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Green synthesis of benzodiazepine derivatives and evaluation for anticancer activity

MOHAMED AHMAD ABDULGADER
POONAM PRAKASH
Department of Chemistry SHIATS
Allahabad, India

Abstract:

An environmentally benign synthesis of biologically potent benzodiazepine has been disclosed. The proposed protocol is highly efficient in terms of product yield reaction time and over all conditions applied was green. In the presented methodology, readily available 3-Acetyl-4-hydroxy-6-methyl-pyran-2-one (2.0 mmol), 2-methyl propane-1,2-diamine (2.0 mmol),and benzaldehyde (2.0 mmol), was stirred in glycol (15 ml) at room temperature to obtain benzodiazepine in an excellent yield. We have synthesized three derivatives and all the derivatives were confirmed by spectral data like ^{1}HNMR, m/z.

Key words: Benzodiazepine, Glycol, Green Protocol, Reusability, Anti-Cancer activity.

The term green chemistry was first given by Pal Traut Anastas **.**Green chemistry is the universally accepted term to describe the movement towards more environmentally acceptable and sustainable (**Tobiszewski *et al.,* 2009)** chemical processes. It encompasses education and promotional work, as well as research and commercial application of cleaner technologies from old and some new sources (**Leitner 1999).** Green Chemistry is defined as environmentally benign synthesis, in which the synthetic schemes are designed in such a way that

there is least pollution to the environment. As on today maximum pollution to the environment is caused by numerous chemical industries. The cost involved in disposal of the waste products is also enormous. Therefore attempts have been made to design synthesis for manufacturing processes in such a way that the waste products are minimized or eliminated. They have no effect on the environment and their disposal is convenient. For carrying out reaction it is necessary that the starting materials, solvents and catalysts should be carefully chosen. Chemicals which are carcinogenic in nature if possible should be avoided **(Li *et al.*, 2006)**. It is best to carry out reaction in the aqueous phase. Methods should be designed in such a way that the starting materials are consumed to the maximum extent in the final product and the reaction should also not generate any toxic by-products **(Aken*et al.*, 2006)**. Green chemistry is one of the most effective approaches for pollution prevention because it applies innovative scientific solution to real world situation.

As part of our continuing interest, in the pursuit of exploring new greener methods to heterocyclic system, herein we wish to describe a practical and environmentally friendly route to a series of benzodiazepine. In this paper, an attempt has also been made to highlight the catalyst free construction of substituted diazipine derivatives which in our knowledge have not been reported yet.

Materials and Methods

The materials and methods used in the present study of "Anticancer activity of heterocyclic scaffold synthesized using green protocol" are as follows:-

Methods:-

Synthesis of 3- [2,2-Dimethyl-7-(4-methylphenyl)-3,6-dihydro-2H-1,4-diazepin-5-yl]-4-hydroxy-6-methyl-2Hpyran-2-one:
A mixture of 3-Acetyl-4-hydroxy-6-methyl-pyran-2-one (2.0 mmol), 2-methyl propane-1,2-diamine (2.0 mmol),and para-4-methyl benzaldehyde (2.0 mmol), was stirred in glycol (20 ml) at room temperature in presence of base for 7 h. After completion of the reaction as indicated by TLC, 30 ml of water was added to the reaction mixture and stirred well. The product was extracted with ethyl acetate (5 - 20 ml). The combined organic layers were dried over anhydrous Na_2SO_4 to afford the crude product. After isolation of the crude product, the analytically pure compound **1** was obtained by column chromatography and characterized by ^{1}HNMR and mass.

Synthesis of 3-(2,2-Dimethyl-7-phenyl-2,3,6,7-tetrahydro-1H-1,4-diazepin-5- yl)- 4-hydroxy-6-methyl-2H-pyran-2- one :
A mixture of 3-Acetyl-4-hydroxy-6-methyl-pyran-2-one (2.0 mmol), 2-methyl propane-1,2-diamine (2.0 mmol),and benzaldehyde (2.0 mmol), was stirred in glycol (15 ml) at room temperature in presence of base KOH for 3 h. After completion of the reaction as indicated by TLC, 20 ml of water was added to the reaction mixture and stirred well. The product was extracted with ethyl acetate (3 - 20 ml). The combined organic layers were dried over anhydrous Na_2SO_4 to afford the crude product which was later purified by Column chromatography in order to obtain analytically pure compound **2** with an (85%) product yield and characterized by ^{1}HNMR and mass spectra.

