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Reaction of 7α-bromo-6-nitrocholest-5-enes with hydrazine: Formation of steroidal pyrazolines and molecular docking against SARS-CoV-2 omicron protease

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ABSTRACT

The present work reports simple and effective protocol for preparing 6α-nitro-5α-cholestano[7α,5-cd] pyrazolines (4–7) by the reaction of 7α-bromo-6-nitrocholest-5-enes (1–3) with hydrazine hydrate under reflux [the substrate (2) gave products (5) and (6) and the later on acetylation with AC_2O/Py gave (7)]. In the case of reaction of 3β-hydroxy analogue (3) with hydrazine, however, 6α-nitro-5α-cholestano [3α,5-cd] pyrazoline (8) and 6α-nitro-3β, 5-oxido-5β-cholestane (9) were obtained. The probable mechanism of the formation of pyrazolines has also been outlined. In the current pandemic coronavirus disease 2019 scenario, the in-silico study was performed with reactants (**1**–**3**), their products (**4**–**9**) against SARS-CoV-2 omicron protease (PDB ID:7T9L) for knowing significant interactions between them. Docking results give information that both reactants and products have binding energies ranges from −5.7 to 7.7 kcal/mol and strong interactions with various hydrophilic and hydrophobic amino acids such as ASP, PRO, PHE, SER and LEU which are significant residues playing important role in SARS-CoV-2 Omicron main protease (Mpro).

1. Introduction

The pyrazoline ring is a nitrogen-containing five-membered heterocyclic molecule with important biological activity [1]. The addition of the pyrazoline ring to the steroid skeleton extended the range of pharmacological activity, resulting in anticancer, anti-inflammatory, antibacterial, antiamoebic, and antiprotozoal effects [2–4]. Several pyrazoline analogues have been noticed or developed for the treatment of neurological diseases such as Alzheimer's, depression, and Parkinson's [5–7]. Furthermore, some non-steroidal diarylpyrazolines with nanomolar AChE inhibitory activity have been touted in the literature as promising treatments for Alzheimer's disease [8]. Cholinesterase (ChE) is made up of two sister enzymes called acetylcholinesterase and butyrylcholinesterase, which are responsible for the breakdown of acetylcholine (ACh) and butyrylcholine (BCh), respectively. Because acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are involved in the pathology of Alzheimer's disease (AD), cholinesterase inhibition (ChE) is a crucial therapeutic approach for AD [9]. Certain natural steroid-skeletoned compounds, such as phulchowkiamide A, hookerianamide I, 2β-hydroxyepipachysamine-D, epipachysamine-D, saracocine, O^2-natafuranamine and buxakashmiramine, have been successfully used as cholinesterase (ChE) inhibitors [10]. Besides that, pyrazolines have been widely used as valuable synthons in organic synthesis. The base catalysed aldol condensation reaction of aromatic ketones and aldehydes is used in the conventional synthesis of these compounds [11]. Since the 1960s, several types of steroids fused to heterocycles have been thoroughly studied, and their chemistry has advanced; based on these findings, a structure–activity relationship between the steroidal structure and their physiological properties has been established [12,13]. In recent years, efforts have been made to rationally modify steroid molecules by incorporating a heteroatom, such as N or O. These compounds have demonstrated a wide range of biological activities, including antimicrobial, anti-inflammatory, hypotensive, hypocholesterolemic, and diuretic properties [14–17]. Pyrazoline

Abbreviations: AC_2O/Py, Acetic anhydride/pyridine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ASP, Aspartic acid; PRO, Proline; PHE, Phenylalanine; SER, Serine; LEU, Leucine.

