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Design, synthesis and biological evaluation of novel inhibitors against cyanobacterial pyruvate dehydrogenase multienzyme complex E1

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ABSTRACT

Cyanobacterial pyruvate dehydrogenase multienzyme complex E1 (PDHc E1) is a potential target enzyme for finding inhibitors to control harmful cyanobacterial blooms. In this study, a series of novel triazole thiamin diphosphate (ThDP) analogs were designed and synthesized by modifying the substituent group of triazole ring and optimizing triazole-benzene linker as potential cyanobacterial PDHc E1 (Cy-PDHc E1) inhibitors. Their inhibitory activities against Cy-PDHc E1 *in vitro* and algicide activities *in vivo* were further examined. Most of these compounds exhibited prominent inhibitory activities against Cy-PDHc E1 (IC_{50} 1.48–4.48 μM) and good algicide activities against *Synechocystis* PCC6803 (EC_{50} 0.84–2.44 μM) and *Microcystis aeruginosa* FACHB905 (EC_{50} 0.74–1.77 μM). Especially, compound **8d** showed not only the highest inhibitory activity against Cy-PDHc E1 (IC_{50} 1.48 μM), but also the most powerful inhibitory selectivity between Cy-PDHc E1 (inhibitory rate 98.90%) and porcine PDHc E1 (inhibitory rate only 9.54%). Furthermore, the potential interaction between compound **8d** and Cy-PDHc E1 was analyzed by a molecular docking method and site-directed mutagenesis and enzymatic analysis and fluorescence spectral analysis. These results indicated that compound **8d** could be used as a hit compound for further optimization and might have potential to be developed as a new algicide.

1. Introduction

With the increasing eutrophication and global warming, harmful cyanobacterial blooms (HCB) have become a major threat to human health and ecological system because HCB have increased all over the world during recent decades and they are possibly expanding further in the recent future[1]. Several approaches have been developed to control HCB, including (1) physical methods, such as nutrient management by phosphorus-binding clays[2], high-frequency sonication for disrupting cell membranes and retarding photosynthetic activity[3]; (2) biological methods, such as viruses, bacteria or fungi[4]; (3) chemical methods, such as herbicides (e.g. diuron) and copper-based compounds (e.g. copper sulfate)[5,6]. Among these approaches, chemical methods could rapidly eradicate HCB and have been used for many decades. However, these chemical compounds are no longer recommended because of their lengthy environmental persistence and toxic effect on both cyanobacteria and other non-target aquatic organisms[5,6]. Therefore, it is very necessary and urgent to develop novel selective and safe algicides targeting cyanobacteria.

Pyruvate dehydrogenase multienzyme complex (PDHc) catalyzes the oxidative decarboxylation of pyruvate, and subsequently acetylates coenzyme A (CoA) to acetyl-CoA during the tricarboxylic acid metabolic pathway using thiamine diphosphate (ThDP) and Mg^{2+} as cofactors[7]. PDHc poses a key role in cyanobacteria cellular metabolism. Pyruvate dehydrogenase complex E1 [PDHc E1, EC1.2.4.1] is the first and most important enzyme during the multienzyme complex composed of three enzymes. The PDHc E1 catalyzes the first step of thiamin diphosphate-dependent oxidative decarboxylation of pyruvate, which is the rate limiting step in the overall reaction catalyzed by the PDHc. It had been reported that PDHc-E1 enzyme was taken as the potent target of antibacterial and antifungal and herbicide for a long time[8,9]. These reports showed that cyanobacterial PDHc E1 (Cy-PDHc E1) possibly could be a potential candidate enzymatic target for searching specific algicide to control HCBs.

So far, few studies reported about the inhibitors or algicides targeted to the Cy-PDHc E1. It has been reported that cofactor ThDP plays an important role in the oxidative decarboxylation catalyzed by PDHc E1. Currently, based on the structure of ThDP, some of its analogs had

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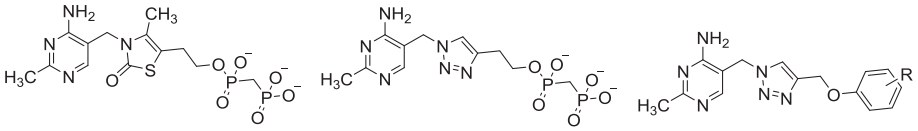


Fig. 1. Structures of known potential ThDP analog inhibitors against PDHc E1.

been reported as effective inhibitors against plant PDHc E1 or *Escherichia coli* (E. coli) PDHc E1 *in vitro* and against herbicide or antifungal or antibacterial activities *in vivo* (Fig. 1)[9–13]. Compound **I** is an effective inhibitor of *Escherichia coli* (E. coli) PDHc E1 *in vitro* in our previous study[16], but we found that it was almost not effective against Cy-PDHc E1.

In order to find the inhibitors of Cy-PDHc E1, structurally diversified inhibitors should be investigated. Based on the structure of the triazole-ThDP analogs and compound I, firstly, considering that benzoic acid and benzoate derivatives have received much attention especially for their structures and biological activities, such as anticancer, antimicrobial activities[17–19], we remained the 2-methylpyrimidine-4-ylamine moiety of triazole ThDP in our new compounds. Then, because the triazole ring moiety has wider biological activities and plays an important role in bio-conjugation due to easily form powerful pharmacophores, we tried to remain the triazole ring from triazole-ThDP analogs in our new compounds. Furthermore, the highly charged pyrophosphate moiety of ThDP analogs leads a bad bioavailability and negligible anti-microbial activity. Our previous studies have been demonstrated that these highly charged pyrophosphate moiety could be replaced with substituted benzene ring to solve the problem and some herbicide and antifungal and antibacterial candidates were found[12–15]. So we used benzene ring to substitute the pyrophosphate group and introduced ester group (O—C═O) as a functional linker to connect the 1, 2, 3-triazole ring with benzene ring. It is very interesting that the optimized compounds showed higher selective inhibition activity against Cy-PDHc E1. These results encouraged us to further design and synthesize a series of ThDP analogs as potential inhibitors of Cy-PDHc E1.

