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Graphical Abstract

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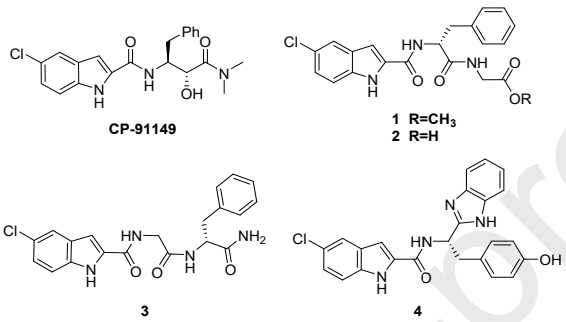

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Synthesis, *in vitro* ADME profiling and *in vivo* Pharmacological Evaluation of Novel Glycogen Phosphorylase Inhibitors

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ABSTRACT

A small set of indole-2-carboxamide derivatives identified from a high-throughput screening campaign has been described as a novel, potent, and glucose-sensitive inhibitors of glycogen phosphorylase a (GPa). Among this series of compounds, compound **2** exhibited moderate GP inhibitory activity (IC_{50} =0.29 μM), good cellular efficacy (IC_{50}= 3.24 μM for HepG2 cells and IC_{50} = 7.15 μM for isolated rat hepatocytes), together with good absorption, distribution, metabolism, and elimination (ADME) profiles. The *in vivo* animal study revealed that compound **2** significantly inhibited an increase of fasting blood glucose level in adrenaline-induced diabetic mice.

With the improvement of people's living standard, diabetes mellitus has gradually become the third major disease endangering human health. In adults, type 2 diabetes accounts for approximately 90-95% of all diagnosed cases of diabetes, and develops most often among middle-aged and older adults.[1] Inhibition of glycogen phosphorylase (GP) has been regarded as a therapeutic strategy for blood glucose control in diabetes, and various studies have shown the efficacy of GP inhibitors in lowering blood glucose in animal models of diabetes and in clinical trials.[2,3]

In the literature, there are several examples of small-molecule allosteric inhibitors of this enzyme, most of them are derived from a high-throughput screening.[4] As this class of molecules contains an indole-2-carboxamide moiety, as exemplified by **CP-91149** (Fig. 1), the binding site is termed the indole site (PDB code 1pyg).[5] **CP-91149** inhibits human liver GP with an IC_{50}=110 nM in the presence of 7.5 mM glucose, and was 5-10 times less potent in the absence of glucose.[6] Thus, it can be concluded that with low concentration of glucose, the inhibitory activity of these molecules is certainly lower than that with high glucose concentration. It is a significant advantage against the risk of hypoglycemia in patients with type 2 diabetes.

Since CP-91149 has poor water solubility (<1 mg/mL) and consequently low bioavaility, there are a lot of analogues have developed Herein, we report the discovery, synthesis, and biological evaluation of a small set of compounds prepared from a high-throughput screening in order to find more potent GP inhibitors with good pharmacokinetic properties. Moreover, *in vitro* DMPK studies and *in vivo* pharmacological evaluation are also presented.

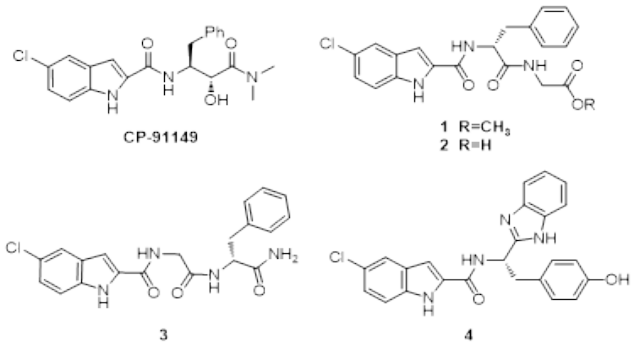
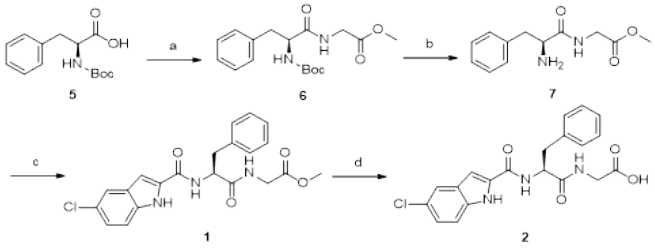


