



GENOTOXICITY, ANTIFUNGAL AND ANTIBACTERIAL ACTIVITY OF NEWLY SYNTHESIZED N-(3-PHTHALIDYL) AMINES AND o-BENZOYL BENZAMIDE DERIVATIVES

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A number of newly synthesized phthalidylamines and o-benzoylbenzamide derivatives were evaluated for some biological activities. Synthesis was established by condensation of 3-acetoxypthalide 1 with morpholine, piperidine, N,N-diisobutyl-N,N-dibenzylamines and piperazine, which afforded N-(3-phthalidyl)amines 3a-d and 4 respectively, while with N,N-diisopropylamine o-formyl N,N-diisopropyl benzamide 5a is formed exclusively. On the other hand the reaction of 3-acetoxy-3-phenylphthalide 2 with secondary amines afforded o-benzoylbenzamide derivatives 5b-c, 6 in high yield. The structure of the reaction products was established from their spectral data. These products were screened for antifungal, antibacterial and genotoxic effect. It was found that all tested compounds have antifungal activity. Compound 1, 2, 3d and 5b were found to be active against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. Genotoxic effects using Ames test showed that compound 1 and 2 have a weak base-pair substitution mutagenicity while a clear base-pair substitution mutagenicity was shown by 3a using TA100-strain of *Salmonella typhimurium*. Compound 4 showed a frameshift mutagenicity while a weak oxidative mutagenic action was revealed by 6. No change in the mutagenicity of the tested chemicals was observed after using the S9 metabolic action system.

Key words: antibacterial; antifungal; mutagenesis; phthalidylamines; o-benzoylbenzamide.

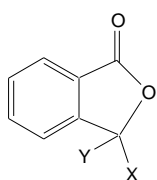
INTRODUCTION

The continuous appearance of resistant and multi-resistant pathogenic bacteria and fungi has promoted a continuous search for new antibacterial and/or antifungal drugs [1]. Phthalides in general including those containing nitrogen moiety and benzamide derivatives are of interest because of their biological activities including antimicrobial activities [2,3], hypertensive and vasorelaxant [4], anti-inflammatory through protection of ischemic sites following ischemic brain injury [5] in addition to their other two compounds.

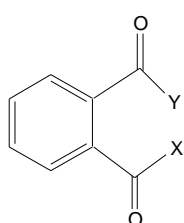
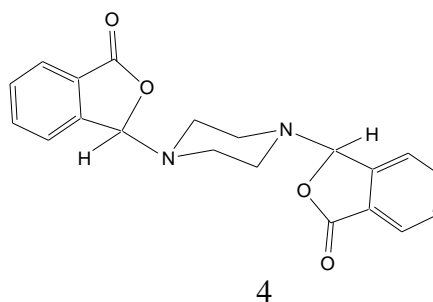
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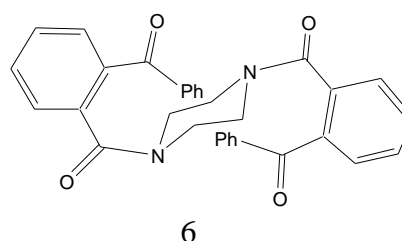
GENOTOXICITY, ANTIFUNGAL AND ANTIBACTERIAL ACTIVITY



- 1- X=H, Y=OAC
2- X=Ph, Y=OAC
3a- X=H, Y= Morphilino
3b- X=H, Y= Piperidino
3c- X=H, Y= NCH₂ (CHCH₃)₂
3d- X=H, Y= N (CH₂Ph)₂



- 5a- X=H, Y= NCH (CH₃)₂
5b- X=Ph, Y=N (CH₂Ph)₂
5c- X=Ph, Y=N (CH₂CH (CH₃)₂)₂



TABEL I Antifungal and antibacterial activities [conc.350 µg/ml]

Compd. No.	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>B.subtilis</i>	<i>S.aureus</i>	<i>C.albicans</i>
1	-	+	+	+	+
2	-	+	+	+	+
3a	-	-	-	-	+
3b	-	-	-	-	+
3c	-	-	-	-	+
3d	-	+	+	+/-	+
4	-	-	-	-	+
5a	-	-	-	-	+
5b	-	+	+	+	+
5c	-	-	-	-	+

