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Research Article

Synthesis, Spectral Characterization, and *In Vitro* Cytotoxicity of N-2′-Hydroxyethyl-Substituted Azacholestanes Prepared from 6-Oxocholestanes by Modified Schmidt Reaction

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The present paper reports the synthesis and spectroscopic characterization of few N-2′-hydroxyethyl-substituted azacholestanes using BF_3-OEt_2, $TiCl_4$, $SnCl_4$, and H_2SO_4 as catalysts in moderate yields by a modified version of Schmidt reaction. A notable feature is the passivity of $SnCl_4$ in case of 3β-acetoxy-N-2′-hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one and 3β-chloro-N-2′-hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one. However, the reaction was unsuccessful in case of N-2′-Hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one. Another striking aspect is the attainment of high yield in case of H_2SO_4 as catalyst. The semisolid compounds are characterized using various spectroscopic techniques such as FT-IR, ^{1}H-NMR and mass spectra, and microanalytical data. A reaction mechanism has been proposed on the basis of previous studies. Moreover, the compounds have also been screened for their *in vitro* cytotoxicity against human colon carcinoma cell line, HCT116, and human liver hepatocellular carcinoma cell line, HepG2, using doxorubicin as standard. On the basis of IC_{50} values, 3β-chloro-N-2′-hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one (**5**) was found to inhibit the cancer cells most effectively.

1. Introduction

Steroidal chemistry has been an area of intense research not from an organic chemist perspective but also for an endocrinologist because of its fundamental importance in an array of biological functions [1]. The replacement of one or more carbon atoms of a steroidal moiety by a heteroatom alters its chemical properties which in certain cases lead to development of useful molecules or drugs [2]. However, insertion of nitrogen into steroidal as well as nonsteroidal nucleus has been effected by the reaction of steroidal ketones as well as nonsteroidal ketones with hydrazoic acid in the presence of protonic or Lewis acids [3–10]. The Beckmann rearrangement of oximes and Schmidt reaction is one of the well-known protocols for the synthesis of lactams. The heterosteroids, particularly azasteroids are obtained by this protocol [11–14]. Lactams are important molecules owing to their versatility as intermediate in the preparation of both synthetically and biologically active compounds [15, 16]. They have also proven to be extremely informative as transition state model in amide reactivity studies [17]. Interestingly simple N-substituted lactams are known to possess significant CNS activity [18]. Similarly, N-substituted hexahydroazapines have also been found to possess significant biological activities particularly they are used as an antitussive, mydriatics, antispasmodics, and oral hypoglycemic [19]. A substantial amount of biologically relevant azasteroids have been reported by Martin-Smith et al.

[20–22]. During the last decade, a number of azasteroids have been synthesized as 5α-reductase inhibitors [23–27]. Guarna and coworkers have also synthesized and explored the biological application of a novel class of potent azasteroidal inhibitors having nuclear nitrogen atom at position C-10 of the steroidal ketones [28]. Several reports pertaining with the preparation of azasteroids have been mentioned in the literature [29–32]. Despite the development of a number of methods, the Schmidt and Beckmann reactions remain the most convenient and general protocol for insertion of nitrogen atom into the steroidal nucleus [33–39]. Besides, the large number of synthetic applications, azasteroids also owes versatile biological activities. For instance, the homo-aza-steroidal esters of [p-[bis(2-chloroethyl)amino]phenyl] acetic acid and [p-[bis(2-chloroethyl)amino]phenyl]butyric acid are successfully used to treat several types of leukemia [40]. Recently, several substituted 4-azasteroids have also been found to be very effective against two human tumor cell lines, cervical carcinoma (HeLa), and chronic myelogenous leukemia (K-562) [41]. These findings prompted us to explore the *in vitro* cytotoxicity of N-2′-hydroxyethyl-substituted azacholestanes. In this paper, a modified version of Schmidt reaction has been adopted as reported by Gracias et al. [42] where hydrazoic acid has been replaced by hydroxyalkyl azide in presence of BF_3–OEt_2, $TiCl_4$, $SnCl_4$, or H_2SO_4 as catalysts. Herein, we report the synthesis of N-hydroxyalkyl lactams from easily accessible steroidal ketones such as 3β-acetoxy-5α-cholestan-6-one (**1**) [43], 3β-chloro-5α-cholestan-6-one (**2**) [44], and 5α-cholestan-6-one (**3**) [45]. On reaction with hydroxyalkyl azide in the presence of different Lewis acids (BF_3–OEt_2, $TiCl_4$, or $SnCl_4$) and protonic acid (H_2SO_4), it gives 3β-acetoxy-N-2′-hydroxyalkyl-6-aza-B-homo-5α-cholestan-7-one (**4**) and its analogues **5** and **6**, respectively.

