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Synthesis and *In vitro* Anticancer Evaluation of Some New 5α-Cholestan-pyrazole Hybrids

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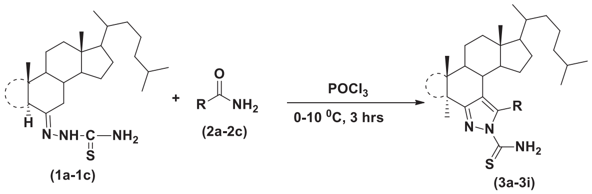
ABSTRACT The synthesis of some new 5α-cholestan-pyrazole hybrids from the reaction of 5α-cholestan-6-one-thiosemicarbazone derivatives with several amides using $POCl_3$ is demonstrated. The structures of newly synthesized hybrids were characterized by ^1H and ^{13}C NMR and mass spectral analysis. These compounds were screened for their *in vitro* anticancer activity over few human cancer cell lines such as ACHN, MCF-7, and A375 using doxorubicin as a positive control. Among them, compounds **3h** and **3i** have shown slightly higher activity than the positive control. The rest of the compounds have displayed moderate to good *in vitro* anticancer activity against all the tested cell lines as compared to the positive control. The IC_{50} values for more active **3h** and **3i** compounds were found to be ranging between 0.71 to 5.48 μM.



KEYWORDS Amides, Vilsmeier reagent, 5α-Cholestan-pyrazole hybrids, *In vitro* anticancer activity.

INTRODUCTION

Steroidal compounds have always gain a special place in medicinal chemistry research due to their key roles in numerous biologically active compounds. The development of new hydrophobic steroid core moieties is due to their proficient binding interaction with cell membranes which then further offers a route for the improved biological activity of respective hybrid compounds.[1] Interestingly, the fusion of heterocycles to steroids often leads to a change in physiological activities or appearance of new interesting biological behavior.[2] Hence, several steroidal-heterocycle-based pharmacologically active compounds such as antitumor, antileishmanial, antimicrobial, antiviral, and antianaphylactic proximities potential inhibitors of cytochrome P450 aromatase have been documented in the literature.[3,4]

Besides, pyrazole moiety can be known as active pharmacophore that shows necessary action in several biologically active compounds and thus became as an essential precursor in combinatorial as well as medicinal chemistry research.[5,6] Pyrazoles also serve as key intermediates for the synthesis of useful heterocyclic compounds in synthetic organic chemistry.[7] Moreover, pyrazole-based

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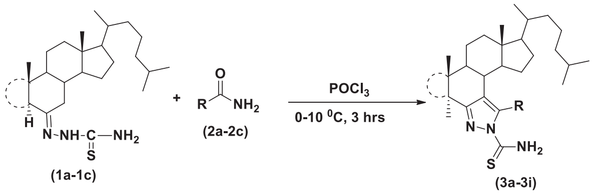
derivatives found to have potential biological activities including antimicrobial, anticancer, antioxidant, analgesic, anti-inflammatory, antipyretic, antiviral, anticonvulsant, antihistaminic, antidepressant, insecticidal, and anti-HIV.[8-13]

In view of the therapeutic importance of the steroidal pyrazoles,[14] interesting behavior of Vilsmeier reagent with simple hydrazones giving pyrazoles and[15-17] in continuation of efforts toward the development of new hetero steroids with an improved biological activity, we herein, report the reaction of some 5α-cholestan-6-one with thiosemicarbazide followed by reaction of resulting thiosemicarbazones with amides in the presence of $POCl_3$ to synthesize some new 5α-cholestan-6-pyrazole hybrids and to evaluate their *in vitro* anticancer activities against three human cancer cell lines, namely, A375, MCF-7, and ACHN.

RESULTS AND DISCUSSION

Chemistry

Synthetic approach to 5α-cholestan-6-one-pyrazole hybrids (**3a–3i**) is presented in **Scheme 1**. Initially, the starting materials were synthesized according to literature procedure (see experimental section). The synthesized 5α-cholestan-6-one thiosemicarbazone derivatives (**1a-1c**) were allowed to react with amide derivatives (**2a-2c**) in the presence of $POCl_3$ in acetonitrile to give corresponding products. The structures of newly synthesized hybrids **(3a-3i)** were characterized by ^{1}H and ^{13}C NMR and mass spectral and elemental analytical data.