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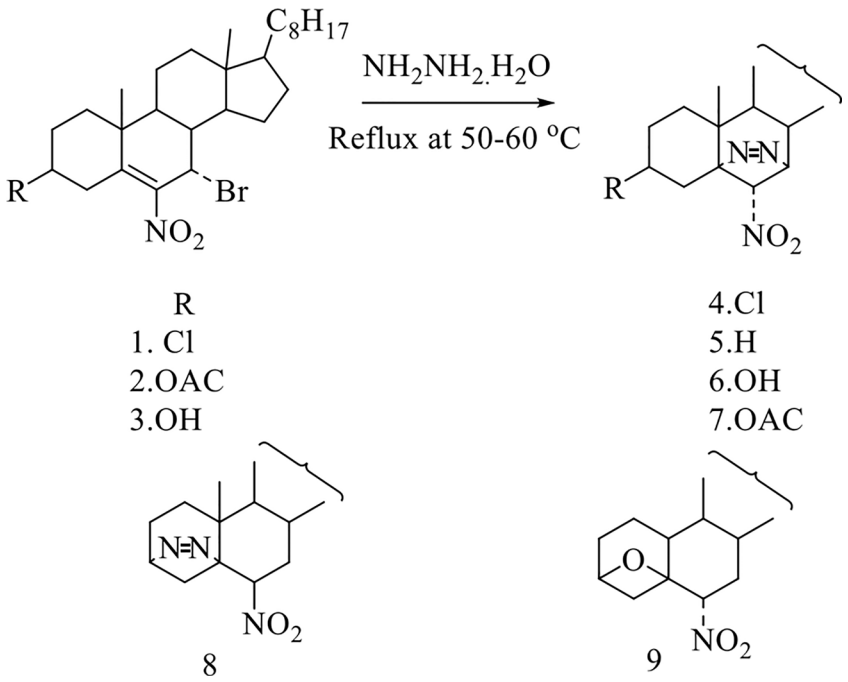
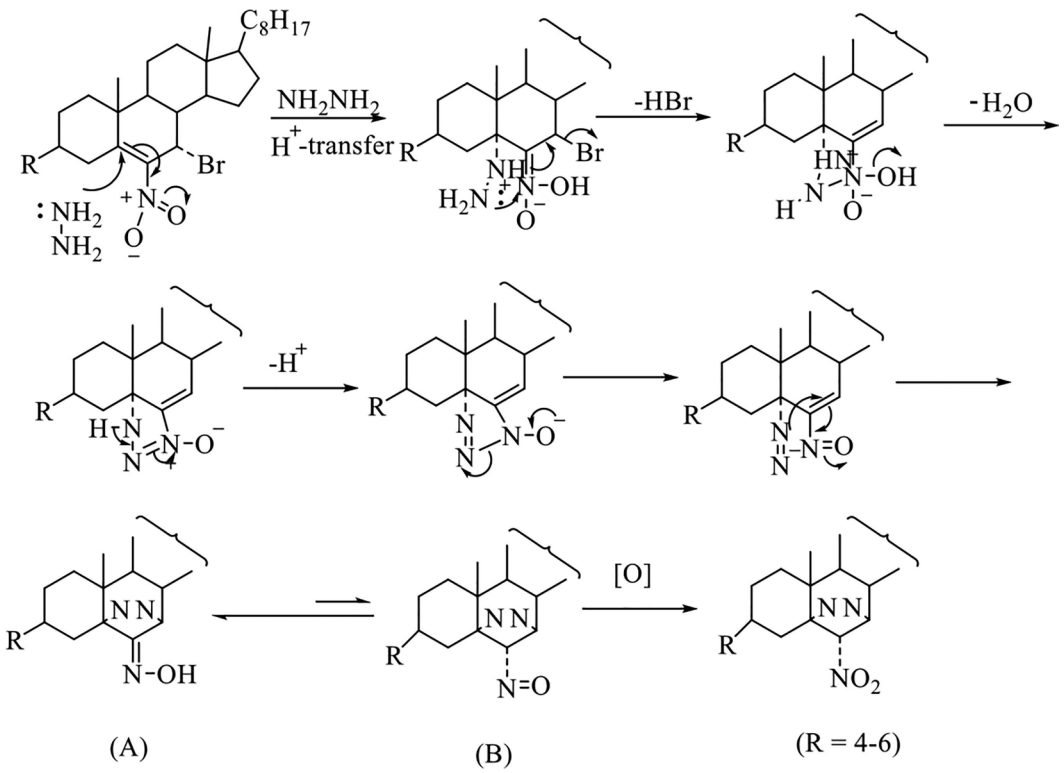


Scheme 1. Synthesis of pyrazolines in cholestane series.