2. Experimental

2.1. Apparatus and Reagents. Melting points were determined on a Kofler apparatus and are uncorrected. IR spectra were recorded as neat with Pye Unicam SP-3-100-spectrophotometer and its values are given in cm^{-1}. Elemental analyses (C, H, and N) were carried out with a Carlo Erba EA-1108 analyzer. ^{1}H-NMR spectra were measured in $CDCl_3$ on a Bruker AC 300 (300 MHz) with TMS as internal standard and its values are given in ppm (δ) (s, singlet; br, broad and mc, multiplet centered at). ^{13}C NMR spectra were recorded on a Bruker Avance II 400 spectrophotometer, with chemical shifts reported in parts per million relative to the residual deuterated solvent. Mass spectra were measured on VG-Micromass model ZAB-IF apparatus at 70 eV ionization voltage. Thin-layer chromatography (TLC) was performed on glass plates precoated with silica gel G and exposed to iodine vapors to monitor the reactions and to certify the purity of the reaction products. Silica gel (mesh size 60–120, BDH) was used for (~25 gram for each gram of material) purification using gravity column chromatography. All the reactions were performed under the anhydrous condition. Petroleum ether refers to a fraction of bp 60–80°C. Sodium sulfate (anhydrous) was used as a drying agent for organic extracts after reaction workup. All the solvents were distilled prior to use.

2.2. In Vitro Cytotoxicity. The *in vitro* cytotoxicity of 3β-acetoxy-N-2′-hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one (**4**), 3β-chloro-N-2′-hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one (**5**), and N-2′-hydroxy-ethyl-6-aza-B-homo-5α-cholestan-7-one (**6**) were performed employing the cell lines HCT116 (human colon carcinoma cell line), HepG2 (human liver hepatocellular carcinoma cell line), and one noncancerous HFL1 (human lung fibroblast) using a standard 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay.

Cells in exponential growth were seeded into 96-well plates at concentration of 5×10^5 cells/200 μL/well and allowed to grow in specific medium containing 5% FCS. After 24 h, cells were treated with various concentrations of test compound at a concentration range of 0–25 μM. Control (ethanol only) and positive control (doxorubicin) cells were cultured using the identical conditions. After 96 h of incubation, the medium was removed and replaced with fresh medium. MTT reagent (5 mg/mL in PBS) was added to each well at a volume of 1 : 10 and incubated for 2 to 3 h at 37°C. After treatment, 100 mL of DMSO was added to each well after carefully aspirating the supernatants. Absorbance was measured at 620 nm in a multiwell plate reader. Triplicate wells were prepared for each individual concentration. Dose-response curves were plotted as percentages of the cell absorbances. Drug sensitivity was expressed in terms of the concentration of drug required for a 50% reduction of cell viability (IC_{50}).

The IC_{50} value was defined as the concentration of test sample resulting in a 50% reduction of absorbance as compared with untreated controls that received a serial dilution of the solvent, in which the test samples were dissolved, and was determined by linear regression analysis.

Caution! Care must be taken while handling H_2SO_4, BF_3–OEt_2, $TiCl_4$, or $SnCl_4$ as these are irritant and corrosive to skin. However, we have used smaller amounts and no such effect was observed.

2.3. General Procedure of Synthesis of N-Substituted Azasteroids [46–56]. Steroidal ketone **1** (0.45 mmol) in CH_2Cl_2 (6 mL) was treated with 2-azidoethanol (3 mmol) and cooled to 0°C. H_2SO_4 (~0.25 mL); BF_3–OEt_2, $TiCl_4$, or $SnCl_4$ (~0.5 mL), respectively, were then added dropwise over a period of 5 to 6 min, and the subsequent evolution of gas was recorded. The reaction was kept at 0°C for 30 min and allowed to attain room temperature and then stirred for 6 h. The resulting solution was concentrated, and saturated solution of $NaHCO_3$ was added to the residual oil. The reaction mixture was then stirred for another 1 h at room temperature. After concentration, 75 mL of CH_2Cl_2 was added and then the organic layer was separated, dried, and concentrated to afford oil **4**, which was chromatographed over silica gel column.

Elution with petroleum ether-acetone (8 : 1) afforded product **4** as a glassy semisolid, which was failed to crystallize in common organic solvents and their mixtures.

