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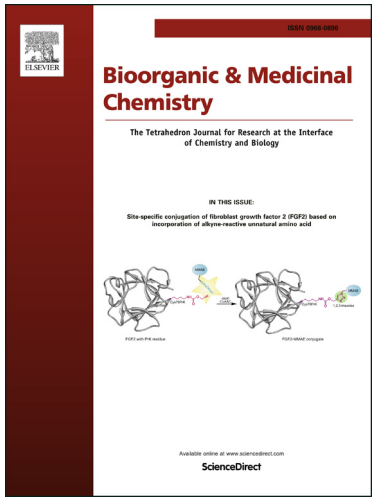

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Design and optimization of *N*-acylhydrazone pyrimidine derivatives as *E. coli* PDHc E1 inhibitors: structure-activity relationship analysis, biological evaluation and molecular docking study

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Abstract

By targeting the thiamin diphosphate (ThDP) binding site of Escherichia coli (*E. coli*) pyruvate dehydrogenase multienzyme complex E1 (PDHc E1), a series of novel 'open-chain' classes of ThDP analogs **A, B,** and **C** with *N*-acylhydrazone moieties was designed and synthesized to explore their activities against *E. coli* PHDc E1 *in vitro* and their inhibitory activity against microbial diseases were further evaluated *in vivo*. As a result, **A1~23** exhibited moderate to potent inhibitory activities against *E. coli* PDHc E1 (IC_{50} = 0.15–23.55 μM). The potent inhibitors **A13**, **A14**, **A15**, **C2,** had strong inhibitory activities with IC_{50} values of 0.60, 0.15, 0.39 and 0.34 μM against *E. coli* PDHc E1 and with good enzyme-selective inhibition between microorganisms and mammals. Especially, the most powerful inhibitor **A14** could 99.37% control *Xanthimonas oryzae pv. Oryzae*. Furthermore, the binding features of compound **A14** within *E. coli* PDHc E1 were investigated to provide useful insights for the further construction of new inhibitor by molecular docking, site-directed mutagenesis, and enzymatic assays. The results indicated that **A14** had most powerful inhibition against *E. coli* PDHc E1 due to the establishment of stronger interaction with Glu571, Met194, Glu522, Leu264 and Phe602 at active site of *E.coli* PDHc E1. It could be used as a lead compound for further optimization, and may have potential as a new microbicide.

Keywords ThDP; *E. coli* PHDc E1; enzyme-selective; molecular docking.

1. Introduction

The pyruvate dehydrogenase complex (PDHc) is an exquisite enzyme that catalyzes the oxidative decarboxylation of pyruvate，and the subsequent acetylation of coenzyme A (CoA) to acetyl-CoA.[1,2] The overall reaction of oxidative decarboxylation can be simply exhibited in **Fig. 1**.

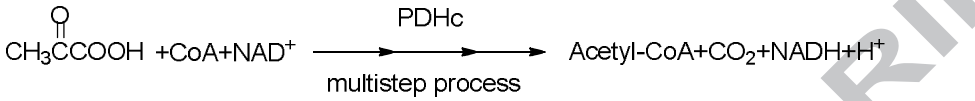


Fig.1 Oxidative decarboxylation catalyzed by PDHc.

The above reactions are carried out by PDHc complex, which is comprised of three different enzyme components: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), dihydrolipoamide dehydrogenase (E3).[3] The pyruvate dehydrogenase complex E1 component (PDHc E1) catalyzes the first step (rate-limiting and irreversible step) of multistep process, under condition of using thiamine diphosphate (ThDP) and Mg^{2+} as cofactors.[4–6] The cofactor of ThDP plays an important role in the enzyme reaction and the catalysis mechanism.[7] As would be expected, blocking the active site of cofactor, for example, by replacing it with ThDP analogue, inactivates the PDHc. Therefore, we selected PDHc E1 as the target enzyme to design new cofactor ThDP analogs as PDHc E1 inhibitors.

Currently, much effort has been made to design and synthesize ThDP analogs (such as ThTDP, ThTTDP, and triazole-ThDP in **Fig. 2**) [8-12] as PDHc E1 inhibitors. These ThDP analog inhibitors, such as ThTDP and ThTTDP, can block the ThDP binding site, and exhibit significantly stronger binding affinities for PDHc E1 than ThDP.[13-14]However, these ThDP analogs exhibit no potential utility and display poor bioavailability, due to their complex structure with highly charged pyrophosphate groups. They also showed poor enzyme-selective inhibition between microorganisms and mammals.[15]

To the best of our knowledge, the crystal structures of the PDHc E1/ThDP or inhibitor complex from *E. coli* (PDB ID:1L8A and 1RP7) have been determined.[16] The crystal structures enable structure-based design of novel inhibitors against PDHc E1. On that basis, we selected *E. coli* PDHc E1 as the target pattern to design new cofactor ThDP analogs as inhibitors of PDHc E1. In our laboratory, we recently had got preliminary progress for finding an effective *E. coli* PDHc E1 inhibitor **I** by structure-based rational design.[17,18] The mechanism was predicted with theory. Base on this work, the linker of **I** was further optimized affording ThDP analogs **II**, **III**, **IV, V** and **VI** as *E. coli* PDHc E1 inhibitors (**Fig. 2**).[19–23] Some of them were demonstrated to be effective inhibition (IC_{50}>0.65 μM) of *E. coli* PDHc E1 with moderate antibacterial or antifungal activity. The results convinced us that the linker (**Fig. 2**) of parent structure **I** plays a vital role in the biological activity of these ThDP analogs. The findings encouraged us to further find useful PDHc E1 inhibitors out with antibacterial or antifungal activity by further optimization of lead structure **I**.

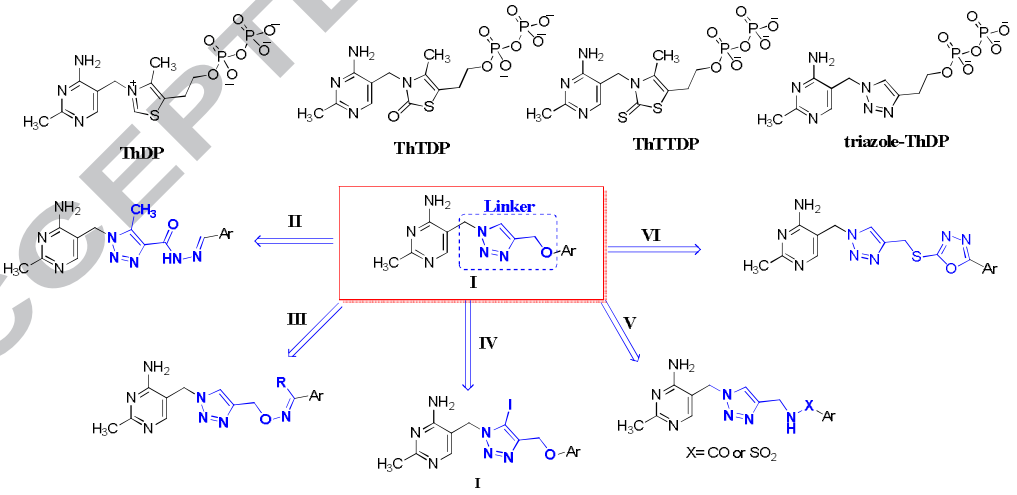


Fig.2 Structures of known PDHc E1 inhibitors.

Structure–activity relationship (SAR) indicated the introduction of hydrogen bond acceptor or hydrogen bond donor structural unit into the linker in parent structure **I** could increase the

inhibitory activity against *E. coli* PDHc E1 and antifungal activity. [22,23] Aiming to explore more optimizing linkages, the *N*-acylhydrazone moiety (-CONHN=CH-), which not only had well-known biological activities,[24–26] but also included hydrogen bond acceptor and hydrogen bond donor structural unit, was introduced to the structure **I** as a 'open-chain' linker to form a new structural class **A** as potential *E. coli* PDHc E1 inhibitors. Furthermore, the effect of Me group on pyrimidine ring was firstly studied, which was replaced by H or NH_2 group. Therefore, three new structural classes of PDHc E1 inhibitors were formed (**Fig. 3**). Considering the binding site of diphosphate of ThDP in the active site of *E. coli* PDHc E1, various chemical groups as Ar were incorporated into the parent compounds **A, B,** and **C** to investigate their preliminary SAR.

