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Research paper

Design, synthesis and biological evaluation of novel 3,4-dihydro-2(1*H*)-quinolinone derivatives as potential chitin synthase inhibitors and antifungal agents

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ABSTRACT

A series of 3,4-dihydro-2(1*H*)-quinolinone derivatives contained butenediamide fragment were designed and synthesized. Their inhibition potency against chitin synthase and antimicrobial activities were screened *in vitro*. The enzymatic assays showed that all the synthesized compounds had inhibition potency against chitin synthase at concentration of 300 μg/mL. Compound **2d** displayed excellent potency with inhibition percentage (IP) value of 82.3%, while IP value of the control polyoxin B was 87.5%. Compounds **2b**, **2e** and **2s** whose IP values were above 70% showed good inhibition potency against chitin synthase. Moreover, the IC_{50} value of **2b** was comparable with that of polyoxin B (0.09 mM). The K_i of compound **2b** was 0.12 mM and the result from Lineweaver-Burk plot showed that **2b** was non-competitive inhibitor to bind chitin synthase. The antifungal experiment showed that these compounds had excellent antifungal activity against fungal strains, especially for *candida albicans*. The antifungal activities against *C .albicans* of compounds **2b**, **2d**, **2e** and **2l** were comparable with that of fluconazole and were superior to that of polyoxin B. Meanwhile, the other compounds against *C. albicans* showed better antifungal activity (MIC 2 μg/mL) than polyoxin B except for compound **2n** (MIC 4 μg/mL). The trial of drug combination use showed that these synthesized compounds had synergistic effects with fluconazole and polyoxin B. It indicated that these compounds were not competing with polyoxin B to bind with chitin synthase, which was also consistence with the result of enzymatic assays. The antibacterial experiment showed that these compounds had no activity against selected strains including three Gram-positive and three Gram-negative bacteria. These results showed that the designed compounds were chitin synthase inhibitors and had selective antifungal activity.

1. Introduction

Fungal infection which range from non-fatal skin and mucous membrane disease to invasive infection involving many organs [1] is a significant worldwide health problem [2]. More than a billion people are affected by fungal infections [3]. In addition, the morbidity and mortality are very high due to invasive fungal infection for immunosuppressive patients [4,5]. The main antifungal drugs used in clinic include echinocandins, polyenes, azoles, pyrimidines and acrylamides [6]. However, these drugs are hampered notoriously with potential toxicity, adverse effects and the increasing drug resistance of the fungi [7]. Therefore, it is an urgent need to explore novel antifungal agents to overcome these disadvantages.

Chitin synthase (CHS) is a fundamental enzyme in the biosynthesis of chitin which is a polymer of N-acetylglucosamine [8] connected by β-(1, 4) glycosidic bonds. Chitin is the scaffold structure of fungal cell walls and absent in mammalian cells [9]. Chitin synthase inhibitors can cause abnormality of cell wall structure, resulting in cell rupture and death [10]. So, chitin synthase is a safe and highly selective target to design new antifungal drugs [11]. Nikkomycins and polyoxins which are two natural-occupying substances possess inhibition potency against chitin synthase owning to their structures similar to that of UDP-GlcNAc which is the substrate of chitin synthase (Fig. 1) [12].

However, despite excellent *in vitro* results, the weak efficacy and high inhibitory concentrations resulting from poor cellular uptake and metabolic instability compromise these natural compounds in