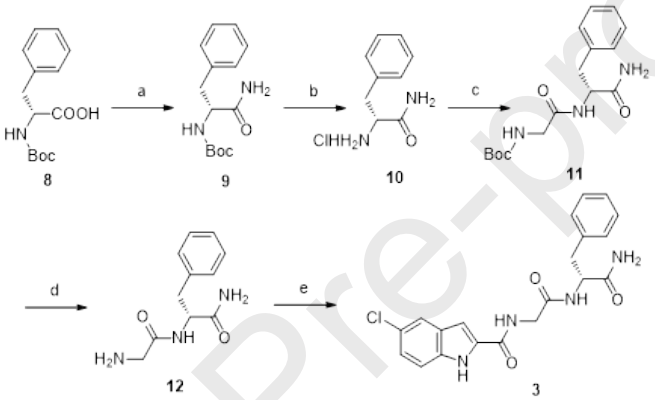
Figure 1. Structures of **CP-91149** and the target compounds **1-4**.

T…ester hydrochloride in the presence of EDCI in anhydrous DMF at room temperature afforded dipeptide ester **6**. Removal of the protected group from **6** with TFA furnished intermediate **7**, which was reacted with 5-chloroindole-2-carboxylic acid through the action of T_3P and DIEA to obtain compound **1**. Hydrolysis of **1** with LiOH provided carboxylic acid derivative **2**.



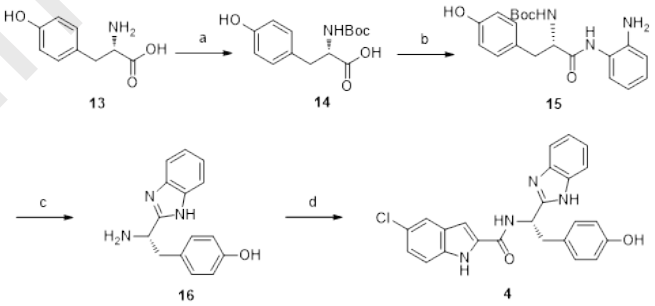
Scheme 1. Reagents and conditions: (a) Glycine methyl ester hydrochloride, EDCI, HOBT, DMAP, DIEA, r.t., 63%; (b) TFA, CH_2Cl_2, r.t.; (c) 5-chloroindole-2-carboxylic acid, T_3P, DIEA, CH_2Cl_2, 0 °C to r.t., 34%; (d) LiOH, THF, reflux, 52%.

As shown in Scheme 2, compound **3** was obtained in a similar manner starting from Boc-D-phenylalanine. In the presence of the condensation agent EDCI, Boc-D-phenylalanine **8** interacts with ammonium hydroxide to afford the corresponding amide **9**. Cleavage of the protecting group of **9** with 2 N HCl afforded hydrochloride salt **10**. Direct coupling of **10** with Boc-glycine through the action of EDCI and DIEA yielded compound **11**. Subsequent deprotection of compound **11** using TFA gave compound **12**, which was reacted with 5-chloroindole-2-carboxylic acid using the condensation agent EDCI to afford target compound **3**.



Scheme 2. Reagents and conditions: (a) ammonium hydroxide (28% in water), EDCI, HOBT, THF, r.t., 46%; (b) 2 N HCl(aq), r.t., 73%; (c) Boc-glycine, EDCI, HOBT, DIEA, DMF, r.t., 63%; (d) TFA, CH_2Cl_2, r.t., 85%; (e) 5-chloroindole-2-carboxylic acid, EDCI, HOBT, DIEA, 0 °C to r.t., 45%.

