INTRODUCTION

Developments of efficient methodologies to access large molecules are of special interest. Steroidal compounds are essential to life in various ways. Synthetic derivatives of steroids have also attracted a good deal of attention for the purpose of developing lead compounds in order to cure different biological disabilities. Especially synthesizing the steroids comprising heterocycles have received much attention of the chemists since many of such compounds have been shown to display important pharmacological properties.

Synthesis of polycyclic skeletons like steroids, have a central place in the development of regio- and stereo chemically controlled synthetic methodology. Boron trifluoride etherate (BF₃·OEt₂), tin chloride (SnCl₂), zinc bromide (ZnBr₂) and p-toluene sulphonic acid (p-TsOH) has been studied for those reactions which involve formation of carbon-carbon or carbon-heteroatom bonds. Because of various advantages incorporated with these compounds, some studies revealed their uses as potential reagents for numerous reactions involving unexpected formation of dioxaspiro compounds. Some representative examples include cycloaddition,
isomerisation, ring contraction, ring expansion and unexpected rearrangement reactions\textsuperscript{4-13}. The Diels-Alder pathway is the most studied reaction, showing numerous uses in synthetic procedures and has been intensively studied\textsuperscript{14}. The important tool in Diels-Alder chemistry is the activation of dienophiles by Lewis acid\textsuperscript{15}. The barrier lies in searching those conditions which promote cycloaddition but without subsequent aromatization. In continuation of our interest in modified steroids\textsuperscript{16}, we herein report the synthesis of desired cholecalciferol derivative in presence of different non-chelating Lewis acids like BF\textsubscript{3}·OEt\textsubscript{2}, SnCl\textsubscript{4}, ZnBr\textsubscript{2}, p-TsOH under Diels-Alder condition and simultaneously study its binding with DNA by gel electrophoresis and molecular docking, and genotoxicity by comet assay.

\textbf{RESULTS AND DISCUSSION}

\textbf{Chemistry}

In synthetic organic chemistry, the Diels-Alder reaction is the convenient procedure for forming the 6-membered ring systems with potential control over regio- and stereo chemical properties. This high regio- and stereo selectivity typically have resulted in various uses of this transformation in the building of highly sneaker targets\textsuperscript{17}. Diels-Alder procedure has shown mature development, depicting the basic importance in synthetic and theoretical sciences. Now a day, this wonderful procedure is one of the most studied and well reviewed, having a beat of incorporations in synthesis. Formulating hetero complexes with different functionalities and their derivatization is the subject of high concern to synthetic scientists. Here we have investigated cyclization behaviour of cholecalciferol acetate with 2, 3-dimethylbutadiene in presence of different reagents like BF\textsubscript{3}·OEt\textsubscript{2}, SnCl\textsubscript{4}, ZnBr\textsubscript{2}, p-TsOH under Diels-Alder condition and simultaneously study its binding with DNA by gel electrophoresis and molecular docking, and genotoxicity by comet assay.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{scheme1.png}
\caption{Pathway for the synthesis of compound 2}
\end{figure}

It was found that after subjugating different Lewis and Brensted acids\textsuperscript{18} like BF\textsubscript{3}·OEt\textsubscript{2}, SnCl\textsubscript{4}, ZnBr\textsubscript{2}, p-TsOH and it was found that the maximum yield was obtained in the order BF\textsubscript{3}·OEt\textsubscript{2} > SnCl\textsubscript{4} > p-TsOH > ZnBr\textsubscript{2}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{S. No} & \textbf{Solvent} & \textbf{Lewis and Brensted acids} & \textbf{Acid} & \textbf{Time} & \textbf{Yield} \\
\hline
1. & Toluene & ZnBr\textsubscript{2} & & 3 h & 55\% \\
2. & Toluene & p-TsOH & & 2.5 h & 60\% \\
3. & Toluene & BF\textsubscript{3}·OEt\textsubscript{2} & & 2.25 h & 83\% \\
4. & Toluene & SnCl\textsubscript{4} & & 3 h & 74\% \\
\hline
\end{tabular}
\end{table}