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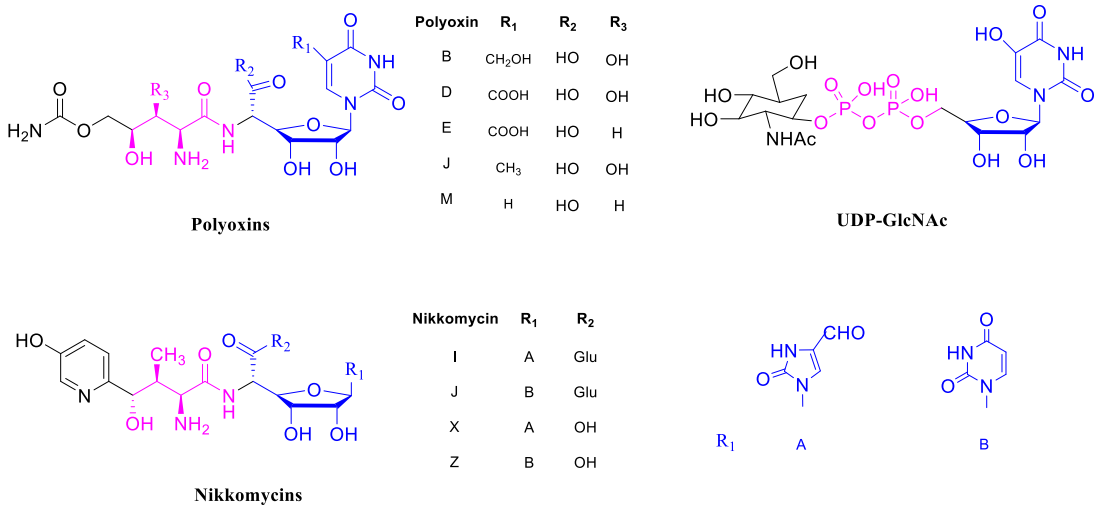


Fig. 1. Structural comparision of Polyoxins, Nikkomycins and UDP-GlcNAc.

clinical utility [13]. Nikkomycin Z is currently the only chitin synthase inhibitor that has undergone clinical trials for curing Valley Fever [14].Therefore, many efforts have been devoted to the synthesis of analogs to improve the pharmacokinetic properties of these compounds since nikkomycins and polyoxins were discovered [15,16]. These efforts have no progress in finding analogs befitting to develop as drug candidates, however, these investigations extended our knowledge of chitin synthase and its binding mode. It also provides information that it is necessary to consider the mechanism of action of chitin synthase to develop new chitin synthase inhibitors.

The quinolinone scaffold is seen as a privileged heterocycle-fused structure [17] and its derivatives exhibited numerous amusingly biological activities such as antipsychotic, antiallergic, antihistaminic or anti-inflammatory [18–20]. These broad biological activities have been a great concern in the field of pharmaceutical chemistry, thus numerous synthetic quinolinone antibacterial agents have been developed and various diseases have been treated with these drugs in clinical trials [21], for example, fluoroquinolones are widely used as antibacterial drugs (Fig. 2). 3-Amino-3,4-dihydro-quinolin-2(1*H*)-one is an interesting core of quinolinones. The amino substituent makes it coordination better and selective with metal ions, and it also be used as an inlay of biological macromolecules and fluorescent probes [22,23]. It is an essential scaffold of many natural products and synthetic products which have a wide range of biological activities. As shown in Fig. 2, compounds **I** and **IV** are potent glycogen phosphorylase inhibitors, and compounds **II** are inhibitors of angiotensin converting enzyme, and compound **III** can exert a radioprotective effect [24–27].

α,*β*-Unsaturated carbonyl fragment which can bind with the DNA, RNA, or enzymes through Michael addition reaction is functional group of many natural products [28]. Precisely because it contains such a special structure, chalcone has excellent biological activity and clinical potential. For example, licochalcone possessed the activity of anticancer and anti-inflammation, while isobavachalcone exerted antifungal activity (Fig. 3) [29–33].