Synthesis of 3-[7-(4-bromophenyl)-2,2-dimethyl-2,3,6,7-tetrahydro-1H-1,4- diazepin-5-yl]-4-hydroxy-6-methyl-2Hpyran-2-one 6:
A mixture of 3-Acetyl-4-hydroxy-6-methyl-pyran-2-one (2.0 mmol), 2-methyl propane-1,2-diamine (2.0 mmol),and 4-bromobenzaldhyde (2.0 mmol), was stirred in glycol (10 ml) at

room temperature using KOH a base for 7 h. after completion of the reaction as indicated by TLC, 30 ml of water was added to the reaction mixture and stirred well. The product was extracted with ethyl acetate (5 _ 20 ml). The combined organic layers were dried over anhydrous Na_2SO_4 to afford the crude product and recrystallized from ethanol to obtain analytically pure compound **3** (87%). After isolation of the product, the compounds **3** was purified by column chromatography and characterized by ^{1}HNMR and mass spectra.

Anticancer activity

Procedure: This procedure is the modification of the method developed by **Abboud*et al*, (2001).**

Cell Culture

HepG2 cells were cultured by using RPMI 1640 (GIBCO) containing 10% fetal bovine serum (GIBCO). Cultured cells were incubated at 37°C in the presence of 5% CO_2.

Trypan Blue Exclusion Test

To detect the cell viability trypan blue exclusion test was chosen. The cancer cells were seeded at a density of 6×10^5 cells/well with different concentration of extract at 37°C in the presence of 5% CO_2. After 72 hr, 20 µl of medium and equal volume of trypan blue were mixed and viable. Dead cells were counted by NeubauerhaemacytometerMosmann (1983).

MTT Assay

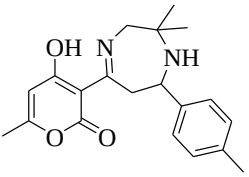
MTT assay as a proper cytotoxicity test was used in this study. Briefly, 10 µl of MTT stock solution [5 mg/ml in phosphate buffer saline (PBS)] was added to 90 µl medium of wells. The microplate was incubated at 37°C for 4 hours and then, the optical density of each well was read by the ELISA reader (TECAN) in 540 nm.

Synthesized compounds were used to study their effect on cancer cells. The 0.1mg of compounds 1, 2 and 3 were used to cell culture plats separately.

Results and Discussion

Characterization of benzodiazepine derivatives by spectral analysis:

Compound 1:-



3-[2,2-Dimethyl-7-(4-methylphenyl)-3,6-dihydro-2H-1,4-diazepin-5-yl]-4-hydroxy-6-methyl-2Hpyran-2-one

^{1}H-NMR (SOLUTION-$CDCl_3$, 250 MHz):

δ 1.32 (s,6H, 2 CH_3), δ 2.34 (s,3H,CH_3), δ 2.51 (s,3H CH_3), δ 3.54 (s, 2H,CH_2-N=), δ 4.17 (t, 1H, CH-Ar), δ 4.70 (d,2H,CH_2), δ 5.03 (s, NH, replaceable with D_2O), δ 5.75 (s, 1H, -CH-), δ 7.17 (d, 2H, Ar-H), δ 7.23 (d, 2H, Ar-H), δ 10.71 (s, OH, replaceable with D_2O), $C_{19}H_{22}N_2O_4$

The calculation of ^{1}HNMR has been done by using δ scale which is started from 0 that indicates highest proton number, Shielding and deshielding can be checked according to the values of this scale. Here the ^{1}HNMR of the compound δ 1.32 singlet of two same methyl groups attached to diazipine ($2CH_3$) and the height of the peak confirmed the presence of 6H atoms. δ 2.34 singlet of CH_3 was a normal value and can be easily identified and singlet of CH_3 at δ 2.51 present on isocumarin and can be easily identified by the peak height and the position by the presence of neighboring groups. Singlet of δ 3.54

