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# Synthesis and phosphodiesterase 5 inhibitory activity of new sildenafil analogues containing a phosphonate group in the 5′-sulfonamide moiety of phenyl ring

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**Abstract**—Synthesis of new sildenafil analogues containing a phosphonate group in the 5′-sulfonamide moiety of the phenyl ring, **12a–e**, **13a–d**, and **14a–d**, and evaluation of their in vitro PDE5 inhibitory activity are disclosed. Enzyme assays revealed that maximum 10-fold increase in PDE5 inhibitory activity, compared with sildenafil, was achieved by introducing a phosphonate group in the 5′-sulfonamide moiety. Docking model of (PDE5: **12d**) complex shows that the PDE5-bound conformation of **12d** matches completely with that of sildenafil, while **12d** is partially overlapped with cGMP with ethyl phosphonate group of **12d** superimposed onto the cyclic phosphate group of cGMP.


Male erectile dysfunction (MED) is a common and important medical problem, and over half of men at 40–70 years of age are reported to suffer from erectile dysfunction according to a random community-based sample study.[1,2] Sildenafil citrate[3] (Viagra; Fig. 1) is the first orally effective phosphodiesterase type 5 (PDE5) inhibitor available for the treatment of MED, and its advent has spurred significant interest in the development of additional PDE5 inhibitors.[4] Despite the efficacy of sildenafil as a treatment for MED, clinically significant adverse side effects such as nausea, headache, facial flushing, and visual disturbances have been reported, and their incidence is dose-dependent. Certain of these side effects are thought to be associated with nonspecific inhibitions of other PDEs, specifically PDE1, PDE3, and PDE6.[5,6] Therefore, the search for more potent and selective PDE5 inhibitors with fewer PDE-related side effects and greater efficacy is of primary interest. In the earlier work of sildenafil series, it was suggested that the 5′-sulfonamide moiety of the phenyl ring might reproduce the role of the cyclic phosphate of cGMP in binding the enzyme active site, thus leading to high and selective inhibition of PDE5.[3c] In addition, it has been proposed by us that introduction of a phosphonate group in the 5′-sulfonamide moiety of the phenyl ring might enhance PDE5 inhibitory activity, probably by mimicking the role of the phosphate of cGMP. In this paper, synthesis of new sildenafil analogues containing a phosphonate group on the phenyl ring, **12a–e**, **13a–d**, and **14a–d**, evaluation of their in vitro PDE5 inhibitory activity, and molecular modeling study of **12d** are disclosed.

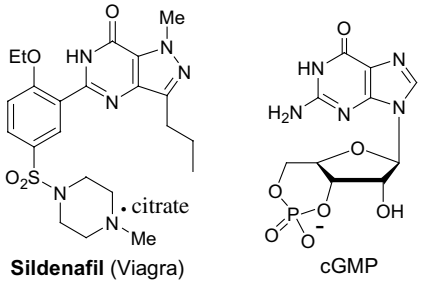


**Figure 1.** Structures of sildenafil and cGMP.

Since it has been demonstrated that a *n*-propoxy group at the 2′-position of the phenyl ring is essential for high PDE5 inhibitory activity,[7a] we decided to focus only on the *n*-propoxy derivatives. Requisite piperidine derivatives, **4c–e** and **5c–e**, were readily prepared according to