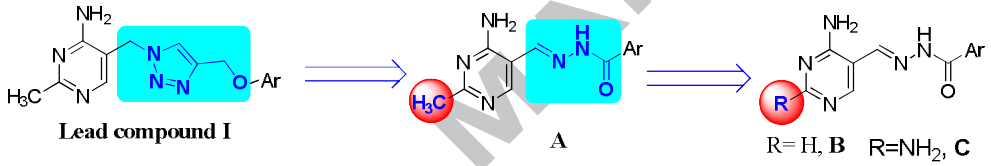
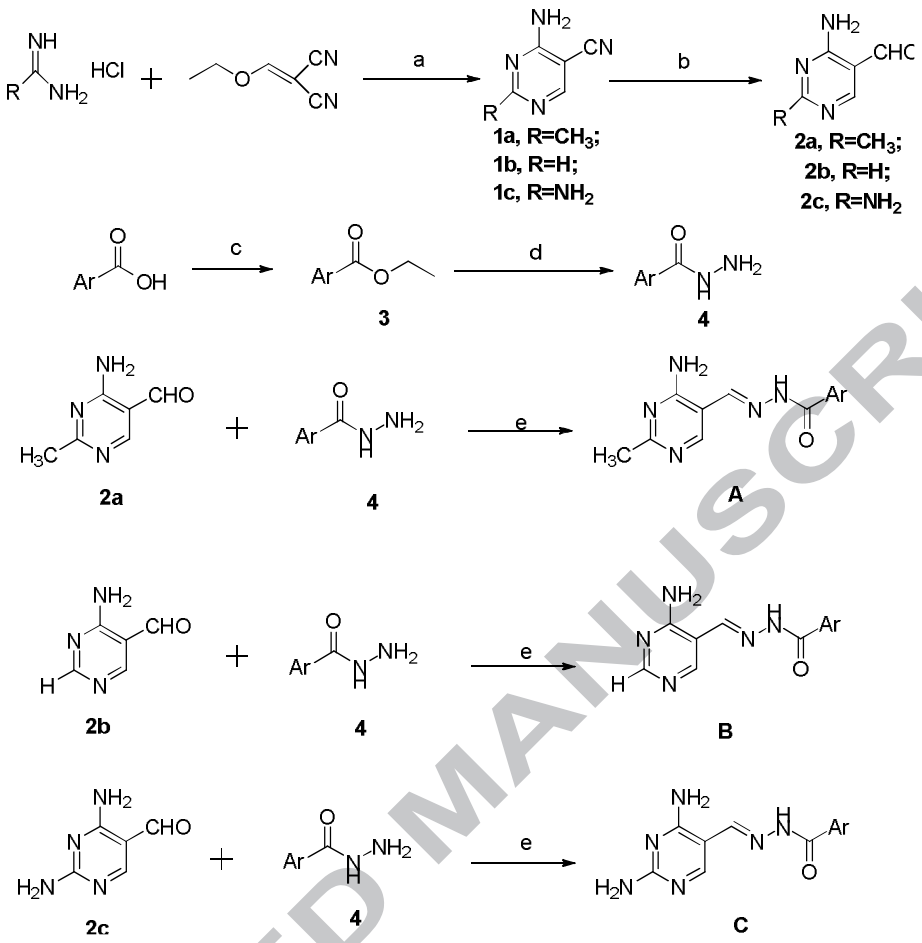


Fig.3 Design of the new *N*-acylhydrazone pyrimidine derivatives **A, B,** and **C**

Herein, the chemical synthesis of these new *N*-acylhydrazone pyrimidine derivatives **A, B,** and **C** as *E. coli* PDHc E1 inhibitors is described in details. The inhibitory activities on *E. coli* PDHc E1 are presented along with their SAR analysis as follows. Interaction between mode of inhibitor and target PDHc E1 was also explored by molecular docking study to identify the critical binding sites of the target PDHc E1. The enzyme-selectivity of representative compounds between microorganisms and mammals was also examined.

2. Chemistry

The synthetic route of **A, B,** and **C** is depicted in **Scheme 1**.



Scheme 1. Reagents and conditions (a) Na, EtOH, rt, 2h; (b) Raney Nickel, HCOOH, 80℃; (c) H_2SO_4, EtOH, reflux; (d) $NH_2NH_2.H_2O$, EtOH, reflux; (e) EtOH, CH_3COOH, reflux;

Substituted-amidine and 2-(ethoxymethylene)malononitrile as starting material was used to prepare 4-amino-2-substitutedpyrimidine-5-carbaldehyde **2** according to the literature method.[27] **2** is the key intermediate for the preparation of title compounds **A, B,** and **C**. The title compounds could be synthesized by a five-step sequence starting from starting material. Various substituted benzoic acid reacted with ethanol in the presence of concentrated sulfuric acid to produce substituted ethyl benzoate **3**, which reacted with hydrazine hydrate in ethanol to produce corresponding hydrazide **4**. Finally, the new *N*-acylhydrazone pyrimidine derivatives **A, B,** and **C** were prepared in good yields by condensing compound **2** with a variety of substituted benzoyl hydrazine in ethanol at reflux. All synthesized compounds were characterized by ^{1}H NMR, ^{13}C

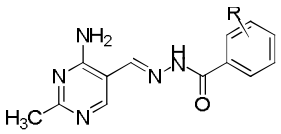
NMR, mass spectrometry and elementary analysis.

3. Results and discussion

3.1. *In vitro* inhibition of *E. coli* PDHc E1

In order to enhance the inhibitory potency against *E. coli* PDHc E1, lead structure **I** was revised by replacing linker with *N*-acylhydrazone moiety (**Fig.3**). Several *N*-acylhydrazone pyrimidine derivatives **A1~23** were firstly synthesized and their inhibitory potency against *E. coli* PDHc E1 was evaluated. The IC_{50} values of **A1~23** are summarized in **Table 1**. It was observed that most of the compounds showed moderate to potent inhibitory activity with IC_{50} values in the range of 0.15–23.55 μM. **A14** was the most potent inhibitor in this series with the activity of IC_{50} at 0.15 μM. All **A** compounds displayed higher inhibitory activity than that of **I** with the same **R** on the benzene ring (**Table 2**). Especially the inhibitory activity of **A22** was 34-folds higher than that of **I22**. The results revealed that the inhibitory potency could be meaningfully improved by replacing linker of **I** with *N*-acylhydrazone moiety.

Table 1 The inhibitory activity against *E. coli* PDHc E1 of *N*-acylhydrazone pyrimidine derivatives **A**

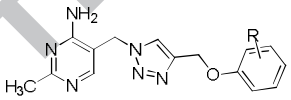


A

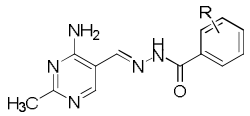
Compd.	R	IC_{50}[a](μM)	Compd.	R	IC_{50}[a](μM)
A1	H	6.29±0.27	**A13**	3-NO_2	0.60±0.03
A2	4-F	8.53±0.43	**A14**	3,5-$(NO_2)_2$	0.15±0.01
A3	2-Cl	21.42±0.76	**A15**	3-NO_2-5-NH_2	0.39±0.01
A4	3-Cl	3.28±0.29	**A16**	4-OH	3.60±0.27
A5	4-Cl	4.92±0.32	**A17**	2-OH	19.56±0.72
A6	2,4-Cl_2	20.95±0.79	**A18**	2-Me	9.34±0.40
A7	2-Br	20.95±0.79	**A19**	3-Me	6.01±0.18
A8	3-Br	2.09±0.14	**A20**	4-Me	5.87±0.25
A9	4-Br	3.17±0.17	**A21**	3,5-Me_2	13.57±0.46
A10	4-NH_2	7.43±0.51	**A22**	4-OCH_3	2.36±0.16
A11	2-Cl-4-NH_2	23.55±0.52	**A23**	3-OCH_3	12.85±0.67
A12	4-NO_2	2.75±0.26			

[a]IC_{50} (μM) value is defined as the micromolar concentration required for 50% inhibition against PDHc E1 from *E. coli in vitro*.

Table 2 The relative activity of compounds **A** and **I**



I



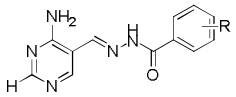
A

Compd.	R	IC_{50}[a](μM)	Relative activity
A1	H	6.29	**8.76**
I1	H	55.15	
A5	4-Cl	4.92	**5.37**
I5	4-Cl	26.44	
A12	4-NO_2	2.75	**3.20**
I12	4-NO_2	8.80	
A22	4-OCH_3	2.36	**34.58**
I122	4-OCH_3	81.62	

[a]IC_{50} (μM) value is defined as the micromolar concentration required for 50% inhibition against PDHc E1 from *E. coli in vitro*

