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Synthesis of pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides, novel Sildenafil analogs with tyrosinase inhibitory activity

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ABSTRACT

Tyrosinase is a multifunctional, glycosylated and copper-containing oxidase which catalyzes the first two steps in mammalian melanogenesis and is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing. Neither hyperpigmentation in human skin nor enzymatic browning in fruits are desirable. These phenomena have encouraged researchers to seek new potent tyrosinase inhibitors for use in foods and cosmetics. This article surveys tyrosinase inhibitors, newly discovered from natural and synthetic sources. The inhibitory strength is comparable to that of the standard inhibitor kojic acid. Also their inhibitory mechanisms are discussed. The new obtained compounds were also tested as PDE5 inhibitors and did not show significant inhibitory effect.

1. Introduction

Azoloazines are biological interesting molecules, and their chemistry is receiving considerable attention.[1–3] Furthermore, the multiple biological activities of pyrazole and its annelated derivatives are of increasing interest as antimycotics,[4] antidepressants,[5] fungicidal[6] or herbicidal agents.[7] Compounds containing the pyrazolo[4,3-*e*][1,2,4]triazine moiety have attracted considerable attention due to their anticancer and antibacterial activity.[8–10] The most important members in this family of naturally occurring purine analogs are pseudoiodinine,[8] nostocine A[9] and fluviols A–E[10] produced by *Pseudomona fluorescens* var. *pseudoiodinine* and *Nostoc spongiaeforme*, respectively. Despite the fact that pyrazolo[4,3-*e*][1,2,4]triazines have been less studied in the group of condensed pyrazolotriazines, some of them show moderate inhibition of purine nucleoside phosphorylase[11] and cytotoxic activity in A549 lung carcinoma cell line at submicromolar range,[12] induction of caspase-dependent cell death, and inhibition of cyclin-dependent kinase 2 (CDK2).[13] Moreover, this system, for example, pyrazolo[4,3-*e*][1,2,4]triazine is structurally related to pyrazolo[4,3-*d*]pyrimidin-7-one present in the structure of Sildenafil and its methyl ether analogs which were found to show tyrosinase inhibitory effect, apart from its well-known orally effective properties for the treatment of male erectile dysfunction.[14,15] It should be noted that so far no analogs of Sildenafil containing the skeleton of 1*H*-pyrazolo[4,3-*e*][1,2,4]triazine ring system have been described in the literature. With regard to the above discussed facts, we envisage a high potential of the pyrazolo[4,3-*e*][1,2,4]triazine system regarding the discovery of new biological activities.

In this context and in continuation of our program aimed at the discovery and development of new bioactive molecules, we represent here the preparation of novel analogs of Sildenafil and *iso*Vigra[16] (termed *aza*-analogs of Sildenafil **8a–j** and *aza-iso*Viagra **9**) in which the carbonyl group, present in the structures of Sildenafil and *iso*Viagra, is replaced with the nitrogen atom N7, present on the triazine ring (Fig. 1), to test their PDE5 and tyrosinase inhibitory activity. Moreover, we have described synthesis, spectral properties and biological activity of new bis-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides **10a–c** related to lodenafil carbonate[17] (Fig. 2). Lodenafil carbonate, a new phosphodiesterase type 5 (PDE5) inhibitor is used in treatment of erectile dysfunction. It is formulated as a dimer, composed of two lodenafil molecules linked via a carbonate bridge and behaves as a prodrug which releases lodenafil as active metabolite. Preclinical and clinical studies have shown that this dimer has higher oral bioavailability than the parent drug (Lodenafil), low toxicity and is safe and efficient in the treatment of erectile dysfunction.

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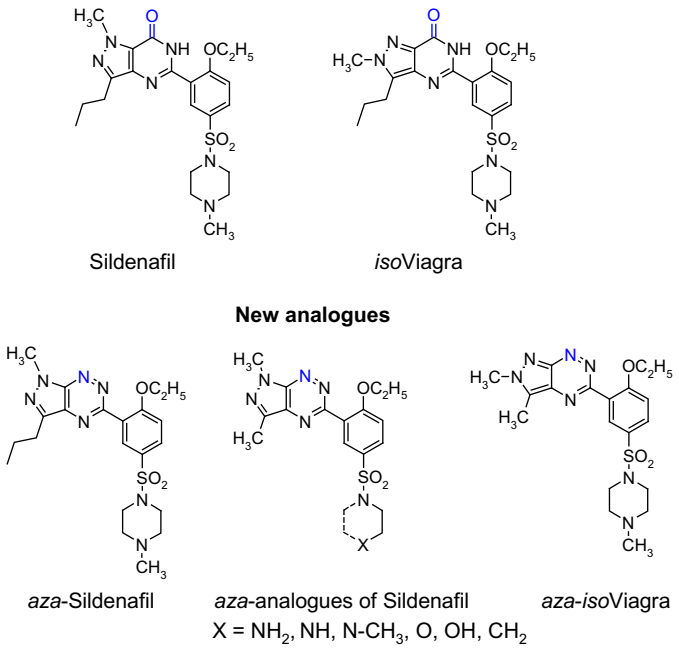


Figure 1. Structures of Sildenafil, *iso*Viagra and new analogs: *aza-iso*Viagra, *aza*-Sildenafil, and its *a*za-analogs.

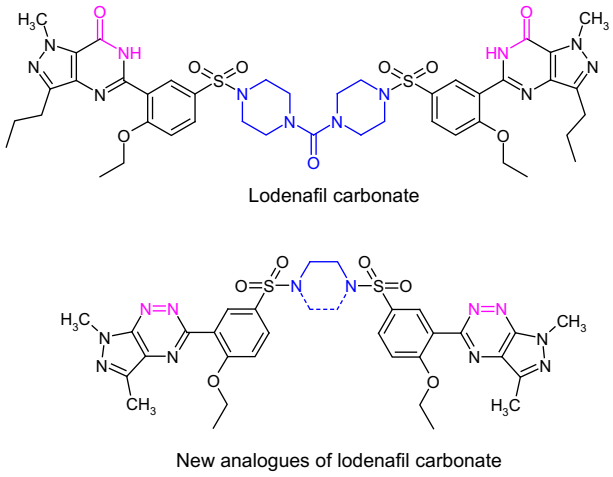


Figure 2. Structure of lodenafil carbonate and its new analogs.

2. Results and discussion

2.1. Synthesis

The synthesis of *aza*-analogs of Sildenafil **8a–j** and *aza*-isoViagra **9** are outlined in Scheme 1, and the preparation could be achieved in two pathways (Scheme 1). The key starting derivative **1** was obtained using our previously discovered procedure.[18] Starting our study on the intended synthesis of new sulfonamides with the pyrazolo[4,3-*e*][1,2,4]triazine core, we decided to exploit N_1- and N_2-methyl derivatives of the pyrazolo[4,3-*e*][1,2,4]triazine ring system (**2** and **3**)[18] to realize our target. Thus, unsubstituted pyrazolotriazine **1** was smoothly converted into the corresponding isomeric structures **2** and **3** upon the reaction with methyl iodide in the presence of potassium carbonate in an EtOH/H_2O mixture (1:1, v/v) at rt. Derivative **2** can be prepared also by cyclization of the methylhydrazone of 5-acyl-1,2,4-triazine under acidic conditions according to our previously published procedure.[18] In the next step, using Guillaumet's and co-workers palladium-catalyzed cross-coupling reaction of 3-methylsulfanyl-1,2,4-triazine with boronic acid derivatives,[19] we have reacted 5-methylsulfanyl-pyrazolo[4,3-*e*][1,2,4]triazines **2** and **3** with 2-ethoxyphenylboronic acid in the presence of copper(I) 3-methylsalicylate to obtain appropriate derivatives **4** and **5**. Compound **4** was collected in excellent yield (90%), but derivative **5** was produced in 30% yield only. Chlorosulfonylation of compounds **4** and **5** in neat chlorosulfonic acid at 0 °C proceeded smoothly and selectively at 5′-position of the phenyl ring to give the desired products **6** and **7** in excellent yield. However, it should be noted that chlorosulfonyl derivative **7** is not stable and after isolation was immediately reacted with amine. The final analogs of Sildenafil **8a–j** and *aza-iso*Viagra **9** were prepared by stirring chlorosulfonyl derivatives **6** and **7** with three equivalents of appropriate amines in acetonitrile at room temperature overnight. The structures of Sildenafil and *iso*Viagra as well as their new analogs are shown in Fig. 1.