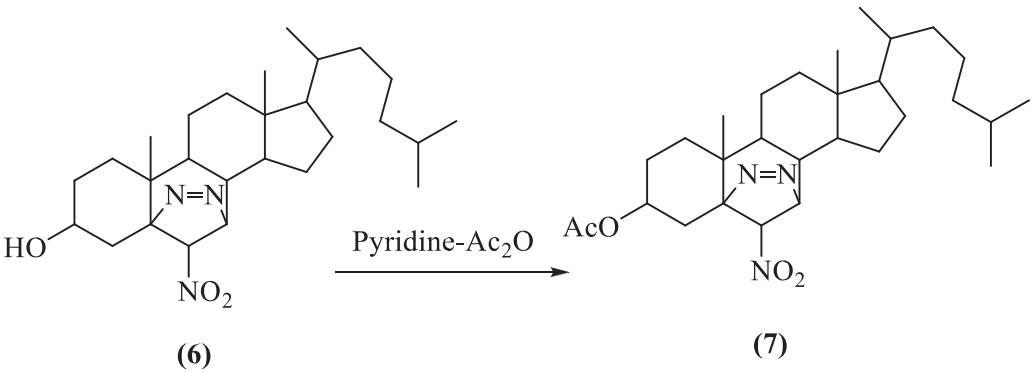


Scheme 2. Tentative mechanism for the formation of product (4–6) from 7α-bromo-6-nitro cholest-5-enes.

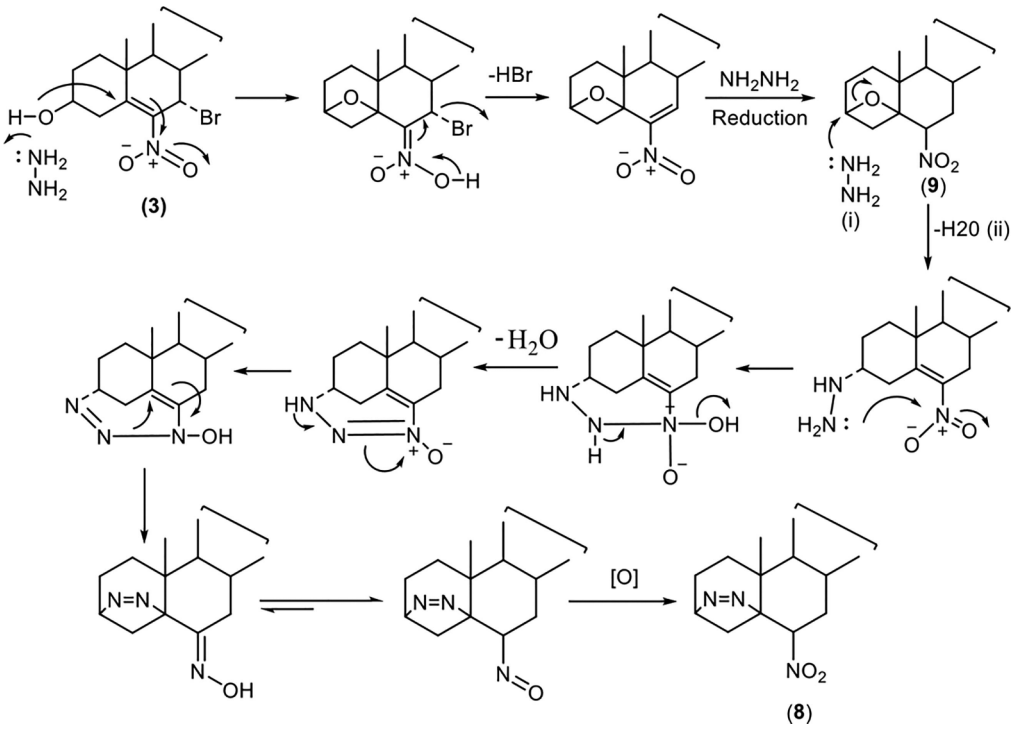
derivatives are electron-rich nitrogen heterocycles with intriguing pharmacological properties, including analgesic, antipyretic, antirheumatic [18,19], anti-inflammatory [20], anti-diabetic agents [21], anticancer [22–25], and antimicrobial activities [26].

Molecular docking is a well-known calculation technique for predicting the interaction energy of two molecules. So that, docking is important in rational drug design because predicting the mode of protein ligand interaction can assume the active site of the protein molecule and aid in protein annotation. Molecular docking has recently emerged as an intriguing tool for drug design and discovery.

Herein, we describe a simple and good yielding synthesis of steroidal pyrazolins with rings A and B, starting from 3β-substituted-7α-bromo-6-nitrocholest-5-enes through a substitution and addition of hydrazine at unsaturated centre of ring B of reactants (Schemes 1-4). Two nitrogen atoms that form a bridge with the corners of the A (C3-C5) or B (C5-C7) inside rings of the cholestane skeleton make up the five-membered



Scheme 3. Preparation of compound **7** by reacting compound **6** with pyridine and acetic anhydride.