In this study, we designed and synthesized a series of (1-((4-amino-2-methyl pyrimidin-5-yl) methyl)-1H-1,2,3-triazol-4-yl) methyl substituted benzoate by remaining the 2- methylpyrimidine-4-ylamine moiety and the triazole ring and using the ester group (O—C═O) as a functional linker between 1,2,3-triazole ring and substituted benzene ring (Fig. 2). These compounds not only exhibited higher inhibitory activities against Cy-PDHc E1 *in vitro*, but also had good algicide activities against *Synechocystis* PCC6803 and *Microcystis aeruginosa* FACHB905 than that of triazole-ThDP or compound **I** *in viro*. Especially compound **8d** showed the highest inhibitory activity against Cy-PDHc E1 and the most powerful inhibitory selectivity between Cy-PDHc E1 and porcine PDHc E1. Furthermore, the potential interaction between compound **8d** and Cy-PDHc E1 was analyzed by a molecular docking method and site-directed mutagenesis and enzymatic analysis and fluorescence spectral analysis.

2. Results and discussion

2.1. Design and synthesis of the new ThDP analogs 8a-8g

As shown in Scheme 1, the (1-((4-amino-2-methyl pyrimidin-5-yl) methyl)-1H- 1,2,3- triazol −4-yl) methyl substituted benzoate compounds **6a-6g** and **8a-8g** were synthesized by thiamine hydrochloride (Vitamin B1) as starting materials[16,20,21].

The **3a-3g** compounds were synthesized by the reaction of various substituted benzoic acid with oxalyl chloride using dichloromethane (DCM) as solvent. **3a-3g** compounds were reacted with propynol to prepare substituted benzoate **4a-4g** using triethylamine (Et_3N) as base. Then the substituted benzoate **4a-4g** was used to prepare **5a-5g** in the presence of CuI, Et_3N and THF. Compounds **5a-5g** were further converted into corresponding hydrochloride **6a-6g** by using hydrochloric acid for increasing the water solubility. Substituted benzoate **4a-4g** reacted with *N*-iodomorpholine to produce compounds **7a-7g** in the presence of catalyzer CuI and solvent tetrahydrofuran (THF). **8a-8g** were synthesized using intermediates **7a-7g** as materials in the same methods.

All title compounds **6a-6g** and **8a-8g** were characterized by ^1H NMR, ^{13}C NMR, mass spectrometry (MS), and confirmed by elementary analysis.

2.2. Inhibitory activity and selectivity of the new ThDP analogs against Cy-PDHc E1 and Porcine PDHc E1 in vitro

As shown in Table 1, the compounds **Ia-Id, 6a-6g** and **8a-8g** were evaluated for their inhibitory activities against Cy-PDHc E1 *in vitro*. The lead compounds **Ia-Id** showed strong inhibitory activity against *E. coli* PDHc E1 in our previous study[16]. However, when we determined the inhibitory activities of lead compounds **Ia-Id** against Cy-PDHc E1, the results showed that compounds **Ia-Id** had very slight inhibitory activity against Cy-PDHc E1 (Table 1). Then compounds **5a-5g** were designed and synthesized by introducing ester group (O—C═O) as a linker to connect the 1, 2, 3-triazole ring with benzene ring. But due to their poor water solubility, compounds **5a-5g** were further converted into corresponding hydrochloride **6a-6g** by using hydrochloric acid to increase their solubility. The most exciting part to us was that all the compounds **6a-6g** had much better inhibitory activity against Cy-PDHc E1 (IC_{50} 3.37–4.48 μM) than that of lead compounds **Ia-Id**.

Subsequently, compounds **8a-8g** were synthesized in order to investigate the effect of introduction of iodine on the inhibitory activity against Cy-PDHc E1. As shown in Table 1, compounds **8a-8g** with iodine (**I**) showed higher inhibitory activity (IC_{50} = 1.48–2.35 μM) against Cy-PDHc E1 than that of compounds **6a-6g** (IC_{50} 3.37–4.48 μM). Especially compound **8d** exhibited highest inhibitory activity (IC_{50} = 1.48 μM).

Based on these results, it can be speculated that the ester group of compounds **6a-6g** and **8a-8g** was the most important factor for their inhibitory activities against Cy-PDHc E1, at the same time, the introduction of iodine group to the 5-position of 1, 2, 3-triazole is to the benefit for increasing the inhibitory activity of compounds **8a-8g** against Cy-PDHc E1.

Furthermore, in order to examine the selectivity of these compounds, the inhibitory activities of compounds **6a-6g** and **8a-8g** against Porcine PDHc E1 were determined. As showed in Table 1, our results

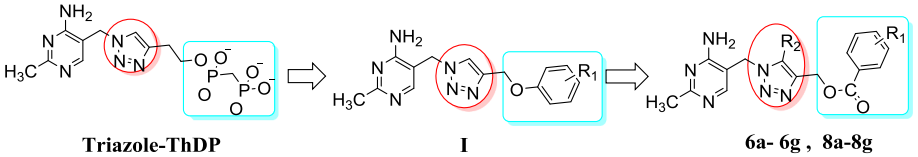
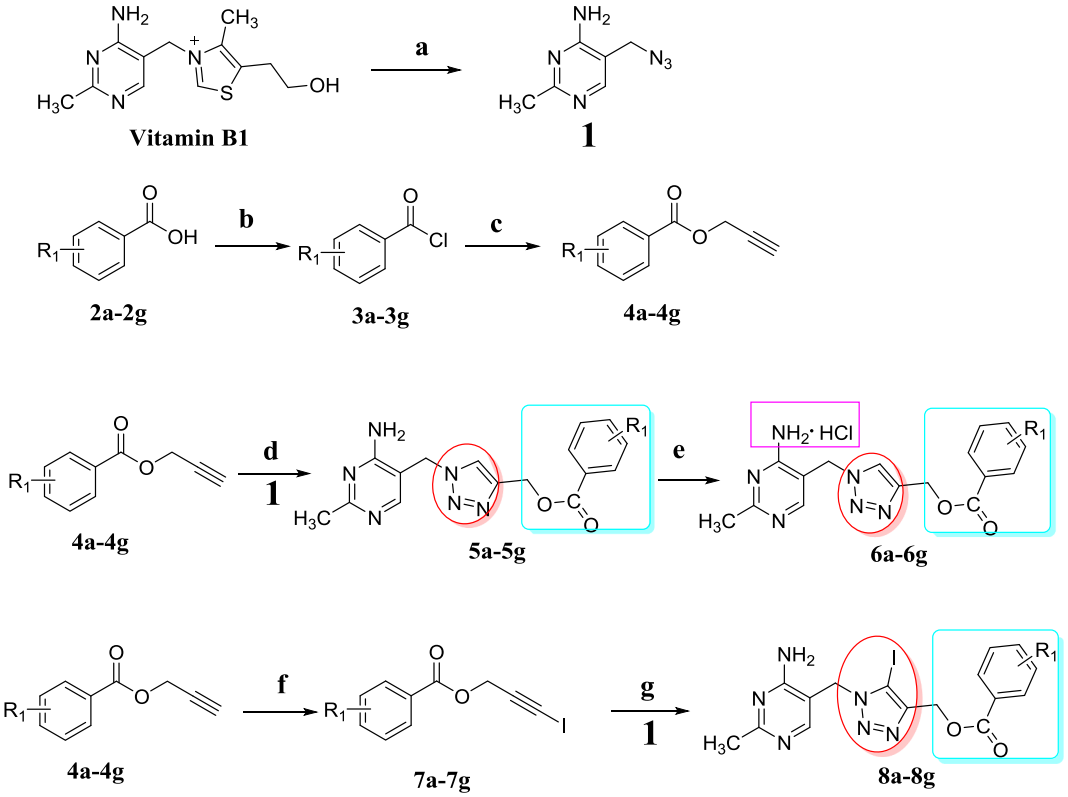
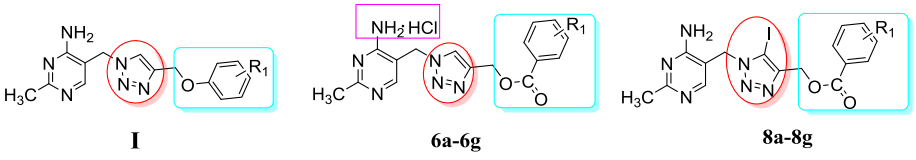


