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Design and Synthesis of Pyrazolo[3,4-d]pyrimidinone Derivatives: Discovery of Selective Phosphodiesterase-5 Inhibitors

Mohamed A. Shaaban[a], Yaseen A. M. M. Elshaier[b,c], Ali H. Hammad[d], Nahla A. Farag[e] , Haredy Hassan Haredy[f], Ahmed A. AbdEl-Ghany[g,h], Khaled O.Mohamed[a,i].

[a]Pharmaceutical Organic Chemistry Department, Faculty of Pharmacy, Cairo University, 11562,Egypt.

[b]Pharmaceutical Organic Chemistry Department, Faculty of Pharmacy, Sadat University, 32958, Sadat City, Menoufia, Egypt.

[d]Pharmaceutical Organic Chemistry Department, Faculty of Pharmacy (Boys), Al-Azahar University, Cairo, 11884,Egypt.

[e]Pharmaceutical Chemistry Department, Faculty of Pharmacy, Misr International University, Cairo Egypt, 11431, Egypt.

[f]Department of Pharmacology, Faculty of Medicine, Al-Azher University, 71524, Assiut, Egypt

[g]Biochemistry Department, Faculty of Pharmacy, Al-Azher University, 71524, Assiut, Egypt.

[h]Biochemistry Department, Faculty of Pharmacy, Nahda University, Benisuif, Egypt

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i Corresponding authors.

E-mail address: khaled.mohamed@pharma.cu.edu.eg

Tel.: +201145822851

[c]Corresponding co-auther.

E-mail address: yaseenelshaier@azher.edu.eg

Tel.: +201028582031

Abstract

A novel series of 1,6-disubstituted pyrazolo[3,4-*d*]pyrimidin-7-one derivatives,**2a-h, 4a-d, 5** and **6**, were successfully synthesized, which showed promising, and potent inhibition of phosphodiesterase 5 (PDE5).The inhibitory activities of **5, 4b**, **2a**, **2d**, **2f**, **4d** and **4a** against PDE5 were similar to that of sildenafil (100%). These compounds exhibited potent relaxant effects on isolated rat cavernosum tissue with pEC_{50} values ranging from 8.31-5.16 μM. Pyrazolo[3,4-*d*]pyrimidin-7-one scaffolds have been rationally designed via consecutive molecular modelling studies prior to their synthesis and biological evaluation. In addition, the results of the pharmacophore-based virtual screening revealed that **1v0p_PVB** might have promising activity as a PDE-5 inhibitor.

Keywords

pyrazolo[3,4-*d*]pyrimidin-7-one,phosphodiesterase 5 (PDE5)**,** sildenafil**,** cavernosum tissue**,** molecular modelling, PDE-5 inhibitors.

1. Introduction

Several orally available phosphodiesterase 5 (PDE5) inhibitors have been developed and are prescribed as first-line therapies for the treatment of erectile dysfunction (ED) [1].

The PDEs are a superfamily contain 11 subfamilies (PDE1-11) and each PDE plays an important role in various tissues [2-6].

Treatment of ED using PDE5 inhibitors occurs via catalyse the inactivation of intracellular mediators of signal transduction such as cAMP and cGMP. PDEs cleave the 3′,5′-cyclicphosphate moiety of cAMP and/or cGMP to produce the corresponding 5′-nucleotide (inactive forms). PDEs are critical modulators of cellular levels of cAMPand/or cGMP based on many stimuli [6,7].

Erection results when the muscarinic receptors are activated by acetylcholine, which leads to increased production, release and diffusion of nitric oxide into vascular smooth muscle cells activates guanylate cyclase, resulting in increased synthesis of cyclic guanosine monophosphate (cGMP), leading to muscle relaxation and vasodilatation of the penis tissues [8,9] (**Figure 1).**

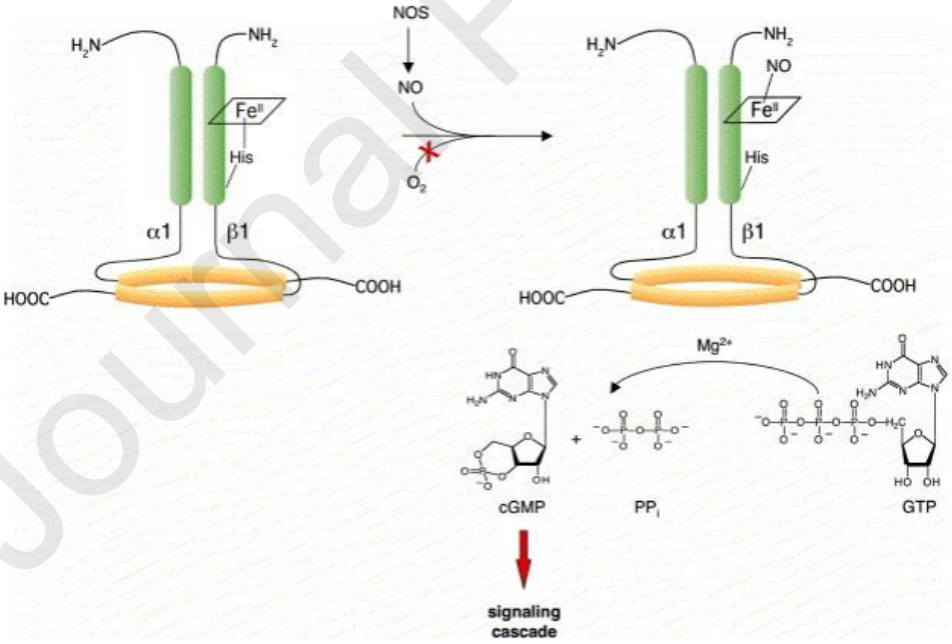
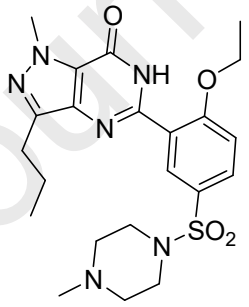


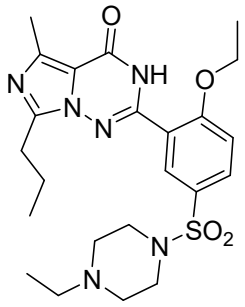
Figure 1.Schematic representation of the structure and function of the α1β1 isoform of soluble guanylate cyclase (sGC).

PDE5 inhibitors protect cGMP from degradation by PDE5, which is specific to cGMP, in the corpus cavernosum. Nitric oxide (NO) in the corpus cavernosum of the penis binds to guanylate cyclase receptors, which results in increased levels of cGMP, decreasing the intracellular calcium concentration, leading to smooth muscle relaxation (vasodilatation) of the intimal cushions of the helicine arteries. This vasodilatation increases the inflow of blood into the sponge tissue of the penis, causing an erection. Sildenafil is a potent and selective inhibitor of cGMP-specific PDE5, which is responsible for the degradation of cGMP in the corpus cavernosum [8, 9].

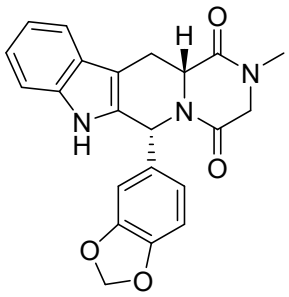
The molecular structure of sildenafil is similar to that of cGMP, and it acts as a competitive binding inhibitor of PDE5 in the corpus cavernosum, resulting in more cGMP and better erections. Without sexual stimulation and therefore no activation of the NO/cGMP system **(Figure 1)**, sildenafil should not cause an erection. Other drugs that operate by the same mechanism include vardenafil (Levitra®) and tadalafil (Cialis®) (**Figure 2).**



Sildenafil (Viagra®)



Vardenafil (Levitra®)



Tadalafil(Cialis ®)

Figure2. Structures of PDE5 inhibitors.

Despite its potency, sildenafil has many clinical side effects, such as headache, blurred vision, facial flashing, visual disturbances, back pain and cardiovascular side effects [2, 10-12]; therefore, discovery of a novel PDE5 inhibitor with an improved safety profile would be a welcome contribution to ED management. [13-15].

In this study, we report the discovery of novel and potent PDE5 inhibitors to potentiate the relaxant effect. Three series of pyrazolo[3,4-*d*]pyrimidin-7-ones bearing 1,6-disubstituted aryl moieties were designed using consecutive computer-aided protocols and then synthesized. The details of the computational design protocols, synthesis and structure-activity relationships are described. Moreover, the *in vitro* PDE inhibitory activities of these compounds were evaluated using sildenafil as a reference drug, and their relaxant effects on isolated rat corpus cavernosum tissue were determined.

2. Results and discussion

2.1. Molecular Modeling Studies

Computer-aided drug design (CADD) is the science of using computers to optimize commercially available drugs [lead compounds] to identify new ligands with potential biological activities against certain biological targets. CADD is based on quantum mechanics and molecular modelling techniques. The traditional method of discovering new drugs is a very hectic process that consumes substantial amounts of effort, money and time [16-18]. Computer-aided programs help to narrow the infinite number of options for lead optimization. Therefore, only the drug compounds with promising activities will be synthesized.

In the current study, we designed a pyrazolo[3,4-*d*]pyrimidin-7-one scaffold as a novel PDE5 inhibitor *via* consecutive protocols of molecular modelling. First, we studied the docking of sildenafil in the binding site of phosphodiesterase 5 and then generated predictive pharmacophore models using a 3D quantitative structure-activity relationship (3D-QSAR) pharmacophore generation protocol from a set of ligands involving 24 compounds with known activities on our biological target (PDE-5). The generated pharmacophores were validated to select the best model for the virtual screening of different databases for searching of additional sildenafil analogues. Then, the resulting hits were analysed. The hits were then docked into the binding site of the crystal structure of PDE-5 obtained in complex with sildenafil as an inhibitor (protein data bank code (**2H42**) to clarify the key structural features required in the design of novel candidates of this class. The compatibility between the fit values of the pharmacophore model and its docking affinity indicates the biological binding mode, offering a relevant basis for the development of new PDE-5 inhibitors.

2.1.1. Docking study of sildenafil

Molecular docking was performed using Accelrys DiscoveryStudio 4.0 software using the Dock Ligands (CDOCKER) protocol, which is an implementation of the CDOCKER algorithm. CDOCKERis a grid-based molecular docking method that employs a CHARMm-based molecular dynamics (MD) scheme to dock ligands into a receptor-binding site. Random ligand conformations are generated using high-temperature MD. This method allows a refinement docking of any number of ligands with a single protein receptor. Various scoring functions were applied to the ligands, including -CDOCKER_ENERGY (CHARMm energy: interaction energy plus ligand strain) and -

CDOCKER_INTERACTION_ENERGY (interaction energy only). The poses were sorted by CHARMm energy, and the top scoring poses were calculated.

The molecular docking was based on the crystal structures of PDE-5 (PDB ID: **2H42**) [19], which is a crystal structures of PDE-5 complexes with sildenafil **(**5-[2-ethoxy-5-[(4-methylpiperazin-1-yl)sulfonyl]phenyl]-1-methyl-3-propyl-1H,6H,7H-pyrazolo[4,3-*d*]pyrimidin-7-one) as the ligand. The protein was prepared using a prepare protein parameter that cleans up common problems in the input protein structure in preparation for further processing; it inserts and minimizes missing atoms in residue as hydrogen, and it also removes alternate conformations. All of the water molecules are removed.

Sildenafil was prepared using the ligand preparation tool, which adds hydrogen's, fix bad valences and generates 3D coordinates using a catalyst. Docking was performed using the CDOCKER protocol, where conformational search tools used in discovery studio software for docking of small flexible to a macromolecule target of known structure is simulated annealing algorithms for conformation searching with a rapid grid-based method of energy evaluation. The pose cluster radius was set to 0.5 Å, the 10 top hits were used, and other docking parameters were kept at their default values. The results were analysed according to their -CDOCKER_ENERGY values. The docking protocol itself was validated through the re-docking of the co-crystallized structure of the reference ligand (Sildenafil). Then, the resulting docking results/poses are compared to that of the crystallized reference ligand. This comparison was carried out by calculating the RMSD (root mean square deviation), and a RMSD of 1.144 was found, confirming the validity of the CDOCKER protocol.