TABLE II Mutagenic activity of the studied compounds

Compd. No.	TA 98	TA 100	TA 102
1	-	~ +	-
2	-	~ +	-
3a	-	+	-
3b	-	+	-
3c	-	-	-
3d	-	-	-
4	+	-	-
5a	-	-	-
5b	-	-	-
5c	-	-	-
6	-	-	~ +



TABLE III Reversion of tester strains by compounds 1, 2, 3a, 4 and 6

Compound	Tester strains	concentration $\mu\text{g}/\text{plate}^{\text{a}}$	No. of revertants/plate ^b
1	TA 100	260	94
2	TA 100	215	107
3a	TA 100	55	218
4	TA 98	27	74
6	TA 102	74	182

^aThe concentration at which the highest mutagenic activity was obtained; ^bThe average of two experiments after subtracting the spontaneous revertants.

The concentration that gave the maximum number of his⁺ revertants were 74,260,215,55 and 27 $\mu\text{g}/\text{plate}$ for compounds 1, 2, 3a, 4 and 6 respectively. Using the S9 metabolic activation system did not lead to any significant increase or decrease in the mutagenicity of the investigated compounds. Concerning the toxicity of the investigated compounds, it could be detected at a concentration $\geq 500 \mu\text{g}/\text{plate}$ in the S.typhomurium strains.

DISSCUION

Kenneth et al.[13] reported that the reaction of 3-halophthalides with cyclic secondary amines afforded N-(o-formylbenzoyl)amines; a product of type 5. To our best knowledge the reaction of amines with 3-acetoxypthalide 1 has not been reported. In our laboratory, we realized that the reactions of secondary amines (open and/or cyclic)[13] with 3-acetoxypthalide 1 afforded exclusively; in opposite manner to that published by Kenneth et al.[13], N-(3-phthalidyl)amines 3a-d except with diisopropylamine, the reaction afforded compound 5a.

The two series of compounds investigated in the present study were synthesized as potential antimicrobial agents. The results of antimicrobial activity showed clearly that all tested compounds exhibited antifungal activity against yeast-like fungi, *C.albicans*. This indicates that such types of compounds are good candidates to be used as fungicidal drugs. The antibacterial activity of the above compounds revealed a rather good activity against three out of four used strains. However, such activity was shown by four compounds out of ten. This may be used as an indication to further improve such active compounds in order to both increase their activity against the fungi and the three strains of bacteria (table I) and to broaden their spectrum against Gram positive and Gram negative bacteria. Development of the other related series of organic compounds may also be recommended.

In the genotoxicity studies we used Ames test, a great advantage of which is that it allows the investigator to differentiate between frameshift, oxidative and base-pair substitution mutagens [14-16]. In the present investigation five compounds were found to be mutagenic although at relatively high concentrations. The results indicated also that the toxicity of such compound is not high in the salmonella strains used in this study. However, it is worthy mention that the mutagenicity of compounds 1, 2, 3a and 6 is weak. Compound 4, on the other hand, showed a clear frame shift mutagenic activity in TA98 strain. These findings may be attributed to the differences in chemical structures and/or chemical properties of the investigated compounds. Although four out of five



compounds were weakly mutagenic, we recommend: first ,further investigation using different systems to confirm their genotoxic activity ; second ,to take these results into consideration if any practical use of these compounds is under discussion . however ,the rest of the compounds including those of potential promising activity as antimicrobial agents have not shown any genetic material-hazardous effect in the used system. accordingly ,further studies of these compounds concerning their mutagenicity using different system such as SOS chromo test [17,18] or other test is high recommended . using mammalian system for further investigation of the formerly described compounds such as toxicity testing ,mouse micronucleus test, rat river UDS test,SCE in human lymphocytes and others is highly recommended [19-21].

CONCLUSION

In the present investigation, series of phthalidylamines and o-Benzoylbenzamide derivatives were synthesized in a distinguish type of reactions. The compounds, which were synthesized as potential antimicrobial agents were found to exhibit both antibacterial and antifungal action. Some of the tested compounds showed a weak mutagenic activity. Using the S9 metabolic activation system failed to induce changes in the mutagenic effects of the investigated compounds.

EXPERIMENTAL

Chemistry

Infrared spectra were recorded using perkin-Elmer FTIR SP2000 spectrometer. ¹H-NMR spectra were run on Bruker WP80-SY instrument using CDCL₃ containing TMS as internal standard. Mass spectra were recorded using VG 7070 EGC mass spectrometer .compounds were analyzed at M.H.W.laboratories, phoenix, Arizona, USA.melting points were determined on an electro thermal melting point apparatus and were uncorrected.