Scheme 4. Tentative mechanism for the formation of product (**8** and **9**) from 3β-hydroxy-7α-bromo-6-nitrocholest-5-ene.

heterocyclic ring structure known as pyrazoline. The formation of a bridge by two nitrogen atoms distinguishes steroid compounds from other steroidal pyrazolines that are arranged adjacently outside the cholestane A or B rings. The steroidal compounds used in this paper were carried out for docking studies against the COVID-19 receptor; PDB ID: 7T9L (Cryo-EM structure of Omicron SARS-CoV-2 spike protein complex with human ACE2, EMD-25,761).

2. Experimental section

2.1. General experimental

All melting points are uncorrected. UV spectra were recorded on a Pye-Unicam PU 8800 spectrophotometer and IR spectra on a Pye-Unicam SP3-100 spectrophotometer. PMR spectra were run on a Varian A-60D and Perkin Elmer R-32, 90 MHz instrument, with TMS as the internal standard. TLC plates were coated with silica gel G and sprayed with a 20 % aq. solution of perchloric acid. Starting materials such as 7α -bromo-3β-chloro-6-nitrocholest-5-ene (**1**), 3β-acetoxy-7α-bromo-6-nitrocholest-5-ene (**2**) and 7α -bromo-3β-hydroxy-6-nitrocholest-5ene (**3**) were synthesized using standard methods described elsewhere [27–28].

2.2. General procedure for steroidal pyrazolines

7α -Bromo-3β-chloro-6-nitrocholest-5-ene (**1**) (2 g) was added to hydrazine hydrate (80 %, 60 ml) and the reaction mixture was heated under gentle reflux at a low temperature (50–60 °C) for 8 h. and poured into water. The product separated as a brown solid was dissolved in chloroform. The chloroform layer was washed repeatedly with water, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed on a steam bath and an oily residue thus obtained was chromatographed over a silica gel column (40 g BDH Bombay) to obtain a crude product which was crystallized from methanol to give the pure 3β-chloro-6α-nitro-5α-cholestano[7α, 5-cd] pyrazoline (**4**). Using a similar synthesis method, 7α-bromo-3β-acetoxy-6-nitrocholest-5-ene (**2**) was reacted with hydrazine, after usual work and column chromatography on silica gel column furnished two compounds (**5** and **6**). Similarly, 7α-bromo-3β-hydroxy-6-nitrocholest-5-ene (**3**) was reacted and usual workup, evaporation of the solvent gave an oil which was chromatographed on a silica

Table 1
Binding energy, hydrogen bonds and residues of interaction of proteins with steroid reagents and their products.

Compounds	Binding energy (Kcal/mol)	Hydrogen bond interaction	Interacting residues
7α-Bromo-3β-chloro-6nitrocholest-5-ene (**1**)	−6.3	SER514-UNK	THR430, LEU517, ARG355, PHE464, PRO463, TYR396, PRO426, PHE515, GLU516
7α-Bromo-3β-acetoxy-6nitrocholest-5-ene (**2**)	−5.7		PHE342, ASN343, ASN437, PRO373, TRP436, LEU441, PHE374, PHE375, LYS440
7α-Bromo-3β-hydroxy-6nitrocholest-5-ene (**3**)	−6.6	ARG509-UNK, ARG509-UNK	LYS440, LEU441, PRO373, ALA344, ASN343, PHE374, PHE342, TRP509
3β-Chloro-6α-nitro-5αcholestano[7α,5-cd] pyrazolines (**4**)	−6.9	SER514-UNK	THR430, LEU517, ARG355, PHE464, PRO463, TYR396, PRO426, PHE515, GLU516, ASP428
6α-Nitro-5αcholestano[7α,5-cd] pyrazolines (**5**)	−6.8	SER514-UNK	ASP428, PHE515, THR430, LEU517, TYR396, PHE464, PRO463, ARG355
3β-Hydroxy-6α-nitro-5αcholestano[7α,5-cd] pyrazolines (**6**)	−6.9	PHE515-UNK, SER514-UNK	THR430, ASP428, GLU516, ARG355, PHE464, TYR396, PRO463, PRO426
3β-Acetoxy-6α-nitro-5αcholestano[7α,5-cd] pyrazolines (**7**)	−7.3	THR430-UNK, LEU517-UNK	PHE515, ASP428, SER514, GLU516, ARG355, TYR396, PRO426, PHE464, PRO463
6α-Nitro-5αcholestano[3α,5-cd] pyrazolines (**8**)	−7.7	SER514-UNK	PHE464, PRO426, ASP428, VAL382, LEU390, THR430, GLY381, PHE392, PHE515, LEU517, GLU516
6α-Nitro-3β,5-oxido-5βcholestane (**9**)	−7.6	SER514-UNK	PHE464, GLU516, LEU517, PRO426, ASP428, THR430, VAL382, LEU390, GLY381, PHE392, PHE515

