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SYNTHESIS OF (BENZIMIDAZOL-2-YL)ANILINE DERIVATIVES AS GLYCOGEN PHOSPHORYLASE INHIBITORS

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Abstract

A series of (benzimidazol-2-yl)-aniline (**1**) derivatives has been synthesized and evaluated as glycogen phosphorylase (GP) inhibitors. Kinetics studies revealed that compounds displaying a lateral heterocyclic residue with several heteroatoms (series 3 and 5) exhibited modest inhibitory properties with IC_{50} values in the 400-600 µM range. Arylsulfonyl derivatives **7** (Ar: phenyl) and **9** (Ar: *o*-nitrophenyl) of **1** exhibited the highest activity (series 2) among the studied compounds (IC_{50} 324 µM and 357 µM, respectively) with stronger effect than the *p*-tolyl analogue **8.**

Keywords: Benzimidazole ; Heterocycles ; glycogen phosphorylase ; enzyme inhibition

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1. Introduction

Alkaloids and generally nitrogen heterocycles are natural and synthetic compounds, displaying a wide range of properties and activities. They are frequently used as chemicals and for crop or health protection. This applies to indole [1] and benzimidazole derivatives, [2, 3] two sub-classes having natural representatives, as tryptophan and neurotransmitter serotonin or *N*-ribosyl-dimethylbenzimidazole the axial ligand for cobalt in vitamin B_{12}. Indole and benzimidazole scaffolds are important pharmacophores for drug discovery as they are good bioisosteres of adenine and guanine present in nucleosides and nucleotides. As revealed by academic and industrial researches, indole [4] and benzimidazole derivatives, [5-7] represent privileged substructures which may interact with proteins, enzymes, and biomolecules. We currently developed synthetic routes toward a number of benzimidazole derivatives exhibiting cytotoxic, [8] antitumor [9-12] antiangiogenic, [13, 14] or analgesic properties, [15, 16] while others showed potent activity against HSV-1, [17] or led to metal complexes studied as topoisomerase II inhibitors.[17] Novel benzimidazole derivatives, found activators of AMP-protein kinase, have been patented for use in the treatment, prevention and suppression of diseases mediated by the AMPK-activated protein kinase. [18, 19] A collaborative work has offered the opportunity for evaluating new benzimidazole derivatives as potential antidiabetic drugs.

Diabetes, particularly its predominant form type 2 diabetes mellitus (T2DM) represents a global health problem, characterized by elevated circulating glucose (hyperglycemia). Although the etiology of T2DM is unclear, the overall metabolic dysfunction is attributed to both relative insensitivity of glucose-metabolizing tissues (muscles, liver, fat tissues) to insulin, and to deficient insulin production from the pancreas. Diabetes management involves diet control, exercise, and pharmacological treatments. [20] As they may fail in normalizing glycemia, thus increasing the risk of severe long-term complications, ongoing investigations from both academia and pharmaceutical companies are addressing the potential identified targets, particularly glycogen phosphorylase (GP). This enzyme catalyzes glycogen degradation to glucose-1-phosphate in the muscles, and in the liver with final release of glucose to the blood stream. [21-25] Therefore, GP inhibition is considered a validated pharmacological approach, as a means of limiting hyperglycemia. [26- 29] Due to extensive studies based mostly on kinetics and X-ray crystallography, of the muscles, and liver isoforms (a brain isoform also exists[30]), GP (Fig. 1) is a well-known homodimeric enzyme [31] which displays a phosphorylation site and various binding sites. They have been characterized as the catalytic site (referred to as the active), the inhibitor, the allosteric, the new allosteric or indole site and the glycogen storage site.[32-34] A binding site, called benzimidazole binding site [35] was also

identified and recently, a quercetin-binding site. [36] However, the role of these two sites for the design of new potent GP inhibitors is yet to be elucidated.

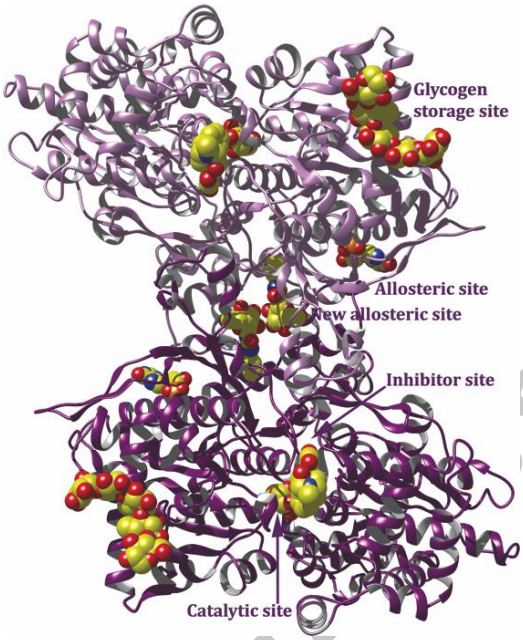
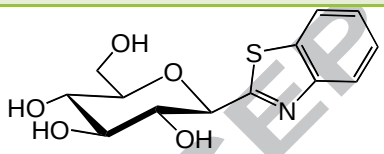
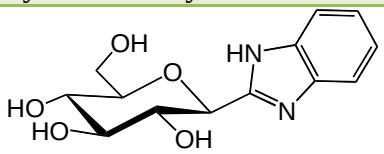
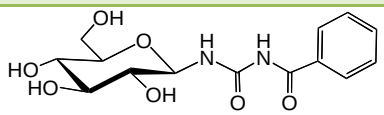
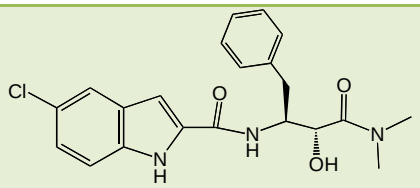
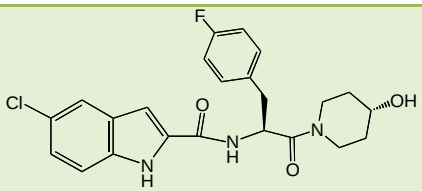
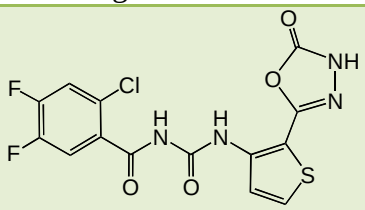


Fig. 1: The three dimensional structure of T-state rabbit muscle GP*b* shown where the distinct binding sites targeted for design of new potential antidiabetics are indicated. The figure was prepared using *Chimera*. [31]

The active site of GP accommodates the substrates glucose-1-phosphate and glycogen, and the inhibitors glucose and glucose analogues. Not surprisingly, glucose-based derivatives represent the most populated class of GP inhibitors. Among them, 2-(β-D-glucopyranosyl)-benzothiazole and benzimidazole (**A**, **B**), [37] proved to be moderate GP inhibitors (K_i against the unphosphorylated Rabbit Muscle GP, RMGP*b* are 229 µM [38] or 76 µM [35] and 11 µM [38] or 9 µM, [35] respectively). Both were found to bind at the enzyme catalytic site (as expected for glucose-based inhibitors) while promoting the inactive T-state [39] of the enzyme, thereby explaining the observed inhibitions. However, 2-(β-D-glucopyranosyl)-benzimidazole **B** was found to bind also at the new allosteric inhibitor site (Fig. 1), and additionally at benzimidazole binding site, located at the protein surface, far removed (~32 Å) from the other binding sites. Interestingly, *N*-benzoyl-*N*-glucosyl-urea **C** was found to bind to the catalytic site, and to the new allosteric inhibitor site, as proved by crystallographic studies. [40] Even for glucose-based inhibitors, crystal analyses of the enzyme-ligand complexes might reveal unusual binding modes as for benzimidazole, and acylurea-derived structures **B** and **C**.