The low yield of Suzuki cross coupling reaction of **3** with 2-ethoxyphenyl boronic acid prompted us to improve the efficiency of the preparation of the useful intermediate **5** as illustrated in Scheme 1 (pathway B). To avoid our problem, another possibility to synthesize 5-2′-ethoxyphenyl-2*H*-pyrazolo[4,3-*e*][1,2,4]-triazine **5** is to use *N*-protecting groups. We decided to exploit previously elucidated ethyl vinyl ether and 3,4-dihydro-1*H*-pyran as new protecting groups for NH-pyrazoles as published in the literature.[20] Exposure of *N*-unsubstituted derivative **1** to both ethers under heating in benzene at 40 °C with catalytic amounts of concentrated HCl gave the appropriate intermediates **11** and **12** in good yield (80–90%). After chromatography and treatment with 2-ethoxyphenyl boronic acid under Guillaumet's procedure,[19] compounds **11** and **12** gave the isomeric derivatives **13** and **14** in 80–85% yield, and their deprotection in methanol with concentrated HCl at room temperature for 12 h gave 1-unsubstituted pyrazolo[4,3-*e*][1,2,4]triazine **15** in excellent yield. *N*-alkylation of **15** with methyl iodide in a mixture of EtOH/water (1:1, v/v) in the presence of potassium carbonate furnished at room temperature isomeric target molecules **4** and **5** in the ratio 1:1.2 with a total yield of 90%.

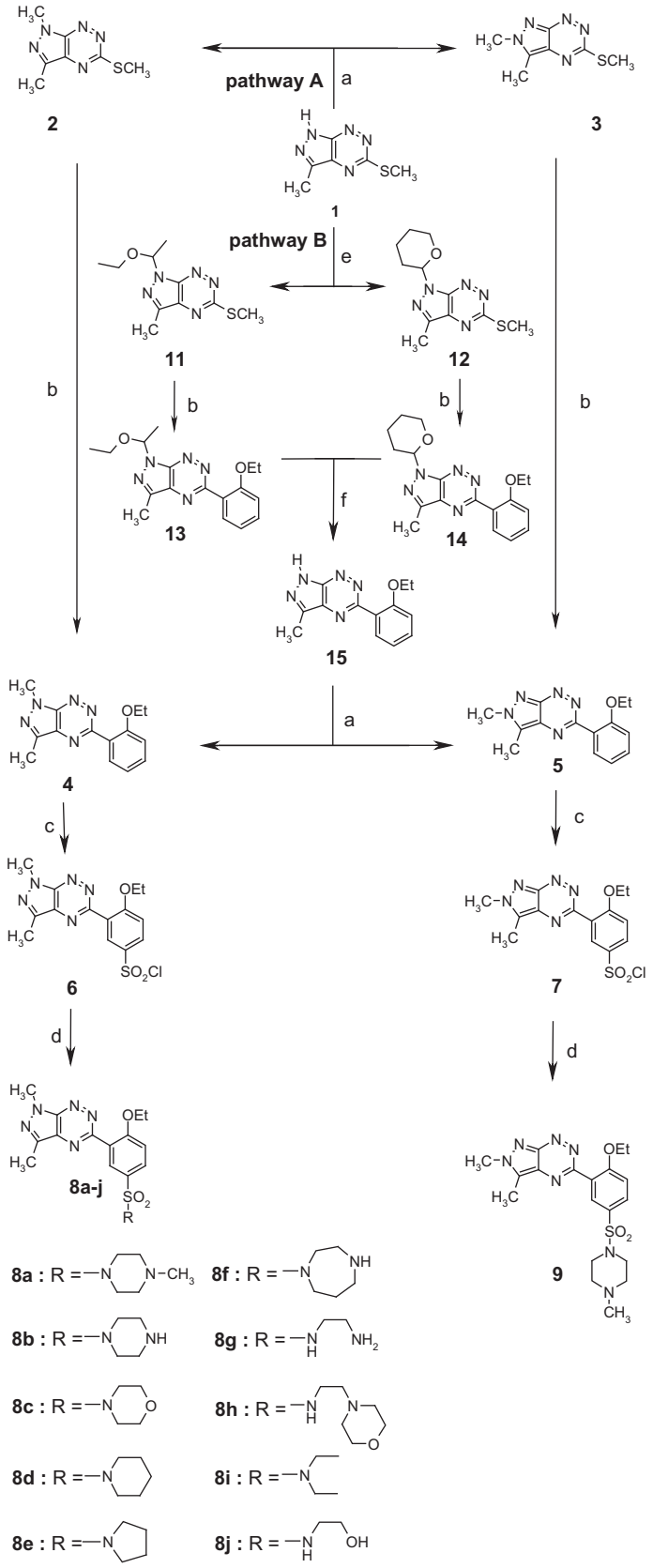
The dimeric sulfonamides **10a–c** were also synthesized following two synthetic pathways as shown in Scheme 2. In the first way, two equivalents of chlorosulfonyl derivative **6** were reacted with one equivalent of bifunctional amine (piperazine, homopiperazine and ethylenediamine) (pathway A, Scheme 2), and in the second way, an equal molar amount of derivative **6** and appropriate monosulfonamide **8b** and **8f–g** were stirred in acetonitrile at room temperature (pathway B, Scheme 2). Structures of lodenafil carbonate and its new analogs **10a–c** are shown in Fig. 2.

2.2. Pharmacology

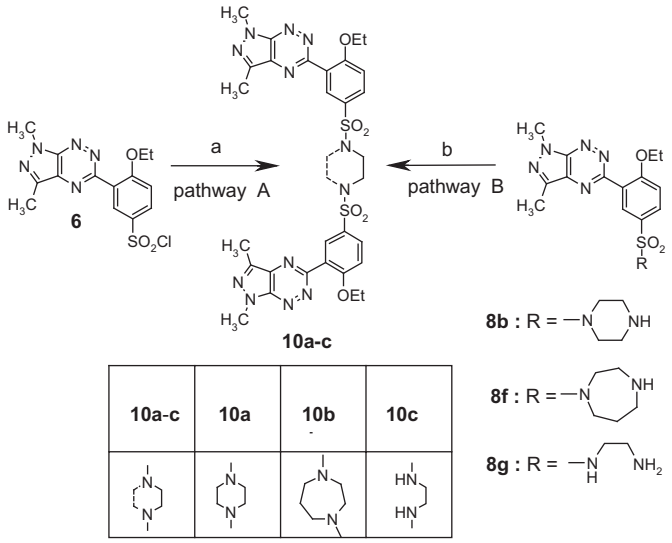
2.2.1. Effect of inhibitors on tyrosinase

For further characterization of the obtained inhibitors were incubated with the enzyme L. sacchari tyrosinase, and their properties were examined (See Table 1).

The enzyme was purified from L. sacchari starting by anion-exchange material (Servacell, DEAE 52), and the obtained supernatant was further purified by size exclusion chromatography, on a FPLC system equipped with a Sephacryl S-100 column (16 × 60 mm). The column was eluted with the same buffer at a flow rate of 0.4 ml/min. The fraction with the highest activity was additionally purified after chromatography on the same column and used for kinetic measurements. The inhibition was determined by measuring the enzymatic activity of this fraction and 5 mM L-DOPA in the presence of the inhibitor at different concentrations. L. sacchari tyrosinase was inhibited completely by 1 mM pyrazolo[4,3-*e*][1,2,4]triazine, but to only 48% by 0.1 mM of the



Scheme 1. Synthetic path to the *aza*-analogs of Sildenafil **8a–j**. Reagents and conditions: (a) CH_3I, $EtOH/H_2O/K_2CO_3$; (b) ethoxyphenylboronic acid, $Pd(PPh_3)_4$, CuMeSal, THF, under argon atmosphere, reflux, overnight; (c) $ClSO_3H$, 0 °C to rt, 2 h; (d) appropriate amine, anhydrous MeCN, rt, overnight; (e) ether (ethyl vinyl ether or 2*H*-pyran, benzene, conc. HCl, 40 °C, 8 h; (f) conc. HCl, MeOH, rt, 12 h.



Scheme 2. Synthetic pathway to dimeric sulfonamides **10a–c**. Reagents and conditions: (a) appropriate amine, anhydrous MeCN, rt, overnight; (b) derivative **6**, anhydrous MeCN, rt, overnight.