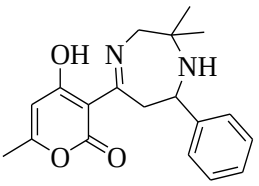
indicated 2H atoms with carbon attached to some electronegative group where N is the only possibility. Triplet at δ 4.17 peak indicated 1H with two neighboring protons in the synthesized heterocyclic compound. The peak at δ 4.70 gave doublet which is in coupling with neighboring 1H.The height of the peaks indicated the presence of 2H. The peaks ranges from 3-6 are due to N-H. The peak at δ 5.75 was from IH of coumarin ring with no neighboring hydrogen. The doublet at δ 717 of same 2H atoms were obtained due to Ar-CH, and doublet at δ 7.23 for 2H of Ar-CH. δ 10.71 peak indicated OH group in the compound.

Mass:

M/Z: 339 (1), 215 (2), 127 (3), 125 (4), 91 (5)

Mass spectra was calculated by intensity of the peaks which explained the stability of the anion aswell. In the above compound peaks were observed at 339,215,127,125, 91 and that are also the expected ions of the base compound. All other fragments were also confirmed as all these form stable ring compounds. The 2,2-dimethyl-7-p-tolyl-2,3,6,7-tetrahydro-1H-1,4-diazepine (215), 4-hydroxy-6-methyl-2H-pyran-2-one (127), 2,2-dimethyl-2,3,6,7-tetrahydro-1H-1,4-diazepine (125), and toluene (91) were the possible and stable fragments of the parent compound.

Compound 2:



3-(2,2-Dimethyl-7-phenyl-2,3,6,7-tetrahydro-1H-1,4 diazepin-5-yl)-4-hydroxy-6-methyl-2H-pyran-2- one.

^{1}H-NMR (SOLUTION-$CDCl_3$, 250 MHz):

δ 1.35 (s, 2 CH_3), 2.57 (s, CH_3), 3.53 (s, 2H,CH_2-N=), 4.25 (t, 1H, CH-Ar), 4.75 (d,2H,CH_2), 5.08 (s, NH, replaceable with D_2O), 5.75 (s, 1H, -CH-), 7.29-7.45 (m, 5H, Aromatic region), 10.73 (s, OH, replaceable with D_2O), $C_{19}H_{22}N_2O_3$

The ^{1}HNMR of the compound showed expected peaks of protons. δ 1.35 singlet of two same methyl groups attached to diazipine (2CH_3) was observed and the height of the peak confirmed the presence of 6H atoms. δ 2.57 singlet of CH_3 which was a normal value and can be easily identified by the peak height and also the position by, the presence of neighboring groups. Singlet of δ 3.53 indicated 2H atoms with carbon attached to some electronegative group where nitrogen is the only possibility. Triplet at δ 4.25 peak indicated 1H with two neighboring protons which can be easily predicted in the synthesized heterocyclic skeleton. The peak at δ 4.75 gave doublet which is in coupling with neighboring 1H, and the height of the peaks state 2H are present. The peaks ranges from 3-6 were due to N-H. The peak at δ 5.75 was of 1H of coumarin ring with no neighboring hydrogen. δ 7.29-7.45 peak (m, 5H) was observed for Aromatic region, and δ 10.73 peak indicated OH group.

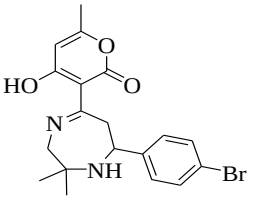
Mass:

M/Z: 325 (1), 201 (2), 127 (3), 125 (4) , 77 (5),.

Mass spectra was calculated by intensity of the peaks which explain the stability of the anion as well. In the above compound peaks were obtained at 339,215,127,125, 91 and that are also the expected ions of the base compound. All other ion peaks were also found stable after fragmentation. The 2,2-dimethyl-7-p-tolyl-2,3,6,7-tetrahydro-1H-1,4-diazepine (201), 4-

hydroxy-6-methyl-2H-pyran-2-one (127), 2,2-dimethyl-2,3,6,7-tetrahydro-1H-1,4-diazepine (125), and benzene (77) were the possible and stable fragments of the parent compound.