Lead discovery by library screening revealed indole-carboxamide derivatives as **D** CP-91149, [41] **E** CP320626 and others, [42] as highly potent GP inhibitors (IC_{50} in the nanomolar range) showing synergism with glucose, e.g. lower IC_{50} in the presence of glucose (Table 1). Crystallographic studies showed their binding to the new allosteric site,[43, 44] also referred to as the indole-binding site, as it accommodates many indole-2-carboxamide-derived potent inhibitors with stabilization of the inactive T-conformation of GP. Located at the interface of two GP subunits, it is a 30 Å long central cavity with an indole site on each subunit. Consequently, bis-indole derivatives are among the most potent known allosteric indole inhibitors, [45] but for other potent allosteric inhibitors (e.g. **F**), the binding site or mechanism of inhibition have not been established. [46] Compounds of the chloroindole series have reached phase II clinical trials, but their development has been discontinued, as *in vivo* tests have shown glycogen accumulation in both liver and muscle.[24,45,48] While 2-nitrobenzimidazole has been recently reported to exhibit significant antihyperglycemic activity in alloxan-induced diabetic rats comparable to that of the antidiabetic sulfonylurea, glibenclamide, [48] the need for leads with an improved pharmacological profile still exists.[49] Although the pharmaceutical industries have continued their efforts in targeting glycogen phosphorylase it is more of the academic research groups that have a continuous interest in designing and developing new glycogen phosphorylase inhibitors. [50, 51]

Table 1: Selected GP inhibitors and their binding sites: glucose-based heterocycles, chloroindole-carboxamide derivatives and an analogue.

Glucose-derived inhibitors found to bind mostly at the catalytic site of GP		
 **A**	 **B**	 **C**
K_i (RMGP*b*) 229 µM, [38] 76 µM [35]	K_i (RMGP*b*) 11 µM [38] 9 µM [35] binding to GP was also observed at the indole-binding site and at the benzimidazole site [35]	K_i (RMGP*b*) 4.6 µM [39] binding to GP was also observed at the indole-binding site
Potent heterocyclic GP's inhibitors and binding sites:		
new allosteric / indole site	new allosteric / indole site	binding mode unknown
		

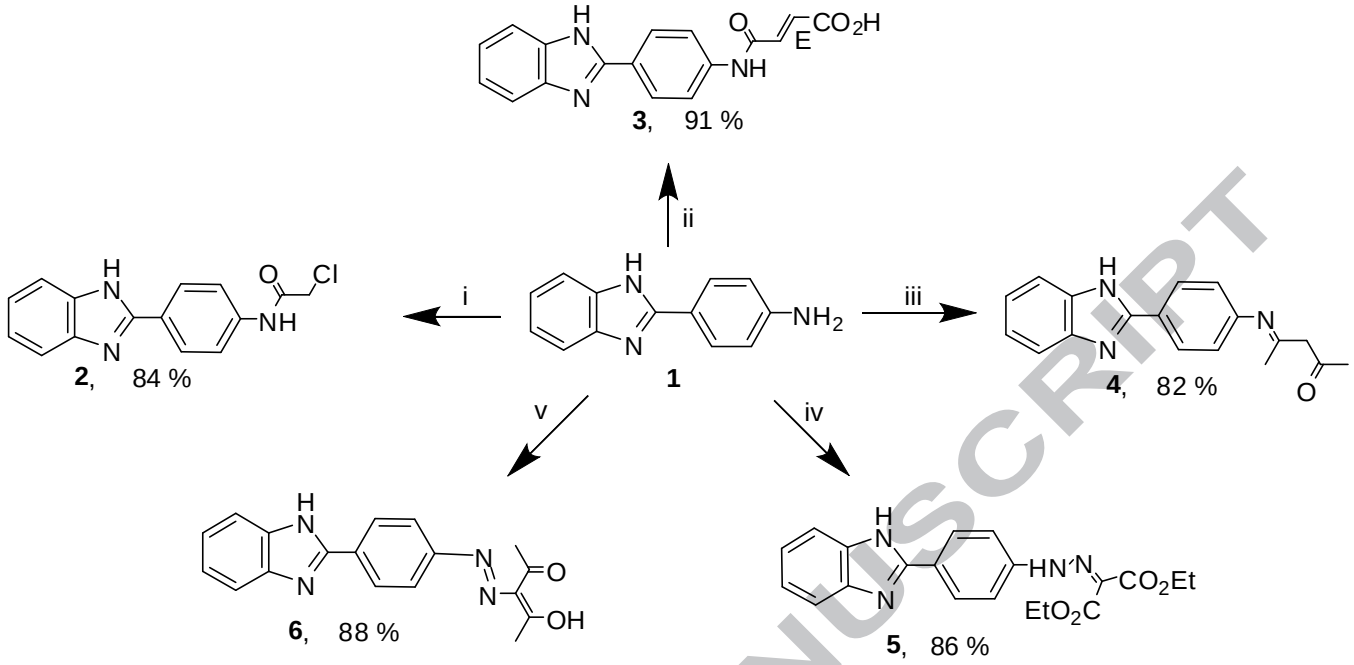
D CP-91149 Pfizer IC_{50} (rHLGP*a*) [41] 0.082 µM with [glucose] = 7.5 mM	**E** CP-320626 Pfizer IC_{50} (rHLGP*a*) [42], 0.2 µM with [glucose] = 7.5 mM IC_{50} (RMGP*b*) [43] 0.178 µM with [glucose] = 10 mM	**F** IC_{50} 30 µM (GP*a* potent cpd displaying a 1,3,4-oxadiazol-5-oxo moiety similar to the polyazacycles found in inhibitors (series 3 & 5)
RMGP*b*: unphosphorylated rabbit muscle glycogen phosphorylase. rHLGP*a* : phosphorylated recombinant human liver glycogen phosphorylase		

Therefore, on the basis of the above information, a series of new (benzimidazol-2-yl)aniline derivatives were synthesized by grafting various acyclic, arylsulfonyl, and heterocyclic residues to the amino group, and they were evaluated as potential GP inhibitors. The results obtained are presented hereafter.