We also synthesized the weak hydrophilic benzimidazole derivative **4** by the method shown in Scheme 3. Reaction of L-tyrosine with $(Boc)_2O$ produced intermediate **14**. Direct coupling of **14** with *o*-phenylenediamine dihydrochloride in the presence of HATU and DIEA afforded the expected amide derivative **15**. Subsequently, in the presence of acetic acid, cyclization of amide **15** was completed via aldol reaction to obtain benzimidazole derivative **16**. Finally, amidation of **16** with 5-chloroindole-2-carboxylicacid yielded target compound **4**.



Scheme 3. Reagents and conditions: (a) $(Boc)_2O$, triethylamine, 50% 1,4-dioxane/water, 0 °C to 80 °C, 91%; (b) *o*-phenylenediamine dihydrochloride, HATU, DIEA, DMF, r.t. to 45 °C, 50%; (c) acetic acid, 60 °C, 59%; (d) 5-chloroindole-2-carboxylic acid, HATU, DIEA, DMF, r.t. to 45 °C, 50%.

The above synthesized derivatives were evaluated in an enzyme inhibition assay against rabbit muscle glycogen phosphorylase a (RMGPa) (Table 1) according to previous literature reports.[7] As described previously, the activity of rabbit muscle GPa was measured by detecting the release of phosphate from glucose-1-phoaphate in the direction of glycogen synthesis.[6] All the synthesized compounds showed good inhibitory activity. Among the compounds, dipeptide ester **1** (IC_{50} = 0.05 μM) showed the most efficient inhibition with a lower IC_{50} value than that of the positive control **CP-91149** (IC_{50} = 0.0922 μM). Derivative **2** with a free carboxylic function and derivative **3** with a weak hydrophilic benzimidazole group diminished inhibitory potency than the **CP-91149**. This may be due to the lipid/water partition coefficient and the group flexibility.

To evaluate the effects of all compounds in cells, glycogenolysis assays were conducted in both rat and human liver cells based on a

publ...tory activities. Interestingly, their inhibitory activities in both HepG2 cells and isolated rat hepatocytes showed a very similar trend with their activities in the GP inhibition assay. Among the compounds, compound **1** showed the best activity in HepG2 cells and isolated rat hepatocytes, with IC_{50} values of 0.67 μM and 0.72 μM, respectively. It was 5-fold more potent than the positive control **CP-91149** in HepG2 cells and 4-fold more potent than **CP-91149** in rat hepatocytes cells.

The effect of varying glucose concentration on the IC_{50} values of both compounds **1** and **2** is shown in Fig. 2. The data were normalized by dividing the IC_{50} obtained at different glucose concentrations by the IC_{50} observed in the absence of glucose. At high glucose concentrations, the relative IC_{50} values of both compounds **1** and **2** were decreased by 2- to 5-fold, respectively. Therefore, glucose had the same relative effect on the IC_{50} of both compounds **1** and **2**.

In order to explore molecular mechanism of action, compound **1** and **4** were docked into the binding site of human liver glycogen phosphorylase A (Fig. 3). The docking score values reflected the differences between their bioactivity (Table 1). These two compounds shared common protein-ligand interactions, including the hydrogen bonds generated with Arg60 and Glu190. However, because of their chirality, different orientations were taken by the two compounds. Additional two hydrogen bonds with Thr38' and Arg60' strengthened the protein-ligand interactions between compound a and the binding site. But for compound 4, only one hydrogen bond with Tyr185' was found. Therefore, the differences of bioactivity between compound **1** and **4** could be attributed to the protein-ligand interactions caused by the chirality.

Table 1. Bioactivity assay for compounds **1-4** and Glide Docking Score of compounds **1**, **4**.

Compound	IC_{50} [a] (nM, RMGPa)	IC_{50} [a] (nM, HepG2 cells)	IC_{50} [a] (nM, rat hepatocytes)	Glide Docking Score
1	48.7±4.2	661.5±82.4	719.5±79.5	-9.114
2	289.4±1.5	3238.9±703.0	7150.0±2421.6	ND [c]
3	555.2±24.0	6472.9±327.7	7650.0±1320.0	ND [c]
4	578.3±18.3	8391.8±5706.5	9460.0±2035.9	-8.683
CP-91149 [b]	92.2±8.8	3310.4±257.5	3172.5±105.4	

[a] Each value represents the mean ± SEM of three determinations.