Synthesis of 3-acetoxypthalide (1) and 3-acetoxy-3-phenylphthalide (2)

General Procedure

A mixture of o-formyl-or o-benzoylbenzoic acid (5 g, 33mmole); fused sodium acetate (5 g)and acetic anhydride (50ml)was refluxed for 5h. The mixture was then poured into ice-cold water (50ml),stirred well until complete precipitation occur. the obtained solid was washed with water ,NaHCO₃ and water,then dried.according to this procedure the following compounds were prepared.

3-acetoxypthalide (1) The obtained solid product (5.5g,85%)was crystallized from n-hexane to afford a white crystalline material m.p.=68-70°C,IR (KBr cm⁻¹)1772 (acetoxo group), 1765(lactonic group), ¹H-NMR (CDCL₃),δ: 2.19 (s,3H,CH₃),7.43 (s,1H,H₃),7.69(dd,2H,H₅+H₆,J=6.2Hz),7.73(s,1H,H₄) and 7.9 (d,1H,H₇,J₁=6.0,J₂=0.3 Hz).mass spectrum m/z= 192(M⁺),149,133 (100%),132,122, 104.



3-phenyl 3-acetoxypthalide (2) From *o*-benzoyl benzoic acid the obtained solid products was recrystallized from cyclohexane to afford white crystals (1.8g,81%),m.p.=108-110 °C ,IR (KBr,cm-1)1777(acetoxy group) and 1753 (lactonic group),¹H-NMR (CDCl₃) δ:2.11 (s,3H,CH₃), 7.3-7.8 (m,8H,Ar-H) and 7.94 (d,1H,H₇, J= 5.1 Hz).mass spectrum m/z = (M⁺) not detected ,266,209(100%),180,152,105.

Reaction of 3-acetoxypthalide (1) with secondary Amines

General Procedure

A mixture of (11mmole)of 3-acetoxypthalide, (10mmole of the amines and ethanol 25ml was refluxed for about (16-20h)then cooled ,the separated crystals were collected otherwise the solvent was evaporated under vacuum to dryness. According to this procedure, the following syntheses were carried out.

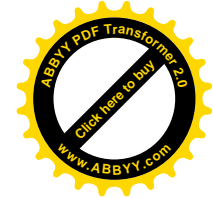
3-(4-morpholino)phthalide (3a) The obtained solid product was crystallized from *n*-hexane to afford a white crystalline material 3-(4-morpholino)phthalide (3a) (2.1g,87%)m.p.=(131-132) °C,IR (KBr cm-1) shows 1748 (lactonic group).¹H-NMR (CDCl₃) δ:2.56-2.78 (m,4H,2CH₂), 3.70 (t, 4H, 2CH₂-O , J=4.6 Hz) , 6.19(s,1H,H₃) 7.56-7.63 (dd,2H, H₅+ H₆) ,7.65 (s, 1H,H₄) ,7.78 (d,1H,H₇).mass spectrum shows m/z= 219 (M⁺)174,133,(100%),130,117,105.

3-(1-piperidyl) phthalide (3b) The obtained product was oily, ¹H-NMR of the crude product indicate the presences of the two components 3-(1-piperidyl)phthalide (3b); 73%; and *N*-(*o*-formyl benzoyl)piperidine (5d), 24%.Treating the mixture with hot petroleum ether (40-60) °C,afforded 3-(1-piperidyl)phthalide (3b) (1.6g, 70%) m.p = 94-95°C ,whose molecular formula is C₁₃H₁₅NO₂, IR (KBr cm-1),1745(lactonic group),¹H-NMR (CDCl₃) δ:1.52 (t,6H,3CH₂), 2.69 (t, 4H,2CH₂),6.23(s,1H,H₃), 7.4 - 7.82(m,3H,Ar-H),7.84-7.94 (d,1H,H₇, J=6.0 Hz).Mass spectrum ,m/z=(M⁺) 217,188 ,172,(100%),144, 133 .Elemental analysis calculated (found):C,71.87(71.95);H,6.96 (6.92);N,9.45(9.42)

3-(N,N-disobutylamino)phthalide (3c) The obtained semisolid product was crystallized from *n*-heptane to afford a white solid crystals3-(*N,N*- disobutylamino) phthalide (3c)(2.3g,80%),m.p= (56-58) °C,IR (KBr cm-1)1784 (lactonic group) .¹H-NMR (CDCl₃) δ:0.86 (d,12H,4CH₃,J=6.3 Hz),1.7-2.6 (m, 6H,2CH₂, 2CH),6.38 (s, 1H,H₇,J=6.1Hz).Mass spectrum m/z= 261(M⁺),218,174,160,133 (100%).