*2.4. 3β-Acetoxy-N-2′-hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one (**4**).* [Found: C 73.95; H 10.54; N 2.78.$C_{31}H_{53}NO_4$ calcd: C 73.92; H 10.49; N 2.72]. IR (KBr) ν_{max}: 3480 (OH), 1730 (ester), 1670 (amide) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H: 4.4 (m, 1H, C3–αH, $W_{1/2}$ = 16 Hz), 3.6 (t, 2H, J = 5.3 Hz, CH_2–N), 3.1 (t, 2H, J = 5.1 Hz, CH_2–OH), 2.9 (br, 1H, C5–αH), 2.2 (d, 2H, J = 6.6 Hz, C7a–H), 2.1 (s, 3H, CH_3COO), 2.0 (s, 1H, OH), 1.2 (C10–CH_3), 0.67 (C13–CH_3), 0.91, and 0.85 (for side chain methyl groups). ^{13}C NMR ($CDCl_3$) δ_C: 170.7 ($OCOCH_3$), 169.9 (N–C=O), 74.0 (C-3), 56.7 (C-14), 56.2 (C-17), 55.5 (C′-1), 50.0 (C-9), 47.3 (C′-2), 42.9 (C-13), 39.7 (C-5), 39.6 (C-12), 38.2 (C-8), 37.1 (C-1), 36.6 (C-20), 36.2 (C-22), 35.8 (C-10), 34.2 (C-7a), 31.9 (C-4), 27.9 (C-16), 28.1 (C-24), 27.8 (C-2)*, 24.4 (C-23), 23.9 (C-15), 22.8 (C-25)*, 22.6 (C-11), 21.9 (C-27), 21.5 (C-21), 21.0 ($OCOCH_3$), 19.4 (C-26), 18.8 (C-19), and 11.9 (C-18), Asterisks denote assignments that may be interchanged EI-MS(m/z): 503 [$M^+_\bullet$], 390 [M-C_8H_{17}]$^+$.

Under similar reaction conditions steroidal ketones **2** and **3** gave compound **5** and **6**, respectively, as semisolid after elution with petroleum ether-acetone (8 : 2) in the silica column, respectively.

*2.5. 3β-Chloro-N-2′-hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one (**5**).* [Found: C 72.71; H 10.45; N 2.80. $C_{29}H_{50}NO_2Cl$ calcd: C 72.65; H 10.43; N 2.92]. IR (KBr) ν_{max}: 3500 (OH), 1660 (amide) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H: 4.2 (m, 1H, C3–αH, $W_{1/2}$ = 17 Hz), 3.7 (t, 2H, J = 5.4 Hz, CH_2–N), 3.4 (m, 2H, CH_2–OH), 2.9 (m, 1H, C5–αH), 2.6 (d, 2H, J = 6.5 Hz, C7a–H), 2.2 (s, 1H, OH), 1.1 (C10–CH_3), 0.66 (C13–CH_3), 0.91, and 0.85 (for side chain methyl groups). ^{13}C NMR ($CDCl_3$) δ_C: 168.9 (N–C=O), 59.5 (C-3), 53.5 (C′-1), 50.0 (C-9), 47.8 (C′-2), and other ^{13}C NMR signals are in close accord with compound **4**. EI-MS(m/z): 479/481 [$M^+_\bullet$], 366/368 [M-C_8H_{17}]$^+$.

*2.6. N-2′-Hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one (**6**).* [Found: C 78.51; H 11.57; N 3.25. $C_{29}H_{51}NO$ calcd: C 78.28; H 11.46; N 3.14.]. IR (KBr) ν_{max}: 3480 (OH), 1685 (amide) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H: 3.8 (t, 2H, J = 5.2 Hz, CH_2–N), 3.4 (t, 2H, J = 5.1 Hz, CH_2–OH), 2.8 (m, 1H, C5–αH), 2.5 (d, 2H, J = 6.5 Hz, C7a–H), 2.0 (s, 1H, OH), 1.2 (C10–CH_3), 0.67 (C13–CH_3), 0.95, and 0.85 (for side chain methyl groups). ^{13}C NMR ($CDCl_3$) δ_C: 167.3 (N–C=O), 55.1 (C′-1), 50.3 (C-9), 48.2 (C′-2), 25.5 (C-3), and other ^{13}C NMR signals are in close accord with compound **4**. EI-MS(m/z): 445 [$M^+_\bullet$], 332 [M-C_8H_{17}]$^+$.