The docking score of sildenafil is -12.41, it showed a CDOCKER_Interaction_Energy of -56.04, and it forms four hydrogen bonds: two hydrogen bonds to the C=O groups of the pyrimidine moiety, one between the CH of the ethoxy moiety and glutamine 817, and one hydrogen bond between the N-methyl of the piperidine ring and asparagine 662. Additionally, the planar regions of pyrazolopyrimidine form hydrophobic interactions with the hydrophobic pocket formed by amino acids valine 782, phenylalanine 786, and phenylalanine 820; the phenyl ring forms another van der Waals interaction with leucine 804, alanine 783, and methionine 816; and the CH of the ethoxy moiety forms hydrophobic interactions with isoleucine 813.These interactions resembled the reported binding mode of sildenafil with PDE5 (2H42) but with additional hydrogen bonds (**Figure 3).**

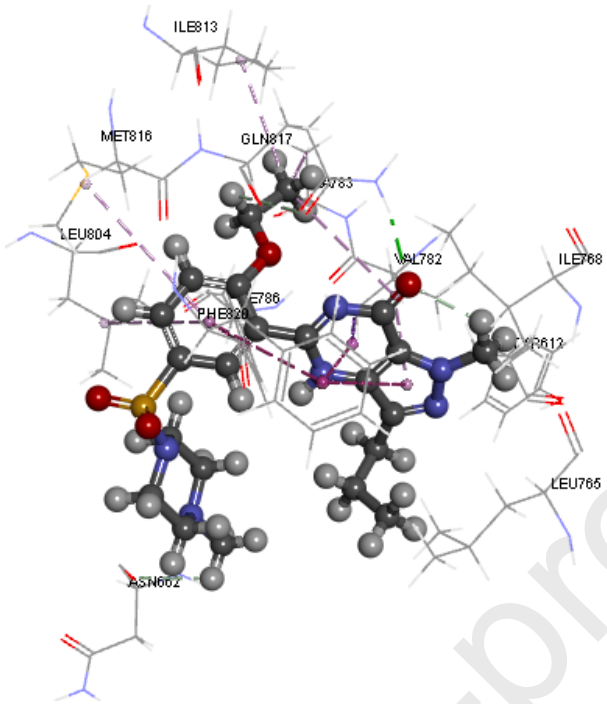
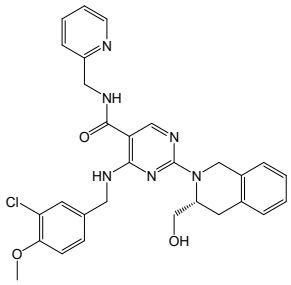


Figure 3. Docking of Sildenafil inside binding site of PDE5 (PDB code 2H42) in 3D style

2.1.2. 3D-QSAR pharmacophore model generation and validation

2.1.2.1. Generation of the PDE5 inhibitor library

A set of 24 PDE-5 inhibitors with known activities (IC_{50}values ranging from 0.9–54 μM) were collected from the literature [20] (**Figure 4).** The ligands in the library were prepared using Accelrys Discovery Studio 4.0 software. Ligand preparation is essential for protonation, minimization of energy, fixing bad valences and converting the 2D structures of the ligands into 3D structures.



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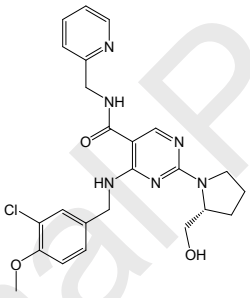

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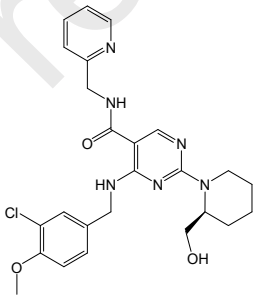

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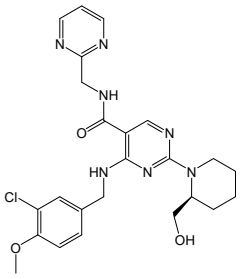

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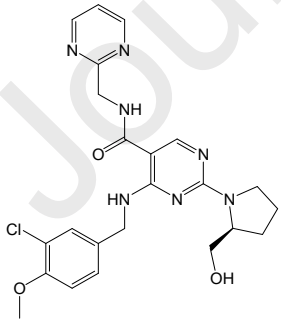
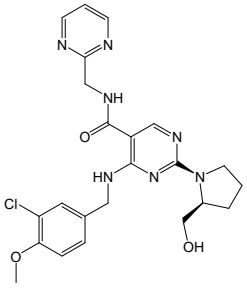


T5 = 25



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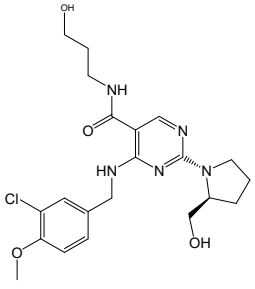
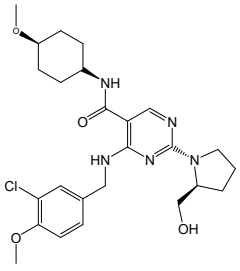


T7 = 6.6



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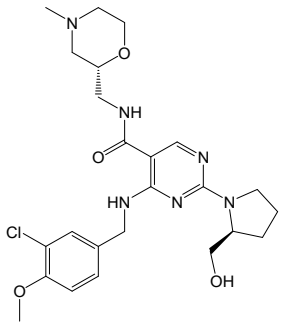

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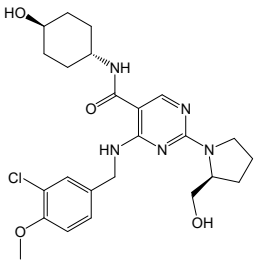

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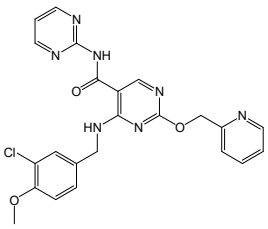

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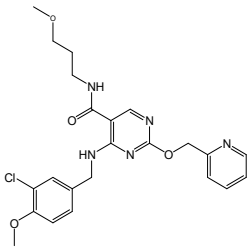

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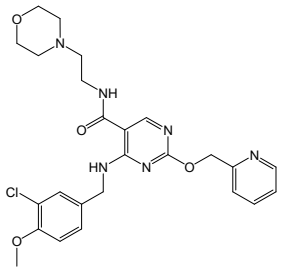

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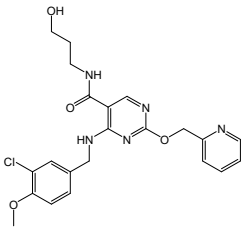

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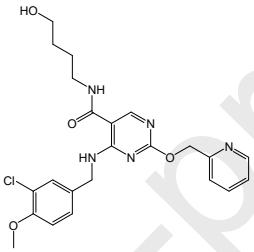

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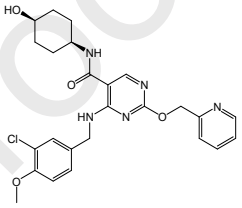

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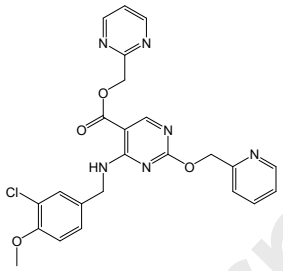

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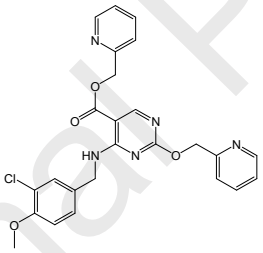

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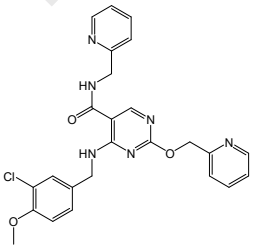

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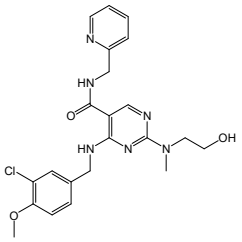

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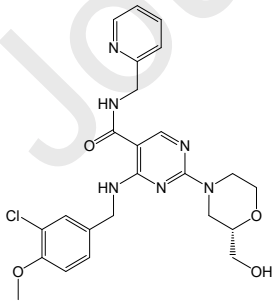

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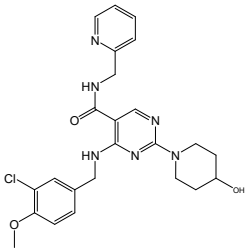

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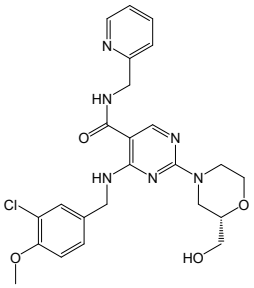

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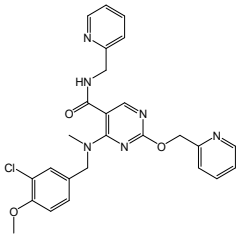


S5 = 85

Figure 4.Training set T1-T19, test set S1-S5, and their PDE-5 inhibitory activity inμM for 3D-QSAR pharmacophore model generation.

2.1.2.2. 3D-QSAR pharmacophore model generation

The term pharmacophore refers to the functional groups essential for the biological activity of a compound [21]; therefore, it is a model generated depending on the relationship between the structure and the activity of a compound (3D-quantitative structure-activity relationship pharmacophore model). The generation of a pharmacophore model can be either ligand-based or structure-based. Ligand-based pharmacophores are generated according to structural features that are common between active compounds in a library, and common features of inactive compounds are omitted.

In the present study, a ligand-based pharmacophore model was generated using Accelrys Discovery Studio 4.0 software using a HypoGen algorithm. The collected library of 24 PDE5 inhibitors with known activities [20] was divided into T 1-19 compounds and S 1-5 (**Figure 4**). The chemical features selected for developing the protocol were hydrogen bond acceptors (HBAs), hydrogen bond donors (HBDs), hydrophobic moieties (HYPs), positive ionizable groups (PIs) and aromatic rings (ARs). The uncertainty was set as1.5 instead of the default value of 3, and IC_{50} was selected as the measure of activity.

2.1.2.3. Validation of the pharmacophore models

The generated pharmacophore model must be validated. The validity of any model is based on its statistical significance and ability to estimate the biological activity of unknown compounds. The model must be validated based on cost difference, the accuracy of the predicted activity and the mapping of a reported reference compound.

The HypoGen algorithm generates different hypotheses, and the best hypothesis is identified based on cost difference [21]. The total cost of each hypothesis is the summation of three factors: weight, error and configuration cost. The fixed cost, the minimum possible cost of a model that fits perfectly, and the null cost, the maximum cost of a model that has no features, were also calculated by the algorithm.

Cost difference = (null cost –fixed cost) - (total cost – fixed cost)

For the model to be statistically significant, the difference between the null and fixed costs must be greater than that between the total and fixed costs. In addition, the cost difference between the null and total costs must be between 40 and 60. The hypothesis with the highest cost difference is the one that will be chosen (**Table 1).**

Table 1. Ten generated hypotheses with their Maximum fit, total cost, Cost difference, RMS, and Correlation coefficient.

Hypothesis	**Maximum Fit**	**Total Cost**	**Cost difference**	**RMS**	**Correlation Coefficient**
1	7.44472	73.6690	45.808	0.84102	0.955074

2	6.78785	85.9444	33.533	1.44232	0.857448
3	7.22863	88.7377	30.739	1.55108	0.832749
4	5.38059	91.5160	27.961	1.64170	0.810324
5	7.30776	92.7914	26.686	1.68426	0.799066
6	6.41761	93.3148	26.162	1.70233	0.794165
7	8.46594	94.2733	25.204	1.73268	0.785713
8	9.25339	94.3107	25.166	1.52816	0.857580
9	5.73834	95.9774	23.500	1.73347	0.786242
10	6.93117	97.0297	22.447	1.80526	0.764812

The pharmacophore model also calculates an estimated activity for the compounds in the training set (**Table 2**). Additionally, for the model to be valid, the estimated activity of each compound in the training set must be close to its reported experimental activity (IC_{50} value).

Further validation of the pharmacophore model can be carried out by mapping a reference ligand (sildenafil) into the generated model and comparing its fit value to that of the tested library. The reference ligand must have a known biological activity on the same target.

Table 2. Training set with their experimental IC_{50} value, their estimated activity and fit value by the valid hypothesis 1.

Training set	Experimental IC_{50}	Estimated IC_{50}	Fit value
T8	0.9	0.932396	6.8514
T2	1.2	1.27609	6.71512
T13	5.6	2.27806	6.46343
T18	2.1	2.28145	6.46279
T10	1.8	2.30912	6.45755
T19	2.7	3.33562	6.29782
T17	4.2	5.38998	6.08941
T16	3.5	5.79737	6.05777
T9	6.1	5.84478	6.05423
T7	6.6	6.10227	6.03551
T6	5.2	7.30281	5.95751
T1	6.5	9.04331	5.86467
T11	12	9.25853	5.85446
T15	12	10.2543	5.81009
T12	15	13.3901	5.69422
T14	13	18.2587	5.55953
T5	25	21.8454	5.48164
T3	54	26.4795	5.39809
T4	63	52.7211	5.09902

Ten hypotheses were generated, and the analysis of these hypotheses revealed that the first model was the best. This model showed the highest cost difference of 45.808, which indicates the statistical significance of the model with a high predictive probability of 75-90% and that it is well correlated with the data. Its RMS value of 0.841and correlation coefficient 0.955 confirming that this model is suitable for predicting the activity of novel compounds (**Table 1).**

In addition to cost analysis, the model was validated by comparing the activity predicted for each compound by the model to their experimental IC_{50} values. As shown in **Table 2**, the difference between the estimated and reported IC_{50} values were quite small, confirming the validity of the model. The valid model has one hydrogen bond acceptors (HBA), and two ring aromatic features (RA), (**Figure 5a, Table 3)**.

