRSA; ATCC 43300), and 2 fungi: *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (ATCC 208821).

Sample preparation and Antimicrobial Assays

Solutions of the samples were made to 10mg/mL in DMSO and stored at -20 ºC. An aliquot of each sample was further diluted to 320 µg/mL in water in 384-well polypropylene plates (PP), and 5 µL was plated in duplicate (n=2) into a 384-well nonbinding surface plate (NBS) for each strain assayed against. All bacteria were cultured in Cation-adjusted Muller Hinton broth (CAMHB) at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh broth and incubated at 37 °C for 1.5-3 h. The resultant mid-log phase cultures were diluted (CFU/mL measured by OD600),, then 45 µL was added to each well of the compound containing plates, giving a cell density of 5×105 CFU/mL and a final compound concentration of 32 µg/mL for the tested samples. All the plates were covered and incubated at 37 °C for 18 h without shaking. Inhibition of bacterial growth was determined using resazurin as a marker for cell viability. Resazurin was added to each well, at 0.001% final concentration, and plates incubated at 37 °C for 2h. Fluorescence intensity is measured, using F (top read), ex 560/10 nm, em 590/10 nm (F560/590), using a Tecan M1000 Pro monochromator plate reader. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. The significance of the inhibition values was determined by Z-scores, calculated using the average and standard deviation of the sample wells (no controls) on the same plate. Samples with inhibition value above 50- 80% and Z-Score above 2.5 for either replicate (n=2 on different plates) were classed as actives.

Fungi strains were cultured for 3 days on Yeast Extract-Peptone Dextrose (YPD) agar at 30 °C. A yeast suspension of 1 x 106 to 5 x 106 cells/mL (as determined by OD530) was prepared from five colonies. These stock suspensions were diluted with Yeast Nitrogen Base (YNB) broth to a final concentration of 2.5 ×103 CFU/mL. Then, 45 µL of the fungi suspension was added to each well of the compound-containing plates, giving a final concentration of 32 µg/mL for the tested samples. Plates were covered and incubated at 35 °C for 24 h without shaking. Growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm (OD530), while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm (OD600-570), after the addition of resazurin (0.001% final concentration) and incubation at 35 °C for additional 2 h. The absorbance was measured using a Biotek Synergy HTX plate reader. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate. The significance of the inhibition values was determined by Z-scores, calculated using the average and standard deviation of the sample wells (no controls) on the same plate. Samples with inhibition value above 50- 80% and Z-Score above 2.5 for either replicate (n=2 on different plates) were classed as actives.

Colistin and Vancomycin were used as positive bacterial inhibitor standards for Gram-negative and Gram-positive bacteria, respectively. Fluconazole was used as a positive fungal inhibitor standard for *C. albicans* and *C. neoformans*. The antibiotics were provided in 4 concentrations, with 2 above and 2 below its MIC value,and plated into the first 8 wells of column 23 of the 384-well NBS plates. The quality control (QC) of the assays was determined by the antimicrobial controls and the Z'-factor (using positive and negative controls). Each plate was deemed to fulfil the quality criteria (pass QC), if the Z'-factor was above 0.4, and the antimicrobial standards showed full range of activity, with full growth inhibition at their highest concentration, and no growth inhibition at their lowest concentration.

2.3 Cytotoxicity

HEK293 cells were counted manually in a Neubauer haemocytometer and plated at a density of 4000 cells/well into each well of the 384-well plates containing the 25x (2 µL) concentrated compounds. The medium used was Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Cells were incubated together with the compounds for 20 h at 37 ^{o}C, 5% CO_2. To measure cytotoxicity, 5 µL (equals 100 µM final) Resazurin was added to each well after incubation, and incubated for further 3 h at 37 ^{o}C with 5% CO_2. After final incubation fluorescence intensity was measured as Fex 560/10 nm, em 590/10 nm ($F_{560/590}$) using a Tecan M1000 Pro monochromator plate reader. CC_{50} values (concentration at 50% cytotoxicity) were calculated by normalizing the fluorescence readout, with 74 µg/ml tamoxifen as negative control (0%) and normal cell growth as positive control (100%). The concentration-dependent percentage cytotoxicity was fitted to a dose response function (using Pipeline Pilot) and CC_{50} values determined. In addition, the maximal percentage of cytotoxicity is reported as DMax, indicating any compounds with marginal cytotoxicity or reduced efficacy. Both replicates were analysed and are reported separately. Hits were classified as cytotoxic by $CC_{50} \leq 32$ µg/mL in either replicate (n=2 on different plates)

