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A Prenylated Flavonol, Sophoflavescenol: A Potent and Selective Inhibitor of cGMP Phosphodiesterase 5

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Abstract—During the search for naturally occurring cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5) inhibitors, it was found that the extracts from *Sophora flavescens* exhibit potent inhibitory activity against cGMP PDE5 prepared from rat diaphragm. Therefore, the inhibitory activities of five flavonoids, kushenol H (**1**), kushenol K (**2**), kurarinol (**3**), sophoflavescenol (**4**) and kuraridine (**5**), isolated from *S. flavescens* were measured against cGMP PDE5 to identify potent cGMP PDE5 inhibitory constituents. Among tested compounds, sophoflavescenol (**4**), a C-8 prenylated flavonol, showed the most potent inhibitory activity ($IC_{50} = 0.013$ μM) against cGMP PDE5 with 31.5- and 196.2-fold selectivity over PDE3 and PDE4, respectively. Kinetic analysis revealed that sophoflavescenol was a mixed inhibitor of PDE5 with a K_i value of 0.005 μM.

Intracellular concentrations of cyclic nucleotides are regulated by two families of enzymes, adenylate and guanylate cyclases, which synthesize adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) from corresponding nucleotide triphosphates, and cyclic nucleotide phosphodiesterases (PDEs) which catalyze the hydrolysis and inactivation of cAMP and/or cGMP.[1] Therefore, the inhibition of PDEs is expected to induce an increase of cAMP and/or cGMP levels and to affect physiologic functions in these tissues.

PDE5, first purified and characterized from rat,[2] is very abundant in vascular smooth muscle cells and appears to play a significant role in modulating smooth muscle tone in general and penile corpus cavernosal smooth muscle tone in particular.[3] The inhibitors of cGMP PDE5 such as sildenafil citrate act in sexual organs to produce enhanced blood flow and an erectile response of sexual organs by increasing the level of cGMP.[4] Although sildenafil citrate is a selective inhibitor of cGMP PDE5, its effects on other body organs produce many side effects such as nausea, headache, and cutaneous flushing. These clinically significant adverse effects are thought to be due to nonspecific inhibition of other PDEs.[3] An improved, second generation of PDE5 inhibitors would be one with greater potency and specificity for PDE5, resulting in an agent with potentially fewer PDE-associated side effects and greater efficacy for the treatment of erectile dysfunction. Accordingly, several types of nitrogen-containing heterocycles such as quinazoline,[5] pyrazolo-pyrimidine,[4,6] isoquinoline,[7] phthalazine,[8] and naphthalene[9] derivatives have been synthesized.

The search of PDE inhibitors from plants can lead to the identification of structurally new scaffolds. In this regard, osthole, a coumarin structure isolated from *Cnidium monnier* (L.) Cusson, was reported to possess a relaxation effect on rabbit corpus cavernosal tissues. The effect is partly attributable to the potentiation of the cGMP and cAMP signal mediating relaxation of cavernosal smooth muscle by inhibiting phosphodiesterase.[10] In continuation of our search program for biologically active compounds from traditional medicine, we have found that a methanol extract of the roots of *Sophora flavescens* had strong inhibitory activity against cGMP PDE5 from rat diaphragm in our screening system ($IC_{50} = 4.77$ μg/mL).

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Sophorae radix, the dried roots of *S. flavescens*, a well-known Chinese traditional medicine, has been used as a diuretic, stomachic, antipyretic, and anthelmintic.[11] The plant *S. flavescens* Ait (Leguminosae) grows as a perennial herb and is widely distributed in Korea.[12] Recently, we reported the phytochemical constituents and their antiviral activities against HSV-1 and HSV-2.[13] It was also reported that some flavonoids such as kushenol A, kurarinone, and kuraridine (**5**) isolated from *S. flavescens* inhibited cAMP phosphodiesterase.[14] However, the inhibitory activity of the extract of *S. flavescens* against cGMP PDE5 was not studied to our best knowledge. In this paper, we wish to describe the identification of sophoflavescenol (**4**), a C-8 prenylated flavonol, as a potent inhibitor of cGMP-specific PDE5.