As shown in **Table 1**, **R** on the benzene ring also had great influence on the inhibitory activity base on the structure skeleton of **A**. Firstly, compounds with NO_2 group as **R** exhibited better inhibitory activity than that of compounds with other substituents, such as **A14** (R=3,5-diNO_2, IC_{50} = 0.15 μM) was the most potent inhibitor in this series, followed by **A15** (R=3-NO_2-5-NH_2, IC_{50} = 0.39 μM) and **A13** (R=3-NO_2, IC_{50} = 0.60 μM). Secondly, compounds **A** with hydrogen bonding acceptor group (NO_2) as **R** exhibited better inhibitory activity than that of compounds with hydrogen bonding donor group (NH_2) substituents, such as **A12** (R=4-NO_2, IC_{50}=2.75 μM) > **A10** (R=4-NH_2, IC_{50}=7.43 μM), **A14** (R=3,5-$(NO_2)_2$, IC_{50}=0.15 μM) > **A15** (R=3-NO_2-5-NH_2, IC_{50}=0.39 μM). Finally, when substituted with the halogen groups on benzene ring, the activity sequence is *meta* > *para* > *ortho*, such as **A4** (R=3-Cl, IC_{50} = 3.28 μM) > **A5** (R=4-Cl, IC_{50} = 4.92 μM)> **A3** (R=2-Cl, IC_{50} = 21.42 μM); **A8** (R=3-Br, IC_{50} = 2.09 μM) > **A9** (R=4-Br, IC_{50} = 3.17 μM)> **A7** (R=2-Br, IC_{50} = 20.95 μM). The results suggest that the 3-position group on the benzene ring is favorable for the PDHc E1 inhibitory activity. When comparing the PDHc E1 inhibitory activity of compounds **A2**, **A5**, and **A9**, the activity sequence is Br > Cl > F, which indicates the larger substituent is better. In the other hand, **R** at 2-position on the benzene ring was unfavorable to inhibitory activity. Inhibitory activity against *E. coli* PDHc E1 decreased in the following order: **A5** (R=4-Cl, IC_{50} = 4.95 μM) > **A6** (R=2,4-diCl, IC_{50} = 20.95 μM) > **A3** (R=2-Cl, IC_{50} = 21.42 μM); **A10** (R=4-NH_2, IC_{50} = 7.43 μM) > **A11** (R=2-Cl-4-NH_2, IC_{50} = 23.55 μM); **A16** (R=4-OH, IC_{50} = 3.60 μM) > **A17** (R=2-OH, IC_{50} = 19.56 μM); **A20** (R=4-Me, IC_{50} = 5.87 μM) > **A19** (R=3-Me, IC_{50} = 6.01 μM)> **A18** (R=2-Me, IC_{50} = 9.34 μM). Above results indicated that the inhibitory activity of **A** was also dependent upon the structure, position and size of **R** on the benzene ring.

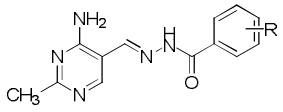
Table 3 The inhibitory activity against *E. coli* PDHc E1 of compounds **B**



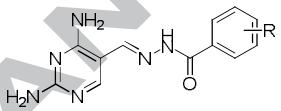
Compd.	R	Inhibition ratio（%）(100 μM)[a]	Compd.	R	Inhibition ratio（%）(100 μM)[a]
B1	H	14.44±0.23	**B5**	2-Me	19.70±1.23
B2	4-F	0	**B6**	3,5-Me_2	4.02±0.32
B3	4-Br	12.07±0.98	**B7**	2,4-Cl_2	26.50±1.21
B4	2-Cl	16.80±0.98			

[a] Inhibitory potency (%) of compounds against enzyme *in vitro* at 100 μM as average of triplicate.

Table 4 The inhibitory activity against *E. coli* PDHc E1 of *N*-acylhydrazone pyrimidine derivatives **C**



A



C

Compd	R	IC_{50}[a](μM)	Compd.	R	IC_{50}(μM)
C1	H	9.81±0.50	**C5**	2,4-Cl_2	30.49±0.97
A1	H	6.29±0.27	**A6**	2,4-Cl_2	20.95±0.79
C2	**3-NO_2**	**0.34±0.02**	**C6**	3-Cl	11.89±0.62
A13	3-NO_2	0.60±0.03	**A4**	3-Cl	3.28±0.29
C3	**4-NH_2**	**5.76±0.25**	**C7**	4-Cl	6.17±0.24
A10	4-NH_2	7.43±0.51	**A5**	4-Cl	4.92±0.32
C4	**2-Cl-4-NH_2**	**10.30±0.79**	**C8**	4-Br	30.44±0.67
A11	2-Cl-4-NH_2	23.55±0.52	**A9**	4-Br	3.17±0.17

[a] IC_{50} (μM) value is defined as the micromolar concentration required for 50% inhibition against PDHc E1 from *E. coli in vitro*.

In order to examine the effect of Me group on the pyrimidine ring on inhibitory activity against *E.coli* PDHc E1, **A** was modified to produce **B** and **C** by replacing Me group on the pyrimidine ring in **A** with H or NH_2 moiety and its inhibitory activity against *E. coli* PDHc E1 was tested (**Table 3 and 4**). The result showed that the substituting of Me group with H group led to a decrease in inhibitory activity against *E. coli* PDHc E1. As show in **Table 3,** all title compounds **B** only showed <30% inhibitory potency against *E. coli* PDHc E1 at 100 μM. It was found that the

inhibitory activity was slight enhanced by changing Me group into NH_2 group on the pyrimidine ring, when **R** included NO_2 or NH_2 group on the benzene ring. As shown in **Table 4**, **C2** (R=3-NO_2, IC_{50} = 0.34 μM), **C3** (R=4-NH_2, IC_{50} = 5.76 μM) and **C4** (R=2-Cl-4-NH_2, IC_{50} = 10.30 μM) displayed better inhibitory activity than **A13** (R=3-NO_2, IC_{50} = 0.60 μM), **A10** (R=4-NH_2, IC_{50} = 7.43 μM) and **A11** (R=2-Cl-4-NH_2, IC_{50} = 23.55 μM). But when **R** was substituted by halogen (Cl or Br) or H group, **C5** (R=2,4-Cl_2, IC_{50} = 30.49 μM), **C6** (R=3-Cl, IC_{50} = 11.89 μM), **C7** (R=4-Cl, IC_{50} = 6.17 μM) and **C8** (R=4-Br, IC_{50} = 30.44 μM) with NH_2 group substituted on the pyrimidine ring displayed lower inhibitory activity than that of corresponding **A6** (R=2,4-Cl_2, IC_{50} = 20.95 μM), **A4** (R=3-Cl, IC_{50} = 3.28 μM), **A5** (R=4-Cl, IC_{50} = 4.92 μM) and **A9** (R=4-Br, IC_{50} = 3.17 μM) with Me group substituted on the pyrimidine ring. Especially, **C8** showed 9-folds activity lower than that of **A9.** Above observation showed the substituted group on the pyrimidine ring in parent structure **I** also played a very important role in inhibitory potency against *E. coli* PDHc E1. Compared with H or NH_2 group, Me as substituent group on the pyrimidine ring was much beneficial to inhibitory activity. These results suggest that the structure skeleton of **A** is better than both **B**, **C** and lead structure **I** for finding more powerful PDHc E1 inhibitor.

3.2. Enzyme-Selective

The literature reported ThDP analogs as PDHc E1 inhibitors exhibited poor enzyme-selective inhibition between mammals and microorganism.[28] In this work, pig PDHc E1 and *E. coli* PDHc E1 were chosen as the target pattern of mammalian and microorganism, respectively. **A13, A14, A15, C2** with good inhibition against *E. coli* PDHc E1 (IC_{50} = 0.15–0.60 μM) were selected to test their inhibition against Pig heart PDHc E1. As shown in **Table 5**, **A13, A14, A15, C2** exhibited weak inhibitory potency (inhibition ratio<20%) against pig PDHc E1. These findings showed that

A13, A14, A15, C2 exhibited better enzyme-selective inhibition between microorganism and mammal.

Table 5 Inhibition of **A** and **C** against *E. coli* and pig PDHc E1

Compd.	*E. coli* PDHc E1		Porcine PDHc E1
	IC_{50}[a] (μM)	Inhibitory potency[b] (%)	Inhibitory potency[b] (%)
A13	0.60±0.03	100.00±0.19	18.46±1.23
A14	0.15±0.01	100.00±1.39	15.46±1.34
A15	0.39±0.01	100.00±2.73	9.11±0.03
C2	0.34±0.02	100.00±3.21	0

[a] IC_{50} (μM) value is defined as the micromolar concentration required for 50% inhibition against *E. coli* PDHc E1 *in vitro*.

[b] Inhibitory potency (%) of compounds against enzyme *in vitro* at 100 μM as average of triplicate.

3.3. Antibacterial activity (*In vivo*)

In order to find useful PDHc E1 inhibitors with antibacterial activity, **A13~15** and **C2** with higher inhibitory activity against *E. coli* PDHc E1 were chosen to further evaluate their antibacterial activity against *Acidovorax avenae subsp. Avenae* and *Xanthimonas oryzae pv. Oryzae* at 500 μg/mL. As shown in **Table 6**, **A14,** with the best *E. coil* PDHc E1 activity (IC_{50} = 0.15 μM), could 99.37% control Xanthimonas oryzae pv. Oryzae. The effect of **A14** was comparable to commercial bactericide streptomycin sulfate (97.58%) as a positive control. Its inhibition against *Xanthimonas oryzae pv. Oryzae* is worth further examination.