[b] **CP-91149** was used as a positive control.

[c] ND is not detected.

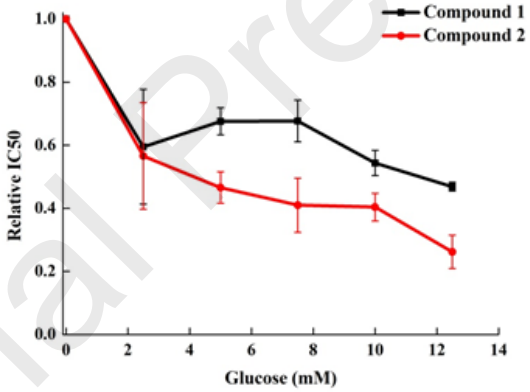


Figure 2. Effect of glucose on the potency of compounds **1**-**2**. Their IC_{50} values for GPa inhibition were determined at varying glucose concentrations, and then normalized by dividing the values by the IC_{50} value obtained in the absence of glucose. The normalized results are plotted as a function of glucose concentration.

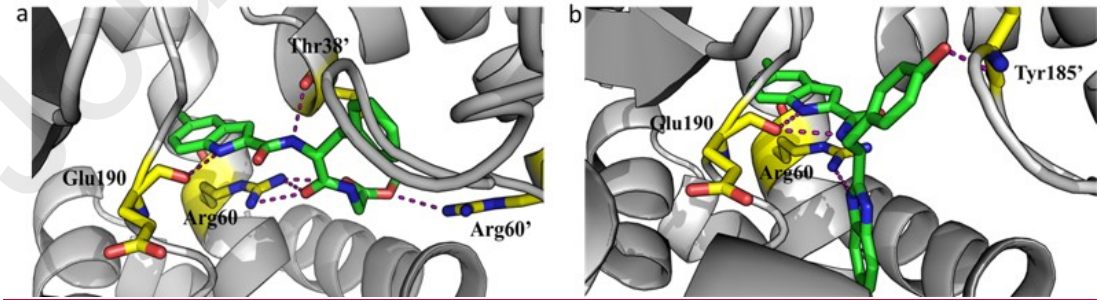


Figure 3. Binding mode of compound 1 (Figure 3a) and 4 (Figure 3b) in the active site of human liver glycogen phosphorylase A (PDB ID: 1EXV).

Kinetic solubility and LogD values are important factors at the discovery stage that provide a rank listing of solubility values with minimal sample requirements.[8] Therefore, the solubility of three selected compounds was assessed using a high-throughput kinetic solubility assay. As shown in Table 2, all the tested compounds showed comparable kinetic solubility. Of these, carboxylic acid derivative **2** (187.82 μM) was slightly less soluble than the reference drug chloramphenicol (199.74 μM) at pH 7.4. However, the initially made ester derivative **1** had low solubility and the best lipophilicities (log $D_{7.4}$: 3.95), suggesting that it might easily pass the

phos the other two compounds. Compound **2**, carrying a carboxylic acid group, had very low lipophilicity among this sub-group.

Table 2. *In vitro* physicochemical properties of compounds **1-3**.

Compound	Kinetic Solubility pH=7.4 (μM)	Compound	*log* $D_{7.4}$ (Oct/buff) result
1	1.89	1	3.95
2	187.82	2	0.72
3	36.84	3	3.32
Amiodarone (PC)	<1.00	Chlorpromazine (PC)	3.23
Chloramphenicol (PC)	199.74	Propranolol (PC)	1.38

Three selected compounds were tested for their chemical and metabolic stability in multiple biological media, including simulated gastric fluid (SGF), simulated interstinal fluid (SIF), mouse plasma (PLS), and mouse liver microsome (MLM).