Compound 3:-



3-[7-(4-bromophenyl)-2,2-dimethyl-2,3,6,7-tetrahydro-1H-1,4-diazepin-5-yl]-4-hydroxy-6-methyl-2Hpyran-2-one.

^{1}H-NMR (SOLUTION-$CDCl_3$, 250 MHz):

δ 1.42 (s, 2 CH_3), δ 2.37 (s,CH_3), δ 3.61 (s, 2H,CH_2-N=), δ 4.29 (t, 1H, CH-Ar), δ 4.81 (d,2H), δ 5.13 (s, NH, replicable with D_2O), δ 5.89 (s, 1H, -CH-), δ 7.25 (d, 2H, Ar-H), δ 7.31 (d, 2H, Ar-H), δ 10.93 (s, OH, replicable with D_2O),.$C_{19}H_{22}N_2O_4$

The calculation of ^{1}HNMR has been done by using δ scale which is started from 0 that indicates highest proton number. Shielding and deshielding can be checked according to the values of this scale. Here the ^{1}HNMR of the compound showed peak at δ 1.42 singlet of two same methyl groups attached to diazipine (2CH_3), and the height of the peak confirmed the presence of 6H atoms. δ 2.37 singlet of CH_3 is a normal value and can be easily identified. Singlet of δ 3.61 indicated 2H atoms with carbon attached to some electronegative group where N is the only possibility. Triplet at δ 4.29 peak indicated 1H with two neighboring protons. All the peaks were easily identified in the synthesized heterocyclic skeleton. The peak at δ 4.81 gave doublet which was in coupling with neighboring 1H and the height of the peaks indicated the presence of 2H . The peaks ranges from 3-6 were due to N-H. The peak at δ 5.***89*** is from ^{1}H of coumarin ring with no neighboring hydrogen. The

doublet at 7.25 of same 2H atoms ofAr-CH and doublet at 7.31 of 2H of Ar-CH were observed. δ 10.93 peak indicated the presence of OH group. $C_{19}H_{21}BrN_2O_3$

Mass:

M/Z: 403 (1), 280 (2), 154(3), 127 (4), 125 (5)

Mass spectra was calculated by intensity of the peaks which explain the stability of the anion aswell. In the above compound δ peaks at 403, 281, 154, 127, 125 were obtained and that are also the expected ions of the base compound. The 7-(4-bromophenyl)-2,2-dimethyl-2,3,6,7-tetrahydro-1H-1,4-diazepine (281), Bromobenzine (154), 2,2-dimethyl-2,3,6,7-tetrahydro-1H-1,4-diazepine (127), 4-hydroxy-6-methyl-2H-pyran-2-one (125) are the possible and stable fragments of the parent compound**.**

Anticancer activity

The anticancer activity of the heterocyclic compounds depend on various factors, the prominent among them are type of substitution, electronic effect, kind of heterocyclic ring present, position of substitution and nature of groups present (**Deng *et al.,* 2013).**

Diazipine and its derivatives have been well known for their anti-cancer activity **(Wang *et al.,* 2006**). The inhibitory properties of synthesized diazipine derivatives were compared with standard controls and showed good anticancer activities against HepG2 cell lines. The percentage cancer cell inhibition profiles were found to be structure dependent of the applied benzodiazepine (Figures 4.2, 4.3 and 4.4). The maximum concentration (µg/ml) used in the study was 1000 µg/ml of all the three derivatives. Following results were obtained when anticancer activity of the synthesized compounds was studied. HepG2 cell lines grown in DMEM, when subjected to different

synthesized compounds resulted in 86.5%, 81.37% and 47.6% inhibition. From table 4.1 a gradual decrease of inhibition was observed in the synthesized compound.