Table 6 Antibacterial activity of **A13~15** and **C2**

Compd.	Inhibitory potency[a] (%) 500 μg/mL	
	Acidovorax avenae subsp. Avenae;	*Xanthimonas oryzae pv. Oryzae*
A13	13.66 ± 0.27	40.56 ± 0.51
A14	25.52 ± 0.34	**99.37 ± 0.30**
A15	12.34 ± 0.33	14.25 ± 0.30
C2	10.73 ± 0.25	19.50 ± 0.24
Streptomycin Sulfate	96.54 ± 0.27	97.58 ± 0.35

[a]Inhibitory potency (%) against the growth of pathogenic fungi at 500 μg/mL, 0 (no effect), 100% (completely kill).

3.4. Analyses of the interaction between inhibitors and *E.coil* PDHc E1

To explore the interaction mode of *N*-acylhydrazone pyrimidine derivative **A, B,** and **C** with the active site of PDHc E1. The SURFLEX module of SYBYL package was used to perform molecular docking simulation of *N*-acylhydrazone pyrimidine derivative **A, B,** and **C**.[17] **A14** as the best PDHc E1 inhibitor (IC_{50} = 0.15 μM) was selected to analyses their interaction with *E. coil* PDHc E1 by molecular docking.

As shown in **Fig. 4**, **A14** could occupy the ThDP-binding pocket with a 'V' conformation. On the right side of the 'V' conformation, the 4-aminopyrimidine ring of **A14** not just displays a π-π stacking with the side chain ring of Phe602，and the nitrogen atom of the pyrimidine ring establish two key hydrogen bonding interaction with the side chain of Glu571 and Met194. The amino group connected to the pyrimidine ring forms a strong hydrogen bond with Glu522, which is an important residue in the stabilization of the enzyme bound LThDP.[10] The oxygen atom of the *N*-acylhydrazone moiety also establishes a hydrogen bond with Leu264. On the left side of the 'V' conformation of **A14**, three strong hydrogen bonds can be formed by the two nitryl groups on benzene ring with His106, Lys392, and Asn260, meanwhile the two nitryl groups coordinate with the Mg^{2+} in the active site. These docking results offered us a reasonable explanation for why compound **A14** had the highest PDHc E1 inhibitory activity.

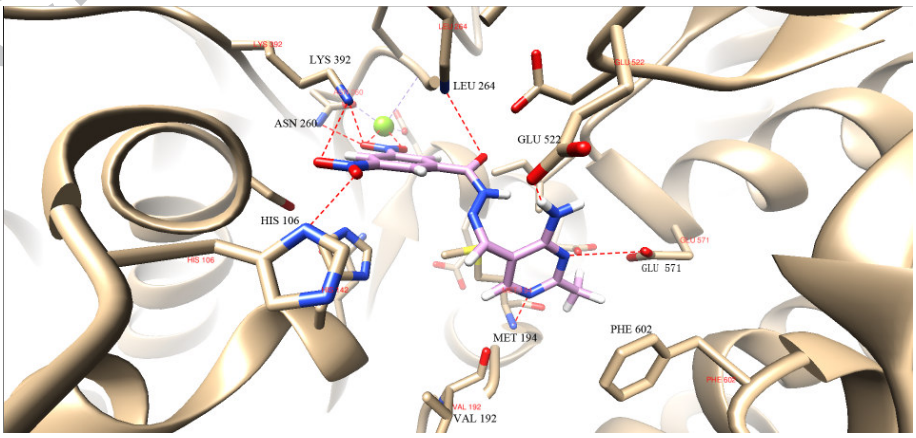


Fig. 4 Optimal binding model for compound **A14** into active site of PDHc E1 from *E. coli* docked

by SURFLEC module, ligand and some key residues are shown in stick, hydrogen bonds are shown in red lines.

In order to verify the prediction of molecular docking, site-directed mutagenesis and enzymatic assays were further performed. As shown in **Table. 7**, the IC_{50} value of **A14** against mutants E571A (36.11 μM), M194A (112.71 μM), E522A (13.31 μM), L264A (2.71 μM), H106A (0.92 μM), K392A (0.50 μM), or F602A (4.82μM) was about 240 times, 751 times, 88 times, 18 times, 6 times, 3 times or 32 times higher than its value against wild-type PDHc E1 (0.15 μM), respectively. The results suggests that the interaction between **A14** and residue Glu571, Met194, Glu522, Leu264, or Phe602 by forming hydrogen bond has a significant contribution for its inhibitory activity against *E. coli* PDHc E1.

Table 7 The IC_{50} values of compound **A14** against wild and mutant *E. coli* PDHc E1

IC_{50} (μM)							
Wild	E571A	M194A	E522A	L264A	H106A	K392A	F602A
0.15±0.01	36.11±2.38	112.71±4.80	13.31±0.52	2.71±0.05	0.92±0.06	0.50±0.01	4.82±0.09
Relative activity	240.7	751.4	88.7	18.1	6.1	3.3	32.1

[a]IC_{50} (μM) value is defined as the micromolar concentration required for 50% inhibition against PDHc E1 from *E. coli in vitro*.

4. Conclusion

In the current study, a series of novel 'open-chain' classes of *E. coli* PDHc E1 inhibitors, *N*-acylhydrazone pyrimidine derivatives **A, B,** and **C** were designed and synthesized. As novel ThDP analogs, all **A** displayed moderate to powerful inhibitory activity with IC_{50} values in the range of 0.15 to 23.55 μM against *E. coli* PHDc E1. The inhibitory potency of compounds against *E. coli* PDHc E1 could be greatly enhanced when the linkage (between pyrimidine and benzene ring moiety) of lead compound **I** was replaced with *N*-acylhydrazone moiety. Moreover the substituted on the pyrimidine ring in parent structure also played a very important role in

inhibitory potency against *E. coli* PDHc E1. The Me group as substituent group on the pyrimidine ring was much beneficial to inhibitory activity, compared with H or NH_2 group. These results recommend that the structure skeleton of **A** is better than both **B**, **C** and lead structure **I** for finding more powerful PDHc E1 inhibitor. Among these title compounds, compounds **A13**, **A14**, **A15**, and **C2** were found to be very effective inhibitors of *E. coli* PDHc E1, with IC_{50} values ranging from 0.15 to 0.60 μM. They also exhibited good enzyme-selective inhibition between microorganisms and mammals. Compound **A14**, with inhibition rates of 99.37% at 500 μgmL^{-1} against *Xanthimonas oryzae pv. Oryzae*, was the most powerful inhibitor of *E. coli* PDHc E1 among title compounds. Binding mode analysis revealed that **A14** displays a π-π stacking with the side chain ring of Phe602，and four key hydrogen bonding interaction is made by the nitrogen and oxygen atom with the side chain of Glu571, Met194, Glu522, Leu264.The two NO_2 groups not only could form three strong hydrogen bonds with Lys392, Asn260, and His106, but also could establish a coordinate-bond with the Mg^{2+}. The above hydrogen bonding interaction in turn seems to be important for enhancing its inhibitory potency. The site-directed mutagenesis and enzymatic assays further verified that the interaction between **A14** with Glu571, Met194, Glu522, Leu264 and Phe602 had a significant contribution for its inhibitory activity against *E.coli* PDHc E1. Therefore, the skeleton of *N*-acylhydrazone pyrimidine derivatives **A** could be as the novel lead structure for further optimization.

5. Experimental

5.1 Chemistry

Melting points (mp) were measured on an electrothermal melting point apparatus and were uncorrected. ^{1}H NMR spectra were recorded at 400 MHz, in DMSO-d_6 solution on a Varian

Mercury-Plus 400 spectrometer and chemical shifts were recorded in parts per million (ppm) with TMS as the internal reference. Mass spectra (MS) were obtained on a QTRAP LC/MS/MS system (API2000; Applied Biosystems, Foster City, CA, USA), and signals were given in *m/z*. Unless otherwise noted, reagents were purchased from commercial suppliers and used without further purification.

5.2 General procedure for preparation of 1a-c.

A solution of sodium ethoxide (1.15g, 50 mmol), and acetamidine hydrochloride (4.7 g, 50 mmol) in ethanol solution (60 mL) was stirred at room temperature for 1h. After this, the reaction mixture was filtered out affording filtrate. Then 2-(ethoxymethylene)malononitrile (6.1 g, 50mmol) was added to the filtrate, and the mixture was stirred at room temperature for 3h. After this, the reaction mixture was filtered and washed with 10 mL ethanol and dried to give the desired compound 4-amino-2-methylpyrimidine-5-carbonitrile **1a**, which was used directly for the next step. Under the same condition, the intermediate compounds **1b** and **1c** were also prepared.

5.3 General procedure for preparation of 2a-c.

A solution of 4-amino-2-methylpyrimidine-5-carbonitrile **1a** (3.04 g, 30 mmol) and raney nickel (3.0 g) in formic acid (20 mL) was stirred at 80 ^{0}C for 4 h. After this, the reaction mixture was filtered and washed with 10 mL formic acid. The filtrate and washings were collected together and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel and eluted with ethyl acetate/petroleum ether (1:1, v/v) to give white solid **2a**, which was used directly for the next step. Under this same condition, the intermediate compounds **2a** and **2c** were also prepared.