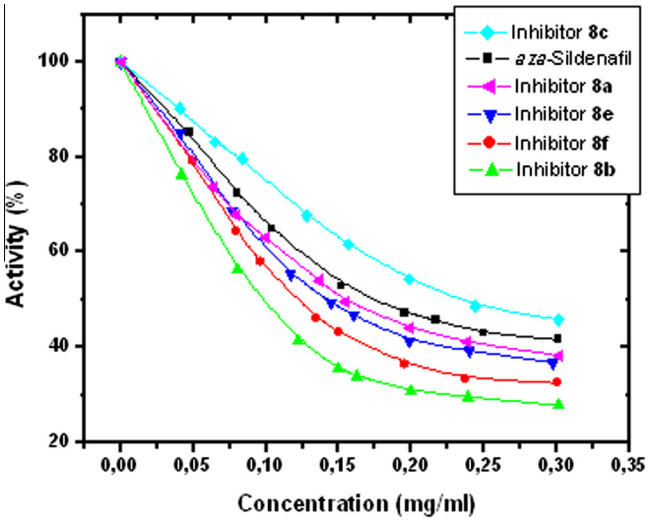


Figure 3A. Kinetic measurements of tyrosinase activity with L-DOPA as substrate and different concentrations of inhibitors in 50 mM phosphoric acid buffer solution (pH 6.8, 1.8 mL), an aqueous solution of tyrosinase (1000 U/mL, 0.1 mL), and DMSO (0.1 mL) at 25 °C for 10 min. The reaction was monitored at 475 nm for 10 min.

Table 1
Tyrosinase inhibitory activities of the tested compounds with concentration of 0.30 mg/ml

Compounds	IC_{50} (mg/ml)	Inhibition (%)	Activity (%)
aza-Sildenafil	0.12	57.1	42.9
8a	0.11	58.8	41.2
8b	0.08	70.5	29.5
8c	0.15	51.4	48.6
8d	0.12	59.8	40.2
8e	0.09	60.4	39.6
8f	0.10	62.1	33.5
8g	0.07	96.3	3.7
8h	0.15	62.1	37.9
8i	0.12	68.3	31.7
8j	0.10	39.5	60.5
9	0.14	54.8	45.2
10a	nt	nt	nt
10b	nt	nt	nt
10c	0.90	79.7	20.2

nt = not tested.

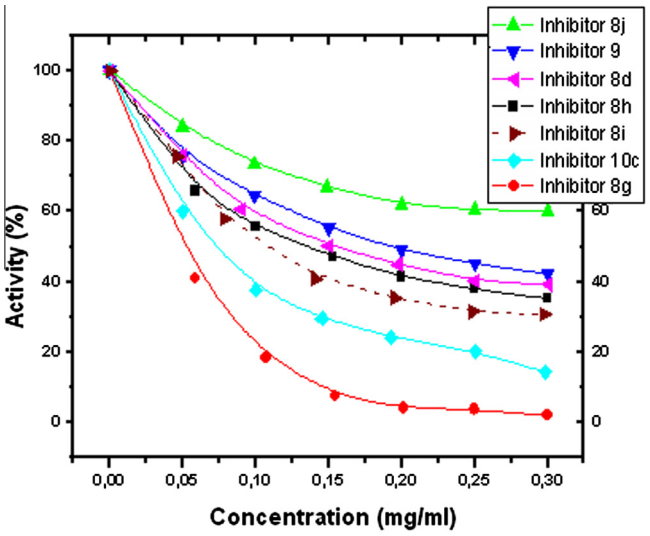


Figure 3B. Kinetic measurements of tyrosinase activity with L-DOPA as substrate and different concentrations of inhibitors in 50 mM phosphoric acid buffer solution (pH 6.8, 1.8 mL), an aqueous solution of tyrosinase (1000 U/mL, 0.1 mL), and DMSO (0.1 mL) at 25 °C for 10 min. The reaction was monitored at 475 nm for 10 min.

same inhibitor. The enzyme was inhibited strongly by glutathione, β-mercaptoethanol, but EDTA and sodium chloride did not inhibit the enzyme very efficiently. Thiol compounds, such as glutathione, inhibit the enzyme and may also affect the subsequent non-enzymatic reactions by reducing the quinones to diphenols and cause retarded browning. These findings are comparable with other tyrosinases from bacterial, fungal and plant origins.[21,22] Low inhibition of L. sacchari tyrosinase by EDTA is similar to tyrosinases from other organisms such as *Trichoderma reesei*.[22,23]

The inhibition effects of synthesized inhibitors on tyrosinase activity were performed with L-DOPA as substrate and different concentrations of inhibitors in 50 mM phosphoric acid buffer solution (pH 6.8, 1.8 mL). The activity of tyrosinase (1000 U/mL, 0.1 mL) was determined by spectrophotometric techniques after an incubation time of 0.5 and 1.0 min (Fig. 3A and B).

Aza-Sildenafil and its isomeric analogs **8a** and **9** are structurally similar and represent comparable range of tyrosinase activity, 42.9%, 41.2% and 45.2%, respectively. Lower inhibition exhibited derivatives **8e** (39.6%) and **8d** (40.2%) with a pyrrolidine and a piperidine ring in the sulfonamide group, respectively. After the treatment of tyrosinase with inhibitors **8c** and **8h,** that possess a morfoline ring attached to the skeleton, the enzyme activity remains high: 48.6% and 37.9%, respectively. Very high enzymatic activity (60.5%) was measured after treatment of tyrosinase with the inhibitor **8j** which contains a *N*-2-hydroxyethyl substituent (Fig. 3B). However, our study showed that inhibitors **8b** and **8f** with one NH group in the sulfonamide part are more active inhibitors and reduce the enzyme activity of tyrosinase to 29.5% and 33.5%, respectively. It is suggested, that NH or NH_2 groups are involved in the inhibition effect of tyrosinase activity. Our suggestion was confirmed by the activity of inhibitor **10c** (20.2%) with two NH groups. The most active inhibitor in the tested group was compound **8g** that reduced the tyrosinase activity to 3.7%. In consideration of our results, the $-NHCH_2CH_2NH_2$ group in the structure of the inhibitor **8g** seems to be the most important residue for the inhibition of tyrosinase activity.

Tyrosinase contains one binuclear copper complex in the catalytic centre and the catechol hydroxyl groups of the substrate are

suggested to be bound to the copper atoms during the enzymatic oxidation process.[24] Phenolic hydroxyl groups are generally capable to coordinate to copper atoms causing inhibition of the enzyme in competition to the catechol substrate. The competitive inhibition the compounds **8g** may arise with strong participation both NH and NH_2 group. However, other tested derivatives were only weak tyrosinase inhibitors. Therefore, we suggest that primary binding to one copper atom should be strengthened by additional interactions between the inhibitor (e.g., the NH group and π electrons of the ring) and another copper atom or the amino acid residues of the enzyme's active site to accomplish the inhibition. Probably flexibility and structure $-NHCH_2CH_2NH_2$ group allows to create strong interactions between the inhibitor **8g** and the amino acid residues of the enzyme's active site. Similar results were obtained by molecular docking study for unsymmetrical curcumin analogs.[25]

2.2.2. PDE5 assay

The newly synthesized *aza*-analogs of Sildenafil were subjected also to the PDE5A (isolated from rabbit platelets) inhibition assay for structure–activity relationship studies. The inhibition activities of the compounds were tested at 1 μM using a tritium scintillation proximity assay (SPA) system (Amersham Biosciences). PDE5A inhibitory potency of the *aza*-Sildenafil[26] and compounds **8a**–**j** are presented in Table 2 in comparison to Sildenafil.

The tested new compounds did not show significant inhibitory effects on PDE5 at 1 μM. The most active compounds are **8d**, **8b**, and **8e** (inhibition: 24.1–29.5%) and the dimers **10c** and **10b** (inhibition: 26.7–28.1%). The *aza*-Sildenafil in which nitrogen atom N7 replaces the carbonyl group present in the pyrimidinone ring of Sildenafil shows very low inhibitory effect (inhibition 5.8%) in comparison with Sildenafil (inhibition 98.9%). The decrease in activity of the tested new Sildenafil analogs probable is due to the lack of two common features that define the scaffold of all known PDE5 inhibitors with the pyrazolo[4,3-*d*]pyrimidin-7-one core. Based on the literature, the first common feature for most of the tested PDE5 inhibitors is a planar ring structure of the molecule that is held tightly in the active site by a pair of hydrophobic residues, and the second feature is that the inhibitor always forms one or two hydrogen bonds with the purine-selective glutamine position at the enzyme.[27] Taking the above into account, the low activity of the new analogs of Sildenafil toward PDE5 might be due to the lack of a planar structure of the sulfonamides resulting on the lack of an intramolecular hydrogen bond between the ethoxy group at the phenyl ring and nitrogen atom N6 in the triazine ring (Fig 4).