Fig. 2. Design of novel ThDP analog inhibitors against cyanobacterial PDHc E1.



Scheme 1. Reagents and conditions (a) NaN_3, Na_2SO_3, H_2O, 60–65 °C, 5 h; (b) $C_2O_2Cl_2$, DCM/DMF, rt, 2–3 h; (c) propynol, Et_3N, DCM, rt; (d) CuI, Et_3N, THF, rt, 12 h; (e) HCl, CH_3OH,rt; (f) *N*-iodomorpholine, CuI, THF, rt, 1 h; (g) CuI, Et_3N, THF, rt, 12 h.

Table 1
Structures and activities against Cy-PDHc E1 or Porcine PDHc-E1 of compounds **6a-6g** and **8a-8g**.



Compd.	R_1	Cy-PDHc-E1 IC_{50}[a] (μM)	Inhibitory rate[b] (%)		EC_{50}[c] (μM)	
			Cy-PDHc-E1	Porcine PDHc-E1	PCC6803[d]	FACHB905[d]
Ia	H	> 100	19.0	–	> 100	> 100
Ib	4-NO_2	> 100	8.64	–	> 100	> 100
Ic	4-F	> 100	16.2	–	> 100	> 100
Id	4-Cl	> 100	29.6	–	> 100	> 100
6a	H	3.58 ± 0.25	95.5	15.9	1.66 ± 0.11	1.17 ± 0.06
6b	4-NO_2	3.57 ± 0.14	95.7	13.5	1.64 ± 0.38	1.33 ± 0.06
6c	4-F	3.88 ± 0.05	99.5	17.8	2.11 ± 0.20	1.22 ± 0.05
6d	2-Cl	3.37 ± 0.16	97.8	7.31	2.44 ± 0.13	1.43 ± 0.11
6e	4-Cl	3.55 ± 0.12	94.2	–	2.25 ± 0.24	1.11 ± 0.05
6f	3-Cl	3.73 ± 0.19	93.2	9.62	1.81 ± 0.56	1.29 ± 0.21
6g	4-CH_3	4.48 ± 0.27	95.3	–	1.43 ± 0.11	1.20 ± 0.13
8a	H	1.94 ± 0.19	96.5	21.0	1.44 ± 0.18	0.99 ± 0.08
8b	4-NO_2	1.69 ± 0.06	99.2	10.4	1.09 ± 0.05	0.95 ± 0.02
8c	4-F	1.61 ± 0.19	95.4	15.3	0.84 ± 0.15	0.78 ± 0.05
8d	2-Cl	1.48 ± 0.05	98.9	9.50	1.17 ± 0.11	0.90 ± 0.07
8e	4-Cl	2.35 ± 0.16	95.6	–	0.90 ± 0.24	1.06 ± 0.07
8f	3-Cl	1.79 ± 0.10	98.7	5.13	1.16 ± 0.15	0.74 ± 0.08
8g	4-CH_3	1.88 ± 0.08	97.1	–	1.14 ± 0.06	0.85 ± 0.03
CuSO4	–	–	–	–	1.47 ± 0.08	1.77 ± 0.14

[a] IC_{50} (μM) is defined as the concentration required for 50% inhibition against Cy-PDHc E1 *in vitro* and were estimated statistically by origin7.5 software. Values are means ± SD (n = 3).
[b] Inhibitory rate (%) of compounds against Cy-PDHc E1 or Porcine PDHc E1 *in vitro* at 100 μM.
[c] EC_{50} (μM) is defined as the concentration required for 50% inhibition against PCC6803 or FACHB905. Values are means ± SD (n = 3).
[d] PCC6803, *Synechocystis* sp. PCC6803; FACHB905, *Microcystis aeruginosa* FACHB905.

showed that compounds **6a-6g** and **8a-8g** had obviously selective inhibition between Cy-PDHc E1 and Porcine PDHc-E1. All the compounds **6a-6g** and **8a-8g** had stronger inhibition against Cy-PDHc E1 (inhibitory rate > 90%) than that of their inhibition against porcine PDHc E1 (inhibitory rate only 7.31–21.0%) *in vitro* at the same concentration (100 μM). It indicated that compounds **6a-6g** and **8a-8g** showed lower toxicity and owed more eco-friendly characterizations as potential algicide.

2.3. Algicide activities of the new ThDP analog inhibitors against cyanobacteria in vivo

Most known ThDP analogs showed antibacterial activity or antifungal or herbicide activity[8,9,12–16], but few researches reported about ThDP analogs with algicide activities. In order to examine the practicality of compounds **6a-6g** and **8a-8g**, we further evaluated their algicide activities against two model cyanobacteria strains *Synechocystis* sp. PCC6803 (PCC6803) and *Microcystis aeruginosa* FACHB905 (FACHB905). As shown in Table 1, compounds **Ia-Id** did not display algicide activities (EC_{50} > 100 μM), conversely the corresponding compounds **6a-6g** and **8a-8g** exhibited dramatically high algicide activities (EC_{50} = 0.74–2.44 μM). Especially compounds **8a-8g** showed higher algicide activities than that of $CuSO_4$, a kind of widely used algicide. These results indicated that the algicide activity of these ThDP analogs also could be significantly enhanced by introducing ester group (O−C═O) as a linker to connect the 1, 2, 3-triazole ring with benzene ring and introducing iodine group to the 5-position of 1, 2, 3-triazole.