IR spectrum exhibited absorption band at 1026 cm\textsuperscript{-1} for C-O, 1735 cm\textsuperscript{-1} for carbonyl group of acetate and other band at 1655 cm\textsuperscript{-1} for (-C=C-...
C=\text{C}. The $^1$H NMR spectrum displayed a multiplet centred at $\delta$ 5.2 for one proton can be ascribed to C$_3$-aH, axial. A distorted signal at $\delta$ 5.8–6.8 can be assigned to the two vinylic protons at C$_6$ and C$_7$. $^{13}$C NMR depicted signals at $\delta$ 36.3 for C$_3$, $\delta$ 138.2 for C$_5$, $\delta$ 126.2 for C$_6$, $\delta$ 128.2 for C$_9$. The strategy can also be applied to diverse steroidal derivatives.

**Evaluation of In vitro DNA damage using plasmid PBR322**

*In vitro* DNA damage of compound 2 was assessed using agarose gel electrophoresis as shown in figure 1. Lane 1 shows control plasmid, lane 2 depicts plasmid with incubation of 100 µM drug and lane 3 reveals plasmid incubated plus 200 µM drug. It is evident from the disappearance of the band and lytic products observed in the gel that the drug is capable of causing DNA damage.

At higher concentration, the disappearance in the band intensity might be the asset of acting the target steroid closely with duplex moiety, which makes the Ethidium bromide to replace.

![Agarose gel electrophoretic patterns with lanes 1, 2 and 3 depict DNA only (control), DNA and 100 µM comp. 2 and DNA and 200 µM comp. 2, respectively](image)

**Fig. 1:** Agarose gel electrophoretic patterns with lanes 1, 2 and 3 depict DNA only (control), DNA and 100 µM comp. 2 and DNA and 200 µM comp. 2, respectively

![Depict the interaction of compound and 5-fluorouracil at the various concentrations on the DNA of lymphocytes](image)

**Fig. 2:** Depict the interaction of compound and 5-fluorouracil at the various concentrations on the DNA of lymphocytes

![Graph confirming the enhancement of apoptotic DNA fragmentation in lymphocytes with the increase in concentration of compound 2. The level of damage caused by 5-Fu (100 µM) was more when compared to the steroid derivative](image)

**Fig. 3:** Graph confirming the enhancement of apoptotic DNA fragmentation in lymphocytes with the increase in concentration of compound 2. The level of damage caused by 5-Fu (100 µM) was more when compared to the steroid derivative
Evaluation of Ex vivo DNA damage using human lymphocytes

Using human lymphocytes, we assessed DNA damage caused by the compound 2 on human genomic DNA ex vivo by comet assay. Figure 2a shows control lymphocyte in which no comet pattern was observed, figure 2b shows lymphocyte incubated with 100 µM drug, figure 2c shows lymphocyte incubated with 200 µM drug and figure 2d shows lymphocyte incubated with 200 µM 5-fluorouracil. The compound 2 did not exhibit apoptotic damage to the level of 5-fluorouracil. Tail length migration presented in (Table 1) show that the molecule is capable of causing severe damage to human genomic DNA. The enhancement in DNA damage suggested that steroid derivative induced dose-dependent chromosomal fragmentation leading to apoptosis. The slides were screened for measuring the length of tail shown by the comet after acting with steroid and the outcome is shown in Figure 3.

<table>
<thead>
<tr>
<th>Tail Length (µm)</th>
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<tbody>
<tr>
<td>Control lymphocyte</td>
</tr>
<tr>
<td>Lymphocyte + 100 µM compound 2</td>
</tr>
<tr>
<td>Lymphocyte + 200 µM compound 2</td>
</tr>
<tr>
<td>Lymphocyte + 200 µM 5-fluorouracil</td>
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Table 1: Tail length Migration in µm

DNA- compound interaction by molecular docking method

Here the DNA nitrogen base sequence (taken from Protein Data Bank ID: 1BNA) was docked with compound 2 so as to clarify the target point as well as the expected stable interaction of the steroid in the DNA groove. As evident from figure 4 that compound 2 interacts in the minor groove and in this configuration the 3β-acetoxy group intercalates with DNA via a phosphodiester bond. The compound did not show any hydrogen bond but literature reveals that existence of intercalating forces like van der Waals forces or hydrophobic are potentially significant than hydrogen bonding. The resulting decrease in energy of steroid-DNA complex is –276.5 KJ mol⁻¹ which proves the stable and potent interaction between duplex and steroid molecules.