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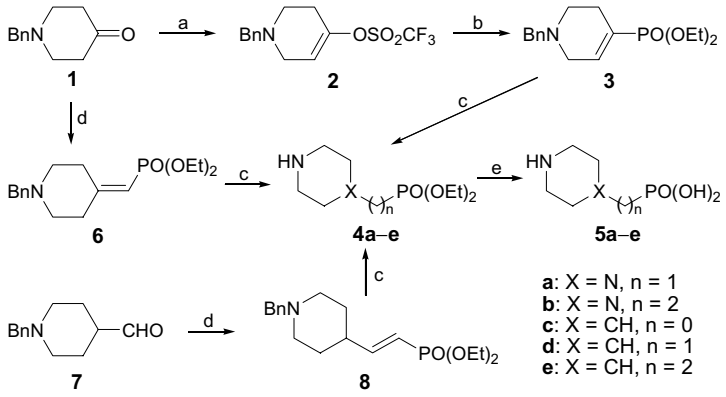
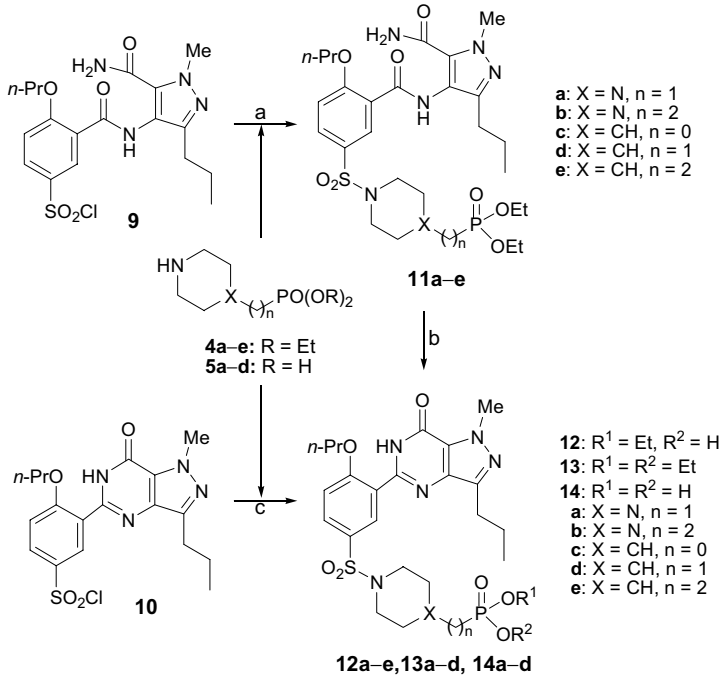



**Scheme 1.** (a) LDA, DME; then $PhN(SO_2CF_3)_2$; (b) diethyl phosphite, cat. $Pd(PPh_3)_4$, DMF; (c) Pd/C, $H_2$ (1 atm), MeOH, rt; (d) $CH_2[PO(OEt)_2]_2$, NaH, toluene; (e) 4 N aqueous HCl, reflux.

Scheme 1 from the intermediates, **3**, **6**, and **8**. Compound **3** was prepared from 1-benzyl 4-piperidone (**1**) in two steps, formation of the enol triflate **2** and Pd-catalyzed coupling with diethyl phosphite, by employing the literature procedure.[8] Olefinations of **1** and **7**[9] using tetraethyl methylenediphosphonate ($CH_2[PO(OEt)_2]_2$) and sodium hydride (NaH) in toluene worked efficiently at room temperature to give the α,β-unsaturated products, **6** and **8**.[10] Simultaneous removal of benzyl group and reduction of the double bond in **3**, **6**, and **8**, occurred under a typical hydrogenation condition to afford the piperidine derivatives **4c**–**e**, respectively. Treatment of the diethyl phosphonates **4c**–**e** with 4 N aqueous HCl solution under reflux produced the corresponding phosphonic acids **5c**–**e**. Diethyl piperazinyl phosphonate derivatives **4a**–**b** were obtained by applying the reported synthetic methods,[10,11] and converted to the phosphonic acids **5a**–**b** as described in the preparation of **5c**–**e**. All the target compounds, **12a**–**e**, **13a**–**d**, and **14a**–**d**, were synthesized in two straightforward manners from the known chlorosulfonyl derivatives, **9** and **10**,[7b] as depicted in Scheme 2. Mono-ethyl phosphonate compounds **12a**–**e** were prepared in two steps from compound **9**. First, coupling reaction of **9** with an appropriate cyclic amine **4a**–**e** in EtOH readily produced the sulfonamides **11a**–**e** in 75–97% yields. Subsequent cyclization of compounds **11a**–**e** and the concomitant partial hydrolysis of the diethyl phosphonate group were efficiently affected under basic conditions at reflux using a mixture of aqueous 2 N NaOH solution and EtOH (2:1, v/v), and the corresponding mono-ethyl phosphonate **12a**–**e** were produced in 71–85% yields. Diethyl phosphonate and phosphonic acid derivatives, **13a**–**d** and **14a**–**d**, were prepared in 61–90% yields by simply mixing compound **10** with a cyclic amine, **4a**–**d** or HCl salt of **5a**–**d**, in the presence of $Et_3N$ in a protic