2. Results and discussion

2.1. Chemistry

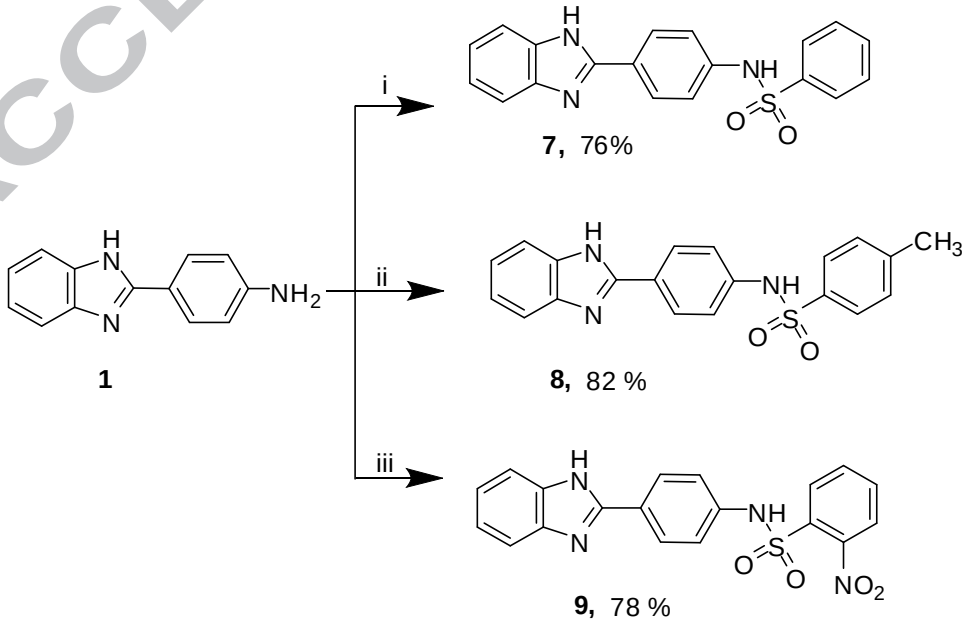
The starting material 4-(*1H*-benzo[*d*]imidazol-2-yl)aniline (**1**) [52] and compounds **2**, **3**, **6, 16, 17**, **18** and **21**[53, 54] (Schemes 1- 3) were known compounds which were synthesized according to described procedures. For the purpose of our study, we firstly prepared a series of derivatives of **1** by attaching a chain to its amino group (Scheme 1). Treatment of **1** with chloroacetyl chloride in DMF and using Et_3N, as a modification of the procedure described by Shahare *et al*, [52] led to 2-chloroacetamide derivative **2**, [52] while its reaction with maleic anhydride afforded compound **3**. [53] Compound **4** was obtained by treating compound **1** with acetylacetone in glacial acetic acid. Compounds **5** and **6** [54] were produced via two steps, involving firstly diazotization of compound **1** achieved by using HCl and $NaNO_2$ at 0 °C. The second step was the reaction of the crude diazonium chloride with diethyl malonate or acetyl acetone to form compounds diethyl 2-(2-(4-(1*H*-benzo[*d*]imidazol-2-yl)phenyl)hydrazono)malonate **(5)** or 3-((4-(1*H*-benzo[*d*]imidazol-2-yl)phenyl)diazenyl)-4-hydroxypent-3-en-2-one **(6),** respectively.[53]



Conditions: i- **1**, $ClCOCH_2Cl$, Et_3N, DMF; ii- **1**, maleic anhydride, toluene, reflux, 6h; iii- **1**, acetylacetone, glacial acetic acid, reflux, 10h; iv- **1** then a, 6*N* HCl, aq. $NaNO_2$, 0°C; b, diethyl malonate, ethanol, 5-10 °C; c, aq. NaOAc, rt; v- **1** then a, 6*N* HCl, aq. $NaNO_2$, 0°C; b, acetyl acetone, acetone, 5-10 °C; c, aq. NaOAc, rt.

Scheme 1: Synthetic routes to compounds **2-6 (**Series 1)

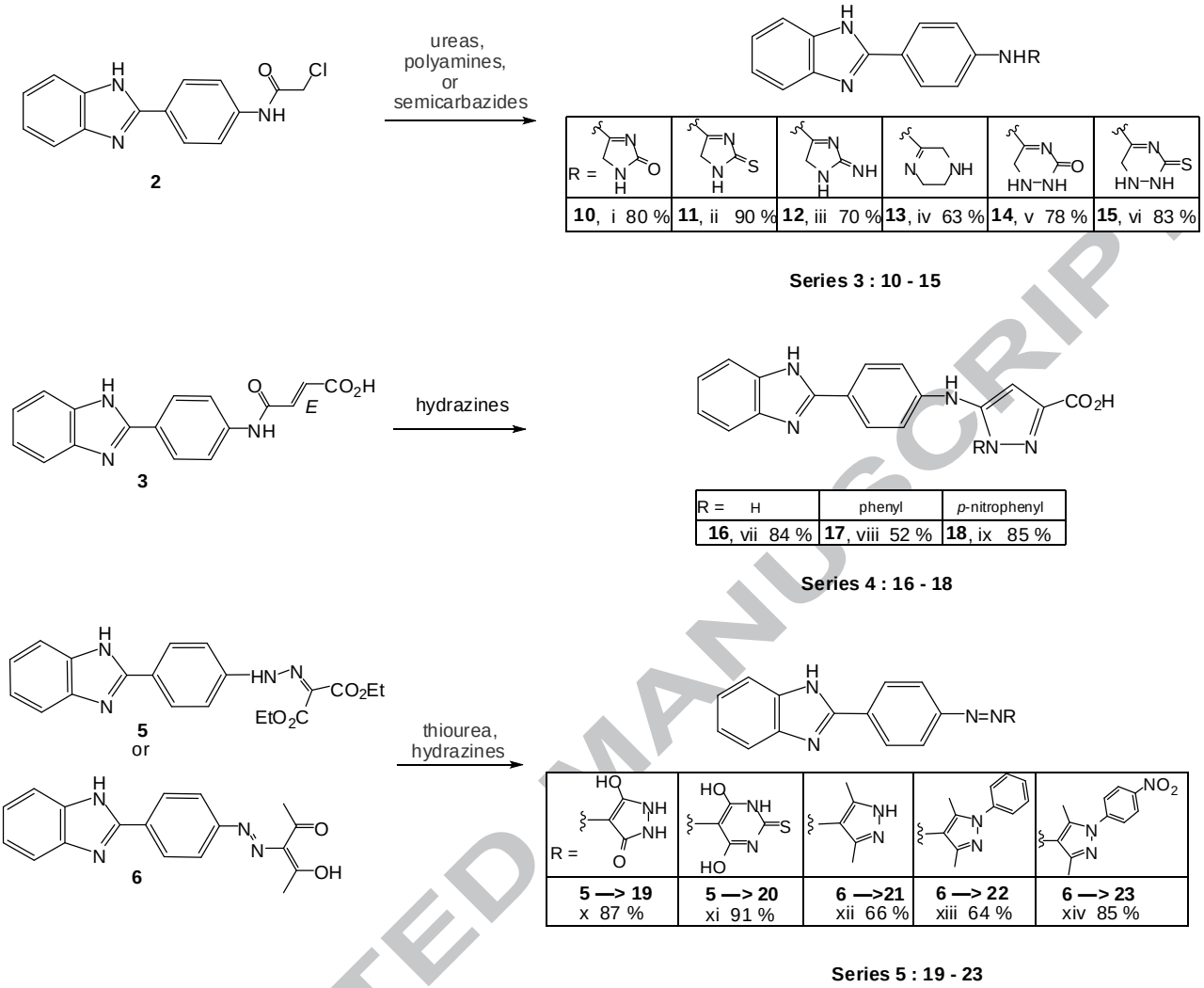
N-Sulfonyl derivatives **7-9** were synthesized by reacting compound **1** with benzene sulfonyl chloride, 4-tosyl chloride, 2-nitrobenzene sulfonyl choride in the presence of Et_3N **(**Scheme 2).



Conditions: i- **1**, benzenesulfonyl chloride, acetone, Et_3N, 2 h; ii- **1**, 4-toluenesulfonyl chloride, acetone, Et_3N, 2 h; iii- **1**, 2-nitrobenzenesulfonyl chloride, acetone, Et_3N, 2 h.