Fig. 4: Model of compound 2-nitrogen bases (downloaded from protein data bank) done by HEX 6.1 software
Physicochemical properties

Bioactivity score

The bioactivity scores of the compound were also calculated for six parameters, GPCR ligand activity, ion channel modulation, kinase inhibition activity, protease inhibitor, enzyme inhibitor and nuclear receptor ligand activity. As we know for organic molecules, if the bioactivity score is more than 0.00 then, the compound is active, but if it is between -0.50 and 0.00 then the compound is moderately active and if the compound has -0.50 then it is inactive compound. The potential bioactivity score of the compound is given in (Table 2) which clearly show that compound show those properties which are required for the characteristics of compound for acting as a drug.

PASS analysis

Molecular properties such as membrane permeability and bioavailability are always associated with some basic molecular descriptors such as log P (partition coefficient), molecular weight (MW), hydrogen bond acceptors and donors count in a molecule. Lipinski used these molecular properties in formulating his ‘Rule of Five’. This rule states that most molecules with good membrane permeability have log P $\leq$ 5, molecular weight $\leq$ 500, number of hydrogen bond acceptors $\leq$ 10 and number of hydrogen bond donors $\leq$ 5. This rule is widely used as a filter for drug-like properties. Although the required molecule did not showed better permeability by showing log P $>$ 5 but other parameters like topological surface area, molecular weight, hydrogen bond acceptor and hydrogen bond donor show that compound has potential to act as drug (Table 3). Also by considering the bioactivity score, the overall potential of the compound can be said to be physiologically active.

Fluorescence spectral studies

At room temperature, compound emit luminescence at 324 nm respectively in 0.01 tris-HCl/50mM NaCl buffer when excited at 260 nm. Fixed amount of compound was titrated with increasing amount of CT DNA, over a range of 5.3 - 2.6 x 10^-5 M DNA concentration. The addition of DNA caused a gradual decrease in fluorescence emission (Figure 5) intensity being consistent with the non-intercalative binding mode such as electrostatic binding mode (surface binding) and be protected by

<table>
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<th>Table 2: Depicting the bioactivity score of the compound</th>
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<tr>
<td><strong>Parameters of Bioactivity score</strong></td>
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<tr>
<td>GPCR ligand 0.08</td>
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<tr>
<td>Ion channel modulator 0.17</td>
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<tr>
<td>Kinase inhibitor -0.64</td>
</tr>
<tr>
<td>Nuclear receptor ligand 0.81</td>
</tr>
<tr>
<td>Protease inhibitor -0.03</td>
</tr>
<tr>
<td>Enzyme inhibitor 0.48</td>
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<tr>
<th>Table 3: Showing the physicochemical properties of the compound</th>
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<tr>
<td><strong>Lipinski rule of 5 parameters</strong></td>
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<tr>
<td>1. mi Log P 7.767</td>
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<tr>
<td>2. TPSA 26.305</td>
</tr>
<tr>
<td>3. MW 426.685</td>
</tr>
<tr>
<td>4. HBA 2</td>
</tr>
<tr>
<td>5. HBD 0</td>
</tr>
<tr>
<td>6. No-of violations 1</td>
</tr>
<tr>
<td>7. N0-of rotatable bonds 7</td>
</tr>
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1. The log P value calculated using mol inspiration server
2. Topological polar surface area (defined as a sum of surfaces of polar atoms in a molecule)
3. Molecular weight
4. Hydrogen bond acceptor (expressed as the sum of O and N atoms)
5. Hydrogen bond donor (expressed as the sum of OH and NH)