**Scheme 2.** (a) $Et_3N$, EtOH, rt (for **5a**–**e**); (b) 2 N NaOH in $H_2O$, EtOH, reflux; (c) $Et_3N$, $DMF/H_2O$ (for **5a**–**d**), or EtOH (for **4a**–**d**), rt.

solvent such as EtOH or DMF/water mixture. Due to the solubility problem of **5a–d**·HCl salts in an alcoholic solvent, it was essential to use very polar system such as a mixture of DMF and water.

PDE5 inhibitory activity was assayed in vitro using [$^3$H]-cGMP SPA kit,[12] and the $IC_{50}$ values for the target compounds, **12a–e**, **13a–d**, and **14a–d**, were determined from concentration–response curves.[13] As shown in Table 1, most of the compounds turned out to be about 2–10-fold more potent PDE5 inhibitors than sildenafil. Concerning the structure–activity relationship, three aspects are worthy of comments. First, the mono-ethyl phosphonates **12a–e** were found to exhibit higher PDE5 inhibitory activity than the diethyl phosphonate and phosphonic acid derivatives, **13a–d** and **14a–d**. All the mono-ethyl phosphonate derivatives **12a–e** were 6–10-fold more potent than sildenafil, and **12a** and **12d** proved to be most active among them. Second, there seemed to be no activity difference between the piperazinylsulfonamide (X = N) and the corresponding piperidinyl (X = CH) derivatives (e.g., **12a–b** vs **12d–e**). Thirdly, it was obvious that the PDE5 inhibitory activity was affected by the length ($n$) of the methylene chain linking the phosphonate group. Compounds with one methylene group ($n = 1$) showed higher activity than the corresponding derivatives with no ($n = 0$) or longer ($n = 2$) chain, regardless of the variations in the phosphonate (R) and cyclic amine part (X).

Compound **12d** showing the highest PDE5 inhibitory activity was chosen, and its inhibitory activity toward PDE1, PDE3, and PDE6 was determined, and its selectivity (PDE1/PDE5, PDE3/PD5, PDE6/PDE5) over these isozymes was compared with that of sildenafil. As shown in Table 2, there were small increases in its selectivity over PDE1 (2.2-fold), PDE3 (1.9-fold), and PDE6 (1.4-fold) compared with those of sildenafil.

**Table 1.** In vitro PDE5 inhibitory activity of compounds, **12a–e**, **13a–d**, and **14a–d**[a]

| Compound | $IC_{50}$ (nM) |
|---|---|
| | PDE5 |
| **12a** | 0.18 |
| **12b** | 0.28 |
| **12c** | 0.30 |
| **12d** | 0.17 |
| **12e** | 0.29 |
| **13a** | 0.58 |
| **13b** | 1.05 |
| **13c** | 0.41 |
| **13d** | 0.34 |
| **14a** | 0.28 |
| **14b** | 0.94 |
| **14c** | 0.70 |
| **14d** | 0.32 |
| Sildenafil | 1.78 |

[a] PDE5 was prepared from rabbit platelet and assayed using [$^3$H]-cGMP SPA kit. $IC_{50}$ values were determined from the logarithmic concentration–inhibition curve. The value is the mean from three independent experiments.