2.4. The interaction between the novel ThDP analog inhibitors and Cy-PDHc E1

Because it showed the highest inhibitory activity (IC_{50} = 1.48 μM) against Cy-PDHc E1 and higher inhibitory activity against *Synechocystis* sp. PCC6803 (PCC6803) and *Microcystis aeruginosa* FACHB905 (FACHB905), compound **8d** was selected as a hit compound for the study of the interaction mode of the novel ThDP analogs (**8a-8g**) with the potential target Cy-PDHc E1. Firstly, the modeling 3D structure of Cy-PDHc E1 was generated on SwissModel server (https://swissmodel.expasy.org/)[22] by homology modeling using the crystal structure of *Geobacillus stearothermophilus* PDHc E1 (Geo-PDHc E1) (PDB: 1 W88) as a template which has 38.4% identity with Cy-PDHc E1[23]. Then the reliability of the 3D model structure of Cy-PDHc E1 was further confirmed by Ramachandran plot analyses of PROCHECK and QMEAN[24–26]. Molecular docking simulation analysis of compound **8d** with the active site of Cy-PDHc E1 was performed by using the SURFLEX module of SYBYL package.

The binding mode of compound **8d** is shown in Fig. 3. It can be seen that **8d** could occupy the ThDP-binding pocket of Cy-PDHc E1, which has the 'V'conformation[27,28]. The aminopyrimidine ring of compound **8d** has similar interactions with amino acid residues to ThDP or ThTDP or compound I in the 'V' conformation, which can form backbone hydrogen bonds with the amino acid residues Ala129 and Ile131. Moreover, strong π-π stacking can be formed between aminopyrimidine ring and amino acid residue Phe85. It was extraordinary that the ester group of compound **8d** obviously owned hydrogen bond interaction with amino acid residues Tyr80 and Arg81 in the active site of Cy-PDHc E1. This is the possibly important reason that the inhibition activity of compound **8d** with ester group against Cy-PDHc E1 was higher than that of compound **I**. To validate the predictive interaction between compounds **8d** and the amino acids binding with ester group, we further performed site-directed mutagenesis and fluorescence spectral analyses as shown in Fig. 4. The binding constant (Kb) against the Y80A, R81A and F85A were investigated using fluorescence spectral analysis. In line with our theoretical prediction, Kbs of the three mutants are obviously lower than the Kb value of the wild type enzyme (Fig. 4), suggesting that Y80, R81 and F85 have stronger interaction with compound **8d**.

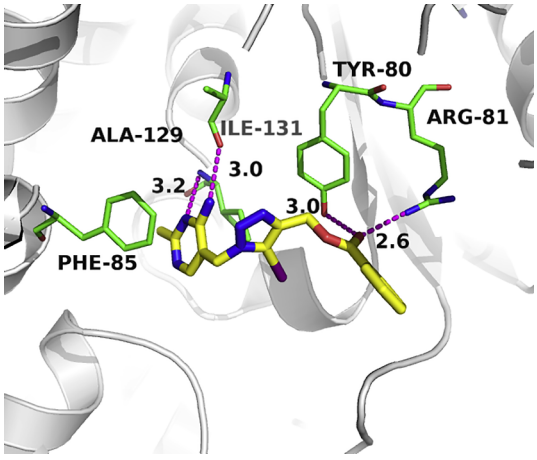


Fig. 3. Binding modes of compound **8d** target into active site of Cy-PDHc E1, PDHc E1 is shown in cartoon, compound **8d** is shown in stick, some key residues are shown in lines and hydrogen bonds are shown in dotted lines.

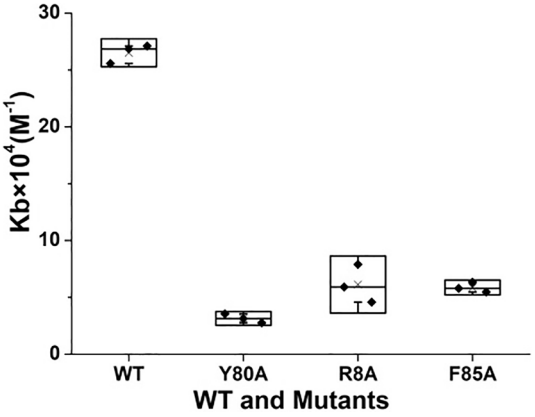


Fig. 4. Binding constants (Kb) determined by fluorescence spectral analyses for the binding of compound 8d to the wild type (WT) and its mutants. Values are means ± SD (n = 3).

3. Conclusion

Based on the structure of ThDP, in this study, a series of novel ThDP analogs **6a-6g** and **8a-8g** were designed by optimizing triazole-benzene linker and modifying the substituent group of triazole ring. Then they were synthesized as potential inhibitors of Cy-PDHc E1 by using click chemistry. All the compounds **6a-6g** (IC_{50} 3.37–4.48 μM) had much better inhibitory activity against Cy-PDHc E1 than that of lead compounds **Ia-Id. C**ompounds **8a-8g** with iodine (**I**) in the 5-position of 1, 2, 3-triazole showed higher inhibitory activity (IC_{50} = 1.48–2.35 μM) against Cy-PDHc E1 than that of compounds **6a-6g.** Especially compound **8d** exhibited highest inhibitory activity (IC_{50} = 1.48 μM). These results showed that the ester group of compounds **6a-6g** and **8a-8g** was the most important factor for their inhibitory activities against Cy-PDHc E1, at the same time, the introduction of iodine group to the 5-position of 1, 2, 3-triazole was to the benefit for increasing the inhibitory activity of compounds **8a-8g** against Cy-PDHc E1.

It is more interesting that all the compounds **6a-6g** and **8a-8g** had stronger inhibition against Cy-PDHc E1 (inhibitory rate > 90%) than that of their inhibition against porcine PDHc E1 (inhibitory rate only 7.31–21.0%) *in vitro* at the same concentration (100 μM), indicating that they owned possibly lower toxicity and more eco-friendly characterizations as potential algicide. Furthermore, the corresponding

compounds **6a-6g** and **8a-8g** exhibited dramatically high algicide activities (EC_{50} = 0.74–2.44 μM). Especially compounds **8a-8g** showed better algicide activities even than that of $CuSO_4$.