UNK = reactants/products.

gel column to afford two products (8 and 9). The obtained compound 6 was then heated on a steam bath with purified pyridine and freshly distilled acetic anhydride, and the reaction was monitored using TLC. Following the completion of the reaction, usual workup, and crystallization, compound 7 was obtained. The structures of these compounds (Schemes 1-3) were confirmed by the analytical and spectral data presented below.

3β-Chloro-6α-nitro-5α-cholestano[7α, 5-cd] pyrazoline (4): Yield (60 %); mp. 160 °C; Anal. Calcd for $C_{27}H_{44}N_3O_2Cl$: C, 67.7, H, 9.2, N, 8.7; found; C, 67.9, H, 9.2, N, 8.8; IR (KBr) ν cm^{-1} 1550 (NO2 and N═N), 770(C-Cl); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 5.30 (s, 1H, C7-βH), 4.50 (s,

1H, C3-αH), 4.30 (s, 1H, C6-βH); UV (λ^{max} nm) 245(NO2) ε = 695, 340(N═N) ε = 120. The angular methyl protons appeared at normal at δ values.

6α-Nitro-5α-cholestano[7α, 5-cd] pyrazoline (5): Yield (65 %); mp. 210 °C; Anal. Calcd for $C_{27}H_{45}N_3O_2$: C, 73.0, H, 10.2, N, 9.6; found; C, 73.1, H, 10.1, N, 9.4; IR (KBr) ν cm^{-1} 1555(NO2), 1545 (N═N); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 5.13 (s, 1H, C7-βH), 4.30 (s, 1H, C6βH); UV (λ^{max} nm) 244(NO2) ε = 682, 360(N═N) ε = 110. The angular methyl protons appeared at normal at δ values.

3β-Hydroxy-6α-nitro-5α-cholestano[7α, 5-cd] pyrazoline (6): Yield (61 %); mp. 212 °C; Anal. Calcd for $C_{27}H_{45}N_3O_3$: C, 70.7, H, 9.7, N, 9.2; found; C, 70.5, H, 9.8, N, 9.1; IR (KBr) ν cm^{-1} 3350 (OH); 1550 (NO2 and N═N); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 5.30 (s, 1H, C7-βH), 4.60 (m, 1H, W1/2 = 20 Hz, C3– αH), 4.28 (s, 1H, C6-βH), 2.80 (m, 1H, exchangeable with D2O, OH); UV (λ^{max} nm) 244 (NO2) ε = 700, 340 (N═N) ε = 124. The angular methyl protons appeared at normal at δ values.

3β-Acetoxy-6α-nitro-5α-cholestano[7α, 5-cd] pyrazoline (7): Yield (65 %); mp. 200 °C; Anal. Calcd for $C_{29}H_{47}N_3O_4$: C, 69.3, H, 9.5, N, 8.4; found; C, 69.4, H, 9.3, N, 8.3; IR (KBr) ν cm^{-1} 1730 (CH3COOH), 1545 (NO2 and N═N); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 5.45 (m, 1H, W1/2 = 19 Hz, C3-αH), 5.24 (s, 1H, C7-βH), 4.24 (s, 1H, C6-βH); UV (λ^{max} nm) 244 (NO2) ε = 718; 340 (N═N) ε = 114. The angular methyl protons appeared at normal at δ values.

6α-Nitro-5α-cholestano[3α, 5-cd] pyrazoline (8): Yield (50 %); mp. 166 °C; Anal. Calcd for $C_{27}H_{45}N_3O2$: C, 75.0, H, 10.6, N, 3.3; found; C, 75.1, H, 10.4, N, 3.2; IR (KBr) ν cm^{-1} 1550.

(NO2 and N═N); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 5.00 (m, 2 h, W1/2 = 16 Hz; C6-βH and C3-βH); UV (λ^{max} nm) 243(NO2) ε = 670, 340 (N═N) ε = 105. The angular methyl protons appeared at normal at δ values.