Aromatic ring can generate rigid scaffold and could bind with sulfur, hydrogen or oxygen atoms of DNA or enzyme through π interactions, so it is the most frequently encountered ring system in small molecule drugs. Moreover, various substituents on the aromatic rings can adjust the hydrophilic lipophilic coefficient of molecules, which is very important for the absorption and

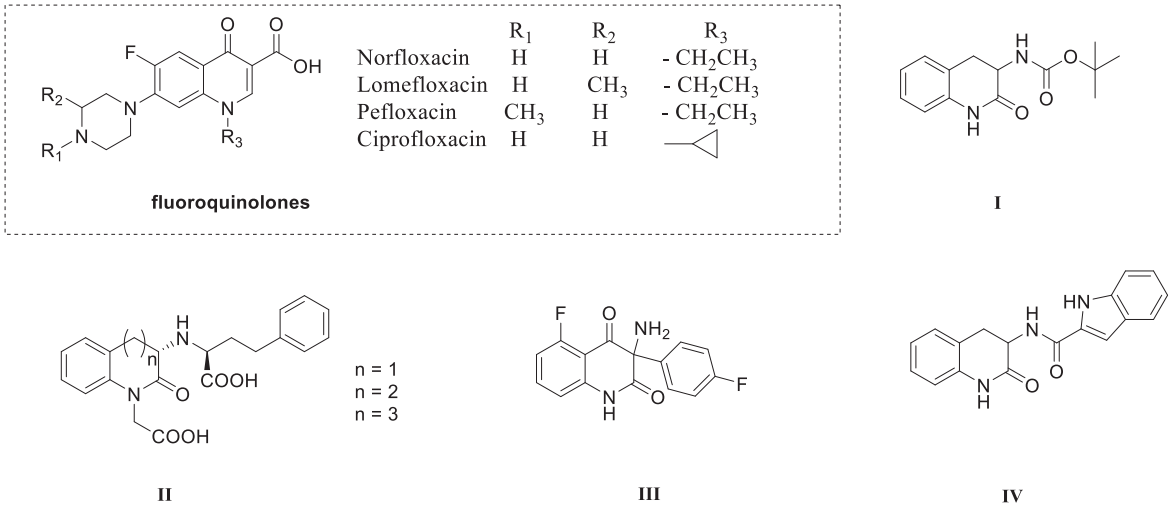


Fig. 2. Representative quinolinones.

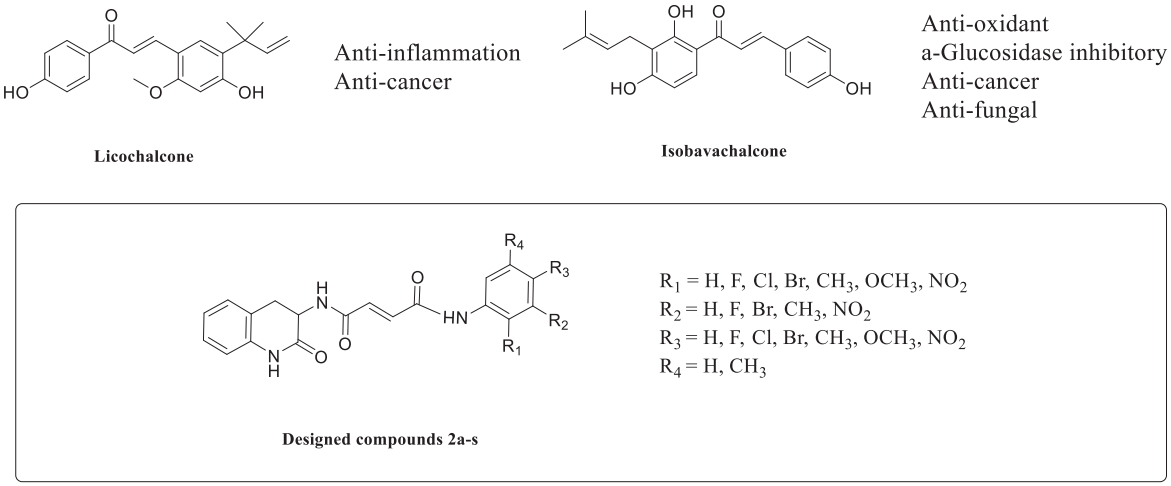


Fig. 3. Representative chalcones and the designed compounds.