(a) (b)

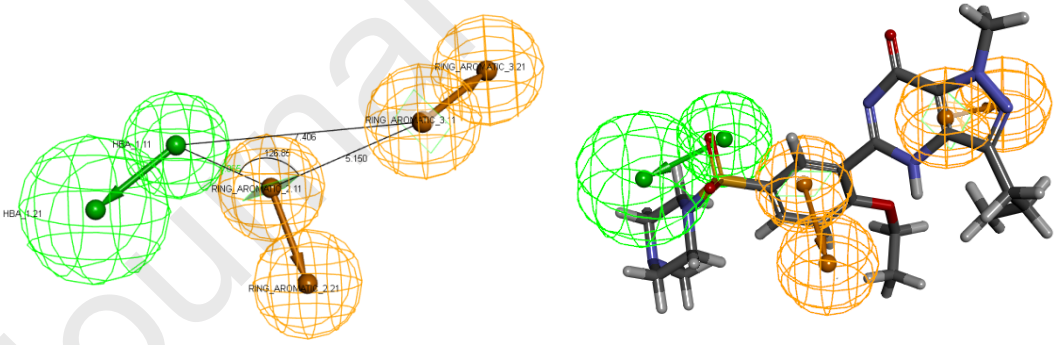


Figure 5a. The best-generated pharmacophore model with the features considered constraint distances and angles between features, considered hydrogen bond donor (HBA) colored in green, ring aromatic (RA) colored in orange, **5b.**Sildenafil viewed as fit reference compound with fit value of 6.5, estimated IC_{50} of 2.08 µM.

Table 3. The valid model of three pharmacophoric features one hydrogen bond acceptors (HBA), and two ring aromatic features (RA) with interfeature distances in angestrum and angles.

Constraint distances (A°)	Constraint angles (°)
(HBA_1) – (RA_2), 3.064; (HBA_1) – (RA_3), 7.406 (RA_2)-(RA_3), 5.150	(HBA_1) – (RA_3) – (RA_2), 126.85

2.1.2.4. Pharmacophore mapping of sildenafil

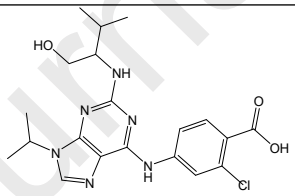
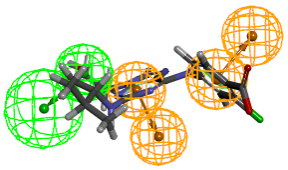
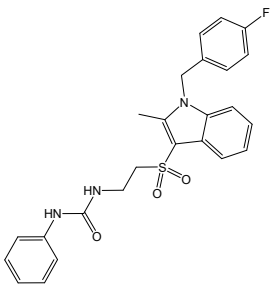
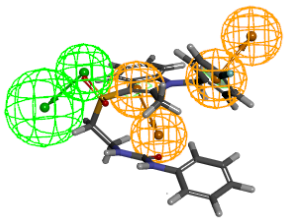
To further validate the model, an active reference drug (sildenafil) was mapped inside the generated model using the ligand-mapping protocol of Accelrys Discovery Studio 4.0software, and scored a fit value of 6.5 and an estimated IC_{50} of 2.08 μM were obtained (**Figure 5b).**

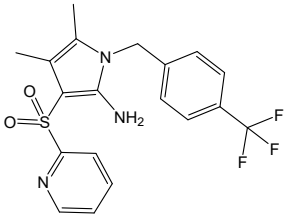
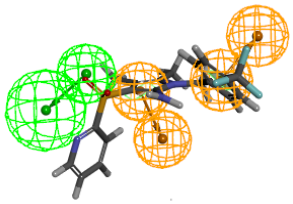
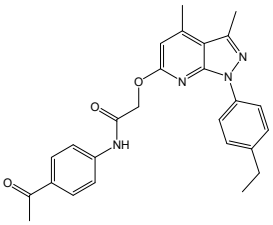
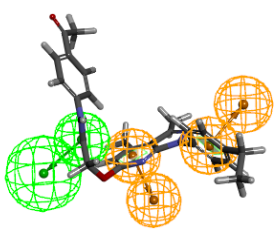
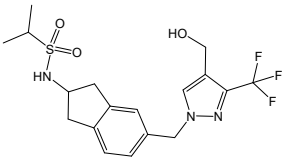
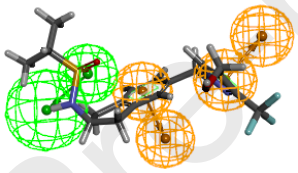
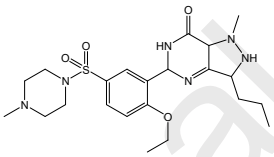
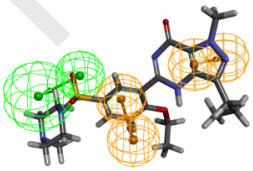
2.1.3. Virtual screening

A virtual screening was conducted to find novel and diverse virtual leads suitable for further optimization as PDE-5 inhibitors. This screening was carried out using the data base searching protocol of Accelrys Discovery Studio 4.0 software with three databases, Drug like diverse, MiniMaybridge, and scPDB, using the validated pharmacophore

model. The significant hits that satisfied the features of the pharmacophore model were then filtered according to their fit values, which yielded 3617 compounds. Compounds with high fit values and estimated activities higher than that of sildenafil were chosen. Five compounds were of particular interest because they fit the pharmacophore model (7.25-7.03) better than mapped sildenafil 6.5, and these five compounds were **1v0p_PVB, LIF172331**, **SPB 02245**, **CDI685470**, and **3pmw_G69 (Table-4)**.

Table 4. Comparison between reference **Sildenafil** and 5 selected top HITs from virtual screening with their pharmacophore fit values, and docking scores, considered pharmacophore features, hydrogen bond donor (HBA) colored in green, ring aromatic (RA) colored in orange.

Selected top HITs	2D structure of 5 top HITs	Mapping on the validated pharmacophore model Hypothesis 1	Estimate activity µM	Vit value	-Cdocker_E	- Cdocker_Interaction_Energy
1v0p_PVB			0.36	7.25	42.57	59.25
LIF172331			0.61	7.03	35.97	53.92

SPB 02245			0.58	7.05	21.90	37.75
CDI685470			0.27	7.37	15.04	52.44
3pmw_G69			0.36	7.26	9.33	46.70
Sildenafil			2.08	6.5	12.41	56.04

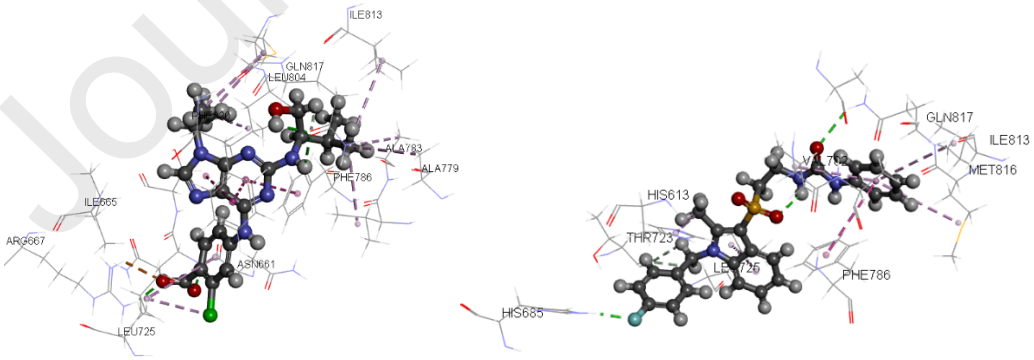
The top 5 selected hits from the virtual screening were then docked in the binding site of the prepared PDE5 crystal structure (PDB code **2H42**) [18]. The docking results of the five selected hits from the pharmacophore-based virtual screening were analysed and filtered according to their binding affinity and binding mode.

2.1.4. Docking studies of top hits from the virtual screening

*2.1.4.1. Docking Study of **1V0P_PVB***

The docking score of **1V0P_PVB** was -42.57, it showed a CDOCKER_Interaction_Energy of -59.25, and its forms six hydrogen bonds; three hydrogen bonds between the NH and OH moieties of the side chain to glutamine 817, one from the carboxylic acid moiety to asparagine 662, and another two hydrogen bonds with arginine 667. Additionally, the planar regions of imidazolopyrimidine form hydrophobic interactions with the hydrophobic pocket formed by the amino acids valine 782, phenylalanine 786, and phenylalanine 820, and the phenyl ring forms a van der Waals interaction with leucine 725, arginine 667, and isoleucine 665. Additionally, the alkyl side chains form hydrophobic interactions with alanine 783, alanine 779, and isoleucine 813, which may increase the affinity of the compound for the binding site of PDE5, resulting in a higher predicted inhibitory activity (**Figure 6a)**.

(a) (b)



(c) (d) (e)

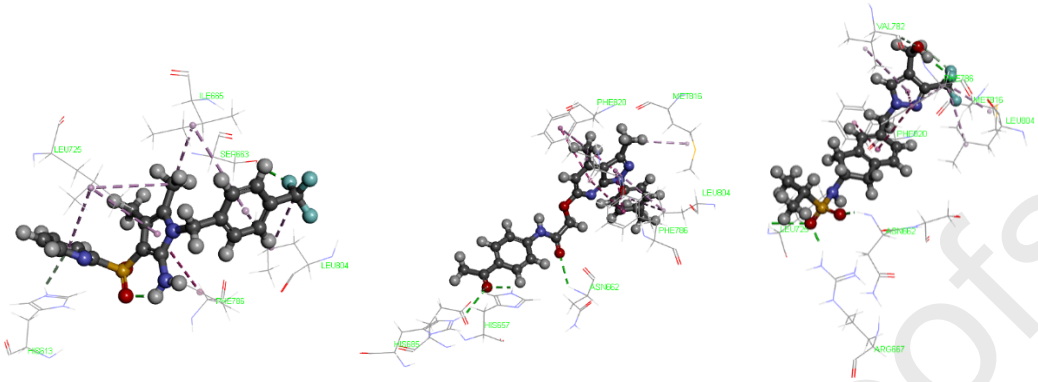


Figure 6a. 3D image of compound **1v0p_PVB** inside **2H42** using Accelrys Discovery Studio 4.5; with docking score of- 42.5735, **6b.** Docking score of **LIF172331** is of -35.97, **6c.** Docking score of **SPB 02245**is of -21.90. **6d.** Docking score of **CDI685470** is of -15.04, **6e.** Docking score of **3pmw_G69** is of -9.33all figures reveal the hydrogen bonds and hydrophobic interaction with the key residues of the receptor. (For interpretation using colors, the reader is referred to the web version of this article).

2.1.4.2. *Docking Study of* **LIF172331.**

The docking score of **LIF172331** is-35.97, it showed a CDOCKER_Interaction_Energy of -53.92, and it forms four hydrogen bonds: one hydrogen bond between the oxygen atom of a carbonyl with glutamine 817, one from the p-fluoro moiety to histidine 685, and two additional hydrogen bonds to threonine723. Additionally, the planar regions of indole form hydrophobic interactions with the hydrophobic pocket generated by leucine 725, histidine 685, threonine723, and the phenyl ring forms another van der Waals

interaction with valine 782, phenylalanine 786, isoleucine 813 and methionine 816. These interactions may increase the affinity of the compound for the binding site of PDE5, resulting in a high predicted inhibitory activity (**Figure 6b)**.

2.1.4.3. *Docking Study of* **SPB 02245**

The docking score of **SPB 02245** is of -21.90, it showed a CDOCKER Interaction_Energy of -37.75, and it forms two hydrogen bonds; one hydrogen bond between the trifluoromethyl moiety and serine 663, and one between the N of the pyridine moiety with histidine 613.Additionally, the phenyl ring forms hydrophobic interactions with the hydrophobic pocket formed by leucine 804and isoleucine 665; the imidazole moiety forms a van der Waals interaction with phenylalanine 786 and leucine 725; and the pyridine ring forms another van der Waals interaction with leucine 725. These interactions may increase the affinity of the compound for the binding site of PDE5, resulting in a high predicted inhibitory activity (**Figure 6c)**.

2.1.4.4. *Docking Study of* **CDI685470**

The docking score of **CDI685470** is -15.04, it showed a CDOCKER_Interaction_Energy of -52.44, and it forms seven hydrogen bonds; four hydrogen bonds between the carbonyl of amide with asparagine 662, and another three hydrogen bonds with histidine 657 and histidine 685. Additionally, the pyrazolopyridine and the phenyl ring form hydrophobic interactions with the hydrophobic pocket formed by phenylalanine 820, leucine 804, methionine 816, and phenylalanine 786.These interactions may increase the affinity of the compound for the binding site of PDE5, resulting in a high predicted inhibitory activity (**Figure 6d)**.