5.4 General procedure for preparation of 4.

We added dense H_2SO_4 (0.098g, 1 mmol) to a solution of substituted benzoic acid (5 mmol) in dry ethyl alcohol (10 mL). The mixture was heated under reflux until completion (as monitored via TLC), and the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (50 mL) and washed with 0.1 M Na_2CO_3, brine, dried and concentrated, respectively. The crude products (intermediate **3**) were used directly for the next step.

5 mL of hydrazine monohydrate (80%) was added to a solution of intermediate **3** (5 mmol) in ethanol (5 mL). The reaction mixture was maintained under reflux for 5 h. was then concentrated under reduced pressure and the resulting solid was collected by filtration, washed with cold water and dried to give the desired intermediate **4** as a white solid.

5.5 General procedure for preparation of target compounds A, B, and C.

Appropriate substituted benzoyl hydrazine **4** (1 equiv) was added to a solution of intermediate **2** (1 mmol) in ethanol (10 mL). The reaction mixture was stirred for 3 h at reflux under the condition of the presence of acetic acid as catalyzer, then poured into cold water and the resulting solid was collected by filtrated, washed with EtOH (10 mL), and dried in the atmospheric pressure to give the desired title compounds **A, B,** and **C**.

5.5.1. *N'*-((4-amino-2-methylpyrimidine-5-yl)methylene)benzohydrazide (A1)

White solid; Yield 78%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 3H, CH_3), 7.53 (s, 2H, NH_2), 7.58 (s, 1H, Ar-H), 7.92 (d, 2H, Ar-H, *J*=4.9Hz), 8.07 (s, 1H, pyrimidine-H), 8.42 (s, 1H, CH=N), 8.42 (s, 2H, Ar-H), 12.08 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.0, 163.2, 160.0, 158.3, 146.3, 133.2, 132.1, 128.7, 127.8, 107.3, 25.8; ESI-MS *m/z*: 256.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{13}N_5O$: C, 61.17; H, 5.13; N, 27.43. Found: C, 61.03; H, 5.22; N, 27.26 .

5.6.2. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-4-fluorobenzohydrazide (A2)

White solid; Yield 89%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 3H, CH_3), 7.38 (s, 2H, NH_2), 7.99 (s, 1H, pyrimidine-H), 7.99 (s, 2H, Ar-H), 8.41 (s, 1H, CH=N), 8.41 (s, 2H, Ar-H), 12.08 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 166.9, 163.1, 161.9, 159.9, 158.3, 146.2, 130.5, 129.5, 115.7, 115.5, 107.1, 25.7; ESI-MS *m/z*: 274.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}FN_5O$: C, 57.14; H, 4.43; N, 25.63. Found: C, 57.04; H, 4.20; N, 25.91 .

5.6.3. *N'*-((4-amino-2-methylpyrimidine-5-yl)methylene)-2-chlorobenzohydrazide (A3)

Light yellow solid; Yield 68%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.35 (d, 3H, CH_3, *J*=34.5Hz), 7.46 (s, 2H, NH_2), 7.52 (d, 1H, Ar-H, *J*=6.4Hz), 7.58 (d, 1H, Ar-H, *J*=6.4Hz), 8.03 (s, 1H, pyrimidine-H), 8.19 (s, 1H, CH=N), 8.25 (s, 2H, Ar-H), 8.29 (s, 2H, Ar-H), 12.14 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 162.5, 159.9, 159.4, 158.6, 146.6, 135.0, 131.7, 130.6, 129.9, 129.6, 127.5, 106.9, 25.8; ESI-MS *m/z*: 290.1 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}ClN_5O$: C, 53.89; H, 4.17; N, 24.17. Found: C, 53.60; H, 4.13; N, 24.25 .

5.6.4. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-3-chlorobenzohydrazide (A4)

White solid; Yield 75%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 7.59 (d, 1H, Ar-H, *J*=7.2Hz), 7.67 (s, 1H, Ar-H), 7.88 (d, 1H, Ar-H, *J*=6.6Hz), 7.96 (s, 1H, Ar-H), 8.07 (s, 1H, pyrimidine-H), 8.32 (s, 1H, CH=N), 8.41 (s, 2H, NH_2), 12.15 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.0, 161.6, 159.9, 158.4, 146.7, 135.0, 133.4, 131.8, 130.6, 127.4, 126.5, 107.0, 25.7; ESI-MS *m/z*: 290.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}ClN_5O$: C, 53.89; H, 4.17; N, 24.17. Found: C, 54.02; H, 3.96; N, 24.50 .

5.6.5. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-4-chlorobenzohydrazide (A5)

White solid; Yield 79%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 3H, CH_3),

7.63 (s, 2H, NH_2), 7.94 (d, 2H, Ar-H, *J*=7.2Hz), 8.07 (s, 1H, pyrimidine-H), 8.30 (s, 1H, CH=N), 8.41 (s, 2H, Ar-H), 12.14 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.0, 162.0, 159.9, 158.4, 146.5, 136.9, 131.7, 129.7, 128.8, 107.2, 25.7; ESI-MS *m/z*: 290.1 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}ClN_5O$: C, 53.89; H, 4.17; N, 24.17. Found: C, 53.86; H, 3.98; N, 24.34.

5.6.6. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-2,4-dichlorobenzohydrazide (A6)

Light yellow solid; Yield 76%; m.p 244-246°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.35 (d, 3H, CH_3, *J*=30.2Hz), 7.53-7.65 (m, 2H, NH_2), 7.75 (d, 1H, Ar-H, *J*=27.0Hz), 8.05 (s, 1H, pyrimidine-H), 8.24 (s, 2H, Ar-H), 8.30 (s, 1H, CH=N), 12.17 (s, 1H, NH); ESI-MS *m/z*: 324.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{11}Cl_2N_5O$: C, 48.17; H, 3.42; N, 21.60. Found: C, 48.12; H, 3.22; N, 21.89 .

5.6.7. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-2-bromobenzohydrazide (A7)

White solid; Yield 69%; m.p 250-251°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.35 (d, 3H, CH_3, *J*=34.9Hz), 7.44 (s, 2H, NH_2), 7.46-7.56 (m, 1H, Ar-H), 7.70 (dd, 1H, Ar-H, *J*=7.6, 7.5Hz), 8.03 (s, 1H, pyrimidine-H), 8.19 (s, 1H, CH=N), 8.24 (s, 1H, Ar-H), 8.29 (s, 1H, Ar-H), 12.12 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.1, 163.3, 159.9, 158.5, 146.5, 137.1, 133.0, 131.8, 129.5, 127.9, 119.6, 106.9, 25.7; ESI-MS *m/z*: 334.3$[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}BrN_5O$: C,46.72; H, 3.62; N, 20.96. Found: C, 46.85; H, 3.98; N, 20.80 .

5.6.8. *N'*-((4-amino-2-methylpyrimidine-5-yl)methylene)-3-bromobenzohydrazide (A8)

White solid; Yield 80%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 7.53 (d, 1H, Ar-H, *J*=7.4Hz), 7.82 (d, 1H, Ar-H, *J*=6.8Hz), 7.92 (d, 1H, Ar-H, *J*=6.8Hz), 8.10 (s, 2H, NH_2), 8.33 (s, 1H, pyrimidine-H), 8.41 (s, 1H, Ar-H), 8.41 (s, 1H, CH=N), 12.16 (s, 1H, NH);

^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 161.5, 159.9, 146.8, 135.2, 134.8, 130.9, 130.3, 127.0, 121.9, 106.1, 25.8; ESI-MS *m/z*: 334.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}BrN_5O$: C, 46.72; H, 3.62; N, 20.96. Found: C, 46.65; H, 3.64; N, 20.83 .

5.6.9. *N′*-((4-amino-2-methylpyrimidin-5-yl)methylene)-4-bromobenzohydrazide (A9)

White solid; Yield 78%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 7.77 (s, 2H, NH_2), 7.86 (s, 2H, Ar-H), 8.06 (s, 1H, pyrimidine-H), 8.34 (s, 1H, CH=N), 8.41 (s, 2H, Ar-H), 12.13 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.0, 162.1, 159.9, 158.4, 146.6, 132.1, 131.7, 129.8, 125.9, 107.2, 25.8; ESI-MS *m/z*: 334.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}BrN_5O$: C, 46.72; H, 3.62; N, 20.96. Found: C, 46.87; H, 3.40; N, 20.71 .

5.6.10. *N′*-((4-amino-2-methylpyrimidine-5-yl)methylene)-4-amino-benzohydrazide (A10)

White solid; Yield 83%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 3H, CH_3), 5.83 (s, 2H, NH_2), 6.60 (d, 2H, NH_2, *J*=8.1Hz), 7.66 (d, 2H, Ar-H, *J*=7.8Hz), 7.96 (s, 1H, pyrimidine-H), 8.25 (s, 1H, CH=N), 8.36 (s, 2H, Ar-H), 11.66 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 166.6, 163.0, 159.9, 157.7, 152.7, 144.3, 129.6, 119.1, 112.8, 107.6, 25.7; ESI-MS *m/z*: 271.4 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{14}N_6O$: C, 57.77; H, 5.22; N, 31.09. Found: C, 57.74; H, 4.91; N, 31.15 .