Table 2
PDE5A Inhibitory potency of *aza*-Sildenafil analogs

Compound	Concentration (μM)	Inhibition (%)
aza-Sildenafil	1	5.8
8a	1	6.0
8b	1	26.5
8c	1	0
8d	1	29.5
8e	1	24.1
8f	1	0.6
8g	1	0
8h	1	13.3
8i	1	16.4
8j	1	Not tested
9	1	0
10a	1	11.4
10b	1	26.7
10c	1	28.1
Sildenafil	1	98.9

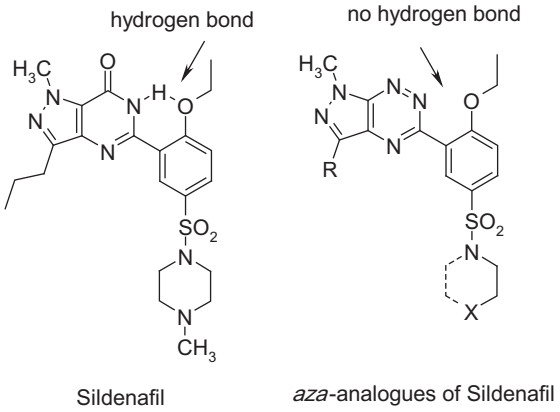


Figure 4. Structure of Sildenafil and its new *aza*-analogs.

3. Summary

A practical, high yielding, and scalable method for the preparation of new pyrazolo[4,3-*e*][1,2,4]triazine derivatives as new *aza*-analogs of Sildenafil from inexpensive commercially available starting materials is described. The new Sildenafil analogs were designed by replacing the carbonyl group of the scaffold by a triazine nitrogen atom. Biotesting experiments for Sildenafil and lodenafil carbonate analogs showed good tyrosinase inhibitory activity, but very low PDE5A inhibitory potency, and further modification of the structure are under way to increase the potency against tyrosinase.

4. Experimental section

4.1. Materials and methods

Melting points were determined on a Mel-Temp apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Varian spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C, respectively). The chemical shift values are expressed in ppm (part per million) with TMS as internal reference. The relative integrals of peak areas agreed with those expected for the assigned structures. The molecular weights of final compounds were assessed by electrospray ionization mass spectrometry (ESI/MS) on an Agilent Technologies 6538 UHD Accurate Mass Q-TOF LC/MS instrument. Elemental compositions are within ±0.4% of the calculated values. Derivatives **1**–**3** were prepared according to the literature procedure.[18a]

4.2. General method for the synthesis of derivatives 4 and 5

To a mixture of **2** or **3** (0.612 g, 3.14 mmol, 1.0 equiv), CuMeSal (1.68 g, 7.85 mmol, 2.5 equiv), 2-ethoxyphenylboronic acid (1.3 g, 7.85 mmol, 2.5 equiv) in dry THF (25 mL), $Pd(PPh_3)_4$ (0.36 g, 0.31 mmol, 0.1 equiv) was added under argon. The reaction mixture was stirred overnight under reflux. The reaction was quenched with a saturated Na_2CO_3 solution and extracted with dichloromethane. The combined organic phases were dried over $MgSO_4$ and concentrated in vacuo. After purification by column chromatography on silica gel (hexane: CH_2Cl_2, 5:1), the desired products **4** or **5** were obtained.

4.2.1. 5-(2-Ethoxyphenyl)-1,3-dimethyl-1H-pyra-zolo[4,3-*e*][1,2,4]triazine (4)

Yield 90%, yellow powder, mp 85–86 °C. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.30 (t, 3H, J = 14.0 Hz), 2.70 (s, 3H), 4.13 (q, 2H,

J = 14.0 Hz), 4.32 (s, 3H); 7.06–7.12 (m, 2H), 7.42–7.46 (m, 1H), 7.75 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.05, 14.69, 34.68, 64.61, 113.47, 120.79, 126.88, 131.09, 131.92, 134.48, 141.97, 146.96, 157.31, 160.10. HRMS (ESI, m/z) calcd. for $C_{14}H_{15}N_5O$ [M+] 269.1276. Found [M+] 269.1284. Anal. calcd. for $C_{14}H_{15}N_5O$: C, 62.44; H, 5.61; N, 26.01. Found: C, 62.30; H, 5.70; N, 25.93.

4.2.2. 5-(2-Ethoxyphenyl)-1,3-dimethyl-2H-pyra-zolo[4,3-*e*][1,2,4]triazine (5)

Yield 30%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.30 (t, 3H, J = 7.2 Hz), 2.75 (s, 3H), 4.11 (q, 2H, J = 7.2 Hz), 4.29 (s, 3H), 7.05–7.11 (m, 2H), 7.41–7.45 (m, 1H), 7.78 (dd, 1H, J_1 = 7.6 Hz, J_2 = 1.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 9.12, 14.69, 39.29, 64.61, 113.44, 120.74, 127.21, 128.83, 131.04, 131.84, 133.52, 154.47, 157.42, 159.32. HRMS (ESI, m/z) calcd. for $C_{14}H_{15}N_5O$ [M$^+$] 269.1276. Found [M+] 269.1284. Anal. calcd for $C_{14}H_{15}N_5O$: C, 62.44; H, 5.61; N, 26.01. Found: C, 62.22; H, 5.74; N, 25.89.

4.3. General method for the synthesis of derivatives 6 and 7

Compounds **4** or **5** (594 mg, 2 mmol) was added portionwise to stirred and cooled chlorosulfonic acid (2 mL) in an ice bath under argon atmosphere. The reaction mixture was then warmed to room temperature gradually for 2 h after the addition. The reaction solution was cautiously added to ice water (15 mL), and the aqueous mixture extracted with dichloromethane. The combined extracts were dried over anhydrous Na_2SO_4 and evaporated under vacuum to give the required sulfonyl chloride **6** or **7**. The crude products were purified by column chromatography (silicagel, CH_2Cl_2).

4.3.1. 4-Ethoxy-3-(1,3-dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)benzene-1-sulfonyl chloride (6)

Yield 85%, yellow crystals, mp 119–120 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.39 (t, 3H, J = 14.0 Hz), 2.72 (s, 3H), 3.64 (s, 3H), 4.26 (q, 2H, J = 14.0 Hz), 7.21 (d, 1H, J = 9.2 Hz), 7.46 (d, 1H, J = 2.4 Hz), 8.14 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.8). ^{13}C NMR ($CDCl_3$) δ: 11.21, 14.48, 34.99, 65.57, 113.15, 127.92, 130.75, 131.71, 134.64, 136.12, 142.45, 147.13, 157.87, 162.69. HRMS (ESI, m/z) calcd. for $C_{14}H_{14}N_5O_3ClS$ [M+] 367.0505. Found [M] 367.0514. Anal. Calcd. for $C_{14}H_{14}N_5O_3ClS$: C, 45.72; H, 3.84; N, 19.04. Found: C, 45.60; H, 3.90; N, 18.90.

4.3.2. 4-Ethoxy-3-(1,3-dimethyl-2H-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)benzene-1-sulfonyl chloride (7)

Yield 80%, yellow-brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.38 (t, 3H, J = 7.2 Hz), 2.79 (s, 3H), 4.24 (q, 2H, J = 7.2 Hz), 4.35 (s, 3H), 7.20 (d, 1H, J = 9.2 Hz), 8.12 (dd, 1H, J_1 = 9.2 Hz, J_2 = 2.4 Hz), 8.48 (d, 1H, J = 2.8 Hz).

4.4. Synthesis of sulfonamides 8a–j and 9

A mixture of sulfonyl chloride **6** or **7** (100 mg, 0.29 mmol) and appropriate amine (1 mmol) in anhydrous acetonitrile (5 mL) was stirred overnight at room temperature and then concentrated in vacuo to afford the crude sulfonamide. The residue was purified on silica gel using a mixture of CH_2Cl_2/EtOH (25:1) as eluent to give the required compounds as a yellow solids.

4.4.1. 5-[2-Ethoxy-5-(4-methylpiperazin-1-ylsulfon-yl)phenyl]-1,3-dimethyl-1H-pyrazolo[4,3-*e*]-[1,2,4]triazine (8a)

Yield 91%, yellow powder, mp 148–153 °C. ^{1}H NMR ($CDCl_3$) δ: 1.36 (t, 3H, J = 14.0 Hz), 2.17 (s, 3H), 2.29 (s, 3H), 2.52 (bs, 4H), 3.13 (bs, 4H), 4.20 (q, 2H, J = 14.0 Hz), 4.34 (s, 3H), 7.16 (d, 1H, J = 8.0 Hz), 7.85 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.18 (d, 1H, J = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.04, 14.43, 34.73, 45.54, 45.76, 53.96, 65.00, 112.84, 127.11, 127.29, 131.01, 132.03, 134.49, 142.22, 146.91, 158.46, 160.79; HRMS (ESI, m/z) calcd. for $C_{19}H_{26}N_7O_3S$ [M+H] 432.1812. Found [M+H] 432.1813. Anal. calcd. for $C_{19}H_{25}N_7O_3S$: C, 52.88; H, 5.84; N, 22.72. Found: C, 52.77; H, 5.90; N,22.60.

