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ORIENTAL JOURNAL OF CHEMISTRY
An International Open Free Access, Peer Reviewed Research Journal

www.orientjchem.org

ISSN: 0970-020 X
CODEN: OJCHEG
2014, Vol. 30, No. (1):
Pg. 385-390

Synthesis and Bioevaluation of 4-[(2/4-chloro-/2, 3-dichloro-/2/4-bromo-/2, 4-dinitro-/4-nitrophenyl) Anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-ones

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http://dx.doi.org/10.13005/ojc/300155

(Received: January 31, 2014; Accepted: February 20, 2014)

ABSTRACT

Synthesis of 4-[(2/4-chloro-/2,3-dichloro-/2/4-bromo-/2,4-dinitro-/4-nitrophenyl) anilinemethyl]-6-t-butyl-2H-1-benzopyran-2-ones have been carried out. Synthesized compounds were characterized by ^{1}HNMR, IR and other physical and analytical data. All of the synthesized compounds were evaluated for their antifungal activity against *Aspergillus awamori* and *Sclerotium rolfsii* and antibacterial activity against a bacterium of *Bacillus* species *in vitro* at 10, 50, 100 and 200 µg/ml concentrations. Compounds 4-[(2-chlorophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one(11) and 4-[(4-chlorophenyl) anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one(12) exhibited good antifungal activity among tested compounds. Compound 4-[(2-bromophenyl) anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one(14) showed significant antibacterial activity against the test bacterium.

Key words: 2H-1-benzopyran-2-one, Antibacterial, Antifungal, *Aspergillus awamori*, *Sclerotium rolfsii, Bacillus species*.

INTRODUCTION

Since a long time, men have been struggling to reduce the adverse effects of pests on crop production and storage by using pesticides. On the other hand, pesticides and their residues are creating environmental problems, which include toxicity to non-target organisms. So there is a need to evolve and assess alternative compounds which may be effective, easily degradable, cheap and ecofriendly to get residues free food. Coumarin derivatives belong to one of the most important classes of natural compounds out of which psoralene and angelicins have been used as medicines[1]. Many biological properties *viz*. anticoagulant[2-3], antibacterial, antifungal[4], antimicrobial[5] have also been reported for a variety of 2H-1-benzopyran-2-ones. Thus, 2H-1-benzopyran-2-one nucleus is the seat of diverse biological activities which may be utilized for preparation of different agrochemicals. Keeping all these in mind and in search of new agrochemicals, the present study involved the synthesis of 4-[(2/4-chloro-/2,3-dichloro-/2/4-bromo-/2,4-dinitro-/4-nitrophenyl)anilinomethyl]-6-

t-butyl-2H-1-benzopyran-2-ones and evaluation of synthesized compounds for their antimicrobial activity to establish structure activity relationship (SAR).

EXPERIMENTAL

Materials and Methods

The melting points were determined in open capillaries on a Genson electrical melting point apparatus. Homogeneity of the compounds was routinely checked on silica gel-G TLC plates using ethyl acetate: hexane (3:7) as irrigant. IR spectra were recorded on Perkin Elmer FTIR spectrophotometer in KBr and frequencies are expressed in cm^{-1}. The NMR spectra were recorded on "Bruker Avance II 400-F" (400MHz) NMR spectrophotometer in DMSO-d_6 using tetramethylsilane (TMS) as internal reference. The chemical shift values are expressed in ´ (ppm) units while J values in Hz and are compatible with the assigned structures. Only those spectral data have been mentioned which have a direct bearing on the assignment of the structure and are discussed here.

Chemistry

4-Chloromethyl-6-t-butyl-2H-1-benzopyran-2-one (3)

A mixture of p-t-butylphenol (**1**, 1.5g, 10 mmol) and ethyl-4-chloroacetoacetate (**2**, 1.64 ml; 10 mmol) was added to sulphuric acid (25 ml; 73%) with constant stirring. After the addition was complete, the reaction mixture was left overnight at ambient temperature. Completion of reaction was monitored by TLC. The reaction mixture was poured on ice water and the solid thus separated, was filtered, washed with water, dried and crystallized from ethyl acetate to afford **3**, Yield 86%, m.p. 122-123°C (lit. 120-121 °C) [5] , IR(KBr) cm^{-1}:1715 (C=O), 745 (C-Cl). ^{1}HNMR ($CDCl_3$): 1.37(s, 9H, $C(CH_3)_3$), 4.72(d, J= 2.0 Hz, 2H, CH_2Cl), 6.55(s, 1H, C_3-H), 7.28(d, J= 8.0 Hz, 1H, C_8-H) 7.61(d, J= 8.0 Hz, 1H, C_7-H), 7.63(s, 1H, C_5H). Analysis Found: C, 67.17; H, 5.83. Molecular formula $C_{14}H_{15}ClO_2$ Required C, 67.07; H, 6.03%.