The methanol extract of the roots of *S. flavescens* was suspended in water and then partitioned consecutively with dichloromethane, ethyl acetate, and butanol. Because ethyl acetate fraction showed the most potent inhibitory activity against cGMP PDE5 ($IC_{50}=1.54$ μg/mL) among other fractions, selected flavonoids, kushenol H (**1**), kushenol K (**2**), kurarinol (**3**), sophoflavescenol (**4**) and kuraridine (**5**), obtained previously from the ethyl acetate fraction of *S. flavescens*, were further tested (Fig. 1).[15]

The cGMP PDE5 inhibition data are summarized in Table 1. The cGMP PDE5 used in the inhibition test was purified from rat diaphragm as described by Lugnier et al.[16] As shown in Table 1, all flavonoids showed moderate to potent inhibitory activity against cGMP PDE5 with a range of IC_{50} values of 0.013–10.6 μM.[17,18] C-8 5-hydroxy-2-isopropenyl-5-methylhexyl group substituted flavanol or flavanone (**1**–**3**) exhibited cGMP PDE5 inhibitory activities at micromolar concentrations. It is interesting that C-8 lavandulylated flavonoid chalcone (**5**) also showed inhibitory activity at submicromolar concentrations. On the other hand, sophoflavescenol (**4**), a C-8 prenylated flavonol, showed greatest inhibitory activity ($IC_{50}=0.013$ μM). Kinetic analysis revealed that sophoflavescenol (**4**) was a mixed inhibitor ($K_i=0.005$ μM) against cGMP PDE5 (Fig. 2),[19] whereas sildenafil was reported as a competitive inhibitor.[20]

PDEs have been classified into at least seven isozyme types. Of these, PDE3 and PDE4 selectively hydrolyze cAMP.[3a] If PDE3 and/or PDE4 activities are inhibited, the level of cAMP is increased to cause various side effects including enhanced myocardial contraction and heart rate and depression of systemic blood pressure.[21] Therefore, inhibitory effects of flavonoid compounds (**1**–**5**) from the ethyl acetate fraction of *S. flavescens* on PDE3 and PDE4 were also evaluated to assess isozyme selectivity (Table 1). Among tested compounds, sophoflavescenol (**4**) showed greatest selectivity toward PDE5, 31.5- and 196.2-fold over PDE3 and PDE4, respectively (Fig. 3). However, other flavonoids showed poor selectivity toward PDE3 and PDE4.

Table 1. IC_{50} values for flavonoids (**1**–**5**) on rat PDE3, PDE4, and PDE5 activities

Compd	IC_{50} (μM)			IC_{50} ratio	
	PDE5	PDE3	PDE4	PDE3/PDE5	PDE4/PDE5
1	4.75	13.49	18.71	2.8	3.9
2	10.6	12.77	33.84	1.2	3.2
3	6.1	2.95	6.59	0.5	1.1
4	0.013	0.41	2.55	31.5	196.2
5	0.64	1.25	8.27	2.0	12.9
Sildenafil	0.003	—	—		
Milrinone		0.74			
Rolipram	—	—	1.12		

PDE3 and PDE5 were isolated from rat diaphragm. PDE4 was isolated from rat liver. IC_{50} values were determined from sigmoidal curves, fitted to plots of enzyme activity versus log compound concentration using a curve fitting program. The activities were assayed with 0.15 μM [^{3}H]-cGMP as substrate.