The results of molecular docking together with site-directed mutagenesis and fluorescence spectral analysis showed that triazole-benzene linker and the substituent group of triazole ring are the possibly important reasons for the higher inhibition activity of compound **8d**. These results indicated that compound **8d** could be used as a hit compound for further optimization and might have potential to be developed as a new algicide.

4. Experimental procedures

4.1. Chemical synthesis procedures

All chemical reagents were commercially available and treated with standard methods. Melting points (mp) were measured on an electrothermal melting point apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz, in DMSO-d_6 solution on a Varian Mercury-Plus 400 MHz 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*. Elemental analysis (EA) was measured on a Vario ELIII CHNSO elemental analyzer. Intermediate 5-(azidomethyl)-2-methylpyrimidine-4-amine **1** was synthesized according to the existing methods[29].

4.1.1. General procedure for preparation of substituted prop-2-yn-1-yl substituted benzoate 4a-4g

Intermediates **3a-3g** were synthesized respectively by the reaction of corresponding substituted benzoic acid (5 mmol) with oxalyl chloride (10 mmol) in the presence of dichloromethane (10 ml). The solution of the substituted benzoyl chloride **3a-3g** was added dropwise to the solution of propynol (6 mmol), trimethylamine (6 mmol) and dichloromethane (20 ml) at 0 °C. Then the reaction mixture was stirred at room temperature until the reaction was completed based on the TLC monitoring. The reaction mixture was washed with water (15 ml) and extracted three times with dichloromethane. Then the organic phases were washed sequentially with 10% NaOH solution, brine, dried with $MgSO_4$ and evaporated under reduced pressure to get crude products. The crude products were purified by column chromatography (silica gel, ethyl acetate: petroleum ether = 1:3 as eluent) to obtain **4a-4g**.

4.1.2. General procedure for preparation of compounds 6a-6g and 8a-8g

Intermediate 5-azidomethyl-2-methylpyrimidine-4-yla mine **1** (1.5 mmol) was reacted with **4a-4g** respectively in the presence of THF (10 ml), CuI (0.075 mmol) and Et_3N (3 mmol)[20]. The reaction was stirred at room temperature until the reaction was complete based on TLC monitoring and then poured into water to form precipitate. The precipitate was collected by filtration, dried under atmospheric pressure and washed with DCM (10 ml) to obtained **5a–5g,** which were used directly to react with 36% hydrogen chloride for preparing compounds **6a-6g.**

Intermediates **4a-4g** (2 mmol) were dissolved in THF (10 ml) and reacted with *N*-iodomorpholine (3 mmol) by using CuI (0.1 mmol) as catalyzer. The reaction mixture was stirred at room temperature for 1 h until white precipitate formed. Then the white precipitate was washed three times with THF (10 ml) to prepare **7a-7g**. Then compounds **8a-8g** were synthesized by **7a-7g** respectively reacting with compound 1 in the same way which was used to synthesize **5a–5g**.

4.1.3. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-1H-1,2,3-triazol-4-yl) methyl-benzoate hydrochloride 6a

Yellow solid: Yield 96%; m.p 172–174 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.50 (s, 3H, CH_3), 5.35 (s, 2H, CH_2), 5.61 (s, 2H, OCH_2), 7.47 (s, 2H, NH_2), 7.61 (s, 1H, Ar-H), 7.88 (s, 2H, Ar-H), 7.95 (s, 1H, 1,2,3-triazol-4-yl-H), 8.40 (s, 2H, Ar-H), 8.67 (s, 1H, pyrimidin-6-yl-H), 14.07 (s, 1H, HCl); ESI-MS *m/z*: 325.2 $[M + 1]^+$; HRMS (ESI): calcd. for $C_{16}H_{16}N_6O_2$ $[M + H]^+$ 325.14075, found: 325.14118; Elemental Anal. Calcd for $C_{16}H_{17}ClN_6O_2$: C, 53.26; H, 4.75; N, 23.29. Found: C, 53.55; H, 4.99; N, 23.54.

4.1.3.1. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl-4-nitrobenzoate hydrochloride 6b. Yellow solid; Yield 90%; m.p 140–142 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.50 (s, 3H, CH_3), 5.44 (s, 2H, CH_2), 5.81 (s, 2H, OCH_2), 8.13 (s, 2H, NH_2), 7.91 (s, 1H, 1,2,3-triazol-4-yl-H), 8.13 (s, 1H, Ar-H), 8.29 (s, 1H, pyrimidin -6-yl-H), 8.29 (s, 2H, Ar-H), 8.60 (s, 1H, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 25.3, 47.0, 58.2, 108.6, 125.5, 127.5, 129.0, 132.0, 138.5, 141.4, 156.8, 160.9, 164.0, 167.6; ESI-MS *m/z*: 370.2 $[M + 1]^+$; HRMS (ESI): calcd. for $C_{16}H_{15}N_7O_4$ $[M + H]^+$ 370.12583, found: 370.12679; Elemental Anal. Calcd for $C_{16}H_{16}ClN_7O_4$: C, 47.36; H, 3.97; N, 24.16. Found: C, 47.19; H, 4.03; N, 24.55.

4.1.3.2. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl-4-fluorobenzoate hydrochloride 6c. Yellow solid; Yield 88%; m.p 126–128 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.50 (s, 3H, CH_3), 5.37 (s, 2H, CH_2), 5.52 (s, 2H, OCH_2), 7.13 (s, 2H, NH_2), 7.32 (s, 2H, Ar-H), 7.98 (s, 2H, Ar-H), 8.01 (s, 1H, 1,2,3-triazol-4-yl-H), 8.26 (s, 1H, pyrimidin-5-yl-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 24.3, 46.4, 57.3, 107.2, 114.8, 115.3, 124.1, 125.0, 131.4, 141.7, 155.2, 160.7, 163.1, 163.9, 165.7, 166.4; ESI-MS *m/z*: 343.3 $[M + 1]^+$; HRMS (ESI): calcd. for $C_{16}H_{15}FN_6O_2$ $[M + H]^+$ 343.13133, found: 343.13204; Elemental Anal. Calcd for $C_{16}H_{16}ClFN_6O_2$: C, 50.73; H, 4.26; N, 22.19. Found: C, 50.88; H, 4.45; N, 22.34.