Scheme 2: Synthetic routes to compounds **7-9 (**Series 2)

The previously obtained derivatives **2**, **3**, **5** and **6** were subjected to nucleophilic reactions with ureas, polyamines, semicarbazides or hydrazines (Scheme 3) to afford compounds **10 - 23** (Series 3 – 5). The dihydroimidazoline derivatives **10-12** were prepared by cyclizing the chloroacetyl side chain of compound **2** with urea, thiourea or guanidine hydrochloride, respectively in the presence of anhydrous K_2CO_3. Reaction of **2**, under the same conditions, with ethylenediamine yielded the tetrahydropyrazine derivative **13**. Cyclization of the side chain in **2** using semicarbazide hydrochloride or thiosemicarbazide afforded compounds **14** and **15,** respectively. Reaction of **3** with hydrazine hydrate, phenyl hydrazine and 4-nitrophenyl hydrazine yielded compounds **16-18**. [53] Compound **5** was reacted with hydrazine hydrate to form compound 4-(2-(4-(1*H*-benzo[*d*]imidazol-2-yl)phenyl)hydrazono)pyrazolidine-3,5-dione **(19).** Reaction of compound **5** with thiourea yielded the pyrimidine derivative **20**. On the other hand, compound **6** was reacted with different hydrazines as hydrazine hydrate, [54] phenylhydrazine and 4-nitrophenylhydrazine to produce the pyrazoles **21**, [54] **22** and **23,** respectively.



Conditions: i- **2**, urea, K_2CO_3, DMF, reflux, 48 h; ii- **2**, thiourea, K_2CO_3, DMF, reflux, 48 h; iii- **2**, guanidine hydrochloride, K_2CO_3, DMF, reflux, 48 h; iv- **2**, ethylenediamine, K_2CO_3, DMF, reflux, 48 h; v- **2**, semicarbazide hydrochloride, K_2CO_3, DMF, reflux, 48 h; vi- **2**, thiosemicarbazide, K_2CO_3, DMF, reflux, 48 h; vii- **3**, hydrazine hydrate, EtOH, reflux, 3 h; viii- **3**, phenylhydrazine, K_2CO_3, DMF, reflux, 8 h; ix- **3**, 4-nitrophenylhydrazine, K_2CO_3, DMF, reflux, 8 h; x- **5**, hydrazine hydrate 98%, EtOH, reflux, 3h; xi- **5**, thiourea, EtONa/EtOH, reflux, 26h; xii- **6**, hydrazine hydrate 98%, EtOH, reflux, 3h; xiii- **6**, phenylhydrazine, K_2CO_3, DMF, reflux, 8h; xiv- **6**, 4-nitrophenyl hydrazine, K_2CO_3, DMF, reflux, 8h.

Scheme 3: Synthetic routes to compounds **10**-**23 (**Series 3-5)

2.2. Kinetic evaluation of the synthesized (benzimidazol-2yl)aniline derivatives as RMGP*b* inhibitors.

The synthesized compounds were assayed for their inhibition potency against RMGP*b,* in the direction of glycogen synthesis. All compounds exhibited very poor solubility in water

therefore, they were initially in 100% DMSO and serial dilutions were then prepared to 10 or 20 % DMSO (1 to 2 % in the reaction). Compounds **2**, **4-6**, **7-9**, **15** were partially soluble in aq. DMSO (20%), while compound **16** was insoluble in DMSO and hence could not be tested. No inhibition was observed for compounds **1**, **2**, **5**, **6**, and **13**, while compounds **3**, **4**, **8**, **17-20** were only weak inhibitors (Table 2). Moderate inhibition was shown by compounds **10** - **12**, **14-15**, **21-23** that exhibited IC_{50} values in range of 400-600 µM. All these modest inhibitors belonged to series 3 and 5, which displayed a lateral heterocycle. The arylsulfonyl derivatives of **1**, **7** (Ar: phenyl) and **9** (Ar: *o*-nitrophenyl) exhibited the strongest inhibition with IC_{50} of 324 µM and 357 µM, respectively. Considering that the solubility of the compounds was poor and the inhibitor solutions were saturated it could be assumed that the IC_{50} values were even less than the ones stated. With the aim to explain the kinetic results obtained, structural studies were performed with the most potent ligands by soaking native preformed crystals of RMGP*b* in inhibitor solution. No binding was detected, though. Cocrystallization studies of (benzimidazol-2-yl)aniline derivatives are under investigation to shed light on their inhibitory effect and binding mode. Introduction of more polar substituents might have enhanced affinity and potency of these compounds.

Table 2: Kinetic data for compounds 1-23 tested as RMGP*b* inhibitors.

Compound	Inhibition (%) or IC_{50} (µM)	Compound	Inhibition (%) or IC_{50} (µM)	Compound	Inhibition (%) or IC_{50} (µM)
Series 1		**Series 3**		**Series 5**	
1	NI	**10**	397.0 ± 91.0	**19**	29.4 % inhibition at 500µM
2	NI	**11**	531.2 ± 89.6	**20**	16.1 % inhibition at 200µM
3	45.0 % inhibition at 1000µM	**12**	515.9 ±16.9	**21**	621.9 ± 99.3
4	7.0 % inhibition at 200µM	**13**	NI	**22**	402.2 ± 19.7
5	NI	**14**	437.0 ± 10.8	**23**	532.3 ± 11.9
6	NI	**15**	508.3 ± 61.0		
Series 2		**Series 4**			
7	324.2 ± 47.9	**16**	-		
8	20.7 % inhibition at 500µM	**17**	25.0 % inhibition		

			at 300µM		
9	357.5 ± 63.5	**18**	8.1 % inhibition at 250µM		
NI: No Inhibition					

2.3. Molecular physiochemical properties

The molecular physiochemical properties (ALOGPS 2.1) of arylsulfonyl derivatives **7** and **9** were evaluated to study how these compounds meet Lipinski rules (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp). Table 3 displays the relevant parameters (molecular mass, hydrogen bond donor, hydrogen bond acceptor, logP, molar refractivity) calculated, and the acceptable range for applicability.

Table 3: Molecular physiochemical properties of arylsulfonyl derivatives 7 and 9

Compound	Molecular mass	H-bond donor	H-bond acceptor	logP	Molar refractivity
7	349	2	4	4.9	98.1
9	394	2	6	5.0	105.5
Applicability	< 500	< 5	< 10	< 5	40 - 130

It appears that both compounds comply with the Lipinsky rules, with a slight advantage for compound **7** (logP = 4.9), compared to **9**. Therefore, both compounds are suitable candidates for further developments aiming at identifying more potent glycogen phosphorylase inhibitors.

3. Conclusion

A series of (benzimidazol-2-yl)-aniline derivatives was investigated as potential RMGP*b* inhibitors, a thoroughly investigated target employed for the development of new antidiabetic pharmacological agents. Compounds **2-23** have been classified into five different series according to their lateral groups. (Benzimidazol-2-yl)-anilines with polyazacycles as in series 3 and 5 exhibited modest effect on RMGP*b* activity with IC_{50} in the 400-600 µM range. Reaction of **1** with arylsulfonyl chlorides produced (benzimidazol-2-yl)-benzene benzenesulfonamide derivatives **7-9**,

series 2, which guide us to compounds of the highest inhibitory effect among the studied derivatives with IC_{50} around 350 µM and could further be exploited as lead molecules for the design of specific inhibitors with increased affinity for the target.