**Table 2.** In vitro PDE inhibitory activity of compound **12d**[a]

| Compound | $IC_{50}$ (nM) | | | |
|---|---|---|---|---|
| | PDE1 | PDE3 | PDE5 | PDE6 |
| **12d** | 156 (918)[b] | 2600 (>15,000)[c] | 0.17 (1) | 2.58 (15)[d] |
| Sildenafil | 739 (415)[b] | 14,600 (>8000)[c] | 1.78 (1) | 19.77 (11)[d] |

[a] PDEs were obtained from bovine heart (PDE1), rabbit platelet (PDE3 and PDE5), and bovine retina (PDE6), respectively, and assayed using [$^3$H]-cGMP or [$^3$H]-cAMP SPA kit. $IC_{50}$ values were determined from the logarithmic concentration–inhibition curve. The value is the mean from three independent experiments.
[b] $IC_{50}$ ratio of PDE1/PDE5.
[c] $IC_{50}$ ratio of PDE3/PDE5.
[d] $IC_{50}$ ratio of PDE6/PDE5.

For the docking analysis of **12d**, the homology model of PDE5 was used. Protein modeling and docking experiments have been performed with the sybyl 6.9 software package (Tripos, Inc., St. Louis, MO, USA) on SGI Octane II workstation. Protein sequence of human cGMP-specific 3′,5′-cyclic phosphodiesterase 5 (PIR Unique id: JW0106) was downloaded from the Protein Information Resource (http://pir.georgetown.edu/pirwww/pirhome.shtml). The amino acid sequences, from Met578 through Gln859, were selected for homology modeling. Homology modeling was based on known crystal structures of PDE4 (PDB entry: 1F0J, 1OYN, and 1MKD),[14] and was performed using an automatic web server, Geno-3D[15] (http://geno3d-pbil.ibcp.fr). Additional modeling and minimization were performed with the Composer and Base module of sybyl 6.9. Reference protein coordinate was prepared by docking of sildenafil into the active site of PDE5 homology model, taking into account the binding mode determined by X-ray crystallography.[16] X-ray data reveals that sildenafil binds into the active site pocket mainly by various hydrophobic interactions between the drug and protein, and with two hydrogen bonds. The

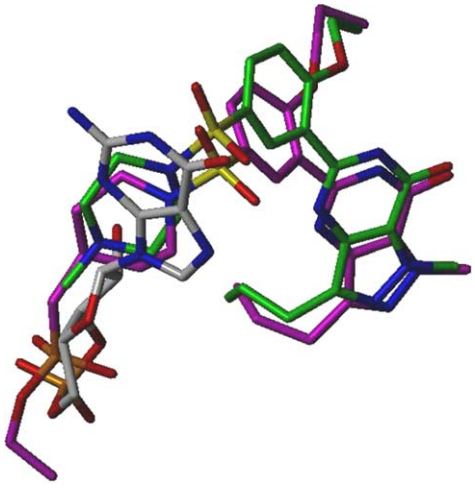


**Figure 2.** Docked alignment of cGMP (gray carbon), **12d** (magenta carbon), and sildenafil (green carbon) within the active site of PDE5. Colors of other atoms are red (oxygen), blue (nitrogen), yellow (sulfur), and orange (phosphorus).

hydrogen bonds are: between carbonyl oxygen of purine ring of sildenafil and Nε2 of Gln 817, and between adjacent NH group of sildenafil and Oε1 of Gln 817.[16b] The ligands, sildenafil, cGMP, and **12d** were flexibly docked into the reference protein structure, respectively, by using the FlexX program.[17] As shown in Figure 3A, FlexX performed well in reproducing the X-ray pose of sildenafil. To compare the binding features of ligands, the docked conformer of each ligand with top rank was retrieved from FlexX results, and overlaid in the active site of PDE5 (Fig. 2).[17] The PDE5-bound conformation of **12d** matches completely with that of sildenafil, while **12d** is partially overlapped with cGMP with ethyl phosphonate group of **12d** superimposed onto the cyclic phosphate group of cGMP. To examine the binding mode of cGMP (Fig. 3B), the molecule is embedded inside the active site pocket, and positioned in line with the polar amino acid residues, Asn 661 through Ser 663, with forming various hydrogen bonding interactions. For **12d** (Fig. 3C), the purine ring moiety forms two hydrogen bonds with Gln 817, just like sildenafil, and additional hydrogen bond between pyrazolyl nitrogen and OH of Tyr 612. The oxygen atoms of ethyl phosphonate group in **12d** also forms two hydrogen bonds with Asn 662, which shows the agreement with our assumption that the phosphonate group of **12d** may substitute for the phosphate of cGMP in the interaction with PDE5. In the binding of **12d** into PDE5, the ligand appears to act like a hybrid of sildenafil and cGMP by utilizing both purine ring and phosphonate moiety for formation of various hydrogen bonds with PDE5. The resulting tight interactions between **12d** and enzyme would be related to the experimental data showing that **12d** has more potent PDE5 inhibitory activity than sildenafil.