2.1.4.5. Docking Study of **3pmw_G69**

The docking score of **3pmw_G69** is of -9.33, it showed a CDOCKER Interaction_Energy of -46.70, and it forms four hydrogen bonds; three hydrogen bonds between the O of the sulfonyl with asparagine 662, leucine 725, and arginine 667, and one between the hydroxyl group and valine 782. Additionally, the phenyl ring of the planar portion of 2,3-dihydro-1H-indene forms a van der Waals interaction with the hydrophobic pocket formed by phenylalanine 786 and phenylalanine 820, and the pyrazole ring forms another van der Waals interaction with valine 782, leucine804, and methionine 816. These interactions may increase the affinity of the compound for the binding site of PDE5, resulting in a high predicted inhibitory activity (**Figure 6e).**

2.1.5. Pharmacophore mapping of the proposed analogues

The proposed analogues were mapped into the validated pharmacophore model to determine their fitting and docked into the crystal structure of PDE5 in complex with a natural inhibitor (sildenafil, protein data bank code **2H42**) to elucidate the key structural features required for the inhibition of PDE5 **Table 4**. The compatibility between their fit values with the pharmacophore model and their docking affinity indicates the biological binding mode, offering a relevant basis for the further development of new PDE-5 inhibitors.

2.1.6. Docking studies of the proposed analogues

In general, a docking study on proposed compounds **2a-h,4a-d,5** and **6** revealed CDOCKER _Energy values from -11.41 to -2.28, CDOCKER_Interaction_Energy values from -56.04 to-39.79 (**Table 5**), and hydrogen bonds between the C=O and NH moieties

of the pyrimidine with glutamine 817. Additionally, the planar region of pyrazolopyrimidine formed hydrophobic interactions with the hydrophobic pocket generated by valine 782, phenylalanine 786, and phenylalanine 820; the phenyl ring forms a van der Waals interaction with leucine 804, alanine 783, and methionine 816and a hydrophobic interaction with isoleucine 813.

Table 5. Mapping of sildenafil and the proposed analogues to pharmacophore hypothesis 1, and their docking scores in the binding site of PDE-5 crystal structure.

Cpd No	- CDocker_ Energy	- CDocker_Interaction _Energy	Fit value by mapping of the proposed analogues to pharmacophore hypothesis 1	Estimated activity with μM using pharmacophore hypothesis 1
Sildenafil	12.41	56.04	6.50	2.80
2h	11.41	56.20	5.49	21.40
2c	8.87	49.92	5.48	21.60
4c	8.50	44.44	5.63	15.46
4d	7.98	44.46	5.63	15.46
2f	7.06	47.38	5.48	21.64
2e	5.87	44.76	5.49	21.38
2g	5.87	46.95	5.48	21.51
5	5.53	46.42	6.06	5.76
4b	5.18	43.65	5.64	15.04
4a	4.82	41.20	5.54	18.86
6	4.30	43.81	6.04	5.99
2b	3.53	43.89	5.48	21.47

2d	3.36	44.08	5.48	21.46
2a	2.28	39.798	5.48	21.57

*2.1.6.1. Docking studies of proposed analogue **2h***

The docking score of compound **2h** is -11.41, it showed a CDOCKER_Interaction_Energy of -56.20, and it forms four hydrogen bonds: three hydrogen bonds between the C=O and NH moieties of the pyrimidine moiety with glutamine 817, and one between the N of the pyrazole ring and tyrosine 612. Additionally, the planar region of pyrazolopyrimidine forms hydrophobic interactions with the hydrophobic pocket formed by valine 782, phenylalanine 786, alanine 783, phenylalanine 820, and alanine 767; the N-phenyl ring forms a van der Waals interaction with leucine 765 and additional interactions with histidine 613; and the p-bromo moiety interacts with leucine 725. The 6-phenyl moiety forms hydrophobic interactions with the hydrophobic pocket generated by phenylalanine 786, leucine 804, methionine 816.In addition, the CH of the butoxy moiety forms hydrophobic interactions with isoleucine 813and interactions with phenylalanine786, phenylalanine787, and alanine783 (**Figure 7a**).The docking study showed that the binding mode of **2h** inside PDE5 (protein code 2H42) resembles that reported for sildenafil with additional hydrogen bonds with tyrosine 612 due to variations in the ring. The replacement of the propyl moiety with an aromatic ring strengthens the interactions with the hydrophobic pocket generated by leucine 765 and histidine613. Moreover, the replacement of the 2-alkoxy moiety with a long chain substituent strengthens the hydrophobic interactions with isoleucine 813and the interactions with phenylalanine786, phenylalanine787, and alanine783.

(a) (b)

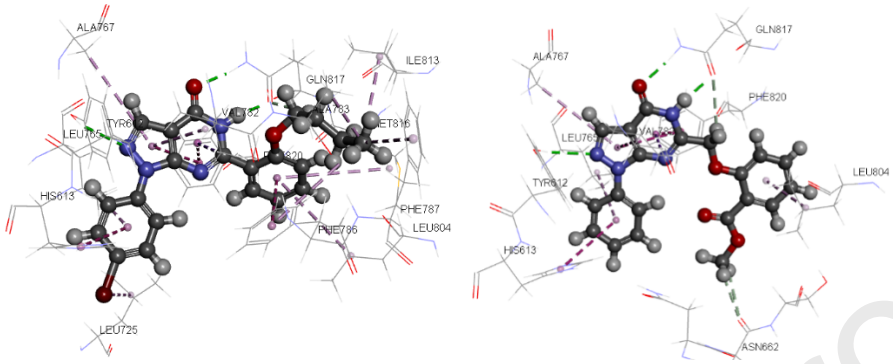


Figure 7a. 3D image of compound **2h**inside **2H42**usingAccelrys Discovery Studio 4.5; with Cdocker energy of -11.41, **7b.** Docking score of **5** is of -5.53, all figures reveal the hydrogen bonds and hydrophobic interaction with the key residues of the receptor. (For interpretation using colors, the reader is referred to the web version of this article).

*2.1.6.2. Docking studies of the designed proposed analogue **5***

The docking score of compound **5** is of -5.53, it showed a CDOCKER_Interaction_Energy of -46.42, and it forms five hydrogen bonds; two hydrogen bonds between the C=O and NH groups of the pyrimidine moiety, one between the H of the side chain with glutamine 817, one between the N of the pyrazole ring with tyrosine 612, and two from the CH_3 group of the ester with asparagine 662. Additionally, the planar portion of the pyrazolopyrimidine forms hydrophobic interactions with the hydrophobic pocket formed by valine 782, phenylalanine 786, alanine 783, phenylalanine 820, and alanine 767; the N-phenyl ring forms a van der Waals interaction with leucine 765 and an additional interaction with histidine 613; and the 6-phenyl moiety forms

hydrophobic interactions with the hydrophobic pocket generated by leucine 804 (**Figure 7b).**

The docking study showed that the binding mode of compound **5** inside PDE5 (2H42) resembles that reported of sildenafil along with an additional hydrogen bond with tyrosine 612 due to variations in the ring. The replacement of the propyl moiety with an aromatic ring strengthens the interactions with the hydrophobic pocket formed by leucine 765, histidine613, and leucine 725.In addition, the 2-methyl ester forms two hydrogen bonds with asparagine 662**.**

2.2. Design Strategy

Different protocols in Accelrys Discovery Studio software version 4.0 were used to design new scaffolds of PDE5 inhibitors and estimate their activities prior to their synthesis. In the current study, we reported molecular modelling studies on PDE5 inhibitors using the 3D-quantitative structure-activity relationship (3D-QSAR) pharmacophore generation protocol, which can be used to generate predictive pharmacophore models from a set of compounds with known activities on PDE5[18].The generated pharmacophore hypotheses were validated to select the best model for the virtual screening of different databases and to predict the activity of the proposed structures to be synthesized.

According to our molecular modelling studies, sildenafil was modified to design new potent PDE5 inhibitors (**Figure 8, 9)**.

a. The ring was varied from pyrazolo[3,4-d]pyrimidine-4-one to pyrazolo[4,3-d]pyrimidine-7-one to study the effect of changing the site of fusion where it

forms three hydrogen bonds between the C=O and NH groups of the pyrimidine moiety with glutamine 817 and an additional hydrogen bond between the N of the pyrazole ring and tyrosine 612. Additionally, planar portion of the pyrazolopyrimidine forms hydrophobic interactions with the hydrophobic pocket generated by valine 782, phenylalanine 786, alanine 783, phenylalanine 820, and alanine 767.

b. The propyl moiety in the pyrazole ring was replaced with an aryl moiety, resulting in stronger hydrophobic interactions with the hydrophobic pocket generated by histidine 613, leucine 765, leucine 725, and tyrosine.
c. The 2-ethoxymoiety was varied to strengthen the hydrophobic interactions with the hydrophobic pocket generated by phenylalanine 786, phenylalanine 787, leucine 804, isoleucine 813, and methionine 816.
d. Additionally, to study the effect of different conformational isomers and their ligand receptor interactions, a linker was inserted at position 6 of the pyrazolo[4,3-*d*]pyrimidine-7-one, and an aminomethyl moiety (CH_2NH) or its bioisosteric methoxy (CH_2O) was installed on the phenyl moiety.
e. In addition, the 2-methyl benzoate ester forms extra hydrogen bonds with asparagine 662.

Therefore, we designed and synthesized a new series of pyrazolo[3,4-*d*]pyrimidinones as sildenafil analogues for the treatment of ED **(Figure 8, 9).**

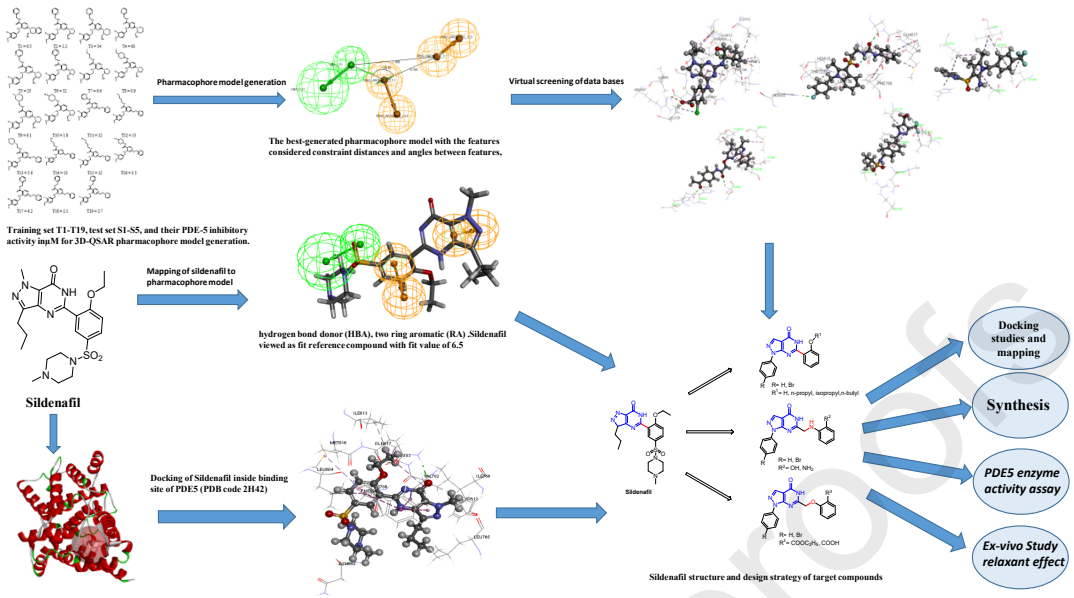


Figure 8. Schematic representation to rational,and design of novel pyrazolo[3,4-d]pyrimidin-7-ones scaffold; synthesis and biological evaluation.