transportation of drugs [34]. Consideration of the mechanism of action of chitin synthase, the α,β-unsaturated carbonyl was hybridized with 3-amino-3,4-dihydro-quinolin-2(1*H*)-one and substituted anilines to form a novel series of quinlinone derivatives (Fig. 3). Two amide fragments were introduced into this series compounds. It is well known that amide is a hydrophilic pharmacophore and it is employed in clinical drug extensively to improve bioavailability [35]. We expected that this series of compounds should have good CHS inhibitory activity and excellent antifungal activity. Herein, we reported the synthesis, biological evaluation of this series of novel 3-amino-3,4-dihydroquinoline-2(1*H*)-one derivatives.

2. Result and discussion

2.1. Chemistry

The 3-amino-3,4-dihydroquinolin-2(1*H*)-one is rarely commercially available in market, but it can be produced from an easily obtained compound *o*-nitrotoluene. *o*-Nitrotoluene reacted with NBS in carbon tetrachloride to produce 1-(bromomethyl)-2-nitrobenzene, and the product combined with diethyl acetylaminomalonate under strong alkaline condition to afford diethyl 2-(2-nitrobenzyl)-2-acetamidomalonate which then was decarboxylation in acidic solution at 100 °C to produce compound 2-amino-3-(2-nitrophenyl) propanoic acid hydrochloride which was esterified with methanol at presence of sulfoxide chloride. The obtained methyl 2-amino-3-(2-nitrophenyl) propanoate hydrochloride was reduced by iron powder to offer the desired compound 3-amino-3,4-dihydroquinolin-2 (1*H*)-one with good yield (see supplementary material).

The compounds 3-(arylcarbamoyl) acrylic acid **1a-s** were prepared by dissolving maleic anhydride and various substituted aniline in dichloromethane and stirring at room temperature. The compounds **1a-s** combined with 3-amino-3,4-dihydroquinolin-2 (1*H*)-one by catalyzation of triethyl phosphite and iodine to produce the target compounds (see Scheme 1).

2.2. Biological analysis

2.2.1. CHS inhibitory activity

The inhibition percentages (IP) of compounds **2a-s** against chitin synthase were screened at concentration of 300 μg/mL (Table 1). The results showed that all compounds exhibited moderate to excellent inhibitory to CHS. Compound **2d** exhibited the highest inhibitory potency among all compounds with the inhibition percentage value of 82.3% which was approximately equal to that of polyoxin B (P.B whose value is 87.5%). Moreover, compounds **2b**, **2e** and **2s** showed significant inhibition against CHS with IP over 70%. The compounds **2a**, **2h** and **2p** exhibited moderated potency whose IP ranged from 60% to 70% (Inhibition percentage = 67.3%, 65.2% and 66.5%, respectively).