Table 4.1 *In-vitro* anticancer effect of compounds on HepG2 cell line

Sample	**Conc. (µg/ml)**	**O.D (optical density)**	**Cell Viability (%)**	**Cell death (%)**
Compound **1**	1000	0.26	13.5	86.5
Compound **2**	1000	0.34	18.63	81.37
Compound **3**	1000	0.36	53.4	47.6
Cell control	-	0.48	100	0.0

The low activities of the compounds against HepG2 cancer cells can be attributed to either expression of the gene expressed targets having minimal affinity to the chemical constituents present in these liver cells or due to the low penetration power of the active principles which resulted in low cell inhibitions **(Wenwei*et al*., 2008).** It can also be explained on the basis of structure activity relationship of the synthesized compounds and the targeted cell lines. As the rate of cell inhibition in the culture plate 1 is more (86.5%) than to plate 2, (81.37%) and 3, (47.6%) using compounds 1, 2, and 3 respectively for cell inhibition control.

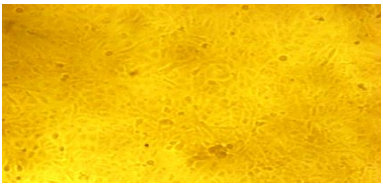


Fig. Control of HepG2 cell line

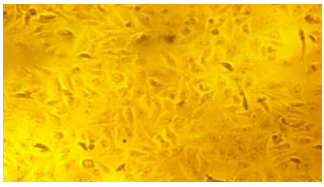


Fig. Anticancer activity of compound 1 on HepG2 cell line

Fig. Anticancer activity of compound 2 on HepG2 cell line

Fig. Anticancer activity of compound 3 on HepG2 cell line

The compound 1 emerged out to be outstanding in its activity as observed 86% control which was the best result and only 14% cancer cells survived in plate 1. The reason of better activity of compound 1 might be that diazipine having electron donating group attached to diazipine. On comparison, compound 1 was observed better than compounds having electron withdrawing groups attached to the diazipine, which showed lower activity. Compound 2 does not have any group attached directly to diazipine ring, also showed good activity but less than compound 1 and more than compound 3 **(Paul *et al*., 2005).** So in the present study, it can be concluded that compound no 1 having electron donating group showed the best anticancer activity.

Summary and Conclusion

Over the course of the past decade, green chemistry has demonstrated how fundamental scientific methodologies can protect human health and the environment in an economically beneficial manner. Significant progress is being made in several key research areas, such as catalysis, the design of safer chemicals and environmentally benign solvents, and the development of renewable feedstocks. Current and future chemists are being trained to design products and processes with an increased awareness for environmental impact. Outreach activities within the green chemistry community highlight the potential for chemistry to solve many of the global environmental challenges. Cancer is a class of diseases characterized by out-of-control cell growth. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected.

Heterocycles are an important class of compounds, making up more than half of all known organic compounds. Heterocycles are present in a wide variety of drugs, most vitamins, many natural products, biomolecules, and biologically

active compounds, including antitumor, antibiotic, anti-inflammatory, antidepressant, antimalarial, anti-HIV, antimicrobial, antibacterial, antifungal, antiviral, antidiabetic, herbicidal, fungicidal, and insecticidal agents. Also, they have been frequently found as a key structural unit in synthetic pharmaceuticals and agrochemicals.

In this work, three derivatives of diazepine i.e. compound 1, 2 and 3, were synthesized adopting green protocol. The process involves mostly eco-friendly starting solvent and conditions making the whole process safe. The methodology offers very attractive features such as reduced time, higher yields and relatively simple procedure and thus has a wide scope in organic synthesis. They have less toxic side effects making them attractive starting compounds for synthesis of pharmacological agents. All the three synthesized compounds were characterized by spectral means and evaluated for their anticancer activity using HepG2 cell lines where they have shown prominent effect control on the growth of cancer cells. Among the synthesized compound the derivative 1 was found to be highly active than that of compound 2 which showed moderate. Compound 3 was found to be least active against targeted cancer cell line. The activities of the compounds were controlled by electronic effect. The derivative 1 has electron donating group hence showed highest activity, Compound 2 showed lesser activity than compound 1 as it is not having any substituent in its moiety. Results showed least activity in compound 3 as electron withdrawing group is attached to it. So that can be concluded that compound 1 showed the best anticancer activity. The present work will be effective in synthetic as well as biological world as the method is new, simple, green and the compounds also showed better anticancer activity. Hence the presented work has a great future demand in scientific community.

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