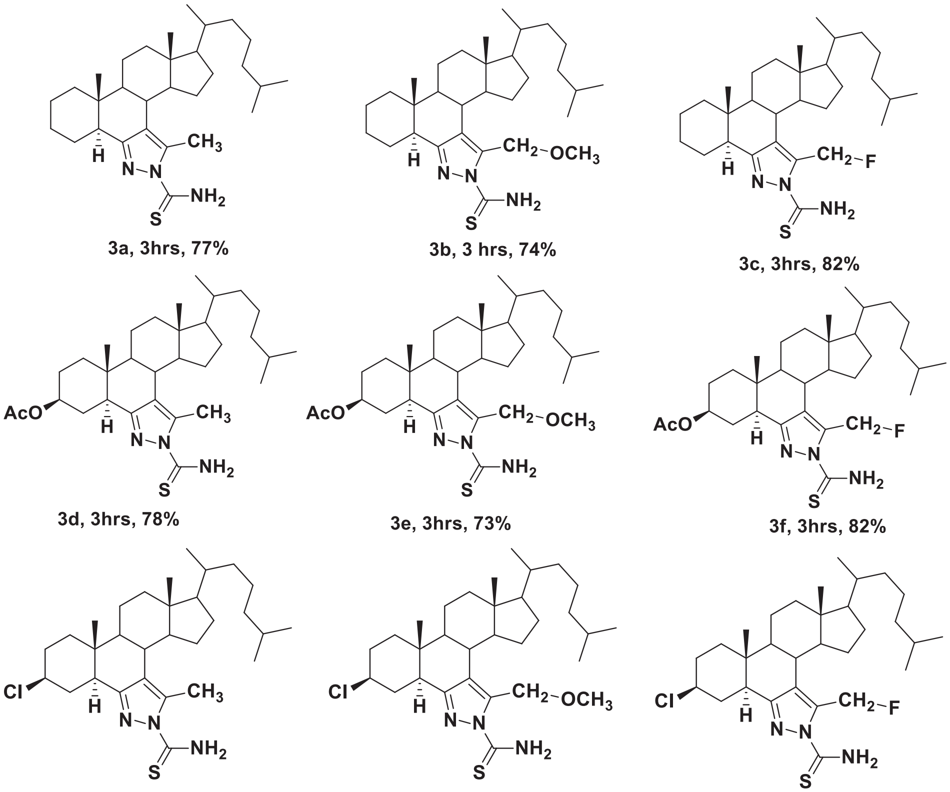
Reaction conditions: **1a-1c** (1 mmol), **2a-2c** (1 mmol), and $POCl_3$ (1 mmol) in 5 mL of CH_3CN

In vitro cytotoxicity

An *in vitro* cytotoxic activity of all the synthesized 5α-cholestan-pyrazole hybrids (**3a-3i**) has been investigated against three human cancer cell lines, namely, A375 (melanoma), MCF-7 (breast), and ACHN (renal) by following the microculture tetrazolium assay (MTT) and the IC_{50} values of compounds (**3a-3i**) are presented in **Table 1**. From **Table 1**, it is evident that the compounds **3h** and **3i** display anticancer activity against all the cell lines comparable to the standard drug doxorubicin. The IC_{50} values of most potent **3h** and **3i** were found in the range of 0.71–5.48 μM. The rest of the compounds were found to have low to moderate activity over all the cell lines when compared with standard drug.

EXPERIMENTAL

All the melting points were determined in degrees celsius on a Kofler apparatus and are uncorrected. ^{1}H and ^{13}C NMR



3a, 3hrs, 77%

3b, 3 hrs, 74%

3c, 3hrs, 82%

3d, 3hrs, 78%

3e, 3hrs, 73%

3f, 3hrs, 82%

3g, 3hrs, 79%

3h, 3hrs, 75%

3i, 3hrs, 81%

Scheme 1: Synthesis of 5α-cholestan-pyrazole hybrids (3a-3i)