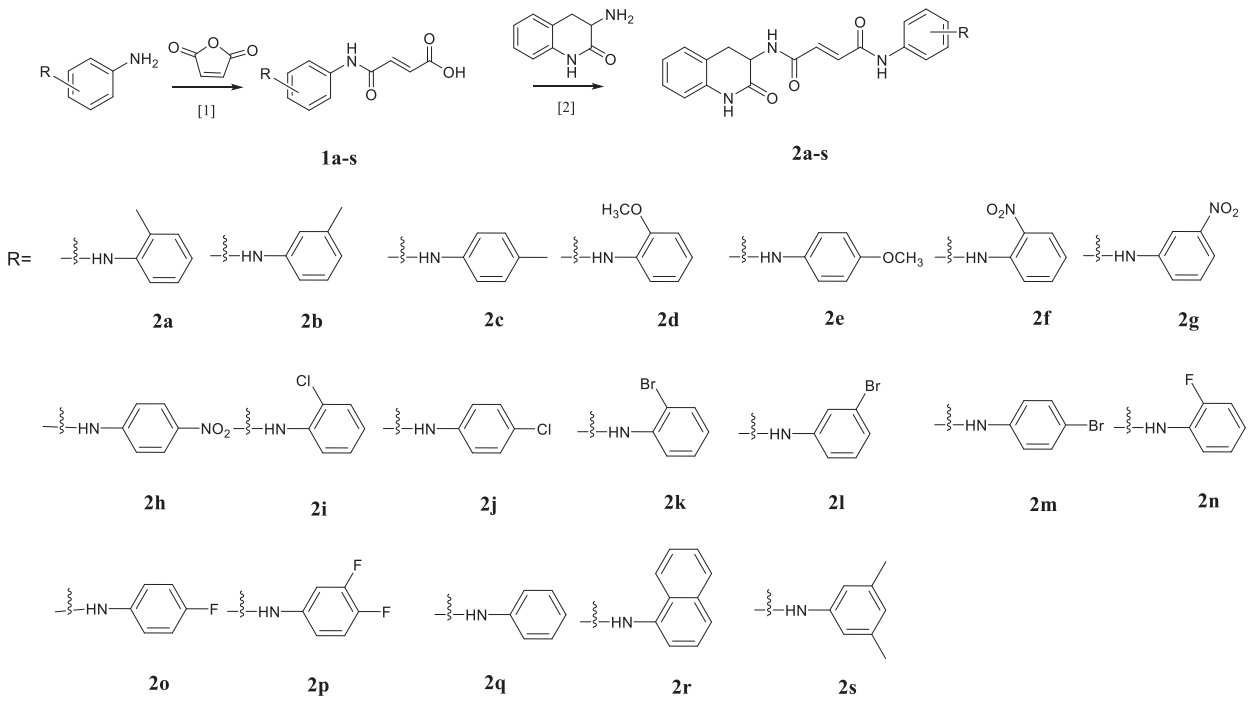
In this series of compounds, the structural difference is the type and the position of the substituents on the aromatic benzene ring. The compounds possessing methyl or the methoxyl substituents had excellent IP values, such as **2b**, **2d**, **2e** and **2s**. The nitro-substituted compounds **2f**, and **2g** showed moderate potency, while the *para*-substitution compound **2h** showed good potency with IP value of 65.2%. The most of the halo-substituted compounds revealed poor to moderate potency except for the difluoro-substituted compound **2p** which exerted good potency with IP value of 66.5%.

To further evaluate the inhibitory effects of these compounds, the IC_{50} (semi-inhibitory concentration) values of compounds whose inhibition percentage (IP) were above 50% were measured. (Fig. 4). The results showed that the IC_{50} values of these test compounds were distributed between 0.09 and 0.40 mM. Among them, compound **2b** had an IC_{50} value of 0.09 mM, which had similar potency to polyoxin B (IC_{50} = 0.09 mM). The IC_{50} values of the compounds **2d, 2s** were 0.16, 0.19 mM respectively, exhibiting moderate potency against CHS.

2.2.2. Kinetic parameters of chitin synthase

The Michaelis-Menten equation $v = \frac{Vmax[S]}{Km+[S]}$ can be expressed the relationship between the enzymatic reaction speed and the substrate concentration. K_m can be used to reflect the affinity between the enzyme and various substrates. The smaller the K_m value is, the stronger the affinity become between the enzyme and the substrate.

2.2.2.1. Determination of Michaelis-Menten constant (K_m). The Michaelis constant K_m is equal to half of the substrate concentration when the reaction rate reaches its maximum. In this experiment, the values of K_m and V_{max} were 4.25 mM, 0.76ΔOD450 ● h^{-1}, respectively. As shown in Fig. 5, the effect of substrate concentration on the enzymatic activities indicated that it was well suited to the *Michaelis-Menten* model.



Scheme 1. Synthetic route of 3-amino-3,4-dihydroquinoline-2(1*H*)-one derivatives.