3-(N,N-dibenzylamino)phthalide (3d) The obtained product was crystallized from *n*-hexane to afford crystalline solid 3-(*N,N*-dibenzylamino)phthalide (3d),(2.2g,81%), m.p.=(104-106) °C whose molecular formula is C₂₂H₁₉NO₂.IR (KBr, cm-1) 1748 (lactonic group).¹H-NMR(CDCl₃) δ: 3.74 (s,4H,2CH₂) ,6.25 (s,1H,H₃),7.32(s,10H, Ar-H),7.21-7.28 (m,1H,H₄),7.57(s,2H,H₅+H₆) and 7.91 (d,1H,H₇,J=6.0Hz). Mass spectrum m/z= 329(M⁺),284,238,223,194(100%),165,133,116.Elemental analysis calculated (found)C₂₂H₁₉NO₂: C;80.24(80.16);H,5.77(5.64);N,4.25(4.28).

N,N-diisopropyl-o-formylbenzamide (5b) After evaporation , a solid product was obtained which was recrystallized form *n*-hexane to afford a white crystal, *N,N*-diisopropyl-*o*-formylbenzamide (5b),(2.2g,84%),m.p.=97°C ,whose molecular formula is C₁₄H₁₉NO₂,IR(KBr, cm-1)1690 (CHO),1633(CONR₂);¹H-NMR (CDCl₃) δ:1.29 (d,12H,4CH₃,J=6.5Hz),3.24-3.38(sept,2H,2CH),7.4-7.8 (m,4H,Ar-H)and 10.56(s,1H, CHO).Mass spectrum m/z=(M⁺) not detected,182,149,132,122,105(100%).Elemental analysis calculated (found);C,66.90(66.40);H,8.36(8.11);N,5.57(5.32).



N,N-Diphthalidyl piperazine (4) The reaction was carried out at room temperature in methanol, filtration of the solid afforded *N,N-Diphthalidyl piperazine (4)*, (3.0g, 76%), m.p.= 299-301°C decompose, whose molecular formula is C₂₀H₁₈N₂O₂. IR (KBr, cm⁻¹) shows 1743 (lactonic group). ¹H-NMR (CDCl₃) δ: 2.75 (s, 8H, 4CH₂-N), 6.19 (s, 1H, H₃), 6.24 (s, 1H, H₃), 7.5-7.9 (m, 8H, Ar-H). Mass spectrum m/z=(M⁺) not detected, 349, 305, 217, 215, 173, 149, 133 (100%), 105.

Reaction of 3-phenyl-3-acetoxypthalide (2) with secondary amines

General procedure

A mixture of (7mmole) of 3-phenyl-3-acetoxypthalide, (7mmole) of amines and ethanol 25ml was refluxed for about 20-46 h, then cooled. The separated solid was collected. Otherwise the solvent was evaporated under vacuum to dryness. According to this procedure the following synthesis were carried out. Each reaction of secondary amines with 3-phenyl-3-acetoxypthalide was found to require longer time about 26-46h in order to obtain a reasonable amount of product.

N,N-dibenzyl-o-benzoylbenzamide (5b) The obtained product was oily (2.9g, 95%). Treating the oily product with n-hexane afforded solid product *N,N-dibenzyl-o-benzoylbenzamide (5b)* (0.8g), m.p.=(150-152) °C, whose molecular formula is C₂₈H₂₃N₂O₂. IR (KBr, cm⁻¹) 1622 and 1666 due to the presence of (CONR₂) and (COPh), respectively. The ¹H-NMR spectrum shows δ: 3.56 (s, 4H, 2CH₂), 7.33-7.89 (m, 15H, Ar-H), 7.89-7.98 (m, 4H, Ar-H). Mass spectrum m/z=266, 209, 196, 182, 149, 121, 91 (100%). Elemental analysis calculated (found): C, 79.49 (79.73); H, 5.91 (5.94); N, 3.30 (3.22).