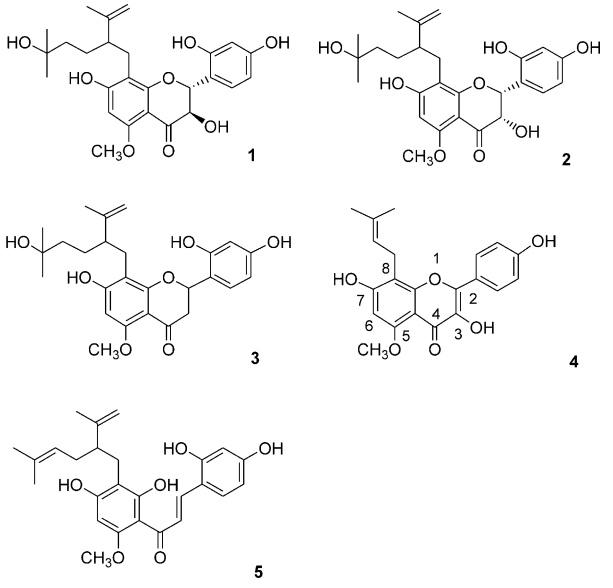

Figure 1. Structures of flavonoid compounds (**1**–**5**) from *S. flavescens*.

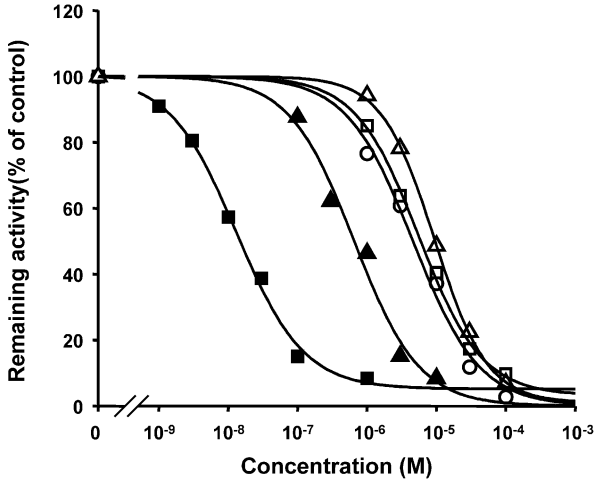

Figure 2. Effects of kushenol H (△), kushenol K (○), kurarinol (□), sophoflavescenol (■) and kuraridine (▲) on PDE5 activity isolated from rat diaphragm. Data shown are mean of two separate determinations and the uninhibited rate of PDE5 activity was 0.072±0.015 pmol product formed/min/mg protein.

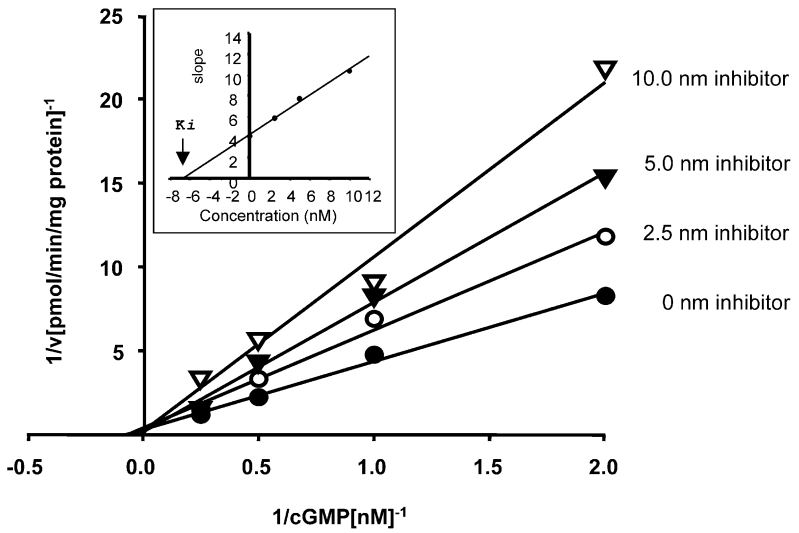

Figure 3. Lineweaver–Burk plot for sophoflavescenol (**4**) induced inhibition of PDE5 activity. PDE5 from rat diaphragm was incubated with various concentrations of cGMP in the presence of increasing amounts of sophoflavescenol (**4**).