Table 1: *In vitro* cytotoxicity of synthesized compounds 3a-3i

Compound	IC_{50} values (μM)		
	A375	MCF-7	ACHN
3a	12.48	11.12	9.18
3b	8.19	6.92	5.23
3c	6.02	3.12	2.73
3d	10.98	7.53	6.78
3e	8.17	4.34	2.64
3f	6.23	2.84	1.48
3g	6.07	2.24	1.23
3h	5.47	1.99	076
3i	5.41	1.83	0.72
Doxorubicin	5.51	2.02	0.79

spectra were run in $CDCl_3$ on a JEOL Eclipse (400 MHz) instrument with tetramethylsilane as internal standard and values are given in ppm (δ). Mass spectra were recorded on a JEOL SX 102/DA-6000 mass spectrometer. Thin-layer chromatography (TLC) plates were coated with silica gel G and exposed to iodine vapors to check the homogeneity as well as the progress of reaction. Petroleum ether refers to a fraction of boiling point 60–80°C. Sodium sulfate (anhydrous) was used as a drying agent.

Synthesis of 5α-cholestan-6-one thiosemicarbazones (1a-1c)

The synthesis of starting materials 5α-cholestan-6-one thiosemicarbazone **(1a),** 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone **(1b),** and 3β-chloro-5α-cholestan-6-one thiosemicarbazone **(1c)** was carried out according to procedure. To a boiling solution of steroidal-ketones (1 mmol) in methanol (5 mL) few drops of conc. HCL were added followed by the addition of thiosemicarbazide (1 mmol) and the resulting reaction mixture was refluxed for one hour and then cooled. The precipitate thus formed was collected via filtration and purified using recrystallization from methanol to achieve thiosemicarbazones **1a-1c** as shining needles.

(5α-cholestan-6-one thiosemicarbazone (**1a**) (m.p. 111–113°C) 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (**1b**) (m.p. 123–126°C) and 3β-chloro-5α-cholestan-6-one thiosemicarbazone (**1c**) (m.p. 118–121°C).

General procedure for the synthesis of 5α-cholestan-pyrazole hybrids (3a-3i)

To a solution of 5α-cholestan-6-one thiosemicarbazone derivative (**1**) (1 mmol) in CH_3CN (5 mL) was added appropriate amide (**2**) (1 mmol) under ice-cold condition. $POCl_3$ (1 mmol) was then added with constant stirring at such a rate that the temperature of the reaction mixture did not exceed 10°C. After the complete addition, the reaction mixture was allowed to attain room temperature; however, the stirring was continued for an additional period of 3 h. The progress of reaction was followed by TLC. After completion of the reaction, the reaction mixture was poured onto crushed ice, left overnight in a refrigerator, and was extracted with diethyl ether. The ethereal layer was further washed with water and dried over anhydrous sodium sulfate. Removal of solvents gave an oil which was crystallized from methanol to give the product **(3)**.

Characterization data of compounds (3a-3i)

5α-cholestano[6,7–c] 5’-methyl-1’-carbothioic acid amido pyrazole (3a)

Yield 77%; m.p. 121°C; ^{1}H NMR ($CDCl_3$): δ 7.6 (*s*, 2*H*, NH_2 exchangeable with D_2O), 2.4 $(s, 3H, C_5^{'} - CH_3)$, 1.19 (*s*, 3*H*), 0.96 and 0.83 (other methyl protons), 0.75(*s*, 3*H*), ^{13}C NMR ($CDCl_3$); δ 179.2, 150.1, 137, 118.2, 42.7,41.8, 39.4, 36.5, 25.2; MS: m/z 483 [M^+]; anal. calcd. for $C_{30}H_{49}N_3S$: C, 74.48; H, 10.21; N, 8.69%. Found: C, 74.46; H, 10.18; N, 8.66%.

5α-cholestano[6,7–c] 5’- methoxymethyl-1’-carbothioic acid amido pyrazole (3b)

Yield 74%; m.p. 127°C; ^{1}H NMR ($CDCl_3$): δ 7.6 (*s*, 2*H*, NH_2 exchangeable with D_2O), 4.54 (*s*, 2*H*), 3.34 (*s*, 3*H*), 1.18 (*s*, 3*H*), 0.76 (*s*, 3*H*), 0.96 and 0.83 (other methyl protons); ^{13}C NMR ($CDCl_3$); δ 179.2, 150.1, 137.2, 118.1, 75.9, 52.2, 42.8, 41.6, 39.5, 36.4; MS: m/z 511 [*M*+]; anal. calcd. for $C_{31}H_{51}N_3OS$: C, 72.46; H, 10.00; N, 8.18%. Found: C, 72.42; H, 9.88; N, 8.16%.