3. Results and Discussion

The present work reports convenient synthesis of N-substituted azacholestanes from a modified version of Schmidt reaction. The easily accessible steroidal ketones (**1**, **2** and **3**) were synthesized by the literature method [43–46]. The experiments were carried out with above ketones (**1**, **2**, and **3**) and 2-azidoethanol in dichloromethane. Thus, when 2-azidoethanol was added to a solution of steroidal ketone (**1**) in BF_3–OEt_2, $TiCl_4$, $SnCl_4$, or H_2SO_4, respectively, vigorous gas evolution was observed immediately. Then a saturated aqueous $NaHCO_3$ solution was added in the reaction mixture followed by standard workup resulted in the formation of desired N-substituted azasteroid (**4**) (Scheme 1).

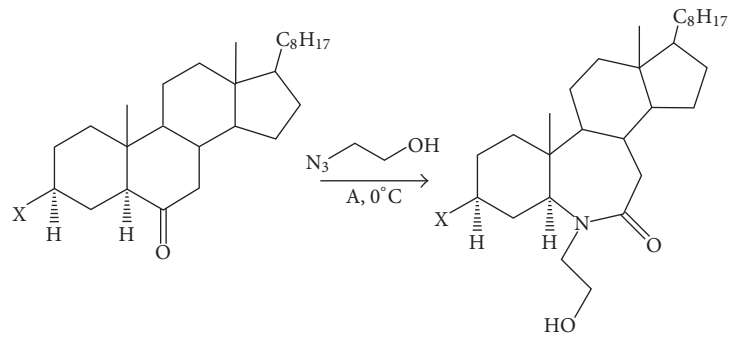
Table 1: Yields of the reaction between protonic acid and lewis acids with 2-azidoethanol starting from ketone **1, 2** or **3**.

Compounds	H_2SO_4 (% yield)	BF_3–OEt_2 (% yield)	$TiCl_4$ (% yield)	$SnCl_4$ (% yield)
4	75	50	45	20
5	70	45	40	12
6	65	45	40	—

The yields of purified compounds (**4**, **5**, and **6**), starting from steroidal ketones (**1**, **2**, and **3**) are given in Table 1. Of the several Lewis acids and protonic acid H_2SO_4 was found to be the most convenient in term of availability, effectiveness, and its relative ease of workup. The structure of compound **4** was ascertained by IR, ^{1}H NMR, ^{13}C NMR and MS spectra, and microanalysis. On the basis of these studies and perusal of literature data, a tentative mechanism has been proposed (Scheme 2).