4.1.3.3. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl-2-chlorobenzoate hydrochloride 6d. Yellow solid; Yield 89%; m.p 136–138 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.50 (s, 3H, CH_3), 5.38 (s, 2H, CH_2), 5.59 (s, 2H, OCH_2), 7.17 (s, 2H, NH_2), 7.43 (s, 1H, Ar-H), 7.55 (s, 2H, Ar-H), 7.75 (s, 1H, Ar-H), 8.00 (s, 1H, 1,2,3-triazol-4-yl-H), 8.26 (s, 1H, pyrimidin-6-yl-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 26.0, 46.5, 58.1, 108.8 125.2, 127.8, 129.0, 130.6, 133.3, 139.0, 140.1, 141.9, 156.7, 162.1, 164.7, 167.5; ESI-MS *m/z*: 359.1 $[M + 1]^+$; HRMS (ESI): calcd. for $C_{16}H_{15}ClN_6O_2$ $[M + H]^+$ 359.10178, found: 359.10356; Elemental Anal. Calcd for $C_{16}H_{16}Cl_2N_6O_2$: C, 48.62; H, 4.08; N, 21.26. Found: C, 48.89; H, 4.44; N, 21.54.

4.1.3.4. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl-4-chlorobenzoate hydrochloride 6e. White solid; Yield 54%; m.p. 188–189 °C; **^{1}H NMR** (400 MHz, DMSO-d_6) δ (ppm): 2.29 (s, 3H, CH_3), 5.37 (s, 2H, CH_2), 5.44 (s, 2H, OCH_2), 6.96 (s, 2H, NH_2), 7.59 (d, 2H, J = 7.2 Hz, Ar-H), 7.94 (d, 2H, J = 7.2 Hz, Ar-H), 8.02 (s, 1H, 1,2,3-triazol-4-yl-H), 8.25 (s, 1H, pyrimidin-5-yl-H); **^{13}C NMR** (100 MHz, DMSO-d_6) δ (ppm): 25.3, 46.8, 59.2, 108.4, 124.3, 127.8, 129.0, 131.5, 138.1, 140.9, 142.0, 156.8, 161.4, 164.5, 167.3; HRMS (ESI): calcd. for $C_{16}H_{15}ClN_6O_2$ $[M + H]^+$ 359.10178, found: 359.10257; Elemental Anal. Calcd for $C_{16}H_{15}ClN_6O_2$ (358.10): C, 53.56; H, 4.21; N, 23.42. Found: C, 53.93; H, 4.15; N, 22.98.

4.1.3.5. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl-3-chlorobenzoate hydrochloride 6f. Yellow solid; Yield 90%; m.p 135–137 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.50 (s, 3H, CH_3), 5.39 (s, 2H, OCH_2), 5.61 (s, 2H, CH_2), 7.23 (s, 2H, NH_2), 8.02 (s, 1H, 1,2,3-triazol-4-yl-H), 7.53 (s, 1H, Ar-H), 7.72 (s, 1H, Ar-H), 7.87 (s, 2H, Ar-H), 8.28 (s, 1H, pyrimidin-6-yl-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 26.2, 47.4, 58.3, 109.5, 124.9, 128.3, 129.2, 131.7, 132.5, 138.9, 140.4, 141.9, 156.3, 161.6, 164.4, 167.5; ESI-MS *m/z*: 359.2 $[M + 1]^+$; HRMS (ESI): calcd. for $C_{16}H_{15}ClN_6O_2$ $[M + H]^+$ 359.10178, found: 359.10229; Elemental Anal. Calcd for

$C_{16}H_{16}Cl_2N_6O_2$: C, 48.62; H, 4.08; N, 21.26. Found: C, 48.55; H, 4.22; N, 21.40.

4.1.3.6. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl-4-methylbenzoate hydrochloride 6g. Yellow solid; Yield 89%; m.p 132–134 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 2.50 (s, 3H, CH_3), 2.65 (s, 3H, CH_3), 5.50 (s, 2H, CH_2), 5.75 (s, 2H, OCH_2), 7.44 (s, 2H, NH_2), 7.44 (s, 2H, Ar-H), 7.96 (s, 2H, Ar-H), 7.96 (s, 1H, 1,2,3-triazol-4-yl-H), 8.44 (s, 1H, pyrimidin-6-yl-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 22.6, 25.0, 47.3, 58.2, 108.2, 125.5, 127.5, 129.2, 131.8, 138.7, 141.8, 156.7, 161.6, 164.8, 167.5; ESI-MS *m/z*: 339.2 $[M + 1]^+$; HRMS (ESI): calcd. for $C_{17}H_{18}N_6O_2$ $[M + H]^+$ 339.15640, found: 339.15706; Elemental Anal. Calcd for $C_{17}H_{19}ClN_6O_2$: C, 54.47; H, 5.11; N, 22.42. Found: C, 54.66; H, 5.34; N, 22.56.

4.1.3.7. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-5-iodo-1H-1,2,3-triazol-4-yl)-methylbenzoate 8a. Green solid; Yield 91%; m.p. 198–199 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.33 (s, 3H, CH_3), 5.36 (s, 2H, CH_2), 5.48 (s, 2H, -CH_2O), 6.95 (s, 2H, NH_2), 7.52 (s, 3H, Ar-H), 7.94(s, 2H, Ar-H), 7.66 (s, H, pyrimidin-5-yl-H Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 25.9, 46.9, 57.7, 108.8, 128.5, 129.4, 131.5, 133.5, 138.5, 142.6, 156.2, 160.2, 164.8, 167.6; HRMS (ESI): calcd. for $C_{16}H_{15}IN_6O_2$ $[M + 1]^+$ 451.03739, found: 451.03703; Elemental Anal. Calcd for $C_{16}H_{15}IN_6O_2$(450.03): C, 42.68; H, 3.36; N, 18.61. Found: C, 42.64; H, 3.43; N, 19.67.