4. Material and methods

4.1. Chemistry

Microanalyses and spectral data of the compounds were performed in National Research Centre, Cairo, Egypt. The IR spectra (4000-400 cm^{-1}) were recorded using KBr pellets in a Jasco FT/IR 300E Fourier transform infrared spectrophotometer on a Perkin Elmer FT-IR 1650 spectrophotometer. The ^{1}HNMR spectra were recorded using 500 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) from the tetramethylsilane resonance in the indicated solvent. Coupling constants are reported in Hertz (Hz); spectral splitting patterns are designed as follow: singlet (s); doublet (d); triplet (t); multiplet (m). Column chromatography was performed on Merck silica gel 60 (200–400 mesh). The mass spectra were recorded using a Finnigan mat SSQ 7000 (Thermo. Inst. Sys. Inc., USA) spectrometer at 70 eV. All chemicals and solvents were purchased from Sigma Chemical Company. All chemicals used were of analytical grade. The petroleum ether had a boiling temperature in the 60-80 °C range.

4.1.1. 4-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenylimino)pentan-2-one (4).

To a solution of compound **1** (1.67 g, 8 mmol) in glacial acetic acid (10 mL), acetylacetone (0.78 mL, 7.6 mmol) was added. The reaction mixture was refluxed for 10 h. The excess solvent was evaporated under reduced pressure. The formed solid was washed with ethyl acetate and recrystallized from acetone (82% yield). M.p.: 302-304 °C, R_f = 0.52 (ethyl acetate/pet. ether, 3:1). IR (KBr) υ_{max}/cm^{-1}: 3430 (NH benzimidazole); 3067 (CH arom); 2992, 2852 (CH aliph); 1674 (C=O); 1626, 1596 (C=N (s)); 1546 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 2.05 (s, 3H,CH_3-C=N); 2.41(s, 3H, CH_3-C=O); 3.53 (s, 2H, CH_2); 7.14 (m, 2H, H_5, H_6 benzimidazole); 7.56 (m, 2H, H_4, H_7 benzimidazole); 7.70 (*d*, 2H, *J* = 8.4 *Hz*, $H_{2`}$, $H_{6`}$ aminophenyl); 8.05 (d, 2H, *J* = 8.4 *Hz*, $H_{3`}$, $H_{5`}$ aminophenyl); 12.84 (br., 1H, NH benzimidazole, D_2O exchangeable). ^{13}C NMR (125MHz, DMSO-d_6): 24.64, 31.02 and 45.46 (aliph. carbons), 115.08, 119.52, 122.46, 125.22, 127.65, 133.67, 135.21, 141.33, 151.77 (Ar-C), 152.87 (C=N), 169.22 (C=O). MS, m/z (%): 292 (M^+ +1, 4%); 291 (M^+, 20%); 248 (54%); 233 (100%); 209 (81%). Anal. Calcd for $C_{18}H_{17}N_3O$ (FW: 291.14): C, 74.20; H, 5.88; N, 14.42. Found: C, 74.28; H, 5.81; N, 14.37.

4.1.2. Diethyl 2-(2-(4-(1*H*-benzo[*d*]imidazol-2-yl)phenyl)hydrazono)malonate (5).

A cold solution of compound **1** (8.36 g, 0.04 mol) in 20 mL 6*N* HCl was prepared. In ice bath, a cold solution of $NaNO_2$ (2.21 g, 0.032 mol) in the minimum quantity of cold water was added portionwise with continuous stirring. The resultant diazonium salt was added to a solution of diethyl malonate (6.16 mL, 0.06 mol) in ethanol. The pH of the mixture was adjusted to 6.5 using sodium acetate solution. The yellow precipitate was collected by vacuum filtration, dried (86% yield) and recrystallized from ethyl acetate. M.p.: 230-232 °C, R_f = 0.68 (ethyl acetate/pet. ether, 1:1), IR (KBr) υ_{max}/cm^{-1}: 3392 (NH benzimidazole); 3332 (NH aminophenyl); 3099, 3038 (CH arom); 2979, 2931 (CH aliph); 1666 (C=O); 1610 (C=N); 1526 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 1.26 (m, 6H, 2 CH_3); 4.23 (q, 2H, CH_2); 4.30 (q, 2H, CH_2); 7.16 (d, 2H, *J* = 8.4 *Hz*, $H_{2`}$, $H_{6`}$ aminophenyl); 7.51 (m, 4H, H_4, H_5, H_6, H_7 benzimidazole); 8.14 (d, 2H, *J* = 8.4 *Hz*, $H_{3`}$, $H_{5`}$ diazenylphenyl); 12.00 (s, 1H, NH aminophenyl, D_2O exchangeable); 12.92 (br., 1H, NH benzimidazole, D_2O exchangeable). ^{13}C NMR (125 MHz, DMSO-d_6): 14.39, 14.63 (aliph. CH_3), 61.42, 61.90 (aliph. CH_2), 111.73, 115.91, 119.10, 122.16, 122.85, 123.16, 125.94, 128.27, 135.54, 143.95, 144.38 (Ar-C), 151.54 (C=N), 162.18, 162.87 (C=O). MS, m/z (%): 380 (M^+, 46%); 335 (13%); 306 (17%); 261 (12%); 207 (100%). Anal. Calcd for $C_{20}H_{20}N_4O_4$ (FW: 380.15): C, 63.15; H, 5.30; N, 14.73. Found: C, 63.17; H, 5.36; N, 14.71.

4.1.3. ***General procedure for the preparation of compounds*** **7-9.**

To a well-stirred solution of compound **1** (10 mmol) and triethylamine (0.5 mL) in acetone, benzenesulfonyl chloride, 4-toluenesulfonyl chloride or 2-nitrobenzenesulfonyl chloride (10 mmol) was added dropwise. The reaction mixture was stirred for 2h at room temperature and left overnight. The solvent was evaporated under reduced pressure. The solid was collected, washed with water, dried. Purification by column chromatography was achieved using ethyl acetate/pet. ether (3:1 ratio) as the mobile phase.

4.1.3.1. ***N*****-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenyl)benzenesulfonamide (7).**

Yield: 76%. M.p. 238^o-240 C. TLC R_f = 0.69 (ethyl acetate/petroleum ether, 2:1). IR (KBr) υ_{max}/cm^{-1}: 3359,3230 (NHs); 3096, 3016, 2985 (CH arom); 1611 (C=N); 1588 (C=C arom); 1495 (υ_{as} SO_2), 1476 (υ_s SO_2). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 7.173(m, 2H, H_3, H_5 benzenesulfonyl moiety); 7.27 (d, 2H, *J* = 7.65 *Hz*, $H_{2`}$, $H_{6`}$ aminophenyl moiety); 7.523-7.832 (m, 3H, H_4 benzenesulfonyl moiety, H_5, H_6 benzimidazole moiety); 7.836-7.853 (d, 2H, H_4, H_7 benzimidazole moiety); 8.022-8.044 (d, 2H, *J* = 7.65 *Hz*, $H_{3`}$, $H_{5`}$ aminophenyl moiety);10.65(s, 1H, NH sulphonamide, D_2O exchangeable), 12.76 (s, 1H, NH benzimidazole, D_2O exchangeable) .

MS, m/z (%): 349(M^+, 15%); 97 (100%). ^{13}C-NMR (DMSO-d_6, 125 MHz, δ *ppm*):112.11, 119.25, 119.77, 120.11, 122.47, 126.23, 126.86, 127.18, 128.03, 129.55, 129.81, 134.65, 135.31, 135.58, 138.93, 139.68, 139.91, 151.31. Anal. Calcd for $C_{19}H_{15}N_3O_2S$ (FW: 349): C, 65.31; H, 4.33; N, 12.03; S, 9.18. Found: C, 65.66 H, 4.47; N, 12.18; S, 9.31.