5α-cholestano[6,7–c] 5’- fluoromethyl-1’-carbothioic acid amido pyrazole (3c)

Yield 82%; m.p. 126°C; ^{1}H NMR ($CDCl_3$): δ 7.6 (*s*, 2*H*, NH_2, exchangeable with D_2O, 5.34 (*s*, 2*H*), 1.19 (*s*, 3*H*), 0.78 (*s*, 3*H*), 0.96 and 0.82 (other methyl protons); ^{13}C NMR ($CDCl_3$); δ 179.2, 150.1, 137.2, 118.1, 85.4, 42.7, 41.8, 39.4, 36.3; MS: m/z 499 [*M*+]; anal. calcd. for $C_{30}H_{48}FN_3S$: C, 71.81; H, 9.64; N, 8.37%. Found: C, 71.79; H, 9.62; N, 8.35%.

3β-Acetoxy-5α-cholestano[6,7–c]-5’-methyl-1’-carbothioic acid amido pyrazole (3d)

Yield 78%; m.p. 147°C; ^{1}H NMR ($CDCl_3$): δ 8.2 (s, 2*H*, NH_2, exchangeable with D_2O), 4.7 $\left(m, 1H, C_3\alpha - HW\frac{1}{2} = 15Hz\right)$, 2.31 (*s*, 3*H*), 2.03 (*s*, 3H), 1.18 (*s*, 3*H*) 0.70 (s, 3*H*) 0.97 and 0.84 (other methyl protons); ^{13}C NMR ($CDCl_3$); δ 181.2, 171.2, 148.3, 134.3, 119.3, 75.1, 40.5, 39.5, 36.7, 25.3; MS: m/z 539 [*M*+]; anal. calcd. for $C_{32}H_{51}N_3O_2S$: C, 70.93; H, 9.49; N, 7.76%. Found: C, 70.90; H, 9.47; N, 7.73%.

3β-Acetoxy-5α-cholestano[6,7–c]-5’-methoxymethyl-1’-carbothioic acid amido pyrazole (3e)

Yield 73%; m.p. 153°C; ^{1}H NMR ($CDCl_3$): δ 8.2 (*s*, 2*H*, NH_2, exchangeable with D_2O, 4.7 $\left(m, 1H, C_3\alpha - HW\frac{1}{2} = 15Hz\right)$, 4.55 (*s*, 2*H*), 3.34 (*s*, 3*H*), 2.03 (*s*, 3*H*), 1.19 (*s*, 3*H*), 0.72 (*s*, 3*H*) 0.97 and 0.85 (other methyl protons); ^{13}C NMR ($CDCl_3$); δ 181.2, 171.1, 148.3, 134.2, 119.3, 75.8, 75.2, 52.2, 40.5, 39.1, 36.6; MS: m/z 569 [*M*+]; anal. calcd. for $C_{33}H_{53}N_3O_3S$: C, 69.31; H, 9.34; N, 7.35%. Found: C, 69.30; H, 9.31; N, 7.33%.

3β-Acetoxy-5α-cholestano[6,7–c]-5'-fluoromethyl-1'-carbothioic acid amido pyrazole (3f)

Yield 82%; m.p. 154°C; NMR ($CDCl_3$): δ 8.2 (*s*, 2*H*, NH_2, exchangeable with D_2O, 5.35 (*s*, 2*H*), 4.7 $\left(m,1H,C_3\alpha-HW\frac{1}{2}=15Hz\right)$, 2.04 (*s*, 3*H*) 1.18 (*s*, 3*H*), 0.71 (*s*, 3*H*) 0.96 and 0.84 (other methyl protons); ^{13}C NMR ($CDCl_3$); δ 181.3, 171.2, 148.2, 134.4, 119.3, 85.6, 42.8, 41.6, 39.5, 36.4; MS: m/z 557 [M^+]; anal. calcd. for $C_{32}H_{50}FN_3O_2S$: C, 68.65; H, 9.00; N, 7.51%. Found: C, 68.63; H, 8.96; N, 7.49%.