4-[(2-Chlorophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (11)

In a dry round bottom flask (2.51 g; 10 mmol) of the 4-Chloromethyl-6-t-butyl-2H-1-benzopyran-2-one (3) and (1.28 g; 10 mmol) of 2-chloroaniline (4) were taken to obtain a solution. The flask was cooled to room temperature and 5 ml glacial acetic acid was added. The solution was then heated in an oil bath between 120-130°C for one h. The cooled product was stirred with 200 ml, 5% hydrochloric acid. Separated solid was washed with water and recrystalized from benzene to afford 11, Yield 79%, m.p. 156°C, IR(KBr) cm^{-1}:1709(C=O), 3403 (N-H). ^{1}HNMR(DMSO-d_6): 1.32(s, 9H, $C(CH_3)_3$), 4.75(bs, 1H, NH), 5.06(d, J = 8.0 Hz,CH_2-NH), 6.56(s, 1H, C_3-H) 7.34(s, 1H, C_8-H), 7.46(s, 1H, C_5-H) 7.51-7.85(m, 4H, C'_3-H, C'_4-H, C'_5-H, C'_6-H). Analysis Found: C, 70.20; H, 5.58; N, 4.06. ;$C_{20}H_{20}ClNO_2$ Required: C, 70.27; H, 5.90; N, 4.10%.

Other compounds 12-17 were prepared similarly from compounds 4-10 respectively.

4-[(4-Chlorophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (12)

Yield 73%, m.p. 165-166°C, IR (KBr) cm^{-1}: 1715 (C=O), 3390 (N-H). ^{1}HNMR(DMSO-d_6) : 1.34(s, 9H, $C(CH_3)_3$), 4.71(bs, 1H, NH), 5.04(d, J = 8.0 Hz, CH_2-NH), 6.43(s, 1H, C_3-H) 7.32(s, 1H, C_8-H), 7.43(s, 1H, C_5-H) 7.44-7.79(m, 4H, C'_3-H, C'_4-H, C'_5-H, C'_6-H). Analysis Found: C, 70.18; H, 5.86; N, 4.02. Molecular formula $C_{20}H_{20}ClNO_2$ Required C, 70.27; H, 5.90; N, 4.10%.

4-[(2,3-Dichlorophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (13)

Yield 81%, m.p. 166-168°C, IR (KBr) cm^{-1}: 1710(C=O), 3404(N-H), Analysis Found: C, 63.64; H, 4.94; N, 3.70%. ; $C_{20}H_{19}Cl_2NO_2$. Required: C, 63.84; H, 5.09; N, 3.72%.

4-[(2-Bromophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (14)

Yield 77%, m.p. 170-171°C, IR (KBr) cm^{-1}:1703(C=O), 3388 (N-H). ^{1}HNMR(DSMO-d_6): 1.37(s, 9H, $C(CH_3)_3$), 4.74(bs, 1H, NH), 5.0(d, CH_2-NH, J= 8.0 Hz), 6.21(s, 1H, C_3-H) 7.30(s, 1H, C_8-H), 7.42(s, 1H, C_5-H) 7.44-7.79(m, 4H, C'_3-H, C'_4-H, C'_5-H, C'_6-H). Analysis Found: C, 61.83; H, 5.42; N, 3.67. Molecular formula $C_{20}H_{20}BrNO_2$ Required C, 62.19; H, 5.22; N, 3.73%.

4-[(4-Bromophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (15)

Yield 82%, m.p. 175-176°C, IR (KBr) cm^{-1} 1714(C=O), 3380(N-H) Analysis Found: C, 62.11; H,

5.07; N, 3.58%. $C_{20}H_{20}BrNO_2$, Required: C, 62.19; H, 5.22; N, 3.63%.

4-[(2,4-Dinitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (16)

Yield 76%, m.p. 164-165°C, IR (KBr) cm^{-1}: 1710(C=O), 3394(N-H), Analysis Found: C, 60.40; H, 4.78; N, 10.50%. Analysis Found: C, 60.26; H, 4.63; N, 10.39; $C_{20}H_{19}N_3O_6$, Required: C, 60.45; H, 4.82; N, 10.57%.