5.6.11. *N′*-((4-amino-2-methylpyrimidin-5-yl)methylene)-2-chloro-4-amino-benzohydrazide (A11)

White solid; Yield 79%; m.p 234-236°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 3H, CH_3), 5.87 (s, 2H, NH_2), 6.54 (d, 1H, Ar-H, *J*=8.2Hz), 6.65 (s, 1H, Ar-H), 7.27 (d, 1H, Ar-H, *J*=8.3Hz), 8.00 (s, 1H, pyrimidine-H), 8.23 (s, 1H, CH=N), 8.23 (s, 2H, NH_2), 11.77 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 166.8, 162.9, 159.9, 158.0, 152.0, 145.3, 132.1, 131.0,

120.6, 114.0, 111.8, 107.3, 25.6; ESI-MS *m/z*: 305.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{13}ClN_6O$: C, 51.24; H, 4.30; N, 27.58. Found: C, 51.36; H, 4.24; N, 27.74 .

5.6.12. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-4-nitrobenzohydrazide (A12)

White solid; Yield 78%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 8.14 (s, 1H, pyrimidine-H), 8.16 (s, 2H, NH_2), 8.32 (s, 1H, CH=N), 8.39 (d, 2H, Ar-H, *J*=8.0Hz), 8.43 (s, 2H, Ar-H), 12.36 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.1, 161.4, 159.9, 158.6, 149.4, 147.3, 138.7, 129.2, 123.8, 106.9, 25.7; ESI-MS *m/z*: 301.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}N_6O_3$: C, 52.00; H, 4.03; N, 27.90. Found: C, 52.02; H, 3.90; N, 27.89 .

5.6.13. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-3-nitrobenzohydrazide (A13)

Red solid; Yield 67%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 7.85 (t, 1H, Ar-H, *J*=7.6Hz), 8.10 (s, 1H, pyrimidine-H), 8.32 (s, 2H, NH_2), 8.36 (d, 1H, Ar-H, *J*=7.3Hz), 8.44 (s, 1H, CH=N), 8.44 (s, 1H, Ar-H), 8.76 (s, 1H, Ar-H), 12.38 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.2, 160.9, 159.9, 158.63, 147.9, 147.3, 134.4, 134.3, 130.5, 126.6, 122.4, 107.0, 25.8; ESI-MS *m/z*: 301.1 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{12}N_6O_3$: C, 52.00; H, 4.03; N, 27.99. Found: C, 51.85; H, 4.05; N, 27.69 .

5.6.14. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-3,5-dinitrobenzohydrazide (A14)

Red solid; Yield 78%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.40(s, 3H, CH_3), 8.58 (s, 1H, Ar-H), 8.75 (s, 1H, Ar-H), 8.89 (s, 1H, Ar-H), 9.06 (s, 2H, NH_2), 9.48 (s, 1H, pyrimidine-H), 8.80(s, 1H, CH=N), 13.35(s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 160.9, 160.4, 159.6, 148.2, 146.6, 144.8, 135.0, 128.3, 121.7, 107.7, 21.3; ESI-MS *m/z*: 346.4 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{11}N_7O_5$: C, 45.22; H, 3.21; N, 28.40. Found: C, 45.15; H,

3.18; N, 28.54 .

5.6.15. *N′*-((4-amino-2-methylpyrimidin-5-yl)methylene)-3-amino-5-nitrobenzohydrazide (A15)

Red solid; Yield 65%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 6.16 (s, 2H, NH_2), 7.48 (s, 1H, Ar-H), 7.56 (s, 1H, Ar-H), 7.88 (s, 1H, Ar-H), 8.08 (s, 1H, pyrimidine-H), 8.30 (s, 2H, NH_2), 8.44 (s, 1H, CH=N), 12.22 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.1, 161.7, 159.9, 158.5, 150.5, 148.9, 146.9, 134.9, 119.0, 110.1, 108.5, 107.1, 25.8; ESI-MS *m/z*: 316.1 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{13}N_7O_3$: C, 49.52; H, 4.16; N, 31.10. Found: C, 49.72; H, 4.28; N, 31.02 .

5.6.16. *N′*-((4-amino-2-methylpyrimidin-5-yl)methylene)-4-hydroxybenzohydrazide (A16)

White solid; Yield 65%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 3H, CH_3), 6.86 (s, 2H, NH_2), 7.80 (d, 2H, Ar-H, *J*=6.7Hz), 8.01 (s, 1H, pyrimidine-H), 8.38 (s, 1H, CH=N), 8.38 (s, 2H, Ar-H), 10.20 (s, 1H, OH), 11.86 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 166.7, 162.7, 161.0, 159.9, 157.9, 145.2, 129.8, 123.5, 115.2, 107.4, 25.7; ESI-MS *m/z*: 272.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{13}N_5O_2$: C, 57.56; H, 4.83; N, 25.82. Found: C, 57.72; H, 4.84; N, 25.97 .

5.6.17. *N′*-((4-amino-2-methylpyrimidin-5-yl)methylene)-2-hydroxybenzohydrazide (A17)

White solid; Yield 82%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 6.97 (s, 2H, NH_2), 7.44 (s, 1H, Ar-H), 7.87 (d, 1H, Ar-H, *J*=6.7Hz), 8.08 (s, 1H, pyrimidine-H), 8.31 (s, 2H, Ar-H), 8.45 (s, 1H, CH=N), 12.00 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.1, 164.7, 160.0, 159.2, 158.5, 147.1, 134.1, 128.7, 119.1, 117.5, 115.8, 107.1, 25.8; ESI-MS *m/z*: 272.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{13}N_5O_2$: C, 57.56; H, 4.83; N, 25.82.

Found: C, 57.73; H, 4.61; N, 26.04 .

5.6.18. *N′*-((4-amino-2-methylpyrimidin-5-yl)methylene)-2-methylbenzohydrazide (A18)

White solid; Yield 76%; m.p. >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 7.77 (s, 2H, NH_2), 7.86 (s, 2H, Ar-H), 8.16 (s, 1H, pyrimidine-H), 8.25 (s, 1H, CH=N), 8.42 (s, 2H, Ar-H), 12.16 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ(ppm): 166.6, 162.1, 159.9, 157.6, 146.3, 136.2, 13.3, 131.7, 129.8, 127.7, 125.9, 107.1, 25.5, 20.9; ESI-MS *m/z*: 270.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{13}N_5O$: C,62.44; H, 5.61; N, 26.01. Found: C, 62.16; H, 5.47; N, 26.06 .

5.6.19. *N′*-((4-amino-2-methylpyrimidin-5-yl)methylene)-3-methylbenzohydrazide (A19)

White solid; Yield 81%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 6H, $2CH_3$), 7.41 (s, 2H, NH_2), 7.73 (s, 2H, Ar-H), 8.05 (s, 1H, pyrimidine-H), 8.28 (s, 1H, CH=N), 8.41 (s, 2H, Ar-H), 12.03 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 166.9, 163.2, 159.9, 158.2, 146.1, 138.0, 133.1, 132.6, 128.5, 128.2, 124.9, 107.2, 25.7, 21.1; ESI-MS *m/z:* 270.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{14}H_{15}N_5O$: C, 62.44; H, 5.61; N, 26.01. Found: C, 62.05; H, 5.44; N, 25.84 .

5.6.20. *N′*-((4-amino-2-methylpyrimidin-5-yl)methylene)-4-methylbenzohydrazide (A20)

White solid; Yield 78%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 6H, $2CH_3$), 7.33 (s, 2H, NH_2), 7.83 (d, 2H, Ar-H, *J*=6.5Hz), 8.04 (s, 1H, pyrimidine-H), 8.28 (s, 1H, CH=N), 8.41 (s, 2H, Ar-H), 12.00 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 166.9, 162.9, 159.9, 158.2, 145.9, 142.2, 130.2, 129.2, 127.8, 107.3, 25.8, 21.2; ESI-MS *m/z*: 270.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{14}H_{15}N_5O$: C, 62.44; H, 5.61; N, 26.01. Found: C, 62.23; H, 5.94; N, 26.38 .

5.6.21. *N'*-((4-amino-2-methylpyrimidine-5-yl)methylene)-3,5-dimethylbenzohydrazide (A21)

White solid; Yield 70%; m.p 246-248°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.34 (s, 6H, $2CH_3$), 2.38 (s, 3H, CH_3), 7.23 (s, 1H, Ar-H), 7.52 (s, 2H, NH_2), , 8.14 (s, 1H, pyrimidine-H), 8.23 (s, 1H, CH=N), 8.41 (s, 2H, Ar-H), 12.00 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 166.7, 163.2, 159.9, 157.8, 145.8, 137.8, 133.3, 133.0, 125.4, 107.2, 25.6, 20.9; ESI-MS *m/z*: 284.1 $[M+1]^+$; Elemental Anal. Calcd for $C_{15}H_{17}N_5O$: C, 63.59; H, 6.05; N, 24.72. Found: C, 63.64; H, 5.73; N, 24.62 .