4.1.3.8. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-5-iodo-1H-1,2,3-triazol-4-yl)methyl-4- nitrobenzoate 8b. Yellow solid; Yield 86%; m.p. 192–193 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ(ppm): 2.39(s, 3H, CH_3), 5.36(s, 2H, CH_2), 5.55(s, 2H, -CH_2O), 6.98(s, 2H, NH_2), 7.36(s, 2H, Ar-H), 8.01(s, 2H, Ar-H), 8.01(s, 1H, pyrimidin-5-yl-H); ^{13}C NMR (100 MHz, DMSO -d_6) δ(ppm): 25.7, 47.9, 57.6, 108.4, 124.9, 127.9, 129.2, 131.3, 138.7, 141.8, 156.6, 161.6, 165.6, 166.7; HRMS (ESI): calcd. for $C_{16}H_{14}IN_7O_4$ $[M + 1]^+$ 496.02247, found: 496.02199; Elemental Anal. Calcd for $C_{16}H_{14}IN_7O_4$(495.02): C, 38.80; H, 2.85; N, 19.80. Found: C, 38.88; H, 3.01; N, 20.05.

4.1.3.9. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-5-iodo-1H-1,2,3-triazol-4-yl)methyl-4-fluorobenzoate 8c. Yellow solid; Yield 73%; m.p. 207–209 °C ; ^{1}H NMR(400 MHz, DMSO-d_6) δ(ppm): 2.30(s, 3H, CH_3), 4.50(s, 2H, CH_2), 5.42(s, 2H, -CH_2O), 6.91(s, 2H, NH_2), 7.53(d, 2H, J = 7.4 Hz, Ar-H), 7.88(d, 2H, J = 7.5 Hz, Ar-H), 8.45(s, 1H, pyrimidin-5-yl-H); ^{13}C NMR(100 MHz, DMSO-d_6) δ(ppm): 24.7, 47.9, 57.6, 108.6, 115.1, 124.2, 127.5, 131.6, 142.5, 157.4, 161.6, 164.2, 165.3, 166.9, 167.9; HRMS (ESI): calcd. for $C_{16}H_{14}FIN_6O_2$ $[M + 1]^+$ 469.02797, found: 469.02847; Elemental Anal. Calcd for $C_{16}H_{14}FIN_6O_2$ (468.02): C, 41.04; H, 3.01; N, 17.95. Found: C, 40.87; H, 3.42; N, 18.01.

4.1.3.10. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-5-iodo-1H-1,2,3-triazol-4-yl)methyl-2-chlorobenzoate 8d. Celadon solid; Yield 86%; m.p. 185–187 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ(ppm): 2.36(s, 3H, CH_3), 4.94(s, 2H, CH_2), 5.50(s, 2H, -CH_2O), 7.00(s, 2H, NH_2), 7.55–7.59(m, 2H, Ar-H), 7.76(d, 2H, J = 4.0 Hz, Ar-H), 8.18 (s, 1H, pyrimidin-5-yl-H); ^{13}C NMR(100 MHz, DMSO-d_6) δ(ppm): 26.1, 48.1, 58.3, 108.6, 125.0, 128.3, 129.0, 130.8, 132.0, 138.7, 141.7, 142.6, 155.8, 161.2, 164.0, 167.5; HRMS (ESI): calcd. for $C_{16}H_{14}ClIN_6O_2$ $[M + 1]^+$ 484.99842, found: 484.99809; Elemental Anal. Calcd for $C_{16}H_{14}ClN_6O_2$(483.99): C, 39.65; H, 2.91; N, 17.34. Found: C, 39.28; H, 3.16; N, 17.79.

4.1.3.11. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-5-iodo-1H-1,2,3-triazol-4-yl)methyl-4-chlorobenzoate 8e. Grass green solid; Yield 84%; m.p. 192–193 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ(ppm): 2.33(s, 3H, CH_3), 5.36(s, 2H, CH_2), 5.49(s, 2H, CH_2O), 6.94(s, 2H, NH_2), 7.59(s, 2H, Ar-H), 7.94(s, 2H, Ar-H), 7.59(s, 1H, pyrimidin-5-yl-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm): 25.9, 46.7, 58.1, 108.2, 124.7, 128.4, 131.3, 136.2, 139.2, 142.5, 157.2, 161.4, 166.0, 168.2; HRMS (ESI): calcd. for $C_{16}H_{14}ClIN_6O_2$ $[M + 1]^+$ 484.99842, found: 484.99794; Elemental Anal. Calcd for $C_{16}H_{14}ClN_6O_2$(483.99): C, 39.65; H, 2.91; N, 17.34. Found: C, 39.38; H, 2.73; N, 17.23.

4.1.3.12. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-5-iodo-1H-1,2,3-triazol-4-yl)methyl-3-chlorobenzoate 8f. Celadon solid; Yield 88%; m.p. 185–186 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ(ppm): 2.50(s, 3H, CH_3), 5.39(s, 2H, CH_2), 5.51(s, 2H, -CH_2O), 6.95(s, 2H, NH_2), 7.57(s, 1H, Ar-H), 7.75(s, 1H, Ar-H), 7.90(s, 2H, Ar-H), ^{13}C NMR(100 MHz, DMSO-d_6) δ(ppm): 25.7, 47.0, 58.1, 108.5, 124.7, 127.9, 128.8, 131.1, 138.3, 142.3, 143.0, 156.4, 161.9, 165.6, 167.7; HRMS (ESI): calcd. for $C_{16}H_{14}ClIN_6O_2$ $[M + 1]^+$ 484.99842, found: 484.99568; Elemental Anal. Calcd for $C_{16}H_{14}ClN_6O_2$ (483.99): C, 39.65; H, 2.91; N, 17.34. Found: C, 39.74; H, 2.86; N, 17.15.

4.1.3.13. (1-((4-amino-2-methylpyrimidin-5-yl)methyl)-5-iodo-1H-1,2,3-triazol-4-yl)methyl-4-methoxybenzoate 8g. Green solid; Yield 75%; m.p. 200-201 °C ; 1H NMR(400 MHz, DMSO-*d6*) δ(ppm): 2.29(s, 3H, CH3), 2.36(s, 3H, -CH3), 5.35(s, 2H, CH2), 5.44(s, 2H, -CH2O), 6.95(s, 2H, NH2), 7.32(d, 2H, J = 8.0 Hz, Ar-H), 7.83(d, 2H, J = 8.0 Hz, Ar-H), 8.01(s, 1H, pyrimidin-5-yl-H); 13C NMR(100 MHz, DMSO-*d6*) δ(ppm): 25.7, 29.7, 46.9, 59.2, 109.1, 124.8, 127.6, 129.0, 131.1, 139.0, 143.3, 155.8, 163.3, 165.1, 167.5; HRMS (ESI): calcd. for C17H17IN6O2 [M +1]+ 465.05304, found: 465.05161; Elemental Anal. Calcd for C17H17IN6O3(464.05): C, 42.51; H, 3.57; N, 17.50. Found: C, 42.44; H, 3.58; N, 17.67.