4.4.2. 5-[2-Ethoxy-5-(piperazin-1-ylsulfonyl)-phenyl]-1,3-dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]-triazine (8b)

Yield 92%, yellow powder, mp 172–176 °C. ^{1}H NMR ($CDCl_3$) δ: 1.38 (t, 3H, J = 14.0 Hz), 2.71 (s, 3H), 2.96–3.09 (m, 8H), 4.20 (q, 2H, J = 14.0 Hz), 4.33 (s, 3H), 7.16 (d, 1H, J = 8.8 Hz), 7.85 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.15 (d, 1H, J = 2,4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.02, 14.41, 34.73, 44.92, 46.35, 64.98, 112.85, 127.16, 127.28, 131.02, 131.96, 134.48, 142.19, 146.90, 158.43, 160.78; HRMS (ESI, m/z) calcd. for $C_{18}H_{24}N_7O_3S$ [M+H] 418.1656. Found [M+H] 418.1656. Anal. calcd for $C_{18}H_{23}N_7O_3S$: C, 51.78; H, 5.55; N, 23.49. Found: C, 51.63; H, 5.66; N, 23.40.

4.4.3. 5-[2-Ethoxy-5-(morpholin-1-ylsulfonyl)-phenyl]-1,3-dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]-triazine (8c)

Yield 87%, yellow powder, mp 170–179 °C. ^{1}H NMR ($CDCl_3$) δ: 1.37 (t, 3H, J = 14.0 Hz), 2.71 (s, 3H), 3.05 (t, 4H, J = 9,2 Hz), 3.74 (t, 4H, J = 9.2 Hz), 4.22 (q, 2H, J = 14.0 Hz), 4.34 (s, 3H), 7.18 (d, 1H, J = 8.8 Hz), 7.86 (dd, 2H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.17 (d, 1H, J = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.04, 14.43, 34.76, 46.04, 65.00, 66.06, 112.84, 126.74, 127.30, 131.15, 132.06, 134.51, 142.23, 146.91, 158.38, 160.89; HRMS (ESI, m/z) calcd. for $C_{18}H_{23}N_6O_4S$ [M+H] 419.1496. Found [M+H] 419.1497. Anal. calcd. for $C_{18}H_{22}N_6O_4S$: C, 51.66; H, 5.30; N, 20.08. Found: C, 51.50; H, 5.47; N.19.94.

4.4.4. 5-[2-Ethoxy-5-(piperidin-1-ylsulfonyl)-phenyl]-1,3-dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]-triazine (8d)

Yield 92%, yellow powder, mp 160–162 °C. ^{1}H NMR ($CDCl_3$) δ: 1.36 (t, 3H, J = 14.0 Hz), 1.42 (t, 2H, J = 11.6 Hz), 1.61–1.67 (m, 4H), 2.71 (s, 3H), 3.02 (t, 4H, J = 10.8 Hz), 4.20 (q, 2H, J = 14.0 Hz), 4.34 (s, 3H), 7.14 (d, 1H, J = 8.8 Hz), 7.86 (dd, 2H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.15 (d, 1H, J = 2,4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.04, 14.45, 23.47, 25.12, 34.76, 46.96, 64.90, 127.08, 127.94, 130.29, 131.00, 131.85, 134.51, 142.20, 146.93, 158.58, 160.48; HRMS (ESI, m/z) calcd. for $C_{19}H_{25}N_6O_3S$ [M+H] 417.1703. Found [M+H] 417.1703. Anal. calcd. for $C_{19}H_{24}N_6O_3S$: C, 54.79; H, 5.81; N, 20.18. Found: C, 54.70; H, 5.90; N.20.04.

4.4.5. 5-[2-Ethoxy-5-(4-pyrrolidin-1-ylsulfonyl)-phenyl]-1,3-dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]-triazine (8e)

Yield 89%, yellow powder, mp 190–193 °C. ^{1}H NMR ($CDCl_3$) δ: 1.35 (t, 3H, J = 13.6 Hz), 1.76–1.79 (m, 4H), 2.71 (s, 3H), 3.26 (t, 4H, J = 13.6 Hz), 4.20 (q, 2H, J = 14.0 Hz), 4.34 (s, 3H), 7.15 (d, 1H, J = 8.4 Hz), 7.93 (dd, 2H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 8.17 (d, 1H, J = 2,4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.04, 14.45, 25.17, 34.76, 47.97, 64.90, 112.71, 127.11, 128.67, 130.83, 131.75, 134.51, 142.20, 146.92, 158.58, 160.49; HRMS (ESI, m/z) calcd. for $C_{18}H_{23}N_6O_3S$ [M+H] 403.1547. Found [M+H] 403.1548. Anal. calcd. for $C_{18}H_{22}N_6O_3S$: C, 53.72; H, 5.51; N, 20.88. Found: C, 53.60; H, 5.66; N, 20.70.

4.4.6. 5-(5-(1,4-Diazepan-1-ylsulfonyl)-2-ethoxy-phenyl]-1,3-dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]-triazine (8f)

Yield 88%, yellow powder, mp 146–157 °C. ^{1}H NMR ($CDCl_3$) δ: 1.35 (t, 3H, J = 13.6 Hz), 1.83 (t, 2H, J = 11.6 Hz), 2.71 (s, 3H), 2.92–2.99 (m, 4H), 3.10–3.13 (m, 2H), 3.35–3.41 (m, 2H), 4.19 (q, 2H, J = 13.6 Hz), 4.33 (s, 3H), 7.13 (d, 1H, J = 8.8 Hz), 7.85 (dd, 2H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.16 (d, 1H, J = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 10.97, 14.38, 30.34, 34.69, 47.14, 47.46, 49.72, 50.56, 64.88, 112.84, 127.16, 130.17, 130.92, 131.08, 134.41, 142.10, 146.85,

158.46, 160.33. HRMS (ESI, *m*/*z*) calcd. for $C_{19}H_{26}N_7O_3S$ [M+H] 432.1812. Found [M+H] 432.1811. Anal. calcd. for $C_{19}H_{25}N_7O_3S$: C, 52.88; H, 5.84; N, 22.72. Found: C, 52.78; H, 5.90; N, 22.60.

4.4.7. *N*-(2-Aminoethyl)-3-(1,3-dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxybezene-sulfonamide (8g)

Yield 65%, yellow powder, mp 149–155 °C. ^{1}H NMR ($CDCl_3$) δ: 1.33 (t, 3H, *J* = 14.0 Hz), 2.69 (s, 3H), 2.95 (t, 2H, *J* = 10.8 Hz), 3.10 (t, 2H, *J* = 10.8 Hz), 4.18 (q, 2H, *J* = 14.0 Hz), 4.31 (s, 3H), 7.16 (d, 1H, *J* = 8.8 Hz), 8.00 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.25 (d, 1H, *J* = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.05, 14.44, 34.77, 40.65, 44.18, 64.96, 113.00, 127.11, 130.53, 131.33, 131.39, 134.51, 142.20, 146.88, 158.51, 160.49. HRMS (ESI, *m*/*z*) calcd. for $C_{16}H_{22}N_7O_3S$ [M+H] 392.1499. Found [M+H] 392.1501. Anal. calcd. for $C_{16}H_{21}N_7O_3S$: C, 49.09; H, 5.41; N, 25.05. Found: C, 48.98; H, 5.55; N, 24.91.

4.4.8. 3-(1,3-Dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]-triazin-5-yl)-4-ethoxy-N-(2-morpholinethyl)-benzenesulfonamide (8h)

Yield 91%, yellow powder, mp 151–153 °C. ^{1}H NMR ($CDCl_3$) δ: 1.36 (t, 3H, *J* = 14.0 Hz), 2.31 (t, 4H, *J* = 8.8 Hz), 2.56 (t, 2H, *J* = 11.2 Hz), 2.72 (s, 3H), 3.04 (t, 2H, *J* = 11.2 Hz), 3.68 (t, 4H, *J* = 8.8 Hz), 4.20 (q, 2H, *J* = 14.0 Hz), 4.34 (s, 3H), 7.15 (d, 1H, *J* = 8.8 Hz), 7.99 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.27 (d, 1H, *J* = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.05, 14.44, 34.79, 38.77, 53.01, 56.48, 65.01, 66.34, 112.82, 127.37, 130.51, 131.17, 131.33, 134.42, 142.19, 146.85, 158.45, 160.58. HRMS (ESI, *m*/*z*) calcd. for $C_{20}H_{28}N_7O_4S$ [M+H] 462.1918. Found [M+H] 462.1920. Anal. calcd. for $C_{20}H_{27}N_7O_4S$: C, 52.05; H, 5.90; N, 21.24. Found: C, 51.97; H, 6.15; N, 21.18.