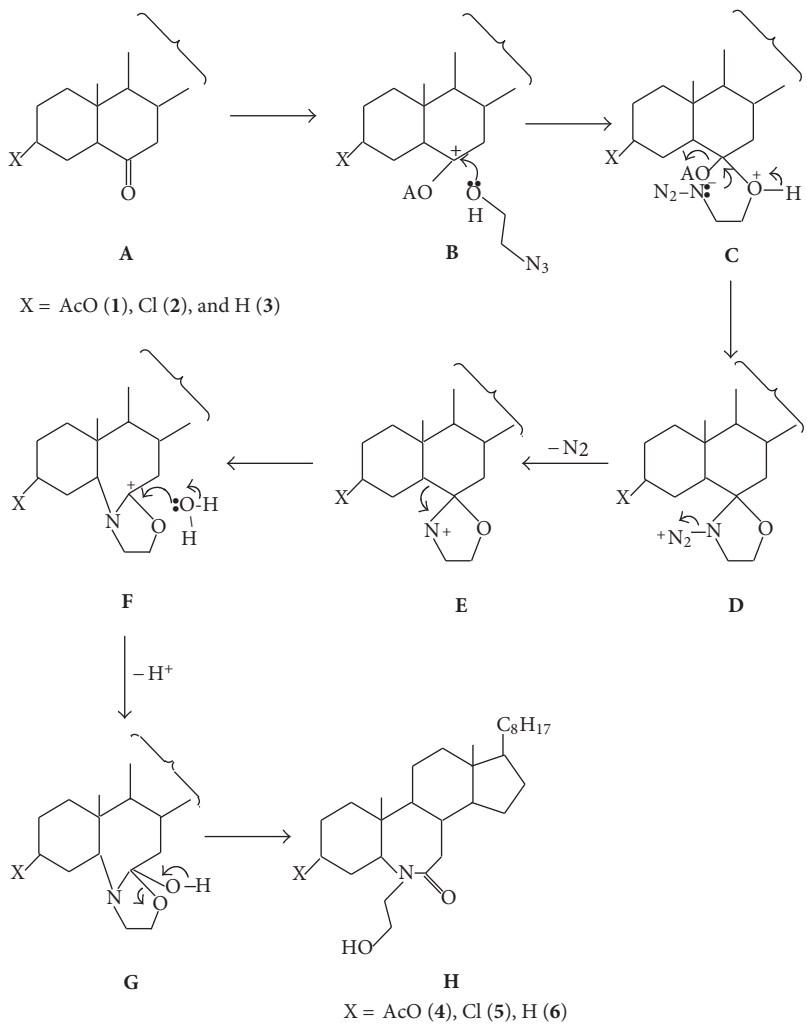
Compound **4** gave diagnostic IR bands at 3480 (OH), 1730 and 1241 (CH_3COO), 1670 (–CO–N), and 1280 cm^{-1} (amide band) supporting the insertion of nitrogen atom into steroidal nucleus. The ^{1}H-NMR spectrum is in conformity with the structure and exhibits diagnostic singles at δ 4.4 ($W_{1/2}$ = 16 Hz) ascribed to C3–αH as a multiplet, suggesting that junction A/B is trans. A peak observed at δ 2.9 may be due to C5–αH while a triplet observed at δ 3.6 (J = 5.3 Hz) may be ascribed to CH_2–N. Similarly a single at δ 3.1 (J = 5.3 Hz) for two protons corresponding to CH_2–OH, while the singlet at δ 2.1 may be due to three hydrogen of acetate. Interestingly one singlet was observed at δ 2.0 which gets vanished when exchanged with deuterium. (The hydroxy proton resonance observed at δ 2.0 was disappeared on the addition of D_2O) However, the angular methyl protons (C10–CH_3) and (C13–CH_3) were observed as singlet and side-chain methyl protons (C21–CH_3) and (C25– $(CH_3)_2$) as doublets at δ 1.2, 0.67, 0.91, and 0.85, respectively. These spectral studies and the proposed mechanism (Scheme 2) are in accordance with the formation of **4**.

The possibility of formation of 7-azacholestanes (**7**) can be ruled out on the basis of the ^{1}H NMR spectrum. The position of C5–αH may be successfully employed to differentiate peak of 6-aza (**4**, **5**, and **6**) and 7-azacholestanes (**7**) (Scheme 3) as evident from a large number of reports. If C5–αH was observed at ≤3.0 ppm, then there is a formation of 6-azacholestanes (**4**, **5**, and **6**) while any value greater than δ 3.0 results in the formation of 7-azacholestanes (**7**). In one of the studies, C5–αH protons were found to resonate

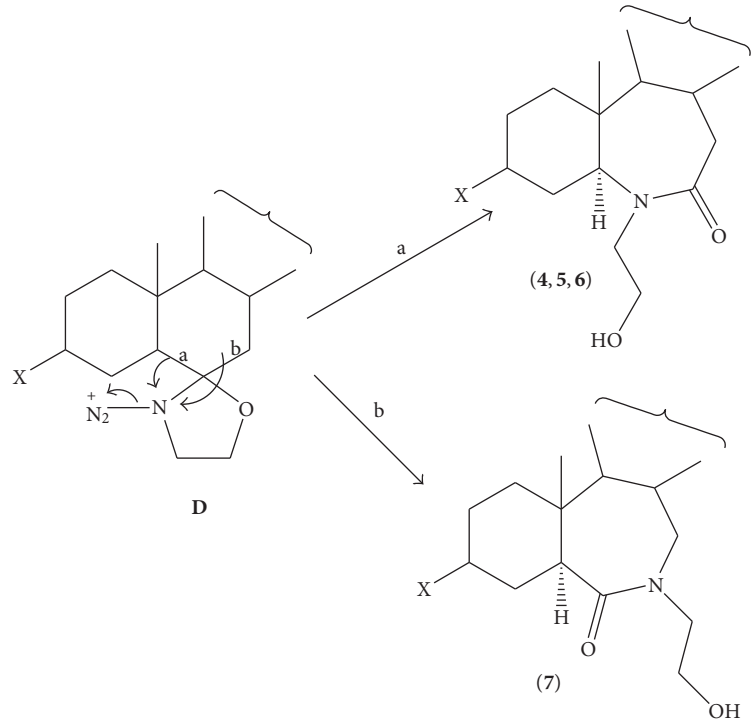


X	A	X
AcO (**1**)	BF_3–OEt_2	AcO (**4**)
Cl (**2**)	$TiCl_4$	Cl (**5**)
H (**3**)	$SnCl_4$	H (**6**)
	H_2SO_4	

Scheme 1: N-substituted Azacholestanes.