4.2. Expression and purification of Cy-PDHc E1 and its activity measurements

The genes encoding Cy-PDHc-E1 (Gene Bank Number: BA000022.2) were directly amplified from genomic DNA of *Synechocystis* PCC6803 and then were inserted into the pETDuet-1 vector (Novagen). Positive transformants were verified by sequencing and then they were co-transformed into *Escherichia coli* BL-21 (DE3) together with plasmid pGro7 for over-expressing chaperonins GroEL and GroES. For expression of the enzyme, the recombination stain was inoculated in 2xYT medium with 100 μg/ml ampicillin and 20 μg/ml chloramphenicol at 37 °C until reaching a cell density to A600 of 0.5–0.6. The culture was induced with a final concentration of 0.5 mM IPTG and 0.5 mg/mL L-Arabinose for 24 h at 22 °C before harvesting. The harvested pellets were resuspended in buffer (300 mM NaCl, 50 mM potassium phosphate buffer, 1% glycerol and 1 mM DTT, 1 mM $MgCl_2$, pH 7.2) and sonicated, subsequently the lysate supernatant containing soluble cellular materials was loaded on a Ni^{2+}-NTA resin (Novagen) in a standard procedure. Proteins were eluted in two steps with 300 mM NaCl, 50 mM potassium phosphate buffer, and 1% glycerol and 1 mM DTT, 1 mM $MgCl_2$, pH 7.2, 75 mM imidazole and 250 mM imidazole. The isolated proteins were dialyzed against 300 mM NaCl, 50 mM potassium phosphate buffer, and 1% glycerol and 1 mM DTT, 1 mM $MgCl_2$, pH 7.2. Purified protein was stored in 50% (v/v) glycerol at −20 °C. The concentrations of purified proteins were determined by the Bradford method[19].

Cy-PDHc E1 activity was determined by monitoring the reduction of 2, 6 – DiChlorophenol IndoPhenol (2,6-DCIP) at 600 nm using a microplate reader (Bioteck Synergy2, USA)[20]. Cofactor ThDP and substrate pyruvate were purchased from Sigma. A standard reaction mixture containing 50 mM K_3PO_4 (pH 7.2), 0.375 mM 2, 6-DiChlorophenolIndoPhenol (DCIP), 0.8 mM sodium pyruvate as substrate, 0.1 mg/ml purified Cy-PDHc E1 enzyme. The reaction mixtures were incubated for 3 min at 37 °C, then added different concentrations of ThDP (ranging from 0 to 200 μM). One unit of activity is defined as the amount of 2, 6-DCIP reduced.

4.3. Evaluation of inhibitory activity of compounds against Cy-PDHc E1 and porcine PDHc E1 in vitro

The inhibitory activities of the compounds were measured by evaluating the Cy-PDHc E1 activity *in vitro*. A standard reaction mixture for the inhibition rate (%) of compounds against Cy-PDHc E1 is that contains 50 mM K_3PO_4 (pH 7.2), 0.375 mM 2, 6-DCIP, 0.1 mg/ml purified PDHc E1 enzyme and 200 μM ThDP and the 100 μM compounds. After the mixture was incubated for 5 min at 37 °C, the saturating substrate pyruvate was added for triggering the reaction. The standard reaction mixture for half maximal inhibitory concentration (IC_{50}) determination is as same as the above except for the compounds ranging from 0 to 100 μM. All the kinetic data were fit to Growth/Sigmoidal model from origin 7.0 software.

Porcine PDHc-E1 was purchased from Sigma. Porcine E1 activity was determined by monitoring the reduction of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 566 nm using a microplate reader (Bioteck Synergy2, USA). The inhibition rate (%) of compounds against porcine PDHc-E1 was assayed at the final concentration (100 μM). A standard reaction mixture containing 1.0 mM $MgCl_2$, 0.2 mM ThDP, 0.5 mM MTT, 6.5 mM phenazine methosulfate (PMS), 50 mM K_3PO_4, pH 7.0, 0.75 mg/mL enzyme and 100 μM compounds. The mixture was incubated for 3 min at 37 °C, and then 2 mM sodium pyruvate was added to initiate the reaction.

4.4. Inhibitory activity evaluation of compounds against Synechocystis PCC6803 and Microcystis aeruginosa FACHB905 in vivo

Inhibitory activities of compounds and half maximal effective inhibitory concentration (EC_{50}) against *Synechocystis* PCC6803 and *Microcystis aeruginosa* FACHB905 was determined *in vivo*. *Synechocystis* PCC6803 and *Microcystis aeruginosa* FACHB905 were cultured in BG11 medium at 28 °C for 7 days at a 12 h ligh/12 h dark cycle until the logarithmic growth phase. The 1% cultured cells were then transferred to a fresh medium containing different compounds. After culturing for 7 days, the OD680 were determined, and the inhibition rate and half maximal effective concentration (EC_{50}) were calculated as described previously[29].

4.5. Structure-based docking

The amino acid sequence of Cy-PDHc E1 obtained from the NCBI database (NCBI Reference Sequence: NC_000911.1) shared 38.4% identity with *Geobacillus Stearothermophilus* PDHc E1 (Geo-PDHc E1, PDB ID: 1W88)[23]. The modeling 3D structure of cyanobacteria PDHc E1 was built by using SWISSMODEL server and taking Geo-PDHc E1 crystal structure as templated[22]. Cofactors ThDP and Mg^{2+} were derived from the template crystal structure. The model 3D structure of Cy-PDHc E1 was further validated by Procheck[25] and Q-mean program[26]. The results of Ramachandran plot were that 89.9% of the residues were distributed in the favored regions, 8.0% in the additionally allowed regions, 1.5% in the generously allowed regions, only 0.6% of the residues were in the disallowed regions. The model 3D structure with cofactors of Cy-PDHc E1 was used for molecular docking analyses. The structure of compound **8d** was prepared with SYBYL7.0 and hydrogen atoms were added to the structure. Then the SURFLEX module of SYBYL7.0 package was performed to explore the interaction model of Cy-PDHc E1 with compound **8d**. All atoms located within 6.5 Å away from the cofactor ThDP were selected into the active site, and the corresponding amino acid residue was involved in the active site if only one of its atoms was selected. Other parameters were set to default. All calculations were performed on the CCNU Grid website http://www.202.114.32.71:8090/ccnu/chem/platform.xml.

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