4.4.9. 3-(1,3-Dimethylo-1H-pyrazolo[4,3-*e*][1,2,4]-triazin-5-yl)-4-ethoksy-*N*,*N*-diethylbenzene-sulfonamide (8i)

Yield 92%, yellow powder, mp 167–170 °C. ^{1}H NMR ($CDCl_3$) δ: 1.15 (t, 6H, *J* = 14.4 Hz), 1.35 (t, 3H, *J* = 14.0 Hz), 2.72 (s, 3H), 3.25 (q, 4H, *J* = 14.4 Hz), 4.20 (q, 2H, *J* = 14.0 Hz), 4.34 (s, 3H), 7.13 (d, 2H, *J* = 8.8 Hz), 7.91 (dd, 2H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.22 (d, 2H, *J* = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.05, 14.23, 14.47, 34.78, 42.14, 64.90, 112.76, 127.08, 130.34, 131.33, 132.14, 134.56, 142.23, 146.91, 158.62, 160.23. HRMS (ESI, *m*/*z*) calcd. for $C_{18}H_{25}N_6O_3S$ [M+H] 405.1703. Found [M+H] 405.1704. Anal. calcd. for $C_{18}H_{24}N_6O_3S$: C, 53.45; H, 5.98; N, 20.78. Found: C, 53.33; H, 6.07; N, 20.63.

4.4.10. 3-(1,3-Dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]-triazin-5-yl)-4-ethoxy-N-(2-hydroxyethyl)benzene-sulfonamide (8j)

Yield 93%, yellow powder, mp 149–152 °C. ^{1}H NMR ($CDCl_3$) δ: 1.35 (t, 3H, *J* = 14.0 Hz), 2.71 (s, 3H), 3.14 (t, 2H, *J* = 8.8 Hz), 3.68 (q, 2H, *J* = 9.6 Hz), 4.20 (q, 2H, *J* = 14.0 Hz), 4.33 (s, 3H), 5.37 (bs, 1H, OH), 7.14 (d, 1H, *J* = 8.8 Hz), 7.96 (dd, 2H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.25 (d, 1H, *J* = 2,4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.05, 14.43, 34.84, 45.34, 61.10, 65.01, 112.89, 126.95, 130.60, 131.35, 131.48, 134.69, 142.26, 146.75, 158.33, 160.53. HRMS (ESI, *m*/*z*) calcd. for $C_{16}H_{21}N_6O_4S$ [M+H] 393.1340. Found [M+H] 393.1341. Anal. calcd. for $C_{16}H_{20}N_6O_4S$: C, 48.97; H, 5.14; N, 21.42. Found: C, 48.90; H, 5.20; N, 21.21.

4.4.11. 5-[2-Ethoxy-5-(4-methylpiperazin-1-ylsulfo-nyl)-phenyl]-2,3-dimethyl-2H-pyrazolo[4,3-*e*]-[1,2,4]triazine (9)

Yield 75%, yellow powder, mp 80–83 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.33 (t, 3H, *J* = 6.8 Hz), 2.47 (t, 4H, J = 4.8 Hz), 2.76 (s, 3H), 3.10 (bs, 4H), 3.67 (s, 3H), 4.16 (q, 2H, *J* = 6.8 Hz), 4.30 (s, 3H), 7.13 (d, 1H, *J* = 8.8 Hz), 7.81 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.0 Hz), 8.18 (d, 1H, *J* = 2.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 9.13, 14.39, 39.42, 45.48, 45.73, 53.90, 64.97, 112.79, 127.03, 127.59, 128.86, 130.93, 131.90, 134.01, 154.40, 157.64, 160.89. HRMS (ESI, *m*/*z*) calcd. for $C_{19}H_{25}N_7O_3S$ [M+] 431.17396. Found [M+] 431.17359. Anal. calcd. for $C_{19}H_{25}N_7O_3S$: C, 52.88; H, 5.84; N, 22.72. Found: C, 52.70; H, 5.95; N, 22.57.

4.5. Synthesis of derivatives 10a–c

Method A: A mixture of the sulfonyl chloride **6** (1 mmol) and amine (piperazine, homopiperazine or ethylenediamine) (0.5 mmol) in anhydrous acetonitrile (5 mL) was stirred at room temperature overnight. Next, the reaction mixture was concentrated in vacuo to afford the crude bisulfonamides **10a**–**c**. The residues were purified on silica gel using a mixture of CH_2Cl_2/EtOH (100:1) as eluent to give the required compounds as a yellow solids.

Method B: An equimolar mixture of **6** and sulfonamide **8c** or **8f**–**g** was allowed to react according to the procedure as described above for **10a**–**c**.

4.5.1. 1,4-Bis(3-(1,3-dimethyl-1H-pyrazolo[4,3-*e*]-[1,2,4]triazin-5-yl)-4-ethoxyphenylsulfonyl)-piperazine (10a)

Yield 92%, mp 172–176 °C. ^{1}H NMR ($CDCl_3$) δ: 1.36 (t, 6H, *J* = 7.2 Hz), 2.70 (s, 6H), 3.16 (s, 8H), 4.19 (q, 4H, *J* = 7.2 Hz), 4.32 (s, 6H), 7.16 (d, 2H, *J* = 8.8 Hz), 7.81 (dd, 2H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.14 (d, 2H, *J* = 2,4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11. 04, 14.40, 34.73, 45.47, 65.03, 112.99, 126.81, 127.42, 130.91, 131.88, 134.46, 142.21, 146.90, 158.43, 161.02. HRMS (ESI, *m*/*z*) calcd. for $C_{32}H_{36}N_{12}O_6S_2$ [M+] 748.2317. Found [M+] 748.2316. Anal. calcd. for $C_{32}H_{36}N_{12}O_6S_2$: C, 51.33; H, 4.85; N, 22.45. Found: C, 51.20; H, 5.00; N, 22.25.

4.5.2. 1,4-Bis(3-(1,3-dimethyl-1H-pyrazolo[4,3-*e*]-[1,2,4]triazin-5-yl)-4-ethoxyphenylsulfonyl)-1,4-diazepane (10b)

Yield 88%, mp 146–157 °C. ^{1}H NMR ($CDCl_3$) δ: 1.34 (t, 6H, *J* = 6.8 Hz), 1.99 (m, 2H), 2.69 (s, 6H), 3.40 (t, 4H, *J* = 6.0 Hz), 3.44 (s, 4H), 4.18 (q, 4H, *J* = 6.8 Hz), 4.31 (s, 6H), 7.13 (d, 2H, *J* = 8.8 Hz), 7.84 (dd, 2H, J_1 = 2.0 Hz, J_2 = 8.8 Hz), 8.16 (d, 2H, *J* = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.00, 14.39, 29.03, 34.70, 47.34, 51.07, 64.94, 112.99, 127.30, 130.07, 130.75, 131.07, 134.41, 142.14, 146.87, 158.39, 160.53. HRMS (ESI, *m*/*z*) calcd. for $C_{33}H_{39}N_{12}O_6S_2$ [M+H] 763.2551. Found [M+H] 763.2548. Anal. calcd. for $C_{33}H_{38}N_{12}O_6S_2$: C, 51.96; H, 5.02; N, 22.03. Found: C, 51.78; H, 5.16; N, 21.91.

4.5.3. *N*,*N*-(Ethane-1,2-diyl)bis(3-(1,3-dimethyl-1H-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-benzenesulfonamide (10c)

Yield 65%, mp 149–155 °C. ^{1}H NMR ($CDCl_3$) δ: 1.32 (t, 6H, *J* = 6.8 Hz), 2.68 (s, 6H), 3.10 (s, 4H), 4.17 (q, 4H, *J* = 6.8 Hz), 4.30 (s, 6H), 7.12 (d, 2H, *J* = 8.8 Hz), 7.90 (dd, 2H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.21 (d, 2H, *J* = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.04, 14.42, 34.79, 42.91, 65.00, 113.04, 126.93, 130.59, 131.26, 131.32, 134.62, 142.24, 146.73, 158.30, 160.58. HRMS (ESI, *m*/*z*) calcd. for $C_{30}H_{34}N_{12}O_6S_2$ [M+] 722.2160. Found [M+H] 722.2156. Anal. calcd. for $C_{30}H_{34}N_{12}O_6S_2$: C, 49.85; H, 4.74; N, 23.25. Found: C, 49.93; H, 4.85; N, 23.13.