Fig. 5: Emission spectrum of compound in Tris-HCl buffer (pH 7.2) in the presence and absence of CT DNA at room temperature. Arrow shows change in intensity with increasing concentration of DNA.
DNA efficiently, since the hydrophobic environment inside the DNA helix reduces the accessibility of solvent molecule to the complex, which as a consequence restrict the complex mobility at binding site, leading to a decrease of the vibrational modes of relaxation. Also, the number of bound steroid molecules per DNA (n) calculated for was found to be 1.06 from fluorescence spectral data. The binding constant K, determined from the Scatchard equation for compound was calculated to be \(1.68 \times 10^3\) M\(^{-1}\).

EXPERIMENTAL

General

IR spectrum was determined in KBr with Perkins Elmer 237 spectrophotometer. \(^1\)H NMR spectrum were run in CDCl\(_3\) on Bruker AV 400. \(^13\)C NMR was also run in CDCl\(_3\) on Bruker DRX 300 and its values are given in ppm. Reaction completion was checked by thin layer chromatography it is cholecalciferol acetate (vit D\(_3\) (commercial substance) was prepared by acetylation of the corresponding sterol with acetic anhydride and pyridine.

Synthesis of 3\(\beta\)-acetoxy-9, 10-seco-19, 8(8)-spiro-5(10), 6-cholestadiene (2)

To a solution of cholecalciferol acetate (1 mol) in toluene, was added 2, 3-dimethylbutadiene (1 mol) and BF\(_3\)-OEt\(_2\)/SnCl\(_2\)/ZnBr\(_2\)/p-TsOH (2 mol) The refluxing was continued for 2-3 h. After completion, the crude yield was obtained after removing solvent under reduced pressure that was later extracted with ether, recrystallized from ethanol to give an oily compound 2.

Characterization

TLC: \(R_f = 0.71\) (Petrol/ether) (3:1); IR (\(\nu cm^{-1}\)) 1026 cm\(^{-1}\) (C-O), 1735 cm\(^{-1}\) (\(\nu CO\)), 3426, 2918, 2844 cm\(^{-1}\) for diene system. \(^1\)H NMR (500 MHz) \(\delta\) 5.2 (m, 1H, C\(_{17}\)-\(\alpha\)H), 5.8 (d, 1H, CH\(_2\)), 6.8 (d, 1H, CH\(_2\)), 2.1 (s, 3H, \(\alpha\)-CH\(_3\)), 0.69 (s, 3H, C\(_{17}\)-\(\alpha\)-CH\(_3\)). 0.96, 0.89 and 0.81 (other CH\(_2\)) \(^13\)C NMR (125 MHz) \(\delta\) (26.5) C\(_{19}\), (27.7) C\(_{20}\), (71.1) C\(_{21}\), (36.3) C\(_{22}\), (135.1) C\(_{23}\), (138.2) C\(_{24}\), (126.2) C\(_{25}\), (35.5) C\(_{26}\), (45.3) C\(_{27}\), (182.2) C\(_{28}\), (21.5) C\(_{11}\), (38.4) C\(_{12}\), (39.5) C\(_{13}\), (55.5) C\(_{14}\), (25.9) C\(_{15}\), (28.8) C\(_{16}\), (56.5) C\(_{17}\), (14.5) C\(_{18}\), (12.7) C\(_{19}\), (29.6) C\(_{20}\), (18.4) C\(_{21}\), (36.4) C\(_{22}\), (42.2) C\(_{23}\), (38.2) C\(_{24}\), (28.2) C\(_{25}\), (22.5) C\(_{26}\), (23.5) C\(_{27}\), (170.9), (170.9) C\(_{1}\), (21.5) C\(_2\).