5.6.22. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-4-methoxybenzohydrazide (A22)

White solid; Yield 79%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.38 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 7.06 (s, 2H, NH_2), 7.91 (d, 2H, Ar-H, *J*=6.3Hz), 8.00 (s, 1H, pyrimidine-H), 8.27 (s, 1H, CH=N), 8.40 (s, 2H, Ar-H), 11.92 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 166.8, 162.6, 162.3, 159.9, 158.1, 145.6, 129.7, 125.1, 114.0, 107.4, 55.6, 25.8; ESI-MS *m/z*: 286.1 $[M+1]^+$; Elemental Anal. Calcd for $C_{14}H_{15}N_5O_2$: C, 58.94; H, 5.30; N, 24.55. Found: C, 59.02; H, 5.10; N, 24.68 .

5.6.23. *N'*-((4-amino-2-methylpyrimidin-5-yl)methylene)-3-methoxybenzohydrazide (A23)

White solid; Yield 79%; m.p 235-237°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 7.17 (d, 1H, Ar-H, *J*=5.6Hz), 7.45 (s, 2H, NH_2), 7.48 (d, 1H, Ar-H, *J*=6.6Hz), 8.06 (s, 1H, pyrimidine-H), 8.29 (s, 1H, CH=N), 8.42 (s, 2H, Ar-H), 12.04 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 167.0, 162.9, 160.0, 159.4, 158.3, 146.3, 134.5, 129.9, 120.0, 117.7, 113.1, 107.2, 55.5, 25.8; ESI-MS *m/z*: 286.1 $[M+1]^+$; Elemental Anal. Calcd for $C_{14}H_{15}N_5O_2$: C, 58.94; H, 5.30; N, 24.55. Found: C, 58.74; H, 5.09; N, 24.40 .

5.6.24. *N'*-((4-aminopyrimidine-5-yl)methylene)benzohydrazide (B1)

White solid; Yield 64%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 7.55 (s, 2H, NH_2), 7.60 (s, 1H, Ar-H), 7.92 (s, 2H, Ar-H), 8.15 (s, 1H, Ar-H), 8.42 (s, 1H, Ar-H), 8.42 (s, 1H, pyrimidine-6-H), 8.45 (s, 1H, CH=N), 8.45 (s, 1H, pyrimidine-2-H), 8.87 (s, 1H, Ar-H), 12.15 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 163.1, 159.7, 157.9, 157.8, 145.9, 132.9, 132.0, 128.6, 127.7, 109.8; ESI-MS *m/z*: 242.4 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{11}N_5O$: C, 59.74; H, 4.60; N, 29.03. Found: C, 59.63; H, 4.68; N, 29.40.

5.6.25. *N'*-((4-aminopyrimidine-5-yl)methylene)-4-fluorobenzohydrazide (B2)

White solid; Yield 74%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 7.40 (s, 2H, NH_2), 8.01 (s, 2H, Ar-H), 8.17 (s, 1H, pyrimidine-6-H), 8.44 (s, 1H, CH=N), 8.44 (s, 1H, pyrimidine-2-H), 8.44 (s, 2H, Ar-H), 12.18 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 165.6, 163.1, 162.0, 159.7, 157.8, 146.0, 130.5, 129.3, 115.7, 115.5, 109.7; ESI-MS *m/z*: 260.4 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{10}FN_5O$: C, 55.60; H, 3.89; N, 27.02. Found: C, 55.83; H, 3.93; N, 27.07 .

5.6.26. *N'*-((4-aminopyrimidine-5-yl)methylene)-4-bromobenzohydrazide (B3)

White solid; Yield 64%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 7.78 (s, 2H, NH_2), 7.81 (s, 2H, Ar-H), 8.17 (s, 1H, pyrimidine-6-H), 8.42 (s, 1H, CH=N), 8.42 (s, 1H, pyrimidine-2-H), 8.44 (s, 2H, Ar-H), 12.22 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 161.9, 159.6, 157.8, 146.1, 131.8, 131.5, 129.6, 125.7, 109.5.; ESI-MS *m/z*: 320.4 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{10}BrN_5O$: C, 45.02; H, 3.15; N, 21.88. Found: C, 45.30; H, 3.29; N, 21.87 .

5.6.27. *N'*-((4-aminopyrimidine-5-yl)methylene)-2-chlorobenzohydrazide (B4)

White solid; Yield 56%; m.p 239-240°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 7.47 (s, 2H,

NH_2), 7.53 (s, 1H, Ar-H), 7.59 (d, 2H, Ar-H, J=7.4Hz), 8.28 (s, 1H, pyrimidine-6-H), 8.31 (s, 1H, CH=N), 8.39 (s, 1H, Ar-H), 8.43 (s, 1H, pyrimidine-2-H), 12.21 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 168.4, 162.5, 159.8, 158.1, 146.4, 143.2, 134.8, 131.7, 130.5, 129.9, 129.5, 127.4, 109.5; ESI-MS *m/z*: 276.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{10}ClN_5O$: C, 52.28; H, 3.66; N, 25.40. Found: C, 52.31; H, 3.91; N, 25.75 .

5.6.28. *N'*-((4-aminopyrimidine-5-yl)methylene)-2-methylbenzohydrazide (B5)

White solid; Yield 51%; m.p 236-238°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.39 (s, 3H, CH_3), 7.29 (s, 1H, Ar-H), 7.32 (s, 2H, NH_2), 7.41 (d, 1H, Ar-H, *J*= 6.9Hz), 7.47 (d, 1H, Ar-H, *J*= 7.0Hz), 8.30 (s, 1H, Ar-H), 8.30 (s, 1H, pyrimidine-6-H), 8.36 (s, 1H, CH=N), 8.41 (s, 1H, pyrimidine-2-H), 12.05 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 165.2, 159.7, 157.9, 145.6, 136.2, 134.7, 130.8, 130.2, 127.6, 126.4, 125.8, 109.7, 19.5; ESI-MS *m/z:* 256.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{13}H_{13}N_5O$: C, 61.17; H, 5.13; N, 27.43. Found: C, 61.24; H, 4.95; N, 27.77 .

5.6.29. *N'*-((4-aminopyrimidine-5-yl)methylene)-3,5-dimethylbenzohydrazide (B6)

White solid; Yield 76%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.35 (s, 6H, $2CH_3$), 7.24 (s, 1H, Ar-H), 7.53 (s, 2H, NH_2), 8.15 (s, 1H, pyrimidine-6-H), 8.44 (s, 1H, CH=N), 8.44 (s, 1H, pyrimidine-2-H), 8.44 (s, 2H, Ar-H), 12.06 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 163.2, 159.7, 157.8, 157.7, 145.7, 137.8, 133.3, 132.9, 125.4, 109.8, 20.9; ESI-MS *m/z*: 270.4 $[M+1]^+$; Elemental Anal. Calcd for $C_{14}H_{15}N_5O$: C, 62.44; H, 5.61; N, 26.01. Found: C, 62.35; H, 5.56; N, 26.15 .

5.6.30. *N'*-((4-aminopyrimidine-5-yl)methylene)-2,4-dichlorobenzohydrazide (B7)

White solid; Yield 77%; m.p 235-236°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 7.57 (s, 2H,

NH_2), 7.65 (s, 1H, Ar-H), 7.77 (d, 1H, Ar-H, *J*=26.5Hz), 8.27 (s, 1H, pyrimidine-6-H), 8.27 (s, 1H, CH=N), 8.27 (s, 1H, Ar-H), 8.44 (s, 1H, pyrimidine-2-H), 8.44 (s, 2H, Ar-H), 12.06 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 161.6, 159.7, 158.0, 146.5, 134.8, 133.4, 131.9, 130.6, 127.4, 126.5, 109.6; ESI-MS *m/z*: 310.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_9Cl_2N_5O$: C, 46.47; H, 2.92; N, 22.58. Found: C, 46.43; H, 2.82; N, 22.67 .

5.6.31. *N'*-((2,4-diaminopyrimidine-5-yl)methylene)benzohydrazide (C1)

Light yellow solid; Yield 79%; m.p 250-252^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 6.55 (s, 2H, NH_2), 7.51 (s, 2H, NH_2), 7.53 (s, 1H, pyrimidine-H), 7.57 (d, 1H, Ar-H, *J*=6.5Hz), 7.89 (d, 2H, Ar-H, *J*=6.7Hz), 7.95 (s, 1H, CH=N), 8.26 (s, 2H, Ar-H), 11.73 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 162.6, 161.0, 160.4, 147.5, 133.4, 131.7, 128.5, 127.5, 101.1; ESI-MS *m/z*: 257.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{12}N_6O$: C, 56.24; H, 4.72; N, 32.79. Found: C, 56.23; H, 4.56; N, 32.69 .