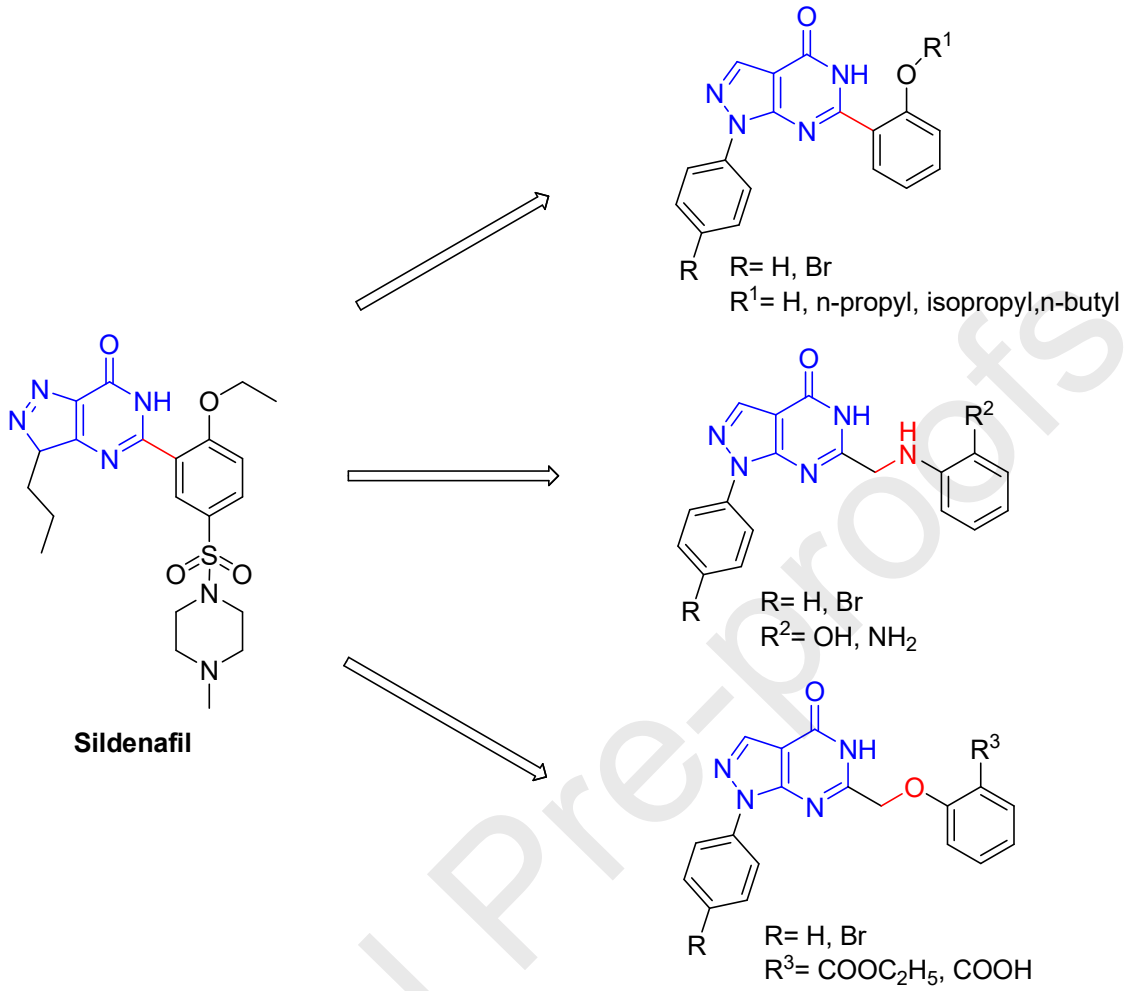
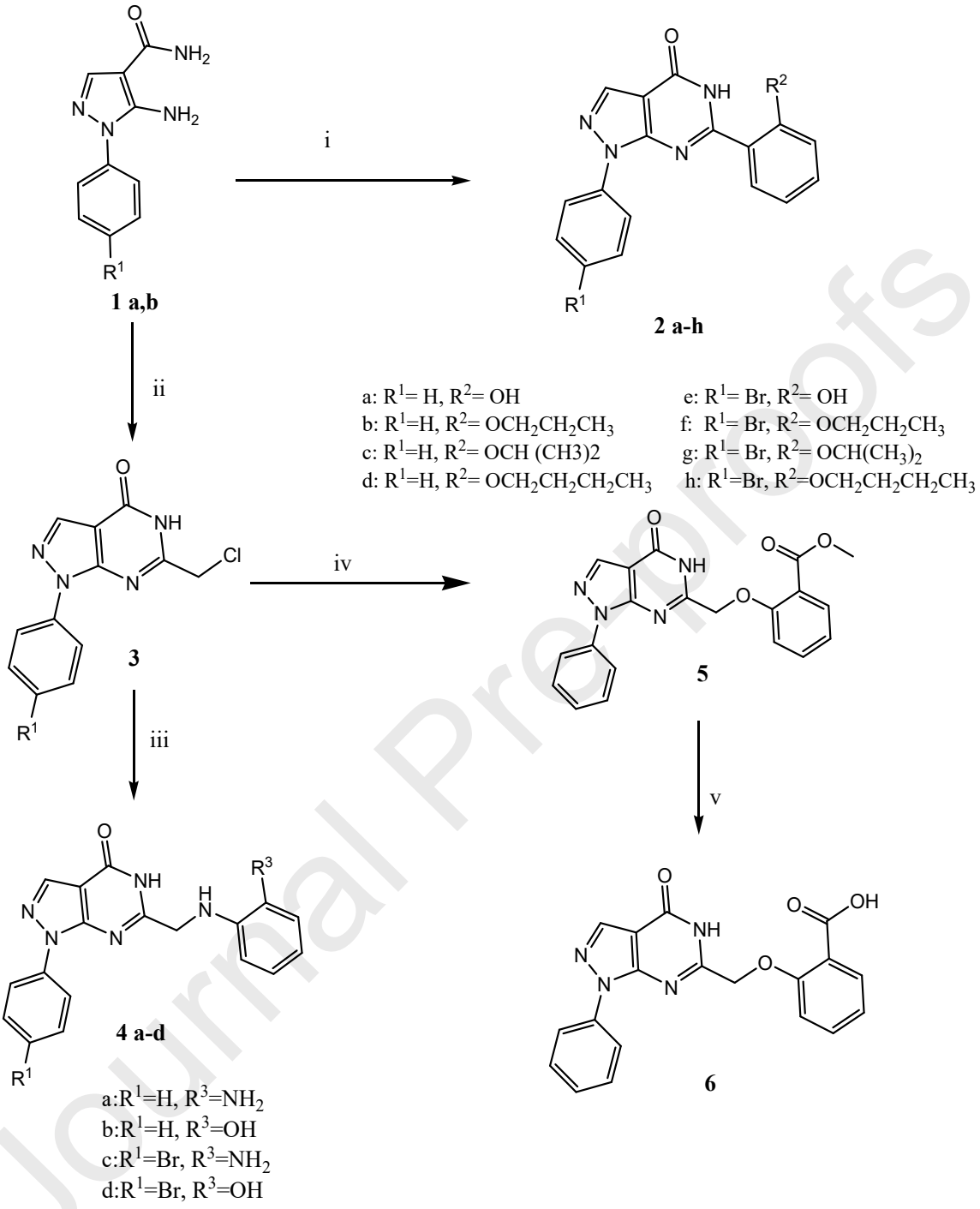


Figure 9. Sildenafil structure and design strategy of target compounds

2.3. Chemistry

1-Substituted-arylpyrazolo[3,4-*d*]pyrimidines **2a-h** were synthesized by the cyclization of 5-amino-1-substituted-phenylyrazolo-4-carboxamides **1a,b** with 2-substituted-benzaldehydes in the presence of iodine. The structures of the obtained compounds were elucidated by spectral and elemental analyses. The ^{1}HNMR spectra of **2a-h** in DMSO-$d_6$showed an increase in the number of aromatic protons in the range of δ: 6.90-8.56 ppm, and the signal of an amino group and a singlet signal in the range δ: 12.04-12.13 ppm

disappeared, corresponding to NH of the pyrimidine ring, as it was exchanged upon treatment with D_2O. The reaction of **1a** with chloroacetyl chloride afforded chloromethyl derivative **3**, and subsequent nucleophilic substitution with an aromatic amine or phenol gave methyl amino derivatives **4a-d** and methoxy derivative **5**, which was hydrolysed with sodium hydroxide to give **6**. The structures of compounds **4a-d** were established by elemental and spectral analyses. The ^{1}HNMR spectra of **4a-d** in DMSO-d_6 exhibited singlet signals for CH_2 in the range 4.34-4.36 ppm and a singlet signal corresponding to NH_2 in the range 5.48-5.46 ppm. Finally, the NH of the pyrimidine ring appears as a singlet signal in the range 12.23-12.38 ppm. The structures of compounds **5** and **6** were confirmed via elemental and spectral analyses. The ^{1}HNMR spectrum of compound **5** in DMSO-$d_6$showed 3 clear singlet's for CH_3 at 3.38 ppm, for CH_2at 4.58 ppm and finally for the NH of the pyrimidine at 12.36 ring, which had exchanged upon treatment with D_2O. While the spectrum of compound **6** offered two singlet signals, at 5.32 ppm due to CH_2 and at 12.68 ppm due to the NH of the pyrimidine ring, exchanged upon treatment with D_2O. The IR spectrum showed a peak at 3446 cm^{-1} due to the acidic OH group **(Scheme 1).**



Scheme 1. Synthesis of 1,6-disubstituted-pyrazolo[3,4-*d*]pyrimidinones. Reagents and solvents: (i) 2-hydroxybenzaldehyde or 2-alkoxybenzaldehyde, I_2, acetonitrile, reflux 6 h, yield: 54% -73% (ii) chloroacetyl chloride, stirring 30 min, then oil bath 5 h, yield: 63%

(iii) o-phenylene diamine or aminophenol, potassium hydroxide, DMSO, heating at 70 ^{0}c for 10 h, yield: 45% -53% (iv) methyl salicylate, potassium hydroxide, DMSO, heating at 70 ^{0}c for 5 h, yield: 52% (v) sodium hydroxide, abs.EtOH, reflux 8 h, acetic acid, yield: 55%.

.

2.4. Biological Analysis

2.4.1. In vitro PDE5 inhibitory activity assay

First, the sildenafil analogues were tested *in vitro* to evaluate their ability to inhibit PDE5 in human platelets by using the reported procedure [22-24]. In this assay, the inhibitory activities of compounds **5, 4b, 2a, 2d, 2f, 4d** and **4a** against PDE5 were similar to that of sildenafil (100% inhibition), and compounds **2e** and **4c** showed 95% inhibition. Compounds **2h, 6,** and **2g** presented less than 50% enzymatic inhibition (**Table 6, Figure 10**).

Table 6. Percent inhibition of the PDE5 inhibitor values in human platelets for compounds (**2a-g,4a-d,5,6),**and their relaxant effects (pEC_{50}) in rat corpus cavernosum preparations and their efficacy in comparison to sildenafil(E_{max}).

Cpd No	% of PDE5 inhibition	EC_{50}(μM) (Potency) in comparison to sildenafil EC 50% ± SE	pEC_{50}*	Efficacy(g) in comparison to sildenafil E_{max}*

Sildenafil	100	1.00± 0.08	6.0000	100
2h	20	0.04 ± 0.03	8.3106	160
5	100	0.01 ± 0.02	7.0000	130
6	20	0.22 ± 0.04	6.6516	**
2b	46.66	0.23 ± 0.03	6.6307	150
4b	100	0.25 ± 0.07	6.6020	125
2g	46.44	0.28 ± 0.05	6.5528	**
2e	95.5	0.33 ± 0.06	6.4710	167
2c	33.33	0.50 ± 0.09	6.3010	130
2a	100	0.50 ± 0.07	6.3010	93
2d	100	0.63 ± 0.06	6.2006	170
4c	95	0.70 ± 0.07	6.1505	148
2f	100	0.83 ± 0.03	6.0809	140
4d	100	5.05 ± 0.05	5.2967	133
4a	100	6.80 ± 0.04	5.1674	113

* Experimental values were calculated relative to the maximal changes from the contraction produced by phenylephrine and represented as –log of molar concentration to produce 50% of maximal relaxation elicited by sildenafil and analogues in contracted tissues. Data represented the mean ±SEM of n experiments

** were not obtained values at 50% of enzymatic inhibition

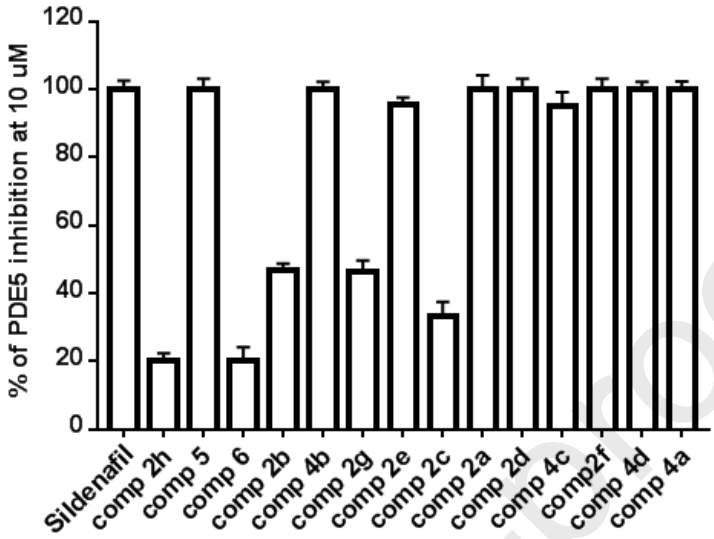


Figure 10. The Phosphodiesterase-5 assay of synthesized compounds at a single dose concentration of 10μM.