4.1.3.2. *N*-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenyl)-4-methylbenzenesulfonamide (8)

Yield: 82%. M.p.: 230-232 °C, R_f = 0.60 (ethylacetate/pet. ether, 2:1). IR (KBr) υ_{max}/cm^{-1}: 3373 (NH aminophenyl); 3061 (CH arom); 2922 (CH aliph); 1610 (C=N); 1596 (C=C arom); 1438 (υ_{as} SO_2), 1375 (υ_s SO_2). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 2.30 (s, 3H, CH_3); 7.07 (d, 2H, *J* = 9.2 *Hz*, $H_{2`}$, $H_{6`}$ aminophenyl); 7.34 (m, 2H, $H_3$and H_5 tosyl); 7.43 (m, 2H, H_2 and H_6 tosyl); 7.71 (m, 2H, H_5, H_6 benzimidazole); 7.84 (m, 2H, H_4, H_7 benzimidazole); 8.01 (d, 2H, *J* = 9.2 *Hz*, $H_{3`}$, $H_{5`}$ aminophenyl); 10.91 (s, 1H, NH sulphonamide, D_2O exchangeable). 12.75, (s, 1H, NH,benzimidazole, D_2O exchangeable). MS, m/z (%):363 (M^+, 14%); 347 (50%); 208 (100%). Anal. Calcd for $C_{20}H_{17}N_3O_2S$ (FW: 363): C, 66.10; H, 4.71 N, 11.56; S, 8.82. Found: C, 66.42; H, 4.95; N, 11.14; S, 8.33.

4.1.3.3. *N*-(4-(1H-Benzo[*d*]imidazol-2-yl)phenyl)-4-nitrobenzenesulfonamide (9).

Yield: 78%. M.p.: 215-217 °C, R_f= 0.42 (EtAc/Pet. ether 2:1). IR (KBr) υ_{max}/cm^{-1}: 3374 (NH aminophenyl); 3029, 2974 (CH arom); 1611 (C=N); 1567 (C=C arom); 1513 (υ_{as} NO_2); 1466 (υ_{as} SO_2); 1437 (υ_s NO_2); 1394 (υ_s SO_2). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 7.39 (d, 2H, *J* = 8.4 *Hz*, $H_{2`}$, $H_{6`}$ aminophenyl moiety); 7.47 (m, 2H, H_5, H_6 benzimidazole moiety); 7.53 (m, 1H, H_4 nitrophenyl); 7.73 (m, 2H, H_4, H_7 benzimidazole moiety); 7.86 (m, 2H, H_5 and H_6 nitrophenyl moiety); 8.00 (m, 1H, H_3, nitrophenyl moiety); 8.06 (d, 2H, *J* = 8.4 *Hz*, $H_{3`}$, $H_{5`}$ aminophenyl moiety); 11.43 (br., 1H, NH aminophenyl, exchangeable). MS, m/z (%): (M^+-1, 60%); 271 (28%); 208 (100%). Anal. Calcd for $C_{19}H_{14}N_4O_4S$ (FW: 394): C, 57.86; H, 3.58; N, 14.21; S, 8.13. Found: C, 57.44; H, 3.41; N, 14.09; S, 8.41.

***4.1.4. General procedure for the preparation of compounds* 10-15:**

Compound **2** (1.99 g, 7 mmol) was added to a solution of urea, thiourea, guanidine hydrochloride, ethylenediamine, semicarbazide hydrochloride or thiosemicarbazide (7 mmol) and K_2CO_3 (7 mmol) in DMF (20 mL) with gentle stirring at r.t. for 1 h. Then, the reaction mixture was refluxed for appropriate time. The products formation was monitored by TLC. After reaction completion, the reaction mixture was poured onto crushed ice with continuous stirring. The formed solids were collected by vacuum filtration. The crude solid was purified by column chromatography using ethyl acetate/pet. ether (2:1) as eluent.

4.1.4.1. 5-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenylamino)-1*H*-imidazol-2(5*H*)-one (10).

Yield: 80%. M.p.: 222-224 °C, crystallized from DMF, R_f = 0.57 (ethyl acetate/pet. ether/ EtOH, 3:1:0.5). IR (KBr) υ_{max}/cm^{-1}: 3419 (NH benzimidazole); 3340, 3279 (NH(s)); 3014 (CH arom); 2924 (CH aliph); 1667 (C=O); 1625, 1602, 1578 (C=N(s)); 1543 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 5.46 (s, 2H, CH_2 imidazolone); 7.09 (s, 1H, NH, D_2O exchangeable); 7.50 (m, 2H, H_5, H_6 benzimidazole); 7.87 (m, 4H, H_4, H_7 benzimidazole and $H_{2`}$, $H_{6`}$ aminophenyl moieties); 8.29 (d, 2H, *J* = 7.65, $H_{3`}$, $H_{5`}$ aminophenyl); 11.41(br., NH, D_2O exchangeable) 12.51 (br., NH benzimidazole, D_2O exchangeable). MS, m/z (%): 291 (M^+, 43%); 234 (73%); 209 (100%). Anal. Calcd for $C_{16}H_{13}N_5O$ (FW: 291.11): C, 65.97; H, 4.50; N, 24.04. Found: C, 65.89; H, 4.44; N, 24.10.

4.1.4.2. 5-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenylamino)-1*H*-imidazole-2(5*H*)-thione (11).

90% Yield; M.p.: 308-310 °C, crystallized from DMF, R_f = 0.6 (ethyl acetate/pet. ether/ EtOH, 3:1:0.5). IR (KBr) υ_{max}/cm^{-1}: 3360 (NH benzimidazole); 3274, 3210 (NH (s); 3053 (CH arom); 2888 (CH aliph); 1627, 1601 (C=N(s)); 1543 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 3.60 (s, 2H, H_2 imidazolethione); 7.07 (s, 1H, NH, D_2O exchangeable); 7.22 (m, 2H, H_5, H_6 benzimidazole); 7.58 (m, 4H, H_4, H_7 benzimidazole and $H_{2`}$, $H_{6`}$ aminophenyl moieties); 8.11 (d, 2H, *J* = 7.65, $H_{3`}$, $H_{5`}$ aminophenyl); 10.36 (s, 1H, NH, D_2O exchangeable); 11.55 (br., 1H, NH benzimidazole, D_2O exchangeable). MS, m/z (%): 309 (M+2, 2%), 307 (M^+, 59%); 275 (33%); 234 (76%); 209 (100%). Anal. Calcd for $C_{16}H_{13}N_5S$ (FW: 307.09): C, 62.52; H, 4.26; N, 22.78; S, 10.43. Found: C, 62.57; H, 4.30; N, 22.75; S, 10.49.

4.1.4.3. N^5-(4-(*1H*-Benzo*[d]*imidazol-2-yl)phenyl)-*4H*-imidazole-2,5-diamine (12).

70% Yield; M.p.: 228-231 °C, crystallized from DMF, R_f = 0.64 (ethyl acetate/pet. ether/ EtOH, 3:1:0.5). IR (KBr) υ_{max}/cm^{-1}: 3431 (NH benzimidazole); 3370, 3308, 3220 (NH(s) and NH_2 of enamine form); 3060 (CH arom); 2859 (CH aliph); 1620, 1604, 1589 (C=N(s)); 1541 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 5.11 (s, 2H, H_2 imidazole amine); 5.57 (br., NH_2, D_2O exchangeable); 7.07 (m, 2H H_5, H_6 benzimidazole); 7.15 (m, 2H, H_4, H_7 benzimidazole), 7.53 (d, 2H, *J* = 7.65, $H_{2`}$, $H_{6`}$ aminophenyl); 8.11 (d, 2H, *J* = 7.65, $H_{3`}$, $H_{5`}$ aminophenyl); 9.95 (s, 1H, NH, D_2O exchangeable); 10.75 (s, 1H, NH, D_2O exchangeable); 12.81 (br., 1H, NH benzimidazole, D_2O exchangeable). MS, m/z (%): 290 (M^+, 16%); 234 (47%); 210 (36%); 209 (100%). Anal. Calcd for $C_{16}H_{14}N_6$ (FW: 290.13): C, 66.19; H, 4.86; N, 28.95. Found: C, 66.23; H, 4.81; N, 28.90.