As shown in Fig. 4, compound **2** exhibited considerable stability in all the media. It was stable in SGF and PLS for 24 h with almost no detectable fragment. It was also stable in SIF for up to 1 h, but degraded within 6 h. Compound **1** is an unstable compound, which was degraded completely within 1 h in SIF, 0.5 h in PLS, and 24 h by almost 50% in SGF. Moreover, compound **3** was a complicated compound, which was relatively stable in SGF and PLS after 24 h of incubation, but degraded rapidly in SIF within 1 h.

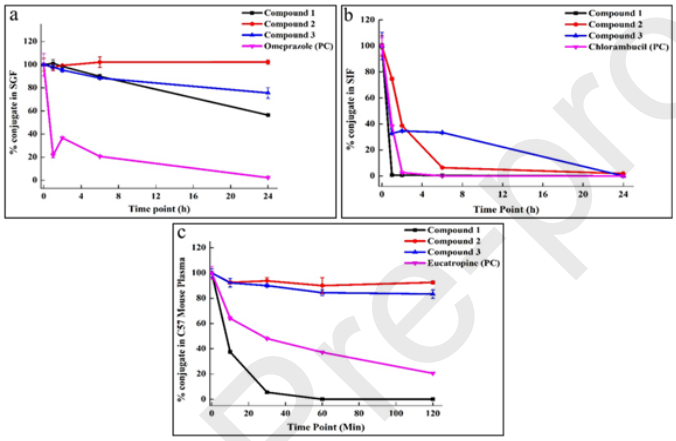


Figure 34. Time-course of stability of compounds **1-3** (n = 3). (a) in SGF; (b) in SIF; (c) in mouse plasma.

Additionally, the compounds' stability in microsome was evaluated by measuring the rate of compound consumption in MLM, and the results are shown in Table 3. Compounds **2** and **3** showed good metabolic stability in MLM with longer half life ($t_{1/2}$ > 145 min) and slower elimination rate (CL_{int} < 9.6 μL/min/mg protein, CL < 38.0 μL/min/mg protein). Compound **1** showed poor metabolic stability with $t_{1/2}$ of 15.4 min and CL_{int} of 90 μL/min/mg protein.

Table 3. MLM stability of compounds **1-3**.

Sample Name	Mouse liver microsomes			
	R^2	$T_{1/2}$ (min)	CL_{int} (μl/min/mg)	CL (mL/min/Kg)
1	0.9780	15.4	90.0	356.4
2	0.9419	>145	<9.6	<38.0
3	0.9247	>145	<9.6	<38.0
Diclofenac (PC)	0.9827	27.1	51.2	202.8

R^2 is the correlation coefficient of the linear regression for the determination of kinetic constant.

$CL = CL_{int}$ • 45 mg microsome/g liver • g liver wt/kg body weight

Liver wt: 88 g/kg relative mouse liver weight

Based on their values in the biological assays and *in vitro* ADME profiling, compound **2** was evaluated for its hypoglycemic activity in adrenaline-induced diabetic mice. Adrenaline is well known to induce high blood glucose level by indirectly stimulating glycogenolysis and therefore increasing hepatic glucose production.[9] Metformin was chosen as a positive control. Not surprisingly, the preliminary animal study results (Fig. 5) showed that compound **2** significantly inhibited an increase in the fasted plasma glucose level of diabetic mice induced by adrenaline. It reduced blood glucose (BG) level to a nadir of 446.25±15.9 mg/dl at 1.5 h vs 486.15±22.65 mg/dl in the vehicle-treated mice ($p < 0.05$), with significant effects also being evident at 1.5 h ($p < 0.05$). Further *in vivo* studies on **2** and related products as hypoglycemic agents are ongoing.

Table 4. Effect of compound **2** on fasted plasma glucose of hyperglycemic mice induced by adrenaline

Group	Dose (mg/kg)	0min	30min	60min	90min	AUC (0 min to 90 min, mg/dL× min)
Vehicle		4.84±0.15	9.58±0.44	8.41±0.48	7.56±0.49	482.63±21.01
Metformin	200	4.76±0.15	8.85±0.58	5.79±0.35***	5.00±0.31***	373.73±19.78***
2	50	4.86±0.16	9.03±0.26	6.83±0.38*	6.05±0.30*	416.33±13.59*
2	25	4.85±0.16	9.52±0.45	7.62±0.44	6.70±0.35	451.13±20.43
2	12.5	4.89±0.16	8.92±0.27	7.40±0.45	7.47±0.50	449.03±15.53

[a] Data are expressed as mean ± SEM (n = 10).
[b] **Metformin** was used as a positive control.
[c] *p < 0.05 and ***p < 0.005 vs vehicle.