2.4.2. Ex-vivo Study (Isolated corpus cavernosum tissue). [22-24]

Additionally, the synthesized compounds were functionally studied in rat corpus cavernosum to evaluate the induction of smooth muscle relaxation by the sildenafil analogues (**2a-h,4a-d,5,** and **6**). In phenylephrine-contracted preparations, cumulative concentration-response curves for the compounds were obtained in corpus cavernosum (0.001-10 μM). Experimental values were calculated relative to maximal changes based on the contraction produced by treating the tissue with phenylephrine, which was taken as

100%. Data represent the mean ± SEM of 3-5 experiments. Our data showed that most of the compounds were as potent as sildenafil in relaxing the corpus cavernosum, with pEC_{50}values between 8.31-5.16 μM. In particular, compounds **2h**,**5**,**6**,**2b**,**4b**,**2g**,**2e**,**2c**,**2a**,**2d**,**4c**, and **2f**showed the best pEC_{50} values for relaxing corpus cavernosum, while compounds **4a** and **4d** were less potent than sildenafil in this assay. The maximal responses for these compounds (E_{max} values between 93-167%) were similar to that of sildenafil (E_{max} 105 ± 3.1%). **Table 6 and Figure 11**show the pE_{50} and E_{max}values for these compounds.

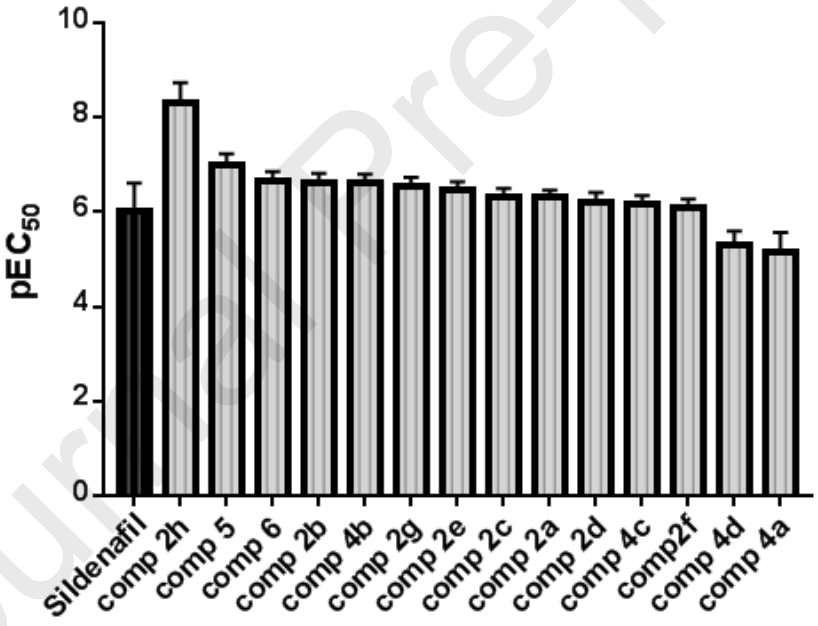


Figure 11. The relaxant effects (pEC_{50}) in rat corpus cavernosum with 10 μM of the synthesized compounds (**2a-g,4a-d,5,6)** and plotted relative to sildenafil.

3. Conclusion

A series of pyrazolo[3,4-*d*]pyrimidinone derivatives were designed, synthesized and evaluated for PDE5 inhibitory activity. Among the synthesized derivatives; **5,2a,2d,2f,4a,4b,4d** possessed high PDE5 inhibitory activities in human platelets relative to that seen with sildenafil. Compound **2h** has an excellent relaxant effect (pEC_{50}) in rat corpus cavernosum preparation, and this result was consistent with the docking study of **2h** in the binding site of PDE5, where it showed a high CDOCKER_Energy of -11.41 and a high CDOCKER_Interaction_Energy of -56.20. These properties may be due to its high lipophilicity from the presence of p-bromo and butyloxy moieties. Also, compounds **2b, 2c, 2g** showing moderate relaxant effect as they have propoxy as lipophilic moiety. These results have inspired us to optimize this class of drug-like compounds as PDE5inhibitors.

4. Experimental

4.1. Chemistry

4.1.1. Material and methods

Melting points were determined on Electro thermal Stuart 5 MP3 digital melting point apparatus and were uncorrected. Elemental microanalyses were performed at the micro analytical center, Al-Azhar University, Cairo, Egypt. IR spectra were recorded on a Bruker Fourier transform (FT) - IR spectrophotometer as KBr discs. NMR spectra (in

DMSO-d_6) were recorded on Bruker AC-300 Ultra Shield NMR spectrometer (Bruker, Flawil, Switzerland, δ ppm) at 400 MHz using TMS as internal Standard and peak multiplicities are designed as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Silica gel used for column chromatography was obtained from Fluka, 70e 230 mesh thin layer chromatography was carried out on silica gel TLC plates with fluorescence indicator (F254). The Chromatographic system was Agilent Technologies 1200 Series, G1315D DAD. The column used was Zorbax Eclipse. A rapid resolution 4.69 150 mm 3.5 lM particle size. The mobile phase employed for the separation (isocratic elution) consisted in 200 mM ammonium acetate pH 6.0 with 2% acetonitrile (v/v). The flow rate was 1.5 cm3 /min; the detector DAD at 254 nm.

4.1.2. Synthesis of compounds ***1, 2a-h, 3,4a-d, 5, 6***

4.1.2.1. 5-Amino-1-(4-substitutedphenyl)-1H-pyrazole-4 carboxamide (**1a, b**). 5-Amino-1-(4-substitutedphenyl)-1H-pyrazole-4-carbonitrile, (1 mM) was refluxed in sodium hydroxide (0.80 g, 2 mM) and ethanol (10 mL) for 30 minutes, upon cooling, the solid precipitated was collected by suction filtration, then dried and crystallized from water.

4.1.2.1.1. 5-Amino-1-phenyl-1H-pyrazole-4 carboxamide **(1a)**

5-Amino-1-phenyl-1H-pyrazole-4 carboxamide (**1a**) was synthesized from 5-Amino-1-(4-substitutedphenyl)-1H-pyrazole-4-carbonitrile according to the general procedure. Yield: 82%; m. p.: 172–174^{0}C as reported [25, 26]

*4.1.2.1.2. 5-Amino-1-(4-bromophenyl)-1H-pyrazole-4 carboxamide(**1b**).* Yield: 74%; m. p.: 180–182 ^{0}C; IR (KBr) cm^{-1}: 3180(NH), 2990(arom.CH), 1678 (C=O), 1585 (C=N), 1519 (C=C); ^{1}HNMR (DMSO-d_6, D_2O) δ: 6.60 (s, 2H, $CONH_2$), 7.70 -8.30(m, 5H, arom.CH), 8.60(s, 2H, NH_2, D_2O- exchangeable).

*4.1.2.2. General synthetic procedure for1-(4-Substitutedphenyl)-6-(2-hydroxy (or alkoxy) phenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one(**2a-h**).* A mixture of 5-amino-1-(4-substitutedphenyl)-1H-pyrazole-4-carboxamide (1mmol,**1a,b**) and 2-hydroxybenzaldehyde or 2-alkoxybenzaldehyde (1.2 mM) and iodine (0.309 g,1.2 mM) in acetonitrile(10 mL) was heated under reflux for 6 hours, the reaction mixture was cooled, treated with sodium thiosulphate (5%), then filtered, washed with acetonitrile, dried and crystallized from ethanol.

*4.1.2.2.1. 1-(4-phenyl)-6-(2-hydroxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**2a**).* Yield: 54%; m. p.: 142–144 ^{0}C; IR (KBr) cm^{-1} : 3350 (OH),3200(NH), 3107 (arom.CH), 1701 (C=O), 1597 (C=N), 1552 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 7.00-8.37(m,10H,arom.CH),12.12 (s,brd,2H,NH and OH, D_2O- exchangeable); ^{13}CNMR(DMSO-d_6):δ: 106.16(C3a,pyrazolopyrimidine) ,116.90 (C2,phenol), 117.84 (C6,phenol), 120.10 (C4,phenol), 122.46 (C2,6,benzene),127.73 (C3,phenol), 129.85(C3,5,benzene),130.05 (C4,benzene), 134.13 (C5,phenol), 136.63(C1,benzene), 138.65 (C1, phenol), 151.89 (C3,C7a,pyrazolopyrimidine), 156.09 (C6,pyrazolopyrimidine), 158.02 (C4,pyrazolopyrimidine,C=O) ; MS (m/z,R.A.%): 306 (M+2,2.47%),305

(M+1,19.70%), 304 (M^+,100 %). Anal. Calc. for $C_{17}H_{12}N_4O_2$ (304). Calculated: C, 67.10; H, 3.95; N, 18.42. Found: C, 67.21; H, 4.03; N, 18.58%.

4.1.2.2.2. 1-(4-phenyl)-6-(2-propoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one ***(2b)****.* Yield: 62 %; m. p.: 167–169 ^{0}C; IR (KBr) cm^{-1}: 3292 (NH), 3070 (arom.CH), 2961 (aliph.CH),1702 (C=O), 1586 (C=N), 1541 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 0.96 (t,3H, $\underline{CH_3}CH_2CH_2$), 1.74 (sext,2H, $CH_3\underline{CH_2}CH_2$), 4.06 (t,2H, $CH_3CH_2\underline{CH_2}$),7.09-8.35(m, 10H, arom.CH),12.08 (s, brd,1H, NH , D_2O- exchangeable); ^{13}CNMR(DMSOd_6):δ:13.81($\underline{CH_3}CH_2CH_2$)19.18($CH_3\underline{CH_2}CH_2$),68.71($CH_3CH_2\underline{CH_2}$),104.57(C3a,pyrazolopyrimidine),113.51(C2,phenylpropylether),119.27(C6,phenylpropylether), 121.10 (C4,phenylpropylether),121.79(C3, phenylpropylether),123.35 (C2,6,benzene), 131.05 (C4,benzene), 132.53(C3,5,benzene), 133.48 (C5, phenylpropylether), 138.15 (C1,benzene),146.75(C3,pyrazolopyrimidine),153.27(C7a,pyrazolopyrimidine),156.19(C1 ,phenylpropylether),157.43(C6,pyrazolopyrimidine),158.27(C4,pyrazolopyrimidine,C=O) ;MS (m/z,R.A.%): 348 (M+2, 2.55 %),347 (M+18,19.32 %), 346 (M^+,1.84%).Anal. Calc. for $C_{20}H_{18}N_4O_2$ (346). Calculated: C, 69.36; H, 5.20; N, 16.18. Found C, 69.56 H, 5.28; N, 16.26 %.

4.1.2.2.3. 1-(4-phenyl)-6-(2-isopropoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one ***(2c)****.* Yield: 65 %; m. p.: 174–176 ^{0}C; IR (KBr) cm^{-1} : 3279 (NH), 3080 (arom.CH), 2975 (aliph.CH),1698 (C=O), 1585 (C=N), 1544 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 1.32 (d,6H, $2CH_3$), 4.75 (sept,1H, CH), 7.09-8.36 (m, 10H, arom.CH),12.10 (s, brd,1H, NH , D_2Oexchangeable);^{13}CNMR(DMSOd_6):δ:22.21($2CH_3$),71.89(CH),106.26(C3a,pyrazolopyrimidine),114.93(C2,phenylisopropylether),121.09(C6,phenylisopropylether),122.13(C2

,6,benzene),122.48(C4,phenylisopropylether),127.43(C3,phenylisopropylether),129.69(C 3,5,benzene),131.26(C4,benzene),133.37(C5,phenylisopropylether),136.45(C1,benzene), 138.86(C3,pyrazolopyrimidine),152.69(C7a,pyrazolopyrimidine),155.91(C1,phenylisopr opylether),156.35(C6,pyrazolopyrimidine),157.65(C4,pyrazolopyrimidine,C=O) ; MS (m/z,R.A.%): 347 (M+1, 1.35 %), 346 (M^+,9.77 %). Anal. Calc. for $C_{20}H_{18}N_4O_2$ (346). Calculated: C, 69.36; H, 5.25; N, 16.18. Found C, 69.44, H, 5.22; N, 16.28 %.