4-[(4-Nitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (17)

Yield 70%, m.p. 159-161°C, IR (KBr) cm^{-1}: 1690(C=O), 3415(N-H). ^{1}HNMR(DSMO-d_6) :1.36(s, 9H, $C(CH_3)_3$), 6.61(s, 1H, C_3-H), 4.73(bs, 1H, NH), 4.97(d, J= 8.0 Hz, CH_2-NH), 7.28(d, J= 8.0 Hz, C_8-H), 7.63(d, 1H, J= 8.0 Hz, C_7-H), 7.73(s, 1H, C_5-H), 7.65(d, 2H, J=8.0 Hz, C'_2-H and C'_6-H), 7.74(d, 2H, J=8.0 Hz, C'_3-H and C'_5-H). Analysis Found: C, 67.96; H, 5.57; N, 7.74. Molecular formula $C_{20}H_{20}N_2O_4$ Required: C, 68.17; H, 5.72; N, 7.95%.

Bioevaluation

Test for fungi toxicity

Amongst the several methods available, poisoned food technique[6] was used to test the antifungal activity of synthesized compounds against pathogenic fungi *Aspergillus awamori* and *Sclerotium rolfsii*. The test fungi was grown on Czapek's agar medium (pH 6.0) containing logarithmic concentrations series of each compound (10-200 µg/ml medium). The required amount of chemical dissolved in 1 ml of acetone was incorporated aseptically into 49 ml aliquotes of sterilized Czapek's agar cooled at 45°C after brief shaking. Each lot of medium was poured into Petri dishes and allowed to solidify. Each dish was inoculated centrally with a 10 mm mycelial disc cut from the periphery of 2-3 days old fungal colonies. Inoculated Petri plates were incubated in the dark at 30±1°C and colony diameters were measured periodically till the central dishes were nearly completely covered with fungus growth. Three replicates were used for each concentration of a chemical together with three dishes containing only the solvent and no toxicant. The degree of inhibition of growth was calculated from the mean differences between treatments and the control as percentage of latter by using the formula

$$\% \text{ Inhibition} = \frac{C-T}{C} \times 100$$

Where

C = mycelial growth in control dish

T = mycelial growth in treated dish

Test of Antibacterial activity

The antibacterial activity of synthesized compounds was tested by zone inhibition method[7] on bacterial culture of bacillus species on agar medium. 30 ml of the medium was taken in 100 ml round bottom flask and autoclaved at 15 lbs pressure for 20 minutes. The bacterial growth (48h old) from five slants was taken and mixed in 100 ml sterilized distilled water aseptically. This gave the concentration of approximately 10^4 cells/ml. The medium was melted and cooled to 43 °C, needed medium was poured aseptically in sterilized Petri dishes and was allowed to solidify. 10, 50, 100 and 200 ppm concentrations of the test compounds were prepared from the stock solution by diluting with acetone.

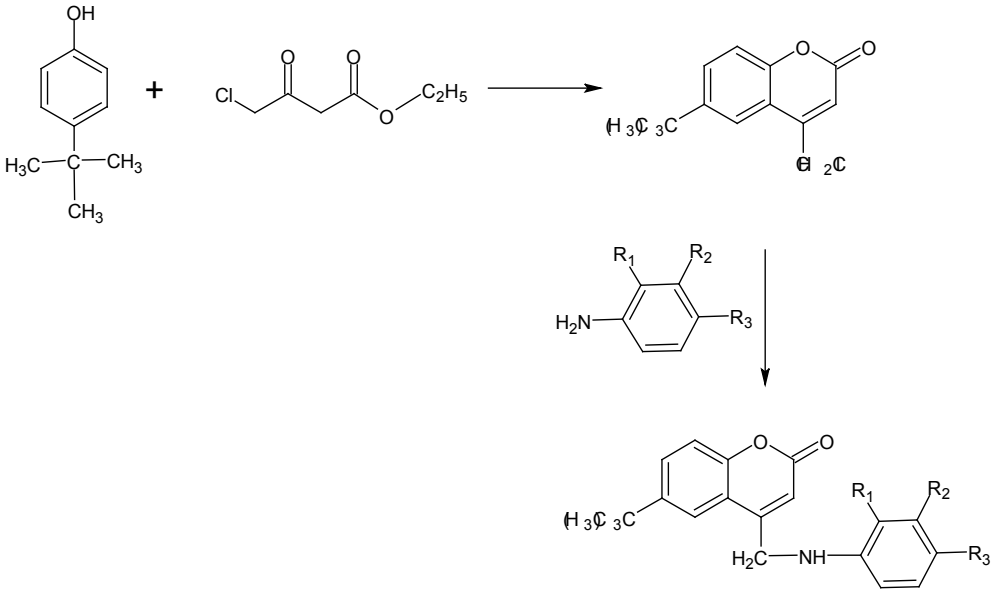
The sterile filter paper discs (Whatman's No. 1) of 10 mm diameter were dipped in different concentrations of the test compounds and a set of two paper discs was dipped in acetone. Such soaked discs were placed aseptically on inoculated Petri plates. Two paper discs were used for each concentration of a chemical. Each concentration and chemical was replicated 4 times. Such Petri plates were inverted and kept at 5°C for two h for better diffusion of the chemicals in agar medium. Later on, the Petri plates were incubated at 30°C ±1 for 48 h and zone of inhibition was recorded in mm, after 48h of incubation.