6α-Nitro-3β,5-oxido-5β-cholestane (9): Yield (55 %); mp. 86 °C; Anal. Calcd for $C_{27}H_{45}NO_3$: C, 75.0, H, 10.6, N, 3.3; found; C, 75.1, H, 10.4, N, 3.2; IR (KBr) ν cm^{-1} 1550 (NO2), 1120, 1050 (C—O); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 4.85 (d, 1H, J = 12 Hz, C6-βH), 3.85 (d, 1H, J = 5 Hz, C3-αH); UV (λ^{max} nm) 242 (NO2) ε = 598. The angular methyl protons appeared at normal at δ values.

2.3. Molecular docking

In-silico study was carried out using docking operation on the steroidal compounds mentioned in this paper with the receptor (PDB ID: 7T9L (Cryo-EM structure of Omicron SARS-CoV-2 spike protein complex with human ACE2, EMD-25,761) downloaded from RCSB PDB: Homepage website using Auto Dock wizard of PyRx virtual screening tool [29]. The chemical structures of the reactants (**1**–**3**) and their products (**4**–**9**) were sketched in ChemDraw, then optimized in MM2 with Chem3D, then saved in PDB format for docking. The active site of the protein was identified by looking at the molecule already bound to the receptor. The grid box was placed to cover the active site where a reference compound was associated with the receptor structure. Vina wizard uses a stochastic gradient optimization technique to estimate the binding affinities between ligands and receptors. Using Discovery Studio Visualizer [30], the docking result with the highest binding affinity was selected to investigate the residual interactions of receptor with steroid compounds.

3. Results and discussion

3.1. Chemistry

One of the representative compounds (4–9) (Scheme 1) synthesized was physicochemical studied, and its melting point was determined to be 160°, correctly identified as $C_{27}H_{44}N_3O_2Cl$ and yielded a molecular ion peak at 477 (Beilstein test positive). The UV spectrum of the product showed two bands at 245 (Ɛ= 695) and 340 nm (Ɛ =120), which correspond to saturated NO_2 and N═N groups. Its IR spectrum revealed an absorption band for saturated NO_2 at 1550 cm^{-1} as well as N═N (merged) stretching frequencies. The PMR spectrum revealed a singlet at δ 5.30 ppm for one proton corresponding to C7-βH. The C3-αH caused a multiplet with W1/2 = 21 Hz centred at δ 4.50 ppm. The C6-axial proton was responsible for another singlet for one proton at δ 4.30 ppm. At δ 1.10, and 0.61, methyl protons were observed at C10 and C13. The remaining methyl protons were observed at δ 0.90 and δ 0.80 ppm, and based on ongoing discussion, the representative compound was proposed as 3βchloro-6α-nitro-5α-cholestanol [7α,5-cd] pyrazoline (4).