*4.1.2.2.4. 1-(4-phenyl)-6-(2-butoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**2d**).* Yield: 69 %; m. p.: 184–186 ^{0}C; IR (KBr) cm^{-1} : 3339 (NH), 3100 (arom.CH), 2910 (aliph.CH),1682 (C=O), 1581 (C=N), 1570 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 0.88 (t,3H, $\underline{CH_3}CH_2CH_2CH_2$), 1.41 (sext,2H, $CH_3\underline{CH_2}CH_2CH_2$), 1.53 (pent,2H, $CH_3CH_2\underline{CH_2}CH_2$), 4.11 (t,2H, $CH_3CH_2CH_2\underline{CH_2}$), 6.96-8.56(m, 10H, arom.CH),12.10 (s, brd,1H, NH , D_2O- exchangeable); ^{13}CNMR (DMSO-d_6):δ: 14.15($\underline{CH_3}CH_2CH_2CH^2$), 19.16 ($CH_3\underline{CH_2}CH_2CH_2$), 31.07($CH_3CH_2\underline{CH_2}CH_2$), 68.68 ($CH_3CH_2CH_2\underline{CH_2}$),106.26 (C3a,pyrazolopyrimidine) ,113.51 (C2,phenylbutylether), 121.08 (C6, phenylbutylether), 122.16 (C2,6,benzene), 122.37 (C4, phenylbutylether), 127.45 (C3, phenylbutylether), 129.70(C3,5,benzene),131.04 (C4,benzene), 133.37 (C5, phenylbutylether), 136.46 (C1,benzene), 138.86 (C3, pyrazolopyrimidine), 152.73 (C7a,pyrazolopyrimidine), 155.97(C1,phenylbutylether),162.19(C6,pyrazolopyrimidine),163.38(C4,pyrazolopyrimid ine,C=O) ; MS (m/z,R.A.%): 362 (M+2, 1.00%),361 (M+1,8.43 %), 360 (M^+, 35.16 %). Anal. Calc. for $C_{21}H_{20}N_4O_2$ (360). Calculated: C, 69.98; H, 5.59; N, 15.55. Found C, 70.13, H, 5.67; N, 15.76 %.

*4.1.2.2.5. 1-(4-bromophenyl)-6-(2-hydroxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**2e**).* Yield: 62 %; m. p.: 152–154 ^{0}C; IR (KBr) cm^{-1}: 3434 (OH), 3210(NH), 3070 (arom.CH), 1701 (C=O), 1672 (C=N), 1593 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 6.90-8.27 (m, 9H, arom.CH), (NH and OH, not appeared in the chart); ^{13}CNMR (DMSO-d_6):δ: 106.26(C3a,pyrazolopyrimidine) ,117.39 (C2,phenol), 118.11 (C6,phenol), 119.58 (C4,benzene and C4,phenol), 123.60 (C2,6,benzene),129.94 (C3,phenol), 132.65(C3,5,benzene), 133.74 (C5,phenol), 136.84(C1,benzene), 138.35 (C3, pyrazolopyrimidine), 153.00 (C7a,pyrazolopyrimidine), 157.76 (C1, phenol), 158.93 (C6,pyrazolopyrimidine), 159.81 (C4,pyrazolopyrimidine,C=O) ; MS (m/z,R.A.%): 385 (M+2, 6.33 %),384 (M+1, 3.26 %), 383 (M^+, 8.41 %). Anal. Calc. for $C_{17}H_{11}BrN_4O_2$ (383). Calculated: C, 53.28; H, 2.89; N, 14.62. Found C, 53.48; H, 2.19; N, 14.75%.

*4.1.2.2.6. 1-(4-bromophenyl)-6-(2-propoxyoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**2f**).* Yield: 68 %; m. p.: 172–174 ^{0}C; IR (KBr) cm^{-1} : 3298 (NH), 3080 (arom.CH), 2967 (aliph.CH),1701 (C=O), 1586 (C=N), 1540 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 0.96 (t,3H, $\underline{CH_3}CH_2CH_2$), 1.74 (sext,2H, $CH_3\underline{CH_2}CH_2$), 4.07 (t,2H, $CH_3CH_2\underline{CH_2}$),7.09-8.37 (m, 9H, arom.CH),12.13 (s, brd,1H, NH , D_2O- exchangeable); ^{13}CNMR(DMSOd_6):δ:10.88($\underline{CH_3}CH_2CH_2$),22.41($CH_3\underline{CH_2}CH_2$),70.41($CH_3CH_2\underline{CH_2}$),106.44(C3a,pyrazolopyrimidine)113.50(C2,phenylpropylether),119.90(C6,phenylpropylether) , 121.11 (C4, phenylpropylether), 121.91 (C4,benzene), 123.79 (C2,6,benzene), 131.09 (C3, phenylpropylether), 132.64(C3,5,benzene), 133.49 (C5, phenylpropylether), 136.82(C1,benzene),138.12 (C3, pyrazolopyrimidine), 152.88 (C7a,pyrazolopyrimidine), 156.18(C1,phenylpropylether),157.41(C6,pyrazolopyrimidine),157.58(C4,pyrazolopyrim

idine,C=O) ; MS (m/z,R.A.%): 427 (M+2, 1.09 %), 426 (M+1,1.60 %), 425 (M^+,1.84 %). Anal. Calc. for $C_{20}H_{17}BrN_4O_2$ (425). Calculated: C, 56.48; H, 4.03; N, 13.17. Found C, 56.60; H, 4.08; N, 13.30 %.

*4.1.2.2.7. 1-(4-bromophenyl)-6-(2-isopropoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**2g**).* Yield: 70 %; m. p.: 183–185^{0}C; IR (KBr) cm^{-1}:3280 (NH), 3080 (arom.CH), 2924 (aliph.CH),1710 (C=O), 1641 (C=N), 1586 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 1.32 (d,6H, 2CH_3), 4.75 (sept,1H, CH), 7.09-8.38 (m, 9H, arom.CH),12.04 (s, brd,1H, NH , D_2O- exchangeable); ^{13}CNMR (DMSO-d_6):δ: 19.16(2 CH_3), 68.69(CH),106.44 (C3a,pyrazolopyrimidine) ,113.52 (C2,phenylisopropylether), 119.90 (C6, phenylisopropylether),121.09(C4,phenylisopropylether),122.01(C3, phenylisopropylether), 123.80 (C2,6,benzene), 131.09 (C4,benzene), 132.65 (C3,5,benzene), 133.46 (C5, phenylisopropylether),136.83(C1,benzene),138.13(C3,pyrazolopyrimidine),152.91(C7a,p yrazolopyrimidine),156.24(C1, phenylisopropylether), 157.42 (C6,pyrazolopyrimidine), 157.65 (C4,pyrazolopyrimidine,C=O) ; MS (m/z,R.A.%): 427 (M+2, 3.49 %), 425 (M^+,5.28 %). Anal. Calc. for $C_{20}H_{17}BrN_4O_2$ (425). Calculated: C, 56.48; H, 4.03; N, 13.17. Found C, 56.60, H, 4.08; N, 13.30 %.

*4.1.2.2.8. 1-(4-bromophenyl)-6-(2-butoxyoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**2h**).* Yield: 73 %; m. p.: 191–193^{0}C; IR (KBr) cm^{-1} : 3295 (NH), 3095(arom.CH), 2956 (aliph.CH),1701 (C=O), 1587 (C=N), 1544 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 0.89 (t,3H, $\underline{CH_3}CH_2CH_2CH_2$), 1.40 (sext,2H, $CH_3\underline{CH_2}CH_2CH_2$), 1.71 (pent,2H, $CH_3CH_2\underline{CH_2}CH_2$), 4.11 (t,2H, $CH_3CH_2CH_2\underline{CH_2}$), 7.09-8.36 (m, 9H,

arom.CH),12.11 (s, brd,1H, NH , D_2O- exchangeable); ^{13}CNMR (DMSO-d_6):δ: 14.15($\underline{CH_3}CH_2CH_2CH^2$),19.16($CH_3\underline{CH_2}CH_2CH_2$),31.06($CH_3CH_2\underline{CH_2}CH_2$),68.69($CH_3CH_2CH_2$ $\underline{CH_2}$),106.44(C3a,pyrazolopyrimidine),113.51(C2,phenylbutylether),119.91(C6, phenylbutylether),121.09(C4,phenylbutyletherandC4,benzene),121.96(C3,phenylbutyleth er), 123.81 (C2,6,benzene), 131.09 (C5, phenylbutylether), 132.65 (C3,5,benzene), 133.48(C1,benzene),136.83 (C3, pyrazolopyrimidine), 138.13 (C7a,pyrazolopyrimidine), 152.89(C1,phenylbutylether),157.42(C6,pyrazolopyrimidine),157.61(C4,pyrazolopyrimid ine,C=O) ; MS (m/z,R.A.%): 362 (M+2, 1.00 %),361 (M+1, 8.43 %), 360 (M^+, 35.16 %). Anal. Calc. for $C_{21}H_{19}BrN_4O_2$ (439). Calculated: C, 57.41; H, 4.32; N, 12.75. Found C, 57.59, H, 4.41; N, 12.90 %.

*4.1.2.2.3. 1-Substitutedphenyl-6-(chloromethyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one(**3a,b**)*. A well stirred mixture of 5-amino-1-phenyl -1H-pyrazole -4-carboxamide (1mmol,**1a**) and choloroacetyl chloride (0.113g (0.079 mL),1mmol) for about 30 minutes at room temperature, which was then heated at 200 ^oC in oil bath for 5 hours, the solid formed was triturated with ethanol, then filtered, dried and crystalized from acetic acid.

*4.1.2.2.3.1 1-phenyl-6-(chloromethyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one(**3a**)*.

Yield: 58%; m. p.: 270–272 ^0C as reported [27].

*4.1.2.2.3.2 1-p-Brromophenyl-6-(chloromethyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one(**3b**)*.Yield: 63%; m.p.: 280–282 ^0C; IR (KBr) cm^{-1}: 3180(NH), 2990(arom.CH), 2838 (aliph.CH), 1678 (C=O), 1585 (C=N), 1519 (C=C); ^{1}HNMR (DMSO-d_6, D_2O) δ: 4.46 (s,2H,CH_2),7.77 -8.36 (m, 5H, arom.CH), 12.87 (s, 1H, NH, D_2O- exchangeable).

*4.1.2.2.4. 6-((2-substitutedaminophenyl or (phenoxy)methyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one(**4a-d**).* A mixture of 6-(chloromethyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (0.266g, 1mmol, **3**), o-phenylenediamine or aminophenol (5mmol) and potassium hydroxide (0.56g, 1mmol) was heated in DMSO (5ml) at 70 ^{o}C for 10 hours, then the reaction mixture was cooled , filtered ,dried and crystallized from ethanol.

*4.1.2.2.4.1. 6-(((2-Aminophenyl)amino)methyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**4a**).* Yield: 48 %; m. p.: 266–268 ^{0}C; IR (KBr) cm^{-1}:3334, 3205 (NH), 3076 (arom.CH), 2900 (aliph.CH),1668 (C=O), 1583 (C=N), 1544 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 4.34 (s,2H, CH_2), 4.60 (s,2H, NH_2, D_2O- exchangeable), 5.19 (t,1H, CH_2*NH*, D_2O- exchangeable), 6.47-8.27 (m, 10H, arom.CH),12.23 (s, brd,1H, NH , D_2O-exchangeable); ^{13}CNMR (DMSO-d_6):δ: 46.49(CH_2), 106.66(C3a,pyrazolopyrimidine) ,111.09(C6,aniline), 115.28 (C3,aniline), 118.20 (C5,aniline), 118.55(C4,aniline), 121.83 (C2,6,benzene), 127.30 (C4,benzene), 129.66(C3,5,benzene), 135.64 (C2,aniline),136.41(C1,aniline),138.80(C1,benzene),152.50(C3,C7a,pyrazolopyrimidine), 158.19(C6,pyrazolopyrimidine),160.79(C4,pyrazolopyrimidine,C=O); MS (m/z,R.A.%): 334 (M+2, 5.66 %),333 (M+1, 35.52 %), 332 (M^+, 100 %). Anal. Calc. for $C_{18}H_{16}N_6O$ (332). Calculated: C, 65.05; H, 4.85; N, 25.29. Found C, 65.24, H, 4.87; N, 25.43 %.

*4.1.2.2.4.2. 6-(((2-Hydroxyphenyl)amino)methyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**4b**).* Yield: 45 %; m. p.: 233–235^{0}C; IR (KBr) cm^{-1} : 3392 (OH),3180 (NH),

3045 (arom.CH), 2972 (aliph.CH),1683 (C=O), 1581 (C=N), 1545 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 4.36 (s,2H, CH_2), 5.46 (s,1H, CH_2*NH* , D_2O- exchangeable), 6.45-8.27 (m, 10H, arom.CH), 9.43 (s,1H,OH, D_2O- exchangeable),12.31 (s, brd,1H, NH , D_2O- exchangeable); ^{13}CNMR (DMSO-d_6):δ: 45.89(CH_2), 106.61(C3a,pyrazolopyrimidine) ,111.02 (C6,phenol), 114.11 (C3,phenol), 117.36 (C5,phenol), 120.01(C4,phenol), 121.68 (C2,6,benzene), 127.27 (C4,benzene), 129.62(C3,5,benzene), 136.40 (C2,phenol), 136.77(C1,phenol), 138.81 (C1, benzene), 144.97 (C3,C7a,pyrazolopyrimidine),158.17(C6,pyrazolopyrimidine),160.35(C4,pyrazolopyrimidine,C=O) ; MS (m/z,R.A.%): 335 (M+2, 5.69 %),334 (M+1, 22.15 %), 333 (M^+, 100 %). Anal. Calc. for $C_{18}H_{15}N_5O_2$ (333). Calculated: C, 64.86; H, 4.54; N, 21.01. Found C, 65.01, H, 4.59; N, 21.13 %.