RESULTS AND DISCUSSION

The Pechmann condensation of 4-t-butylphenol (1) and ethyl-4-chloroacetoacetate (2) was carried out with the help of 73% H_2SO_4 to get 4-chloromethyl-6-t-butyl-2H-1-benzopyran-2-one (3). The compound (3) was then treated with various substituted amines (4-10) in the presence of glacial acetic acid by refluxing on oil bath at temperature 120-130°C for 1h, resulted into good yield of 4-[(2/4-chloro-/2,3-dichloro-/2/4-bromo-/2,4-

dinitro-/4-nitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-ones (11-17) (scheme 1).

The ^{1}HNMR spectrum of (3) was in accordance with the proposed structure. The diagnostic proton at position 3 appeared as triplet (J=2.0 Hz) at 6.55 δ, with chloromethyl protons at position 4 as doublet (J=2.0 Hz) at 4.72 δ due to allylic coupling and nine protons of t-butyl moiety as singlet at 1.37 δ. 1, 2, 4 pattern of aromatic protons could be picked up by the appearance of two ortho coupled doublets at 7.28 and 7.61 δ with J=8.0 Hz and a singlet at 7.63 δ, each. Integrating for one proton at positions 8, 7 and 5 respectively and thus NMR data was in agreement with earlier report[4]. Presence of 2H-1-benzopyran-2-one (coumarin moiety) was further confirmed by characteristic band at 1715 cm^{-1} in the IR spectrum[9]. In the second step of synthesis, condensation of (3) with 2-chloroaniline (4), 4-chloroaniline (5), 2,3-dichloroaniline (6), 2-bromoaniline (7), 4-bromoaniline (8), 2,4-dinitroaniline (9) and 4-nitroaniline (10) were carried out by refluxing on oil bath in glacial acetic acid to yield 4-[(2/4-chloro-/2,3-dichloro-/2/4-bromo-/2,4-dinitro-/4-nitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one[11-17]. The synthesis of these compounds is outlined in scheme 1. In the NMR spectrum of 11 the diagnostic doublet (J=8.0 Hz)



Reagents and Reaction Conditions

(i) 73% H_2SO_4
(ii) Glacial acetic acid, 5% HCl, 120°-130°C

Compound Number	R_1	R_2	R_3
4, 11	Cl	H	H
5, 12	H	H	Cl
6, 13	Cl	Cl	H
7, 14	Br	H	H
8, 15	H	H	Br
9, 16	NO_2	H	NO_2
10, 17	H	H	NO_2

Scheme 1: Synthesis of 4-[(substitutedphenyl)anilinomethyl] -6-t-butyl-2H-1-benzopyran-2-ones(11-17)

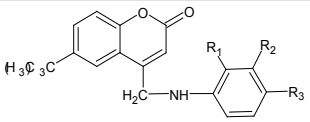
at 5.06δ for C_4-CH_2NH moiety was indicative of the attachment of anilino group to CH_2Cl in 3. The triplet (J=2.0 Hz) due to allylic coupling appeared at 6.56 δ, was assigned to C_3-H proton, while the singlet at 1.32 δ integrating for nine protons was for t-butyl moiety. A singlet for one proton at 7.46 δ was assigned proton at position C_5-H. Other protons were found at their usual positions. In IR spectra it showed absorption at 1709 cm^{-1} showing the presence of carbonyl group which is characteristic for 2H-1-benzopyran-2-one and absorption at 3403 cm^{-1} for NH stretching. Other compounds 12-17 were prepared similarly from compound 4-10 respectively. The structures of all above compounds were assigned by spectroscopic and other physical data.