In conclusion, the present data demonstrate that sophoflavescenol (**4**), a new prenylated flavonol obtained from *S. flavescens*, has a potent and selective inhibitory activity against rat diaphragm PDE5. Sophoflavescenol is not structurally related to the other cGMP PDE5 inhibitors that have been synthesized through the modification of sildenafil structure. Therefore, this compound can be considered as a lead structure for the future synthesis of selective and potent cGMP PDE5 inhibitors with probably more desirable pharmacological profiles.

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17. Isolation of PDE isozymes: PDE3 and PDE5 were purified from rat diaphragm. Briefly, fresh tissue was minced and homogenized using Ultra Turrax in 5 volumes of HEPES buffer [20 mM HEPES, 0.25 M sucrose, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), pH 7.2]. The homogenate was filtered though two layers of surgical gauze to remove any undispersed tissue and fibrous materials. The filtrate was centrifuged at 100,000*g* for 60 min at 4 °C. The supernatant was filtered though a 0.2-μm pore filter. PDE activities in the soluble fractions prepared from rat tissue were separated using a Pharmacia FPLC system (Pharmacia Ltd, Milton Keynes, UK) with a Mono Q anion exchange column (8-mL bed volume, Pharmacia Ltd). The Mono Q column was pre-equilibrated with 50 mM sodium acetate buffer (pH 6.5) containing 20 mM bis-Tris, 2 mM EDTA, 0.2 mM DTT, 1 mM benzamide, 1 μM PMSF following by application of a 20 mL sample of tissue soluble fraction. The PDE isozymes were eluted using a continuous gradient of 0 to 1 M sodium acetate buffer (pH 6.5) at a flow rate of 1.5 min/mL and 3-mL fractions were collected over a 90-min period. PDE3 and PDE5 activities in each fraction were determined and then fractions containing PDE3 and PDE5 activities were pooled. PDE4 was also purified from rat liver according to the above method.
18. Determination of PDE activity: The cyclic nucleotide PDE activity in FPLC fraction was determined using a modification of the two-step radioisotopic procedure. The reaction mixture (total volume = 100 μL) contained column fraction eluate (20–40 μL), [^{3}H]-cGMP (22,000 dpm/μL) or [^{3}H]-cAMP (22,000 dpm/μL), $MgCl_2$ (5 mM) in Tris–Cl buffer (15 mM, pH 7.4). Reactions were initiated by addition of the radiolabeled substrate and incubated in a water bath at 30 °C for 20 min. The reaction was stopped by immersing the sample

tubes in boiling water for 2 min and chilled for 10 min before the addition of 5 μL of 10 mg/mL snake venom protein containing 5′-nucleotidase activity. Samples were further incubated for 10 min at 30 °C and 0.5 mL of cold water was added. Fractions were then eluted on DEAE-Sephacel columns, and the effluent was counted in scintillation cocktail by β-counter. Calmodulin stimulation experiments were performed using a final concentration of 0.5 mM $CaCl_2$ and 3 μg/mL of calmodulin. For studies of inhibition of PDE activities, inhibitors in DMSO were added to the assay buffer containing enzyme and preincubated for 5 min before reactions were initiated by the addition of substrate. [^{3}H]-cGMP was used as the substrate for PDE5 and [^{3}H]-cAMP as substrate for PDE3 and PDE4, ranging from 0.5 to 10 μM. IC_{50} values for inhibition of PDE isozyme were determined from sigmoidal curves, fitted to plots of enzyme activity versus log compound concentration using a curve fitting program.

19. To determine the K_i value for sophoflavescenol as a cGMP PDE5 inhibitor, the slopes of double-reciprocal plots generated from initial rates of cGMP hydrolysis using varying concentrations of cGMP and inhibitor are re-plotted as a function of the corresponding concentration of inhibitor. The point of intersection of each line with the horizontal axis gives K_i values. The reaction rate data were analyzed using Lineweaver–Burk plots.

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