4.1.4.4. *N*-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenyl)-3,4,5,6-tetrahydropyrazin-2-amine (13).

63% Yield; M.p.: 316-318 °C, crystallized from DMF, R_f = 0.64 (ethyl acetate/pet. ether/ EtOH, 3:1:0.5). IR (KBr) υ_{max}/cm^{-1}: 3445 (NH benzimidazole); 3312, 3207; (NH(s)); 3055 (CH arom); 2866 (CH aliph); 1628, 1604 (C=N(s)); 1506 (C=C arom.). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 3.41 (t, 2H, CH_2 pyrazinyl); 3.47 (t, 2H, CH_2 pyrazinyl); 3.54 (s, 2H, CH_2 pyrazinyl); 6.74 (m, 2H, H_5, H_6 benzimidazole); 7.44 (d, 2H, *J* = 7.65, $H_{2`}$, $H_{6`}$ aminophenyl); 7.68 (d, 2H, *J* = 7.65, $H_{3`}$, $H_{5`}$ aminophenyl); 8.01 (m, 2H, H_4, H_7 benzimidazole); 8.31 (s, 1H, NH, D_2O exchangeable); 10.47 (s, 1H, NH, D_2O exchangeable); 12.89 (br., 1H, NH benzimidazole, D_2O exchangeable). MS, m/z (%): 291 (M^+, 46%); 234 (23%); 210 (100%). Anal. Calcd for $C_{17}H_{17}N_5$ (FW: 291.15): C, 70.08; H, 5.88; N, 24.04. Found: C, 70.11; H, 5.85; N, 24.00.

4.1.4.5. 5-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenylamino)-1,2-dihydro-1,2,4-triazin-3(6*H*)-one (14).

78% Yield; M.p. 270-272 °C, crystallized from DMF, R_f = 0.76 (ethyl acetate/pet. ether/ EtOH, 3:1:0.5). IR (KBr) υ_{max}/cm^{-1}: 3400 (NH benzimidazole); 3315- 3280, (NH(s)); 3059 (CH arom); 2928 (CH aliph); 1674 (C=O); 1626, 1606, 1587 (C=N(s)); 1537 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 5.55 (s, 2H, CH_2 triazinone); 6.81 (s, 1H, NH, D_2O exchangeable); 7.54 (m, 2H, H_5, H_6 benzimidazole); 7.90 (m, 4H, H_4, H_7 benzimidazole and $H_{2`}$, $H_{6`}$ aminophenyl moieties); 8.39 (d, 2H, *J* = 7.65, $H_{3`}$, $H_{5`}$ aminophenyl); 10.25 (s, 1H, NH, D_2O exchangeable); 11.01 (br., NH benzimidazole, D_2O exchangeable); 11.77 (s, 1H, NH, D_2O exchangeable). MS, m/z (%): 306 (M^+, 36%); 234 (59%); 209 (100%). Anal. Calcd for $C_{16}H_{14}N_6O$ (FW: 306.12): C, 62.74; H, 4.61; N, 27.44. Found: C, 62.77; H, 4.65; N, 27.42.

4.1.4.6. 5-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenylamino)-1,2-dihydro-1,2,4-triazin-3(6*H*)-thione (15).

83% Yield; M.p.: 202-204 °C crystallized from DMF, R_f = 0.73 (ethylacetate/pet. ether/ EtOH, 3:1:0.5). IR (KBr) υ_{max}/cm^{-1}: 3429 (NH benzimidazole); 3340, 3260 (NH(s)); 3090, 2923 (CH arom); 2857 (CH aliph); 1627; 1609, 1576 (C=N(s)); 1552 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 3.54 (s, 2H, CH_2 triazinethione); 7.15 (m, 2H, H_5, H_6 benzimidazole); 7.47 (m, 1H, H_4 benzimidazole); 7.59 (m, 1H, H_7 benzimidazole); 7.73 (d, 2H, *J* = 7.65, $H_{2`}$, $H_{6`}$ aminophenyl); 8.09 (d, 2H, *J* = 7.65, $H_{3`}$, $H_{5`}$ aminophenyl); 10.38 (s, 1H, NH, D_2O exchangeable); 10.61 (s, 1H, NH, D_2O exchangeable); 12.78 (br., 1H, NH benzimidazole, D_2O exchangeable). MS, m/z (%): 324 (M^+ +2, 4%); 322 (M^+, 89%); 290 (38%); 234 (69%); 209 (100%). Anal. Calcd for $C_{16}H_{14}N_6S$ (FW: 322.10): C, 59.61; H, 4.38; N, 26.07; S, 9.95. Found: C, 59.56; H, 4.33; N, 26.12; S, 9.90.

4.1.4.7. 4-(2-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenyl)diazenyl)-1,2-dihydro-3-hydroxypyrazol-5-one (19).

A mixture of compound **5** (1.33 g, 3.5 mmol) and hydrazine hydrate (0.49 mL, 10 mmol) in ethanol (40 mL) was refluxed for 3h. The excess solvent was evaporated under reduced pressure and then, the reaction mixture was poured onto crushed ice. The formed solid was collected by vacuum filtration, dried and recrystallized (87% yield) from EtOH. M.p.: 318-320 °C, R_f = 0.66 ($CHCl_3$/MeOH, 3:0.5), IR (KBr) υ_{max}/cm^{-1}: 3650-2400 (H-bonded OH & NH); 3404 (NH benzimidazole); 3263, 3220 (NH pyrazole); 3060 (CH arom); 1656 (C=O); 1609 (C=N); 1536 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 7.17 (m, 2H, H_5, H_6 benzimidazole); 7.54 (m, 2H, H_4, H_7 benzimidazole); 7.64 (d, 1H, *J* = 8.4 *Hz*, $H_{2`}$ diazenylphenyl); 7.68 (d, 1H, *J* = 8.4 *Hz*, $H_{6`}$ diazenylphenyl); 8.10 (d, 1H, *J* = 8.4 *Hz*, $H_{3`}$ diazenylphenyl); 8.18 (d, 1H, *J* = 8.4 *Hz*, $H_{5`}$ diazenylphenyl); 9.72 (s, 1H, NH pyrazole, D_2O exchangeable); 10.57 (br., 1H, NH pyrazole, D_2O exchangeable); 13.18 (br., 1H, NH benzimidazole, D_2O exchangeable); 13.93 (s, 1H, OH, D_2O exchangeable). MS, m/z (%): 320 (M^+, 15%); 221 (19%); 208 (100%); 193 (48%); 117 (14%). Anal. Calcd for $C_{16}H_{12}N_6O_2$ (FW: 320.10): C, 60.00; H, 3.78; N, 26.24. Found: C, 60.04; H, 3.83; N, 26.23.

4.1.4.8. 5-(2-(4-(1*H*-Benzo[*d*]imidazol-2-yl)phenyl)diazenyl)-4,6-dihydroxypyrimidine-2(1*H*)-thione (20).