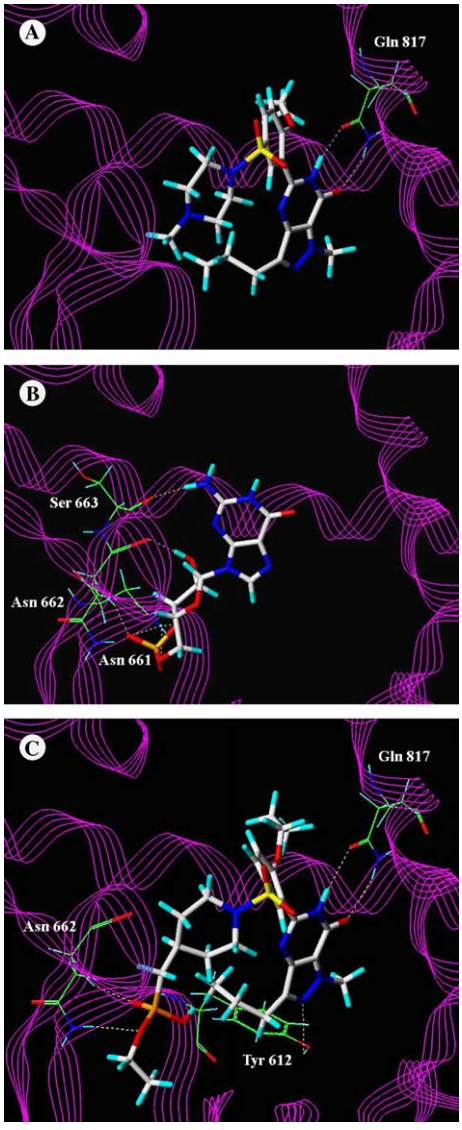



**Figure 3.** Comparison of binding poses of sildenafil (A), cGMP (B), and **12d** (C) in the active site of PDE5 (homology model), generated by FlexX. The amino acid residues, which form hydrogen bonds within the binding site are represented in line form, and the ligands are rendered in capped stick. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å).

In conclusion, our study on new sildenafil analogues demonstrated that maximum 10-fold increase in PDE5 inhibitory activity, compared with sildenafil, could be achieved by introducing a phosphonate group in the 5′-sulfonamide moiety. The piperidinyl derivative **12d**, which showed the highest PDE5 inhibitory activity, has been selected for further biological investigations.