Bioevaluation

Antifungal activity

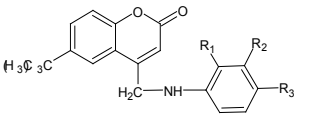
Percentage growth inhibition was plotted on a probit scale against chemical concentrations on a log scale and the percentage providing 50% inhibition (EC_{50} values) were derived from the dosage response curves. None of the compound has shown specificity to tested fungi.

Table 1: Antifungal activity of 4-[(2/4-chloro-/2,3-dichloro-/2/4-bromo-/2,4-dinitro-/4-nitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-ones (11-17)

Compound No.				Fungi	
				Aspergillus awamori	*Sclerotium rolfsii*
				EC_{50} Value (µg/ml)	
	R_1	R_2	R_3		
11	Cl	H	H	117	122
12	H	H	Cl	132	124
13	Cl	Cl	H	>200	160
14	Br	H	H	180	155
15	H	H	Br	160	190
16	NO_2	H	NO_2	>200	>200
17	H	H	NO_2	a	>200

a : No growth inhibition even up to 200 µg/ml

Table 2: Antibacterial activity of 4-[(2/4-chloro-/2,3-dichloro-/2/4-bromo-/2,4-dinitro-/4-nitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-ones (11-17)

Compound No.				*Bacillus* species			
				Zone inhibition in mm			
				Concentration µg/ml			
	R_1	R_2	R_3	1	10	50	100
11	Cl	H	H	a	a	a	8.50
12	H	H	Cl	a	a	19.00	32.00
13	Cl	Cl	H	6.50	13.00	27.00	43.00
14	Br	H	H	7.00	15.00	32.00	48.50
15	H	H	Br	a	a	a	a
16	NO_2	H	NO_2	a	a	a	8.50
17	H	H	NO_2	a	a	a	a

a : No growth inhibition even up to 200 µg/ml

A perusal of the activity data presented in table 1 showed that compounds 4-[(2-chlorophenyl) anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (11) and 4-[(4-chlorophenyl) anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (12) exhibited moderate activity against both the fungi. Compound 4-[(2,4-dinitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran -2-one (16) was found inactive against both the tested fungi upto 200 µg/ml concentration. Compound 4-[(4-Nitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (17) did not showed any activity against *Aspergillus awamori* even upto highest tested concentration.

Presence of chloro group at position-2 in compound 11 of phenyl ring showed appreciable activity and its replacement with NO_2 group at position-2 and 4 in 4-[(2,4-dinitrophenyl) anilinomethyl]-6-t-butyl-2H-1-benzopyran -2-one (16) and 4-[(4-Nitrophenyl)anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (17) reduces the antifungal activity. Thus the introduction of electron withdrawing group resulted into an overall decrease in the antifungal activity.

Antibacterial activity

A perusal of the activity data presented in table 2 revealed that these compounds exhibited differential antimicrobial activity. The compounds 4-[(2,3-dichlorophenyl) anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (13) and 4-[(2-bromophenyl) anilinomethyl]-6-t-butyl-2H-1-benzopyran-2-one (14) showed activity against the tested bacterium. The remaining compounds are either inactive or active at highest tested concentration (200 µg/ml).

The compounds containing halogens such as chlorine and bromine as substitutents showed good antibacterial activity. Among them, Compound 14 containing bromo group at position-2 of phenyl ring showed the comparative best antibacterial activity with inhibition zone of 7mm at lowest concentration, followed by compound 13 with 6.50 inhibition zone containing chloro group at position-2 and 3. On the other hand, addition of bromo group at position 4 of phenyl ring in compound 15 makes it inactive. Again, introduction of NO_2 group in compounds drastically reduces the antibacterial activity with no growth inhibition even up to 200 µg/ml.

ACKNOWLEDGEMENTS

The authors are thankful to the Professor and Head, Department of Chemistry & Physics and Department of Microbiology, CCS Haryana Agricultural University, Hisar for providing laboratory facilities. Thanks also to SAIF, Panjab University, Chandigarh, for providing the spectral data.

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