*4.1.2.2.4.3. 6-(((2-Aminophenyl)amino)methyl)-1-(4-bromophenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**4c**).* Yield: 53 %; m. p.: 280–282^{0}C; IR (KBr) cm^{-1} :3320,3302 (NH), 3076 (arom.CH), 2925(aliph.CH),1704 (C=O), 1586 (C=N), 1546 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 4.35 (s,2H, CH_2), 5.20 (s,2H, NH_2, D_2O- exchangeable), 6.46 (t,1H, CH_2*NH* , D_2O- exchangeable), 7.60-8.29 (m, 9H, arom.CH),12.37 (s, brd,1H, NH , D_2O-exchangeable); ^{13}CNMR (DMSO-d_6):δ:56.41(CH_2), 106.51(C3a,pyrazolopyrimidine) ,112.56 (C6,aniline), 119.89 (C3,aniline), 121.09 (C5,aniline), 122.10(C4,aniline), 123.76 (C2,6,p-bromophenyl), 131.14 (C4,p-bromophenyl), 132.63(C3,5,p-bromophenyl),133.39(C2,aniline),136.79(C1,aniline),138.11(C1,pbromophenyl),152.86(C7a,pyrazolopyrimidine),156.23(C3,pyrazolopyrimidine),157.75(C6,pyrazolopyrimidine),157.81(C4,pyrazolopyrimidine,C=O) ; MS (m/z,R.A.%): 410 (M+, 4.42 %),412 (M+2,

17.52 %), 107 (100 %). Anal. Calc. for $C_{18}H_{15}BrN_6O$ (410). Calculated: C, 52.57; H, 3.68; N, 20.44. Found: C, 52.74, H, 3.74; N, 20.61 %.

*4.1.2.2.4.4. 6-(((2-Hydroxyphenyl) amino) methyl)-1-(4-bromopheny)l-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (**4d**).* Yield: 51 %; m. p.: 247–249^{0}C; IR (KBr) cm^{-1} : 3385 (OH),3200 (NH), 3045 (arom.CH), 2972 (aliph.CH),1678 (C=O), 1533 (C=N), 1518 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 4.36 (s,2H, CH_2), 5.48 (s,1H, CH_2*NH* , D_2O-exchangeable), 6.45-9.45 (m, 9H, arom.CH), 9.87 (s,1H,OH, D_2O- exchangeable),12.38 (s, brd,1H, NH , D_2O- exchangeable); MS (m/z,R.A.%): 411 (M+, 5.69 %),412 (M+1, 17.04 %), 413 (M+2, 7.30 %),414 (M+3, 4.37 %),. Anal. Calc. for $C_{18}H_{15}N_5O_2$ (411). Calculated: C, 52.55; H, 3.40; N, 17.03. Found C, 52.69, H, 3.64; N, 17.05 %.

*4.1.2.2.5. Methyl 2-((4-oxo-1-phenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)methoxy)benzoate (**5**).* A mixture of 6-(chloromethyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (0.266g, 1mmol, **3**), methyl salicylate (0.156g, 1mmol), and potassium hydroxide (0.56g, 1mmol) was heated at 70ºC for 5 hours in DMSO (5ml) then, the reaction mixture was cooled , filtered ,dried and crystallized from ethanol.

Yield: 52 %; m. p.: 205–207 ^{0}C; IR (KBr) cm^{-1} : 3251 (NH), 3078 (arom.CH), 2954 (aliph.CH),1712,1703 (C=O), 1589 (C=N), 1546 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 3.38 (s,3H, CH_3), 4.58 (s,2H, CH_2), 7.31-8.31 (m, 10H, arom.CH),12.36 (s, brd,1H, NH , D_2O- exchangeable); ^{13}CNMR (DMSO-d_6):δ: 58.99 (CH_3),70.49 (CH_2), 106.97(C3a,pyrazolopyrimidine),121.93(C3,benzoate),122.15(C1,5,benzoate),127.28(C2,4,6,benzene),127.52(C6,benzoateandC3,5,benzene),129.67(C4,benzoateandC1,benzene),136.41(

C3,C7a,pyrazolopyrimidine),138.76(C6,pyrazolopyrimidine),152.34(C2,benzoate),158.10(C4,pyrazolopyrimidine,C=O),158.35(C=O,ester);MS (m/z,R.A.%): 378 (M+2, 3.17 %),377 (M+1, 19.25 %), 376 (M^+, 76.64 %). Anal. Calc. for $C_{20}H_{16}N_4O_4$ (376). Calculated: C, 63.82; H, 4.28; N, 14.89. Found: C, 63.94; H, 4.29; N, 15.14 %.

*4.1.2.2.6.2-((4-Oxo-1-phenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)methoxy)benzoic acid (**6**).* Methyl 2-((4-oxo-1-phenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl) methoxy) benzoate (0.1 g, 0.25mmol,) **5** was refluxed with sodium hydroxide (0.040g, 1mmol) and ethanol (10mL) for about 8 hours, then cooled ,neutralized with acetic acid ,the obtained precipitate was filtered ,dried and crystallized from ethanol.Yield: 55 %; m. p.: 308–310^{0}C; IR (KBr) cm^{-1} : 3446 (OH), 3292 (NH), 3078 (arom.CH), 2954 (aliph.CH),1720,1683 (C=O), 1598 (C=N), 1591 (C=C); ^{1}HNMR (DMSO-d_6,D_2O) δ: 5.32 (s,2H, CH_2), 7.08-8.33 (m, 10H, arom.CH),12.35 (s, brd,1H, NH , D_2O- exchangeable); ^{13}CNMR (DMSO-d_6) δ: 67.60 (CH_2), 106.97(C3a,pyrazolopyrimidine) ,115.12(C3,benzoic), 120.47(C1,benzoic), 121.80(C2,6,benzene and C5,benzoic), 127.44(C4,benzene),129.59(C6,benzoate and C3,5,benzene),131.55(C4,benzoic),134.31(C1,benzene),136.51(C3, pyrazolopyrimidine), 138.67(C7a,pyrazolopyrimidine),152.11(C6,pyrazolopyrimidine),156.90(C2,benzoic),157.70(C4,pyrazolopyrimidine,C=O),166.42(C=O, benzoic); MS (m/z,R.A.%): 364 (M+2, 0.76 %),363 (M+1, 4.58 %), 362 (M^+, 20.04 %). Anal. Calc. for $C_{19}H_{14}N_4O_4$ (362). Calculated: C, 62.98; H, 3.89; N, 15.46. Found: C, 63.12; H, 3.94; N, 15.63 %.

4.2. PDE5 enzyme activity assay procedure

The standard enzymatic reaction mixture (total volume of 200 µL) contained 100 mMTris-HCl buffer (pH 8.3), 10 mM $MgCl_2$, 10m MKCl at 37 °C. Alfa casein (2 mg) used as a carrier for protein precipitation when protein concentration is low. PDE enzyme sample (final protein concentration 0.5 mg/mL). [21-24]. A concentration of 10 µMof the agents under study (sildenafil analogues) were prepared in dimethyl sulfoxide (DMSO) and preincubated in the enzymatic mixture for 5 min at room temperature. Reaction was initiated by addition of the substrate cGMP (5 µM) at 35°C for 30 min. The reaction was finished by transferring the reaction mixture tubes in boiling water bath for 3 min. The sample was then centrifuged and filtered through a nylon-66 filter, 0.2 mm (Rainin Corporation). The clear filtrate obtained may be used directly for HPLC assay or stored at -20 °C. A blank with protein, denaturated by boiling water bath for 3 min, with and without substrate was performed

Both incubation time and enzyme concentration were adjusted so that no more than 25% of the substrate was hydrolyzed under the assay conditions. The injection volume was 30 mm^3. Peak identities were confirmed by co-elution with standards all assays were done in duplicate.

4.3. Preparation of Rat Isolated Corpus Carvernosum

Forty adult male albino rats weighing 200 to 250 mg were divided into four groups (10 rats each). Animals were anaesthetized with sodium pentobarbital (Hypnol, 50mg/kg, i.v.), penis were removed. The protocol was approved by the University Ethics Committee. Corpus carvernosum preparations were obtained, following dissection of tunica albuginea and surrounding connective tissues. Tissues were immediately placed in chilled Kreb,s-Henseleit solution of the following composition (mM/L):(NaCl 118.4, KCl 4.69,$KH_2PO_4$1.17, $MgSO_4$ 1.18, $CaCl_2$ 2.52, D-glucose 11.10 and $NaHCO_3$ 25) aerated with carbogen (95% oxygen and 5% carbogen dioxide), penis tissue put under the dissecting microscope, corpus cavernosum, cleaned from the surrounding attached tissues and cut into small rings (about 4mm length).

Corpus cavernosum was suspended in an isolated organ bath (30 ml capacity) containing Kreb,s-Henseleit solution maintained at 37^{o}C and aerated with carbogen. Corpus cavernosum was subjected to an initial tension 1g, and were kept in the organ bath (for equilibration) for approximately 90 minutes before the start of the experiments; the physiological solution was renewed every 15 minutes. Response of the corpus cavernosum to drugs were measured isometrically with a Grass FT O3 force-displacement transducer, and recorded on a polygraph [21-24].

Cumulative concentration response curves of (1×10^{-8}, 1×10^{-6}, 1×10^{-4} and 1×10^{-2}) of **2a-h**, **4a-d**, **5**, and **6**. During performing the dose-response curves of these compounds, each dose was added after reaching the plateau of the response of the previous dose. Sildenafil itself were tested in the concentration range of (0.001–10μM).

5. *References*

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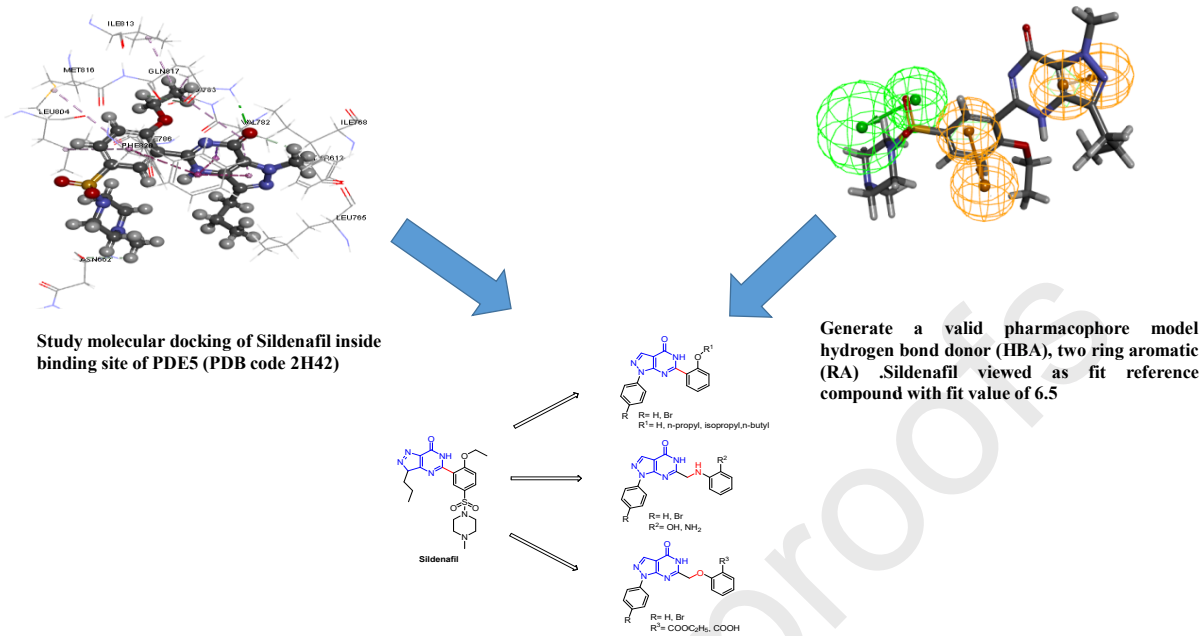
Graphical abstract

Rational to design the pyrazolo[3,4-*d*]pyrimidin-7-ones scafold



Rational to design the pyrazolo[3,4-*d*]pyrimidin-7-ones scafold

Highlights

- Pyrazolo[3,4-*d*]pyrimidin-7-one derivatives were synthesized and evaluated for their biological activity.
- New scaffold with potential phosphodiesterase 5 inhibitory activities were perform protocols of molecular modelling.
- The promising results of pharmacophore were subjected to molecular docking, which were filtered into 5 HITs.
- The structure of the 5 HITs, were used to develop a set of the new proposed PDE-5 inhibitors.