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12. *Determination of PDE1, PDE3, PDE5, and PDE6 inhibitory activity*. Bovine heart PDE1 was purchased from Sigma (St. Louis, MO, USA). PDE3 and PDE5 were prepared from the rabbit platelet using the method described by Hidaka et al. with minor modifications (*Biochim. Biophys. Acta.* **1976**, *429*, 485). Fresh rabbit whole blood was centrifuged at 360*g* to obtain the platelet-rich plasma (PRP). Platelets were isolated from PRP by centrifugation at 1200*g*, sonicated (20 s/mL) in 50 mM tris-HCl buffer (pH 7.4) containing 1 mM $MgCl_2$, and then centrifuged at 40,000*g* for 2 h at 4 °C. The supernatant was loaded on the DEAE-cellulose column with a bed volume of 35 mL (Sigma) pre-equilibrated with equilibration buffer (50 mM tris-acetate containing 3.75 mM 2-mercaptoethanol, pH 6.0). After the column was washed with 60 mL of equilibration buffer, PDE3, and PDE5 were eluted using a continuous gradient of 0–600 mM sodium acetate in equilibration buffer with a total volume of 60 mL. The bovine retina PDE6 was prepared using the method described by Ballard et al. (*J. Urol.* **1998**, *159*, 2164) with minor modifications. Bovine retinas were minced and homogenized in the homogenization buffer (20 mM HEPES containing 0.25 M sucrose, 1 mM EDTA, 1 mM phenylmethyl sulfonylfluoride (PMSF), pH 7.2) using a Polytron PT 10/35 homogenizer (Kinematica AG, Switzerland) at 5000 rpm with two bursts for 10 s. The homogenate was then centrifuged at 40,000*g* for 60 min at 4 °C. The supernatant was recovered and filtered through 0.2 μm filter. The filtered sample was loaded on the Hitrap Q column with a bed volume of 5 mL (Pharmacia, Uppsala, Sweden) pre-equilibrated with 20 mM HEPES buffer (pH 7.2) containing 1 mM EDTA and 0.5 mM PMSF. The column was then washed with 25 mL of equilibration buffer. PDE6 was eluted using a continuous gradient of 0–600 mM NaCl in equilibration buffer with a total volume of 60 mL. Fractions (1.0 mL each) collected at a flow rate of 60 mL/h were characterized for cGMP (PDE5 and PDE6) or cAMP (PDE3) hydrolytic PDE activities as described below. Fractions comprising the main peaks of cGMP hydrolytic PDE activity were pooled and stored at −20 °C in 50% glycerol until the enzyme assay. Enzymatic activity was determined using a PDE scintillation proximity assay (SPA) kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the protocol supplied by the manufacturer. The reaction buffer contained [$^3$H]-cGMP (5 μCi/mL) or [$^3$H]-cAMP (5 μCi/mL), 1.7 mM EGTA, and 8.3 mM $MgCl_2$ in 50 mM Tris–HCl buffer (pH 7.5). After PDE was added to the reaction buffer, the mixtures were incubated at 30 °C for 30 min. The reaction was then stopped by the addition of 50 μL of SPA beads, and the radioactivity was counted on the liquid scintillation counter (Tri-Carb 1500, Packard Inc., Meriden, CT, USA) after each sample was settled for 20 min. For the inhibitor studies, sildenafil and test compounds were dissolved in DMSO and diluted with distilled water. The final concentration of DMSO was less than 0.2% (v/v). All the inhibition experiments were conducted under the conditions where the level of cGMP or cAMP hydrolysis did not exceed 15%, and the product formation increased linearly with time and amount of enzyme. $IC_{50}$ was defined as the concentration of compounds to produce a 50% inhibition of enzyme activity and calculated by quantal probit analysis in Pharmacological Calculation System.

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17. *Preparation of molecular structures*. The structures of cGMP, sildenafil, and **12d** were prepared in MOL2 format using the sketcher module and Gasteiger–Huckel charges were assigned to the ligand atoms. The structures of molecules are optimized by energy minimization, and molecular dynamics using simulated annealing method. The lowest-energy conformer of each molecule was selected and stored in database. *Preparation of target protein structure and flexible docking*. Initially, the reference molecule, sildenafil, was manually docked into the homology model of PDE5, based on the crystallographically-determined binding mode of sildenafil in the active site of PDE5. The resulting sildenafil-PDE5 docked complex was used as a receptor for flexible docking of cGMP, **12d**, and sildenafil. The active site was defined as all the amino acid residues enclosed within 6.5 Å radius sphere centered by the bound ligand, sildenafil. The docking and subsequent scoring were performed using the default parameters of the FlexX programs implanted in the sybyl 6.9. For the docking of ligand into the target active site, the main settings are 1000 solutions per iteration during the incremental construction algorithm. Final scores for all FlexX solutions were calculated by a standard scoring function, and used for database ranking.