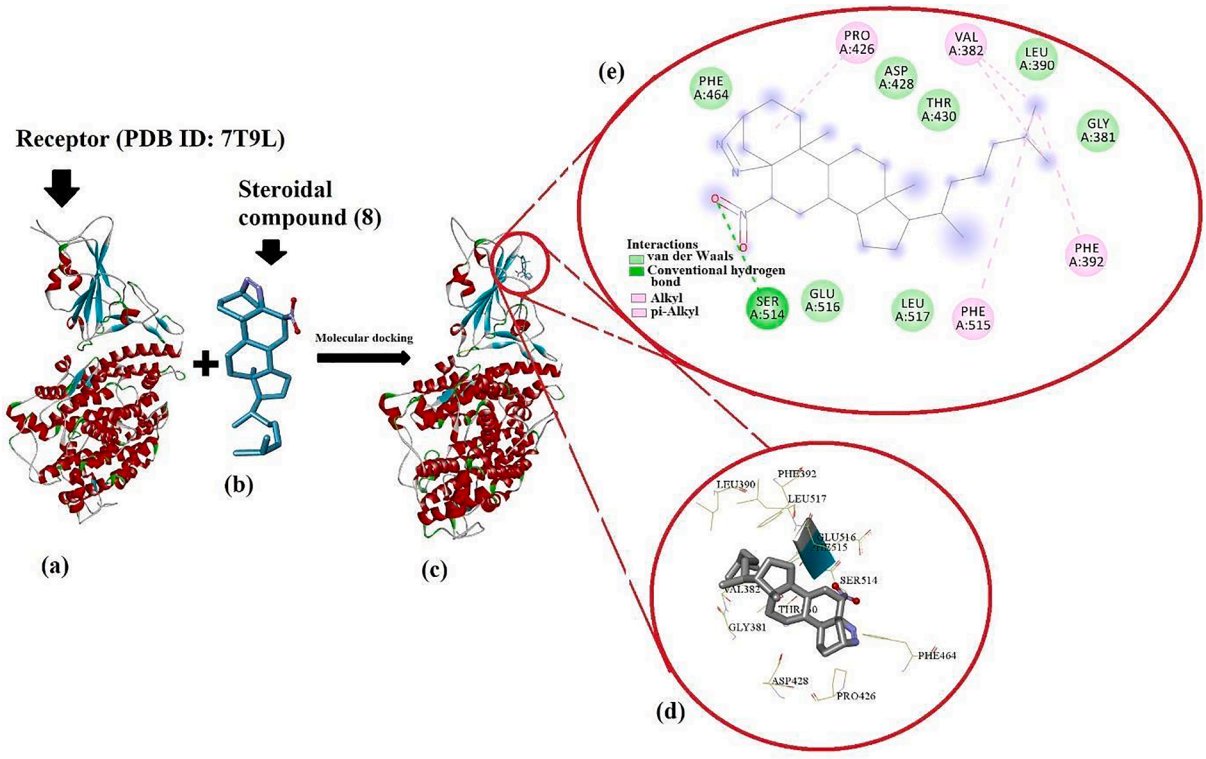


Fig. 1. Molecular docking study of (a) receptor, (b) 6α-nitro-5α-cholestano[3α, 5-cd] pyrazoline (8), (c) best docking pose and (d and e) Different interactions of 6α-nitro-5α-cholestano[3α, 5-cd] pyrazoline with amino acids involving in cavity formation showing with different colors in (e).

Other compounds in this series (5–6) had characteristic IR stretching frequencies and PMR peaks of functional groups at 1555 cm^{-1} (NO2), 1545 cm^{-1} (N═N) for 1555 cm^{-1} (NO_2), 1545 cm^{-1} (N═N) for compound 5; 3350 cm^{1} (OH), 1550 cm^{-1} (NO_2 and N═N) for compound 6; 1550 cm-1 (NO_2 and N═N) for compound 8 and 9, as well as 5.31–5.24 and 4.30–4.24 ppm that could be assigned to C7-βH and C6-αH, respectively, for compounds 5–6. The ^{1}H NMR peaks of the other functionality of cholestane series compounds were given in the experimental section. Steroidal pyrazolines have mainly been prepared by the reaction of suitable steroidal substrates and diazocompounds [31], specially diazomethane [32–34]. But the preparation of steroidal pyrazoline has hitherto not been reported by a reaction between allylic bromo nitro olefin and hydrazine. In fact, the reaction aimed at substituting the C7-bromine by hydrazine to obtain 7β-substituted hydrazino derivative analogous to the preparation of 7β-thiocyanates [35]. But the reaction did not take the expected route as a result of which pyrazolines were formed.

The appearance of a singlet for C7 proton suggest it to be equatorial and its geminal substituent to be axial. This orientation is explicable only when the hydrazine attacks at C5 in way similar to cyanide [36] and subsequently helps to remove the bromine from C7 to yield pyrazoline.

The alternative attack of hydrazine at C7 would have resulted β-oriented product. The unusual upfield shift of C6-proton [37] by δ 0.5 ppm is because of the diamagnetic anisotropic effect of -N═N- which shields the C6-proton falling in the area of conical zone. The mechanism for the formation of pyrazolines has been rationalized below:

The attack of hydrazine at C5 leads to the formation of a likely intermediate [A], which rearranges to give nitroso compound [B]. During this change, the terminal nitrogen of hydrazino moiety attacks C7 from the back side to result into the pyrazolines. The nitroso at C6 as in [B], then oxidizes to a more stable nitro group. The symbol [O] denotes the air oxidation of the nitro group to the nitro group which is a common phenomenon because NO groups are unstable and easily converted to NO_2 groups. It is worthy to mention that during the course of reaction two products are formed (as visualized on TLC plate), which during workup and column chromatography change to a single third product, identified as pyrazoline.