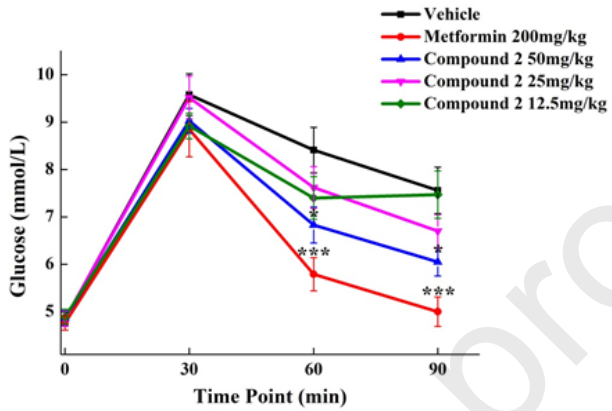


Figure 45. Time-course of the glucose level-lowering effect of oral administration of compound **2** in diabetic mice induced by adrenaline. (n = 10; *p < 0.05 and ***p < 0.005 vs vehicle).

In the process of drug development and clinical application evaluation, the ADME property of compounds is one of the important indicators of drug-forming. Therefore, according to previous literature reports,[10, 11] pharmacokinetic parameters the four target compounds were predicted by Molinspiration online property calculation toolkit (http://www.molinspiration.com/). All compounds meet requirements of Lipinski's rule of five and shows good characterizing drug absorption range from 68.61% to 76.89%. This provides a theoretical basis for further research.

Table 5. The predicted ADME through Molinspiration online software.

Compound	%ABS [a]	MV [b]	MW [c] (< 500)	n(rotb) [d] (< 10)	miLogP [e] (< 5)	n(ON) [f] (< 10)	n(OHNH) [g] (< 5)	Lipinski's violation
1	74.40	355.70	413.86	8	3.01	7	3	0
2	70.60	338.17	399.83	7	1.48	7	4	0
3	68.61	341.44	398.85	7	2.94	7	5	0
4	76.89	367.23	430.89	5	4.75	6	4	0

[a] Percentage of absorption (%ABS); [b] Molecular volume (MV); [c] Molecular weight (MW); [d] Number of rotatable bonds (n-rotb); [e] Logarithm of partition coefficient between n-octanol and water (miLogP); [f] Number of hydrogen bond acceptors (nON); [g] Number of hydrogen bond donors (nOHNH).

In summary, four novel indole-2-carboxamide derivatives were successfully identified from high-throughput screening as novel, potent, and glucose-sensitive inhibitors of GPa. Biological evaluation of the synthesized compounds has been described. Our data showed that compound **2** can inhibit glycogenolysis and then lower plasma glucose levels in diabetic rodents. The potency and ADME values rendered this series worthy of continued exploration and development.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at…