Scheme 2: Synthetic pathway for preparation of N-substituted Azacholestanes (**4**–**6**) (where A = Lewis acid or protonic acid).



Scheme 3: Migration ability of a and b carbon.

Table 2: Cytotoxicity of compounds **4**–**6** against the cell lines.

Compounds	Cytotoxicity of Cell lines (μM)		
	HCT116	HepG2	HFL1
4	4.66	5.02	Not active
5	2.76	3.12	Not active
6	4.51	5.18	Not active
Doxorubicin	2.53	2.88	Not active

The highest concentration tested was 25 μM for compounds **4**–**6** and all the values are an average of three observations.

more than 5.0 ppm [46] while in the present case, we have observed C5–αH at δ 2.9 ppm, which clearly indicates the regioselectivity expansion of B-ring of cholestanes and leads to the formation of 6-azacholestanes (**4**, **5**, and **6**).

Generally, the regioselectivity found in the Schmidt reaction is rooted in the migratory aptitudes of the groups attached to the carbonyl carbon. It is commonly observed that for acyclic or cyclic ketones, the more electrons donating substituent attached to the carbonyl will preferentially migrate.

Moreover, in the ^{13}C NMR spectrum, the peak appearing in the range of δ 167–169 ppm corresponds to –N–C=O of the lactam implying the insertion of nitrogen atom of the azidoethanol reagent into steroid B-ring while other characteristics ^{13}C NMR signals appeared at δ 170.67 (CH_3COO), 55.50 (C$'$-1), 47.37 (C$'$-2), and 34.27 ppm (C-7a), respectively.

Hence, it has been characterized as 3β-acetoxy-N-2$'$-hydroxyalkyl-6-aza-B-homo-5α-cholestan-7-one (**4**). Products **5** and **6** were also characterized on the basis of similar accounts. Moreover, the mass spectrum of compound (**4**) further establishes its formation and gave ion peaks for respective fragments. Molecular ion peak was observed at *m/z* 503 ($M^{+}_{\bullet}$), and other notable peaks include *m/z* 390 (M-C_8H_{17}).

The proposed mechanism for the formation of desired products (**4**, **5**, and **6**) is given on account of previous findings [46–48]. It is believed that the Lewis acids/protonic acid activate the carbonyl group followed by attack of hydroxyl group of reagent to afford **C** (Scheme 2). Subsequent dehydration of **C** leads to the formation of oxonium ion that undergoes intramolecular azide attack, forming iminium ether intermediate in a concerted step, **D** and **E**. After loss of N_2, it results in the formation of cation, **F**, which on hydrolysis yields the titled compounds **H**.

The compounds **4** to **6** were tested against two human cancer cell lines: HCT116 and HepG2 and one noncancerous HFL1 (human lung fibroblast) cell line. The IC_{50} values for these compounds were compared to doxorubicin, a well-known anticancer drug. The result implies that the compounds **4** to **6** inhibit various cancer cell lines in a dose-dependent manner. However, the IC_{50} of 3β-chloro-N-2$'$-hydroxyethyl-6-aza-B-homo-5α-cholestan-7-one (**5**), is

found to be comparable with doxorubicin (Table 2). The antitumor efficacy of compound **5** may be attributed to its binding to cellular Fe pools. This inactivates ribonucleotide reductase, the enzyme that catalyzes the conversion of ribonucleotides to deoxyribonucleotides. A strong positive correlation was established between RR activity and the rate of replication of tumor cells. The inhibition of RR prevents the production of deoxyribonucleotides. As a consequence these compounds interfere with DNA synthesis, thus decreasing the rate of replication of tumor cells and inhibiting tumor growth. The antitumor activity seems to be due to an inhibition of DNA synthesis in cancer cells produced by modification in reductive conversion of ribonucleotides to deoxyribonucleotides [57].

4. Conclusion

The present work describes facile synthesis of N-substituted aza cholestanes starting from conveniently accessible steroidal ketones. The adopted procedure supports the utility of organoazides and expands the scope of these reactions to employ hydrazoic acid as the azide source. The reported compounds have also shown potent *in vitro* cytotoxicity against human colon carcinoma cell line, HCT116, and human liver hepatocellular carcinoma cell line, HepG2.

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