3β-Chloro-5α-cholestano[6,7–c]-5'-methyl-1'-carbothioic acid amido pyrazole (3g)

Yield 79%; m.p. 156°C; ^1H NMR ($CDCl_3$): δ 7.8 (*s*, 2*H*, NH_2, $\left(m,1H,C_3\alpha-HW\frac{1}{2}=17Hz\right)$, 2.4 $(s,3H,C_5'-CH_3)$, 1.19 (*s*, 3*H*, $C_{10}-CH_3$ 0.75 (*s*, 3*H*), 0.97 and 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$); δ 184.2, 144.5, 132.2, 119.2, 52.6, 42.2, 41.9,39.3, 36.3; MS: m/z 515 [M^+ +2]; anal. calcd. for $C_{30}H_{48}ClN_3S$: C, 69.53; H, 9.34; N, 8.11;%. Found: C, 69.51; H, 9.32; N, 8.09;%.

3β-Chloro-5α-cholestano[6,7–c]-5'-methoxymethyl-1'-carbothioic acid amido pyrazole (3h)

Yield 75%; m.p.167°C; ^1HNMR ($CDCl_3$): δ 7.8 (*s*, 2*H*, NH_2, exchangeable with D_2O, 3.9 $\left(m,1H,C_3\alpha-HW\frac{1}{2}=17Hz\right)$, 4.54 (*s*, 2*H*), 3.34 (*s*, 3*H*), 1.19 (*s*, 3*H*) 0.75 (*s*, 3*H*), 0.97 and 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$); δ 184.2, 144.5, 132.2, 119.2, 75.8, 52.6, 52.1, 42.2, 41.9, 39.3, 36.3; MS: m/z 545 [M^+ +2]; anal. calcd. for $C_{31}H_{50}ClN_3OS$: C, 67.91; H, 9.19; N, 7.66%. Found: C, 67.89; H, 9.17; N, 7.64%.

3β-Chloro-5α-cholestano[6,7–c]-5'-fluoromethyl-1'-carbothioic acid amido pyrazole (3i)

Yield 81%; m.p. 165°C; ^1HNMR ($CDCl_3$): δ 7.8 (*s*, 2*H*, NH_2, exchangeable with D_2O), 3.9 $\left(m,1H,C_3\alpha-HW\frac{1}{2}=17Hz\right)$, 5.34 (*s*, 2*H*), 1.18 (*s*, 3*H*), 0.78 (*s*, 3*H*), 0.97 and 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$); δ 184.2, 144.5, 132.2, 119.2, 85.4, 52.3, 42.4, 41.8,39.3, 36.3; MS: m/z 533 [M^++2]; anal. calcd. for $C_{30}H_{47}ClFN_3S$: C, 67.20; H, 8.83; N, 7.84%. Found: C, 67.17; H, 8.80; N, 7.81%.

In vitro anticancer activity

All synthesized compounds were evaluated for their *in vitro* cytotoxic activity against three different cancer cell lines such as ACHN, MCF-7, and A375 by MTT. This assay is a quantitative colorimetric method for determining cell cytotoxicity. The assessed parameter is the metabolic activity of living cells. Metabolically active cells reduce bleached yellow tetrazolium salt (MTT) to a dark blue water-insoluble formazan which can be quantified directly after solubilization with dimethyl sulfoxide (DMSO). The absorbance of the formazan directly correlates with the number of viable cells. ACHN, MCF-7, and A375 were plated into a 96-well plate at density of 1 × 10^4 cells/well. Cells were grown for overnight in the full medium and then switched to the low serum media. DMSO was used as control. After 48 h of treatment with different concentrations of test compounds, the cells were incubated with MTT (2.5 mg/mL) in the CO_2 chamber for 2 h. The medium was then removed and 100 µL of DMSO was added into each well to dissolve formazan crystals. After thoroughly mixing, the plates were read at 570 nm for optical density, which is directly correlated with cell quantity. The results were represented as percentage of cytotoxicity/viability. All the experiments were carried out in triplicates. The IC_{50} values were calculated from the percentage of cytotoxicity and compared with the reference drug doxorubicin.

CONCLUSION

The application of Vilsmeier regent in the synthesis of 5α-cholestan-pyrazole hybrids with good yields was demonstrated. Besides, all the synthesized compounds were screened for their *in vitro* anticancer activity over three human cancer cell lines labeled ACHN, MCF-7, and A375 using doxorubicin as positive control. Among them, compounds **3h** and **3i** at their micromolar concentration have shown superior activity than the doxorubicin standard.

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