To a freshly prepared 0.64 molar solution of sodium ethoxide, thiourea (4 mmol) was added, and then the reaction was stirred at r.t. for one hour. Compound **5** (1.33 g, 3.5 mmol) was added, and then the reaction mixture was refluxed for 16h. The reaction mixture was left to cool, poured onto crushed ice with continuous stirring and the pH was adjusted to (7). The formed solid was collected by vacuum filtration, dried and recrystallized (91% yield) from ethanol. M.p.: 260-262 °C, R_f = 0.36 (ethyl acetate/pet. ether/C_2H_5OH, 1:1:0.5), IR (KBr) υ_{max}/cm^{-1}: 3427 (OH); 3278 (NH benzimidazole); 3211 (NH aminophenyl); 3044, 2994 (CH arom); 1606 (C=N); 1505 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 7.14 (m, 2H, H_5, H_6 benzimidazole); 7.22 (d, 2H, *J* = 8.4 *Hz*, $H_{2`}$, $H_{6`}$ aminophenyl); 7.52 (m, 2H, H_4, H_7 benzimidazole); 8.06 (d, 2H, *J* = 8.4 *Hz*, $H_{3`}$, $H_{5`}$ aminophenyl); 9.48, (s, 1H, NH, D_2O exchangeable), 11.39 (br., 1H, NH benzimidazole, D_2O exchangeable); 12.47, 12.65 (s, 1H, OH, D_2O exchangeable). MS, m/z (%): 366 (M^+ +2, 6%); 364 (M^+, 57%); 208 (100%); 193 (37%); 156 (24%). Anal. Calcd for $C_{17}H_{12}N_6O_2S$ (FW: 364.07): C, 56.04; H, 3.32; N, 23.06; S, 8.80. Found: C, 56.09; H, 3.38; N, 23.11; S, 8.77.

4.1.5. *General procedure for the preparation of compounds* 22 and 23.

Compound **6** (1.12 g, 3.5 mmol) was added to a mixture of phenylhydrazine or 4-nitrophenyl hydrazine (3.5 mmol) and anh. K_2CO_3 (0.48 g, 3.5 mmol) in 10 mL DMF and the reaction mixture was refluxed for 8h. The excess solvent was evaporated under reduced pressure. The reaction mixture was poured onto ice. The formed solid was collected by vacuum filtration, dried and recrystallized from EtOH.

4.1.5.1. 2-(4-((3,5-Dimethyl-1-phenyl-1*H*-pyrazol-4-yl)diazenyl)phenyl)-1*H*-benzo[*d*]imidazole (22).

64% Yield; M.p.: 224-226 °C (EtOH), R_f = 0.62 (ethyl acetate/pet. ether/MeOH, 3:1:0.5), IR (KBr) υ_{max}/cm^{-1}: 3419 (NH benzimidazole); 3154, 2984 (CH arom); 2922 (CH aliph); 1612, 1595 (C=N); 1547 (C=C arom). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 2.48 (s, 3H, CH_3); 2.63 (s, 3H, CH_3); 7.22 (m, 2H, H_5, H_6 benzimidazole); 7.48 (m, 1H, H_{4a} phenyl); 7.55 (m, 2H, H_4, H_7 benzimidazole); 7.59 (m, 4H, H_{2a}, H_{3a}, H_{5a}, H_{6a} phenyl); 7.93 (d, 2H, *J* = 7.6 *Hz*, $H_{2`}$, $H_{6`}$ diazenylphenyl); 8.31 (d, 2H, *J* = 7.6 *Hz*, $H_{3`}$, $H_{5`}$ diazenylphenyl); 12.37 (br., 1H, NH benzimidazole, D_2O exchangeable). MS, m/z (%):393 (M^+ +1, 93%); 285 (37%); 208 (82%). Anal. Calcd for $C_{24}H_{20}N_6$ (FW: 392.17): C, 73.45; H, 5.14; N, 21.41. Found: C, 73.50; H, 5.19; N, 21.44.

4.1.5.2. 2-(4-((3,5-Dimethyl-1-(4-nitrophenyl)-1*H*-pyrazol-4-yl)diazenyl)phenyl)-1*H*-benzo[*d*] imidazole (23).

85% Yield; M.p.: 270-272 °C (EtOH), R_f = 0.58 (ethyl acetate/pet. ether/MeOH, 3:1:0.5), IR (KBr) υ_{max}/cm^{-1}: 3412 (NH benzimidazole); 3048 (CH arom); 2923 (CH aliph); 1606 (C=N); 1594, 1563 (C=C arom), 1509 (υ_{as} NO_2), 1417 (υ_s NO_2). ^{1}H-NMR (DMSO-d_6, 500 MHz, δ *ppm*): 2.40 (s, 3H, CH_3); 2.51 (s, 3H, CH_3); 7.19 (m, 2H, H_5, H_6 benzimidazole); 7.51 (d, 2H, *J* = 8.4 *Hz*, $H_{2`}$, $H_{6`}$ diazenylphenyl) 7.65 (m, 2H, H_4, H_7 benzimidazole); 7.79 (d, 2H, *J* = 8.4 *Hz*, $H_{3`}$, $H_{5`}$ diazenylphenyl); 7.85 (d, 2H, *J* = 7.6 *Hz*, H_{2a}, H_{6a} nitrophenyl); 8.28 (d, 2H, *J* = 7.6 *Hz*, H_{3a}, H_{5a} nitrophenyl); 12.89 (br., 1H, NH benzimidazole, D_2O exchangeable). MS, m/z (%): 438 (M^+ +1, 24%); 361 (17%); 285 (48%); 210 (73%). Anal. Calcd for $C_{24}H_{19}N_7O_2$ (FW: 437.16): C, 65.89; H, 4.38; N, 22.41. Found: C, 65.96; H, 4.43; N, 22.40.

4.2. Kinetic evaluation of compound 1 – 23

RMGP*b* was isolated and purified with successive crystallization and recrystallization steps from rabbit skeletal muscle as described previouly.[55] Evaluation of the inhibitory potency of the compounds on RMGP*b* was performed in the direction of glycogen synthesis at 30 °C, pH 6.8 in

the presence of 5 µg/mL enzyme, 2 mM glucose-1-phosphate, 1 mM AMP and 1 % glycogen. The compounds tested exhibited very poor solubility in water, therefore they were dissolved in 100 % DMSO prior to kinetic experiments. Dilutions were prepared and their concentration in the reaction ranged from 5 µM to 1 mM in the presence of 1-2% DMSO. Enzyme activity was measured by the release of inorganic phosphate.[56, 57]

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SYNTHESIS OF (BENZIMIDAZOL-2-YL)ANILINE DERIVATIVES AS GLYCOGEN PHOSPHORYLASE INHIBITORS

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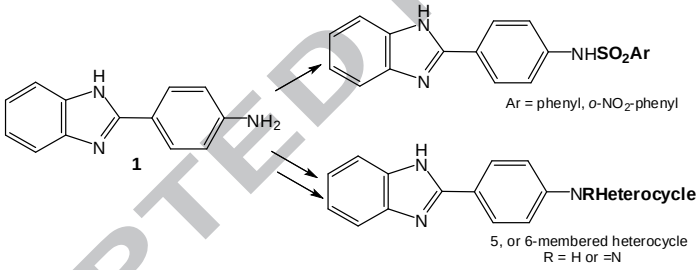

Arylsulfonyl derivatives of **1** showed higher inhibition (IC_{50} *ca* 350 μM) against **glycogen phosphorylase** as compared to other amino or hydrazino heterocyclic analogues (IC_{50} 400 - 600 μM).