4.6. Synthesis of derivatives 11 and 12

To a solution of **1** (1.81 g, 10 mmol) in benzene (140 mL), ethyl vinyl ether (2 ml, 20 mmol) or 3,4-dihydro-2*H*-pyran and one drop of HCl (38%) was added. The reaction mixture was stirred at 40 °C for 8 hours, and then a saturated solution of $NaHCO_3$ was added. The benzene layer was separated, dried over anhydrous $MgSO_4$ and concentrated in vacuo. The product was purified by column chromatography using silica gel and chloroform as eluent.

4.6.1. 1-(1-Ethoxyethyl)-3-methyl-5-methylsulfanyl-1H-pyrazolo[4,3-*e*][1,2,4]triazine (11)

Yield 90%, mp 51 °C. ^{1}H NMR ($CDCl_3$) δ: 1.14 (t, 3H, J = 7.0 Hz), 1.92 (d, 3H, J = 6.0 Hz), 2.65 (s, 3H), 2.74 (s, 3H), 3.19–3.34 (m, 1H), 3.49–3.64 (m, 1H), 6.30 (q, 1H, J = 6.0 Hz); HRMS (ESI) calcd. for $C_{10}H_{15}N_5OSNa$ [M+Na] 276.0890. Found [M+Na] 276.0910.

4.6.2. 1-(Tetrahydro-2H-pyran-2-yl)-3-methyl-5-methylsulfanyl-1H-pyrazolo[4,3-*e*][1,2,4]triazine (12)

Yield 80%, yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.65–1.68 (m, 2H), 1.81–1.87 (m, 2H), 2.08–2.12 (m, 1H), 2.16–2.20 (m, 1H), 2.63 (s, 3H), 2.73 (s, 3H), 2.73–2.81 (m, 1H), 4.13–4.19 (m, 1H), 6.27 (dd, 1H, J_1 = 10.4 Hz, J_2 = 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.03, 14.27, 22.94, 24.83, 29.31, 68.49, 84.11, 135.97, 142.09, 147.14, 167.93. HRMS (ESI, m/z) calcd. for $C_{11}H_{15}N_5OS$ [M^+] 265.0997. Found [M^+] 265.0998.

4.7. Synthesis of derivatives 13 and 14

Compounds **13** and **14** were synthesized following the procedure for the preparation of derivatives **4** and **5** as described above.

4.7.1. 1-(1-Ethoxyethyl)-5-(2-ethoxyphenyl)-3-methyl-1H-pyrazolo[4,3-*e*][1,2,4]triazine (13)

Yield 85%, yellow powder, mp 149–155 °C.^{1}H NMR ($CDCl_3$) δ: 1.17 (t, 3H, J = 7.2 Hz), 1.34 (t, 3H, J = 7.2 Hz), 1.96 (d, 3H, J = 6.0 Hz), 2.73 (s, 3H), 3.33–3.39 (m, 1H), 3.58–3.64 (m, 1H), 4.15 (q, 2H, J = 7.2 Hz), 6.43 (q, 1H, J = 6.0 Hz), 7.07–7.13 (m, 2H), 7.43–7.48 (m, 1H), 7.80 (dd, 1H, J_1 = 7.0 Hz, J_2 = 2.0 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.27, 14.70, 14.78, 20.57, 64.61, 64.70, 83.25, 113.59, 120.85, 126.73, 131.21, 132.02, 135.13, 143.25, 147.36, 157.34, 160.78. HRMS (ESI, m/z) calcd. for $C_{17}H_{21}N_5O_2$ [M+] 327.1690. Found [M+] 327.1691. Anal. calcd. for $C_{17}H_{21}N_5O_2$: C, 62.37; H, 6.47; N, 21.39. Found: C, 62.25; H, 6.52; N, 21.30.

4.7.2. 5-(2-Ethoxyphenyl)-1-(tetrahydro-2H-pyran-2-yl)-3-methyl-1H-pyrazolo[4,3-*e*][1,2,4]-triazine (14)

Yield 80%, yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.34 (t, 3H, J = 6.8 Hz), 1.65–1.68 (m, 2H), 1.81–1.87 (m, 2H), 2.08–2.12 (m, 1H), 2.16–2.20 (m, 1H) 2.73 (s, 3H), 2.73–2.81 (m, 1H), 4.12 (q, 2H, J = 6.8 Hz), 4.13–4.19 (m, 1H), 6.27 (dd, 1H, J_1 = 10.4 Hz, J_2 = 2.4 Hz), 7.07–7.14 (m, 2H), 7.45–7.48 (m, 1H), 7.79 (dd, 1H, J_1 = 7.2 Hz, J_2 = 2.0 Hz). ^{13}C NMR ($CDCl_3$) δ: 11.26, 14.72, 22.98, 24.87, 29.44, 64.72, 68.61, 83.96, 113.45, 120.89, 128.44, 128.56, 131.55, 131.99, 132.06, 132.16, 143.82, 157.35. HRMS (ESI, m/z) calcd. for $C_{18}H_{21}N_5O_2$ [M^+] 339.1695. Found [M^+] 339.1696. Anal. calcd. for $C_{18}H_{21}N_5O_2$: C, 63.70; H, 6.24; N, 20.64. Found: C, 63.80; H, 6.32; N, 20.45.

4.8. Deprotection of 13 and 14

A mixture of **13** or **14** (206 mg, 1 mmol) and concentrated HCl (0.5 mL) in methanol (10 mL) was stirred at room temperature for 12 h. Then the solvent was removed and the residue separated by column chromatography (silica gel, eluent/chloroform/ethanol, 20:1) to afford 124 mg (92%) of **15** as a yellow solid.

4.8.1. 3-Methyl-5-(2-ethoxyphenyl)-1H-pyrazolo-[4,3-*e*][1,2,4]triazine (15)

Yield 75%, yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.31 (t, 3H, J = 7.2 Hz), 2.75 (s, 3H), 4.16 (q, 2H, J = 7.2 Hz), 7.08–7.14 (m, 2H), 7.43–7.48 (m, 1H), 7.80 (dd, 1H, J_1 = 2.0 Hz, J_2 = 8.0 Hz), 11.70 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 11.21, 14.66, 64.62, 113.36, 120.84, 126.23, 131.42, 131.96, 135.14, 144.02, 147.97, 157.26, 160.28. HRMS (ESI, m/z) calcd. for $C_{13}H_{13}N_5O$ [M+] 255.1115. Found [M+] 255.1116. Anal. calcd. for $C_{13}H_{13}N_5O$: C, 61.17; H, 5.13; N, 27.43. Found: C, 61.00; H, 5.29; N, 27.25.

4.9. Reaction of 15 with methyl iodide

A mixture of potassium carbonate (138 mg, 1 mmol) and derivative **15** (123 mg, 0.5 mmol) in ethanol/water mixture (EtOH/H_2O, 4:1, v/v) was stirred for 10–15 min at room temperature. Then a solution of methyl iodide (0.1 mL, 1 mmol) was added and stirred overnight at room temperature. The excess of base was then destroyed by the addition of a saturated solution of ammonium chloride and the solvent was removed in vacuo. The crude product was submitted to column chromatography on silica gel using dichloromethane as eluent to give **4** as the first product. Further elution with CH_2Cl_2/EtOH (25:1) gives pure **5**. Total yield 90% with a ratio of the isomers **4** and **5** 1:1.2.

4.10. Purification of enzyme

The enzyme was purified from L. sacchari starting with 1000 ml of the culture filtrate after saturation by 60% $(NH_4)_2SO_4$ under continuous stirring at 4 °C. Then, 10 g anion-exchange material (Servacell, DEAE 52) was suspended in 500 ml 25 mM sodium acetate buffer, pH 5.5, and equilibrated overnight at 4 °C. The upper layer was decanted, and the sediment was mixed with 10 ml of the sample. The mixture was then adjusted to pH 7.0. The sediment was centrifuged at 2500g for 15 min at 4 °C and the obtained supernatant used for further purification by size exclusion chromatography. A FPLC system was used, equipped with a Sephacryl S-100 column (16 × 60 mm), equilibrated with 25 mM PIPES buffer, pH 7.0, containing 150 mM NaCl. The column was eluted with the same buffer at a flow rate of 0.4 ml/min. The fraction with the highest activity was additionally purified after chromatography on the same column.