3. Results and Discussion

3.1 Synthesis

The desired phenylhydrazones **4a-d** and **5a-d** were synthesized by the reaction of the aniline diazonium salts intermediates formed from the corresponding anilines **2a-d** with diethylmalonate. The condensation reactions gave the corresponding barbiturate-arylazo derivatives **4a-d** and **5a-d** as shown in Scheme 1. Initially we employed the experimental procedures described by others [7-9] to form the barbituric acid aryl hydrazone derivatives. In our hands however, these methods produced only low yields of the target compounds and with many impurities as ascertained by TLC monitoring. The desired compounds however could be produced reproducibly and with increased isolated yields and purity and characterized using the methodology described herein, although the yields which we report were not optimized under different conditions to achieve maximal yields.

The structures of **4a-d** and **5a-d** were determined and confirmed by their ^{1}H, ^{13}C-NMR, MS and HRMS spectra. In particular, a characteristic pair of signals were present in the ^{1}H NMR spectra in the δ = 11.72-11.36 ppm range (D_2O exchangeable) for the NH protons of the pyrimidine-2,4,6-trione rings in **4a-d**, and in the δ = 12.77-

12.56 ppm range (D_2O exchangeable) for **5a-d** for the corresponding NH protons of the thioxodihydropyrimidine-4,6-dione ring protons. [13]

3.2 Antimicrobial Screening and Cytotoxicity

Compounds were deemed to be active at a single concentration of 32 µg/mL, n=2, where one or both replicates showed inhibition of ≥ 50-80% and Z-Score ≥ 2.5, as shown in **Table 1**.

Table 1: Percentage inhibition for compounds **4a-d** and **5a-d** from 2 duplicates at 32µg/mL

	4a	**4b**	**4c**	**4d**	**5a**	**5b**	**5c**	**5d**
S. aureus	30.7;31.5	23.2;26.9	25.3;30.0	23.6;28.6	25.9;30.7	27.2;33.5	**57.6;61.8**	**62.1;67.0**
E.coli	2.0;3.1	0.3-0.4	3.3;2.8	2.6;4.7	12.1;0.5	8.8;0.3	6.0;7.0	8.1;6.4
K.pneumoniae	16.1;1.9	12.3;1.0	6.8;2.7	10.7;10.5	8.5; 2.2	5.4;5.0	5.5;4.9	9.2;4.0
A.baumanii	13.3;5.7	10.0;4.2	7.9;2.1	14.5;3.2	5.3; 7.7	19.0;0.9	2.5;1.3	14.1;5.2
P.aeurginosa	6.6-3.8	8.3;3.0	15.2;3.7	**50.0;100**	13.8;2.5	7.9;1.9	0.2;1.5	4.5;0.1
C.albicans	3.1;6.3	0.5;2.3	0.4;4.1	1.2;4.6	11.9;6.5	5.9;8.9	3.5;6.6	5.6;13.9
C.neoformans	1.0;4.9	3.2;2.1	0.8;1.8	1.2;3.2	0.1;4.3	14.3;7.3	8.0;0.5	1.1;10.0

The results of the antimicrobial screening (at 32 µg/mL) revealed that **4d** had high activity against the Gram-negative bacteria, *P.aeurginosa*, ranging from 50-100% inhibition whereas its bromo analogue **4c**, showed no comparable activity with any of the tested strains. Furthermore, both **5c** and **5d** showed significant activity (58-68% inhibition) against the Gram-positive *S. aureus* (MRSA). No other significant activity was observed for the other compounds which were tested at the concentration levels and with the same bacterial and fungal strains tested. All of the compounds which were tested proved to be non-cytoxic against the human embryonic kidney cell line, HEK293. All of the measured CC_{50} values were > 32 µg/mL.

In conclusion, we report herein a simple, convenient and reproducible synthesis of several new disubstitutedarylazo-barbituric and thiobarbituric acid derivatives of which three of the newly-synthesized compounds showed significant antibacterial activities with no observable cytotoxicity.

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[13] The NMR spectra of all of the new compounds reported herein can also be found in the Supporting Information which can be obtained directly upon request from the corresponding author.