5.6.32. *N'*-((2,4-diaminopyrimidine-5-yl)methylene)-3-nitrobenzohydrazide (C2)

Light yellow solid; Yield 79%; m.p>260 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 6.60 (s, 2H, NH_2), 7.50 (s, 1H, pyrimidine-H), 7.83 (s, H, NH_2), 7.99 (s, H, NH_2), 8.17 (s, 1H, CH=N), 8.29 (s, 1H, Ar-H), 8.34 (s, 1H, Ar-H), 8.43 (s, 2H, Ar-H), 8.74 (s, 1H, Ar-H), 12.05 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 162.9, 161.1, 160.9, 160.4, 148.6, 147.9, 134.9, 134.1, 130.4, 126.3, 122.3, 101.0; ESI-MS *m/z*: 302.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{11}N_7O_3$: C, 47.84; H, 3.68; N, 32.55. Found: C, 47.60; H, 3.76; N, 32.56 .

5.6.33. *N'*-((2,4-diaminopyrimidine-5-yl)methylene)-4-amino-benzohydrazide (C3)

Yellow solid; Yield 87%; m.p 238-240^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 5.76 (s, 2H, NH_2), 6.48 (s, 2H, NH_2), 6.58 (s, 2H, NH_2), 7.39 (s, 1H, pyrimidine-H), 7.63 (d, 2H, Ar-H,

J=5.4Hz), 7.91 (s, 1H, CH=N), 8.20 (s, 2H, Ar-H), 11.32 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 162.6, 162.3, 160.9, 159.6, 152.2, 145.7, 129.2, 119.5, 112.7, 101.4; ESI-MS *m/z*: 272.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{13}N_7O$: C, 53.13; H, 4.83; N, 36.14. Found: C, 53.43; H, 4.55; N, 36.46 .

5.6.34. *N*'-((2,4-diaminopyrimidine-5-yl)methylene)-4-amino-2-chloro-benzohydrazide (C4)

Yellow solid; Yield 87%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 5.82 (s, 2H, NH_2), 6.52 (s, 2H, NH_2), 6.52 (s, 1H, NH_2), 6.63 (s, 1H, NH_2), 7.22 (d, 1H, Ar-H, *J*=7.8Hz), 7.42 (s, 1H, pyrimidine-H), 7.89 (s, 1H, CH=N), 8.07 (s, 2H, Ar-H), 11.42 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 162.6, 162.3, 161.0, 160.3, 151.6, 146.4, 131.8, 130.8, 121.0, 113.7, 111.7, 101.0; ESI-MS *m/z*: 306.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{12}ClN_7O$: C, 47.14; H, 3.96; N, 32.07. Found: C, 47.28; H, 3.75; N, 32.14.

5.6.35. *N'*-((2,4-diaminopyrimidine-5-yl)methylene)-2,4-dichloro-benzohydrazide (C5)

Light yellow solid; Yield 77%; m.p 149-151°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 6.60 (s, 2H, NH_2), 7.50-7.57 (m, 2H, NH_2), 7.61 (d, 1H, Ar-H, *J*=8.1Hz), 7.77 (s, 1H, pyrimidine-H), 7.88 (d, 2H, Ar-H, *J*=8.2Hz), 7.96 (s, 1H, CH=N), 8.07 (s, 2H, Ar-H), 11.81 (s, 1H, NH); ESI-MS *m/z*: 325.1 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{10}Cl_2N_6O$: C, 44.33; H, 3.10; N, 25.85. Found: C, 44.39; H, 3.27; N, 25.82.

5.6.36. *N'*-((2,4-diaminopyrimidine-5-yl)methylene)-3-chloro-benzohydrazide (C6)

Light yellow solid; Yield 79%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 6.58 (s, 2H, NH_2), 7.49-7.58 (m, 2H, NH_2), 7.65 (d, 1H, Ar-H, *J*=7.1Hz), 7.86 (d, 1H, Ar-H, *J*=6.8Hz), 7.94 (s, 1H, pyrimidine-H), 7.97 (s, 1H, Ar-H), 8.16 (s, 1H, CH=N), 8.25 (s, 1H, Ar-H), 11.81 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 162.8, 161.1, 160.7, 158.9, 148.0, 135.4, 133.3, 131.5,

130.6, 127.3, 126.4, 100.9; ESI-MS *m/z*: 291.2 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{11}ClN_6O$: C, 49.58; H, 3.81; N, 28.91. Found: C, 49.74; H, 3.61; N, 28.97.

5.6.37. *N*'-((2,4-diaminopyrimidine-5-yl)methylene)-4-chloro-benzohydrazide (C7)

Light yellow solid; Yield 87%; m.p >260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 6.57 (s, 2H, NH_2), 6.65 (s, 1H, Ar-H), 7.50 (s, 1H, pyrimidine-H), 7.60 (d, 2H, NH_2, *J*=8.0Hz), 7.92 (d, 2H, Ar-H, *J*=7.9Hz), 8.13 (s, 1H, CH=N), 8.25 (s, 1H, Ar-H), 11.79 (s, 1H, NH); ESI-MS *m/z*: 291.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{11}ClN_6O$: C, 49.58; H, 3.81; N, 28.91. Found: C, 49.57; H, 3.98; N, 28.53.

5.6.38. *N*'-((2,4-diaminopyrimidine-5-yl)methylene)-4-bromo-benzohydrazide (C8)

Light yellow solid; Yield 80%; m.p 249-251°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 6.56 (s, 2H, NH_2), 7.75 (s, 2H, NH_2), 7.47 (s, 1H, pyrimidine-H), 7.84 (d, 2H, Ar-H, *J*=6.9Hz), 7.95 (s, 1H, CH=N), 8.25 (s, 2H, Ar-H), 11.80 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 163.2, 159.7, 157.8, 157.7, 145.7, 137.8, 133.3, 132.9, 125.4, 109.8; ESI-MS *m/z*: 335.3 $[M+1]^+$; Elemental Anal. Calcd for $C_{12}H_{11}BrN_6O$: C, 43.00; H, 3.31; N, 25.07. Found: C, 42.85; H, 3.56; N, 24.83 .

5.7. Assay of *E.coli* PDHc E1 (*in vitro*) and site-directed mutagenesis of PDHc E1

The half maximal inhibitory concentration (IC_{50}) values of the title compounds were determined at the PDHc E1enzyme level *in vitro*. The cloning, expression, purification and activity of PDHc E1 were carried out according the literature by the method of Bradford or Ren.[17, 29] The enzymatic assay was used to measure the inhibitory activities of synthesized compounds. PDHc E1 activity was measured by monitoring the reduction of 2,6-DichloroPhenolindophenol (2,6-DCPIP) at 600 nm using a microplate reader (BioTek Synergy 2, USA).[30] Each experiment was performed at least

three times. All data were handled by origin 7.0 software. One unit of activity is defined as the amount of 2,6-DCPIP reduced (μmol/min/mg of PDHc E1). The IC_{50} values of these compounds are shown in **Table 1~4**.

Site-directed mutagenesis of PDHc E1 was accomplished by the introduction of specific base changes into a double stranded DNA plasmid, as described previously. [23]

5.8. Inhibitory activity evaluation of compounds against *Acidovorax avenae subsp. Avenae* and *Xanthimonas oryzae pv. Oryzae in vivo*

Inhibitory activity evaluation of compounds **A13~15** and **C2** were tested against *Acidovorax avenae subsp. Avenae* and *Xanthimonas oryzae pv. Oryzae in vivo* by using the mycelium growth rate method.[31] The DMSO solution of title compounds was diluted in water to the required concentration (500 μg mL^{-1}). The mycelial elongation (mm) of *Acidovorax avenae subsp. Avenae* and *Xanthimonas oryzae pv. Oryzae* settlements was measured after 48 h of culture on potato glucose solid medium. Each test was carried out three replicates. The growth inhibition rates (I) were calculated using the equation: $I = [(C - T)/C] \times 100\%$, where C is the average diameter of mycelia in the presence of these compounds. The result are shown in **Table 6**.

5.9. Molecular docking

The crystallographic coordinates of the PDHc E1 with bound ThDP from *E. coli* (PDB code: 1L8A)[32] were obtained from Brookhaven Data Bank for molecule docking. In order to form hydrogen bonds with the ligand, the protonated state of several important residues, such as Met194, His142, His640, His106, Glu571, and Tyr177 were adjusted by using SYBYL7.3 (Tripos, St. Louis, MO, USA). Molecular docking inquiry was carried out by the SURFLEX module of SYBYL package to study the interaction model for the active site of PDHc E1 with its ligand.

Any atom of the cofactor ThDP, which located within the range of 6.5 Å, were selected into the active site, and the corresponding amino acid residue was involved into the active site when its atoms was selected. All calculations were performed on a CCNU Grid-based computational environment (CCNUGrid website http://www.202.114.32.71:8090/ccnu/chem/platform.xml).

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Graphic abstract for

Design and optimization of N-acylhydrazone pyrimidine derivatives as E. coli PDHc E1 inhibitors: structure-activity relationship analysis, biological evaluation and molecular docking study

N-acylhydrazone pyrimidine **A14** exhibited most powerful inhibitory potency against *E. coli* PDHc E1 (IC_{50} = 0.15 μM).