From the exclusive formation of [7α,5-cd] pyrazolines as the final product, it is evident that hydrazine does not replace bromine present from C7 in the first instance (the C7-β hydrazino derivatives are not at all formed). Rather, it undergoes an addition-elimination reaction which involved the initial reaction of hydrazine with nitroolefins like the addition of HCN [38] and the subsequent removal of bromine from C7.

3β-Hydroxy-6α-nitro-5α-cholestano[7α, 5-cd] pyrazoline (6) obtained from 3β-acetoxy-7αbromo-6-nitrocholest-5-ene by reacting with hydrazine hydrate was acetylated with purified pyridine and freshly distilled acetic anhydride was heated on a steam bath and reaction was monitored by TLC. After the completion of reaction, usual work up and crystallization, a 3βacetoxy-6α-nitro-5α-cholestano[7α, 5-cd] pyrazoline (**7**), m.p. 200 °C was obtained (Scheme 3).

The reaction of 3β-hydroxy-7α-bromo-6-nitrocholest-5-ene (**3**) produced two compounds identified as 6α-nitro-5α-cholestano [3α, 5-cd] pyrazoline (**8**) and 6α-nitro-3β,5-oxido-5 cholestane (**9**). Hydrazine being basic in nature picks up a proton producing an oxanion which attacks the C5 of nitroolefin to form product (8). In the case of formation of product, m.p. 166 °C hydrazine attacks at C3 of oxido compound (3) and then participates with C6-nitro to finally give the above product. Based on the foregoing discussion, a provisional mechanism for the formation of two products (**8** and **9**) is sketched below (Scheme 4).

3.2. Docking analysis

The COVID-19 panic pandemic is a serious threat to humanity and has spread rapidly over the past two years. The WHO first reported the newly identified SARS-CoV-2 Omicron virus variant B.1.1.529 on November 24, 2021 [39]. In order to determine the feasibility of binding affinity between steroid compounds (1–9) and SARS-CoV-2 Omicron PDB ID: 7T9L (CryoEM structure of SARS-CoV-2 Omicron spike protein complex with human ACE2, EMD-25, and 761) downloaded from the

RSCPDB website. PyRx software was implemented for molecular docking, and Discovery Studio was used to visualize the best pose of docked steroidal molecule (**8**) with high negative value, as shown in Table 1. Steroidal compounds may be successful in achieving binding contacts into the omicron PDB binding pocket, and structure-binding experiments suggest that hydrophobic, hydrophilic and non-bonding interactions play a part in the substantial binding of stable complexes. According to the results of molecular docking, the synthetic steroidal compounds **8**, **9**, and **7** obtained the best docking scores, with respective values of −7.7, −7.6, and −7.3 kcal/mol as mentioned in Table 1. Energetically, the Nitro group of steroid compounds involved the formation of hydrogen bonds with SER514 of SARS-CoV-2 Omicron in the majority of the reactants and products, as shown in Fig. 1e. As stated in Table 1, additional amino acids that constitute active sites like ARG509, PHE515, THR430-and LEU517 of SARS-CoV-2 Omicron also engage in non–bonding interactions with hydrophilic and hydrophobic amino acids in addition to participating in the formation of hydrogen bonds (Table 1). Through docking analysis, it was observed that while 7-bromo-3-acetoxy-6-nitrocholest-5-ene (2) did not form a hydrogen bond, other secondary interactions like van der Waals interactions did, in which both hydrophobic and hydrophilic amino acids were revealed to be involved. High negative binding energy (-7.7 kcal/mol) was produced by the molecular docking posture of 6α-Nitro-5α-cholestano [3α,5-cd] pyrazolines (**8**) that closely interact with SARS-CoV-2 Omicron as represented in Fig. 1.

We hypothesize that the reactants and products under theoretical examination may both possess inhibitory qualities based on the interaction and binding energies. However, in order to validate the computational predictions, biological investigations are needed.

4. Conclusions

The present study discloses the practical synthesis of steroidal pyrazolines (4–9) with good yield from readily available cholestane series reactants (1–3). Physicochemical approaches were used to characterize the structures of compounds. In-silico investigation against SARSCoV-2 Omicron was performed with reactants and their products to explore the binding interactions with amino acids that may be involved for disease transmission. Based on docking studies, these are likely to have better interactions with the receptor, leading to the speculative conclusion that these steroid compounds could be used against SARS-CoV-2 Omicron. It will need further exploration and real assays in order to confirm this possibility but it clearly gives a direction to the future developments in this area.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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