References

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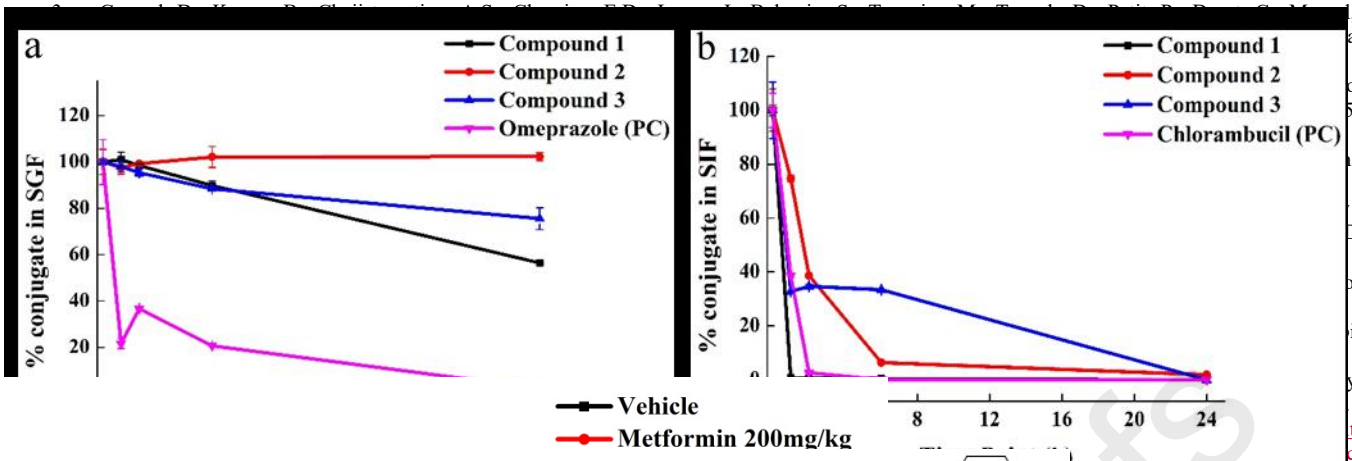

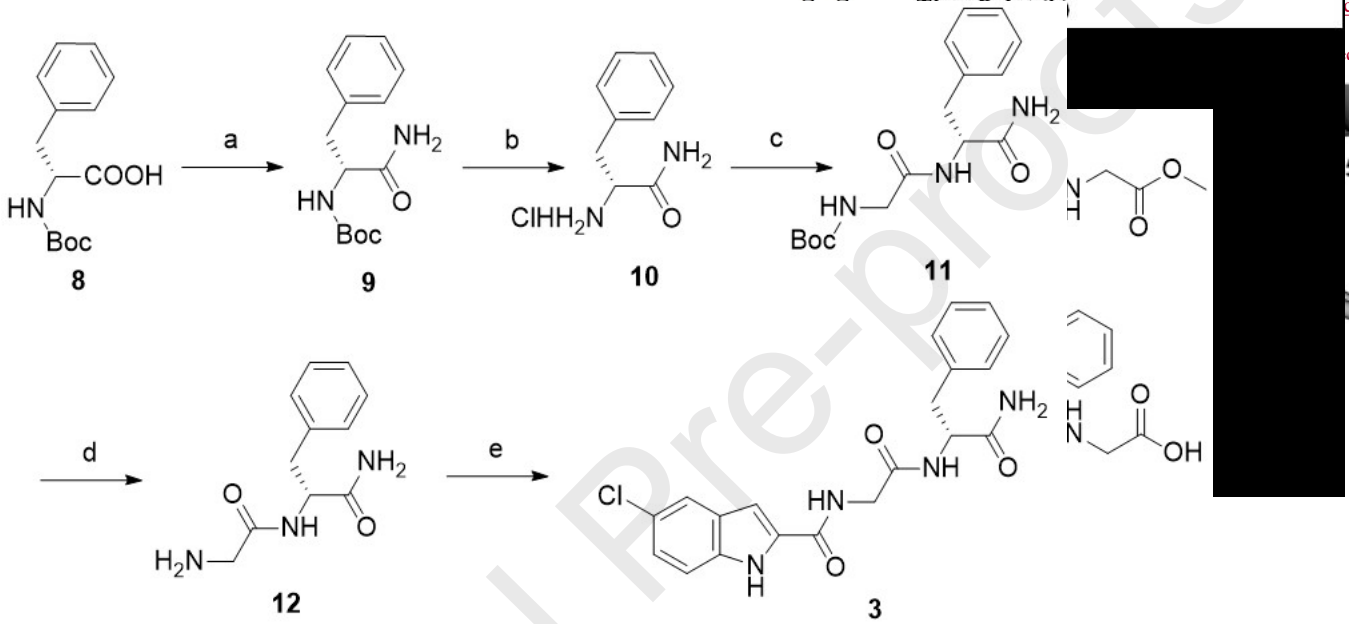

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