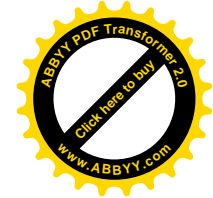
N,N-Diisobutyl-o-benzoylbenzamide (5c) The obtained product was oily (2.4g, 69%). Treating the oily product with n-heptane, afforded a solid product, m.p.=(138-140) °C (5c). ¹H-NMR in (CDCl₃) shows δ: 0.8 (d, 12H, 4CH₃, J=7.0Hz), 1.75-1.9 (sep, 2H, 2CH), 2.39 (d, 4H, 2CH₂, J=7.0Hz), 7.3-7.45 (m, 5H, Ar-H), 7.5-7.98 (m, 4H). The IR spectrum shows 1670 and 1630 cm⁻¹ due to (COPh and CONR₃).

N,N-Di-2-benzoylpiperazine (6) The solid product was recrystallized from methanol to afford *N,N-di-2-benzoylpiperazine (6)* (3.0g, 80%), m.p.=239°C, whose molecular formula is C₃₂H₂₆N₂O₄. IR (KBr, cm⁻¹) 1660 and 1630 indicated that (COPh) group and (CONR) group respectively. ¹H-NMR in (CDCl₃) shows δ: 3.4-3.8 (bd, 8H, 4CH₂), 7.41-7.7 (m, 14H, Ar-H), 7.79-7.87 (dd, 4H, 2H₄+2H₇, J=6.0Hz). Mass spectrum shows m/z=501, 485, 379, 293, 209 (100%), 181, 152. Elemental analysis calculated (found): C, 76.49 (76.43); H, 5.17 (5.0); N, 5.57 (5.54).

Biological activities

Bacterial strains

The following strains were used in antimicrobial study: *B.subtilis* ATCC 6633, *S.aureus* ATCC 25923, *E.coli* ATCC 2592 and *P.aeruginosa*, and *C.albicans*. For mutagenicity test: *S.typhimurium* strains TA98, TA 100 and TA 102 were used. The last strains were kindly supplied by Pro.B.N. Ames (Department of Biochemistry, university of California, Berkeley, U.S.A.).



Antimicrobial Activity

Preparation of the test chemicals: the test chemicals were dissolved in DMSO. Dextrose both was then added to get a starting concentration of 4mg/ml for each compound. The microorganisms were grown overnight in dextrose broth at 35°C and diluted to 10⁻³ just before being used. Plates were prepared by mixing each test chemical solution with the melted nutrient agar to get the desired final concentrations ranging from 450 down to 25µg/ml. The mixture were poured into Petri dishes and allowed to harden at room temperature. As positive controls, nalidixic acid as an antibacterial drug and miconazole as an antifungal drug were used. Each plate including positive and negative once was inoculated with a single streak using a 10µl calibrated loop. The plates were examined after 20h incubation at 35 °C for the presence or absence of bacterial growth [22].

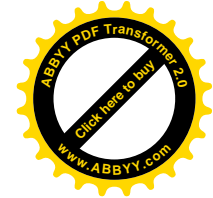
Mutagenicity studies

Stock solution of the test chemicals were prepared by dissolving 10mg of the compound in dimethylsulfoxide .serial dilution ranging from 4 to 0.01mg/ml were made. Vogel-Bonner medium E (50X), histidine –biotin solution (0.5M), top agar, minimal glucose plates, histidine –biotin and ampicillin plates were prepared as described by Maron and Ames [14]. The plate's incorporation test as described by Maron and Ames [14] was followed. The top agar was distributed into capped culture tubes which were held at 45°C in a water bath. To each tube, 0.1ml of a fresh overnight culture of the tester strain was added, followed by the addition of 0.1 ml of the test compound. Sodium azide, nitrophenylene diamine and methyl methane sulfonate were used as appositive controls. The test components were mixed by vortexing the tube for about 3s at low speed and directly poured onto a minimal glucose agar plates. After 45min, the plates were inverted and placed in a dark 37°C incubator. The revertant colonies on the treated as well as on the negative control plates were counted.

The assay with S9mix was carried out as described by Maron and Ames [14]. The rat-liver S9fraction was prepared as described by Ono et.al. [23]. Young male Sprague-Dawley rats, weighing approximately 200mg, were supplied by yaromuk university / animal house unite (Jordan), and were used after induction with Phenobarbital and β-naphtofavone . the S9mix (50ml) contained 5ml of induced rat liver S9, of concentration was used . Triplicate plates were made for each dose, and each experiment was repeated at least twice in to separate days.

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