The purity of the protein was checked after each step of purification by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (10% polyacrylamide) according to Laemmli.[28] The protein concentration was measured after each step of purification by the method of Bradford.[29]

4.11. Tyrosinase inhibition assay

Tyrosinase activity assays were performed with L-DOPA as substrate, as previously described,[30] with slight modifications. Different inhibitor concentrations (**8a**–**j**, **9** and **10c**) in 50 mM phosphoric acid buffer solution (pH 6.8, 1.8 mL) and an aqueous solution of tyrosinase (1000 U/mL, 0.1 mL) and DMSO (0.1 mL) were pre-incubated for 10 min at 25 °C. Then, a 1.5 mM L-DOPA solution (1 mL) was added and the reaction monitored at 475 nm for 10 min. The detected absorbance increases accompanying the oxidation of the substrate (L-DOPA). The final concentration of dopaquinone was measured using the molar absorption coefficient of 3700 M^{-1} cm^{-1}. The percentage of inhibition of tyrosinase activity was calculated as inhibition (%) = $(A - B)/A \times 100$, where A represents the difference in the absorbance of control sample between 0.5 and 1.0 min of an incubation time, and B the difference in absorbance of the test sample between an incubation time of 0.5 and 1.0 min. The activity of tyrosinase was determined by spectrophotometric techniques (Shimadzu UV-2100). DMSO inhibitory activity, was determined to be 2.5% under the same experimental conditions

4.12. PDE5 assay

PDE5A (BPS Bioscience Inc) activity was analyzed using a tritium scintillation proximity assay (SPA) system, and the assay was performed according to the manufacturer's instructions (Amersham Biosciences). Briefly, assays were performed in the presence of 50 mM Tris–HCl (pH 7.5) containing 8.3 mM $MgCl_2$ and 1.7 mM EGTA. Each assay was performed in a 100 µl reaction

volume containing the above buffer (Tris–HCl, pH 7.5), enzyme and around 0.05 μCi [^{3}H]cGMP. The reaction was carried out at 30 °C for 30 minutes and stopped by the addition of 50 μl PDE SPA beads (1 mg) suspended in 18 mM zinc sulfate. The reaction mixture was left to settle at room temperature for 20 min before counting in a MicroBeta TriLux instrument (Perkin–Elmer Life Sciences, USA). For compound inhibition study on PDE5A, a stock solution of the compounds was prepared in 100% DMSO, diluted in water to the appropriate concentrations, and added to the assay buffer to give a final concentration of <1% DMSO. The amount of enzyme used in each reaction was such that the hydrolysis of substrates did not exceed 15%, so that the amount of product increased linearly with time. Duplicates were run in each assay.

PDE inhibitory activity was calculated from equation below:

$$\%\text{PDE inhibition} = \left[1 - \frac{(\text{CPM}_{\text{sample}} - \text{CPM}_{\text{blank}})}{(\text{CPM}_{\text{control}} - \text{CPM}_{\text{blank}})}\right] \times 100$$

Whereby, $\text{CPM}_{\text{sample}}$ represents the radioactive value of sample obtained from the assay (with enzyme), $\text{CPM}_{\text{control}}$ the radioactive value of solvent (water) which was used for diluting the sample obtained from the assay (with enzyme), and $\text{CPM}_{\text{blank}}$ the radioactive value of the mixture of only buffer and substrate (without enzyme).

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References and notes

1. Elnagdi, M. H.; Al-Awadi, N.; Erian, A. W. In *Comprehensive Heterocyclic Chem. II*; Katrizky, A. R., Rees, C. W., Scriven, E. F. V., Eds.; Pergamon Press: Oxford, 1996; p 431.
2. Desenko, S. M.; Komykhov, S. A.; Orlov, V. D.; Meier, H. *J. Heterocycl. Chem.* **1998**, *35*, 989.
3. Kurasawa, Y.; Mitomi, T.; Okamoto, Y.; Kim, H. S. *J. Heterocycl. Chem.* **1998**, *35*, 1333.
4. Youngdle, G. A. U.S. Patent, 4 288 440, 1980; *Chem. Abstr.* **1982**, *96*, 6596c.
5. Todd, A. H. Br. Patent, 1203 149, 1970; *Chem. Abstr.* **1970**, 73, 120 508b
6. Gante, J.; Lust, S. Ger. Offen., 1 908 947, 1970; *Chem. Abstr.* **1970**, 73, 120501.
7. Huppatz, J. L. *Aust. J. Chem.* **1983**, *36*, 135.
8. Lindner, H. J.; Schaden, G. *Chem. Ber.* **1949**, *1972*, 105.
9. Hirata, K.; Nakagami, H.; Takashina, J.; Mahmud, T.; Kobayashi, M.; In, Y.; Ishida, T.; Miyamoto, K. *Heterocycles* **1996**, *43*, 1513.
10. Smirnov, V. V.; Kiprianova, E. A.; Garagulya, A. D.; Esipov, S. E.; Dovjenko, S. A. *FEMS Microbiol. Lett.* **1997**, *153*, 357. *Chem. Abstr.* **1997**, *127*, 231635t.
11. Mojzych, M.; Rykowski, A.; Wierzchowski, J. *Molecules* **2005**, *10*, 1298.
12. Gucky, T.; Frysova, I.; Slouka, J.; Hajduch, M.; Dzubak, P. *Eur. J. Med. Chem.* **2009**, *44*, 891.
13. Gucky, T.; Reznickova, E.; Dzubak, P.; Hajduch, M.; Krystof, V. *Monatsh. Chem.* **2010**, *141*, 709.
14. Khan, K. M.; Maharvi, G. M.; Khan, M. T. H.; Perveen, S.; Choudhary, M. I.; Atta-ur-Rahman *Mol. Diversity* **2005**, *9*, 15.
15. Khan, K. M.; Maharvi, G. M.; Perveen, S.; Khan, M. T. H. *Chem. Biodivers.* **2005**, *2*, 470.
16. El-Abadelah, M. M.; Sabri, S. S.; Khanfar, M. A.; Yasin, H. A.; Voelter, W. *J. Heterocycl. Chem.* **2002**, *39*, 1055.
17. Toque, H. A.; Teixeira, C. E.; Lorenzetti, R.; Okuyama, C. E.; Antunes, E.; De Nucci, G. *Eur. J. Pharmacol.* **2008**, *591*, 189.
18. (a) Mojzych, M.; Rykowski, A. *Polish J. Chem.* **2003**, *77*, 1797; (b) Mojzych, M.; Rykowski, A. *J. Heterocycl. Chem.* **2007**, *44*, 1003.
19. Alphonse, F. A.; Suzenet, F.; Keromnes, A.; Lebret, B.; Guillaumet, G. *Synlett* **2002**, 447.
20. (a) Vasilevsky, S. F.; Klyatskaya, S. V.; Tretyakov, E. V.; Elquero, J. *Heterocycles* **2003**, *60*, 879; (b) Zhang, H.-C. H.; Daves, G. D., Jr. *J. Org. Chem.* **1992**, *57*, 4690.
21. Kongo, K. H.; Park, S. Y.; Hong, M. P.; Cho, S. H. *Comp. Biochem. Physiol., B: Biochem. Mol. Biol.* **2000**, *125*, 563.
22. Selinheimo, E.; Saloheimo, M.; Ahola, E.; Westerholm-Parvinen, A.; Westerholm-Parvinen, A.; Kalkkinen Buchert, N. J.; Kruus, K. *FEBS J.* **2006**, *273*, 4322.
23. Selinheimo, E.; NiEidhin, D.; Steffensen, C.; Nielsen, J.; Lomascolo, A.; Halaouli, S.; Record, E.; O'Beirne, D.; Buchert, J.; Kruus, K. *J. Biotechnol.* **2007**, *130*, 471.
24. (a) Chang, T.-S. *Int. J. Mol. Sci.* **2009**, *10*, 2440; (b) Muñoz-Muñoz, J. L.; García-Molina, F.; García-Ruiz, P. A.; Molina-Alarcón, M.; Tudela, J.; García-Cánovas, F.; Rodríguez López, J. N. *Biochem. J.* **2008**, *416*, 431.
25. Jiang, Y.; Du, Z.; Xue, G.; Chen, Q.; Lu, Y.; Zheng, X.; Conney, A. H.; Zhang, K. *Molecules* **2013**, *18*, 3948.
26. Mojzych, M.; Karczmarzyk, Z.; Kalicki, P.; Ceruso, M.; Supuran, C. T. *Bioorg. Med. Chem.* under preparation.
27. Card, G. L.; England, B. P.; Suzuki, Y.; Fong, D.; Powell, B.; Lee, B.; Luu, C.; Tabrizizad, M.; Gillette, S.; Ibrahim, P. N.; Artis, D. R.; Bollag, G.; Milburn, M. V.; Kim, S. H.; Schlessinger, J.; Zhang, K. Y. *Structure* **2004**, *12*, 2233.
28. Laemmli, U. K. *Nature* **1970**, *227*, 680.
29. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
30. Dolashki, A.; Voelter, W.; Gushterova, A.; Van Beeumen, J.; Devreese, B.; Tchorbanov, B. *Protein Pept. Lett.* **2012**, *19*, 538.