Evaluation of In vitro DNA Damage using plasmid PBR322

The mixture of buffer (pH7.5, 30 µl), 0.5 µg PBR322 DNA and 100 µM compound 2 was prepared. The 20 µl solution containing 40 mM ethylenediaminetetraacetic acid, 0.05% bromophenol blue and 50% (v/v) glycerol was added after incubation for 1 h. The mixture was electrophoresized in submarine 1% agarose paste. UV-transilluminator was used to view and photograph the Ethidium bromide stained gel.

Evaluation of Ex vivo DNA damage by comet assay using human lymphocytes

Comet assay confirmed by the method incarnated and used by Rizvi and co-workers\(^6\) with certain changes. Normal melting agarose was used to precoat the fully frosted slides and to form a cell suspension, few micro litres of agarose (low m.p) and lymphocytes were mixed and put over the former paste and were put hidden. To solidify agarose, the strains were cooled down for few min in a refrigerator. The removal of cover slips was done gently and another coating of 0.5% agarose (low m.p) was drawn over the already solidified layer. For solidification, the covered strains were put on the slides and drawn in cold. The removal of covered slips was done and the ice cold lysis buffer was taken in which slides were immersed in for an hour. After lysis in basic electrophoretic medium, the DNA was allowed to unwind. The gel documentation system was worked out at 4 °C in field strength of 0.7 V/cm and 300 mA current. The strains got neutralized with ice cold 0.4 M buffer pH 7.5, stained with 75 µl EtBr (20 mg/ml) and covered with a cover slip. A fluorescent microscope incorporated with CC camera was used to picture the images of strains. Analysis included images of 25 cells in each triplicate. DNA disintegration was measured in terms of the tail length.

Molecular docking

HEX 6.1 software was used to proclaim the molecular docking studies\(^7\). HEX is a software program for analyzing the binding energy, depicting stereo chemical interacting modes of ligand with DNA and stability of docked complex. It studies the interaction between the heterocycle and the host (DNA \([\text{d(CGCAAAATTTCGC)}]_2\) (PDB ID: 1BNA) / protein) (as input) in protein data bank format and
the output is a stable complex deduced with shape complementary and decrease in binding energy criteria. The compound structure was generated by Avagadro 1.01 software using MMFF94 force field. Visualization of the interacted complex has been done by using PyMol (http://pymol.sourceforge.net/) graphic programming. An Intel CORE i5, 2.6 GHz based computer was used to sort the calculations.

Determination of physicochemical properties  Bioactivity score and PASS (Prediction of biological activity spectra) analysis

The physicochemical parameters including octanol partition coefficients (mLogP), Mw, HBD, HBA, TPSA and Rotatable bonds were calculated using molinspiration server (http://www.molinspiration.com/cgi-bin/properties) and ChemAxon (chemicalize.org). The PASS (Prediction of biological activity spectra) approach is based on the structure-activity correlation, Compound activity prediction is done by “comparing” the structure of query compound with the structure of well-known biological active substrate existing in database of the freely available PASS web service. Molecule activity prediction is done by “comparing” the structure of query compound with the structure of well-known biological active substrate existing in database of PASS web service.

Fluorescence Spectral study

The fluorescence measurements were carried out on a Shimazdu spectrofluorimeter (Model USA), while DNA (5.3 - 2.6 × 10^{-5}) pre-treated with compound (4 × 10^{-5}M) for 30 min in buffer. In the fixed concentration of compound, the emission intensities of DNA were recorded between 274 and 500 nm, at the excitation wavelength of 260 nm.

CONCLUSION

A convenient and facile route for synthesizing new cholestadiene derivative has been reported. The gel electrophoresis depicted that compound (in comparison to 5-fluorouracil) acts to be active DNA cleaving agent. Comet assay depicted that compound exhibited potential cell apoptosis hence proved to be genotoxic nature. The interactive docking studies undertaken revealed minor groove binding of compound to the DNA molecule. The bioactivity score and PASS analysis also predicted the potential of the compound to act as physiologically active drug. The fluorescence studies also agreed the hypochromism behavior of the compound depicting the decrease in the intensity which also correlates the results with the DNA cleaving ability of the compound as shown in gel electrophoresis.

ACKNOWLEDGEMENTS

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