Table 1
The chitin synthase inhibition percent (IP) of designed compounds at 300 μg/mL.

Compound	IP(%)	Compound	IP(%)	Compound	IP(%)
2a	67.3	**2h**	65.2	**2o**	51.2
2b	78.3	**2i**	46.7	**2p**	66.5
2c	58.7	**2j**	50.5	**2q**	35.7
2d	82.3	**2k**	39.9	**2r**	27.8
2e	74.6	**2l**	45.2	**2s**	71.5
2f	53.3	**2m**	40.3	Polyoxin B	87.5
2g	49.3	**2n**	48.9		

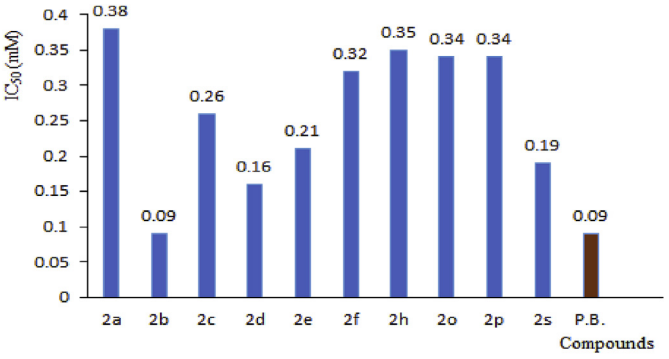


Fig. 4. The IC_{50} values of some compounds.

2.2.2.2. Determination of K_i. The K_i value was determined in the presence of different concentrations of compound **2b** and the K_i value was 0.12 mM, which was the intersection of the straight lines (Fig. 6b). The results proved that compound **2b** was non-competitive inhibitors (Fig. 6a) and had stronger affinity than UDP-GlcNAc.

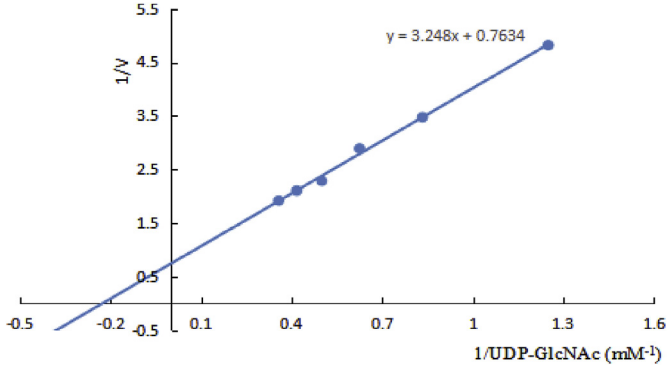


Fig. 5. The Lineweaver-Burk plot of UDP-GlcNAc.

2.2.3. Antifungal activity

Attempts had been made to evaluate the antifungal properties of all synthesized compounds using *two-fold broth dilution method* in 96-well microplate. Five pathogenic fungal strains which were the common pathogen fungi in clinic were selected as the test fungi and fluconazole and polyoxin B were used as positive controls. Each of groups was tested triply. The minimum inhibition concentration (MIC) value of each compound was measured to evaluate the antifungal efficacy. As shown in Table 2, these compounds showed moderate to excellent antifungal activity to the five pathogenic fungi strains. To *C. albicans* (ATCC 90023), compounds **2b**, **2d**, **2e** and **2l** displayed the most excellent potency with MIC values (1 μg/mL) similar to that of fluconazole. All compounds showed superior activity compared with polyoxin B. To *C. albicans* (ATCC 76615), compounds **2a**, **2d** with MIC values of 1 μg/mL exhibited most potency, whose MIC values were equal to that of fluconazole and superior to that of polyoxin B. Compounds **2b**, **2e**, **2l**, **2m**, **2o** and **2r** with MIC values of 2 μg/mL respectively exhibited similar potency comparable with fluconazole. To *A. fumigatus* (GIMCC 3.19), the

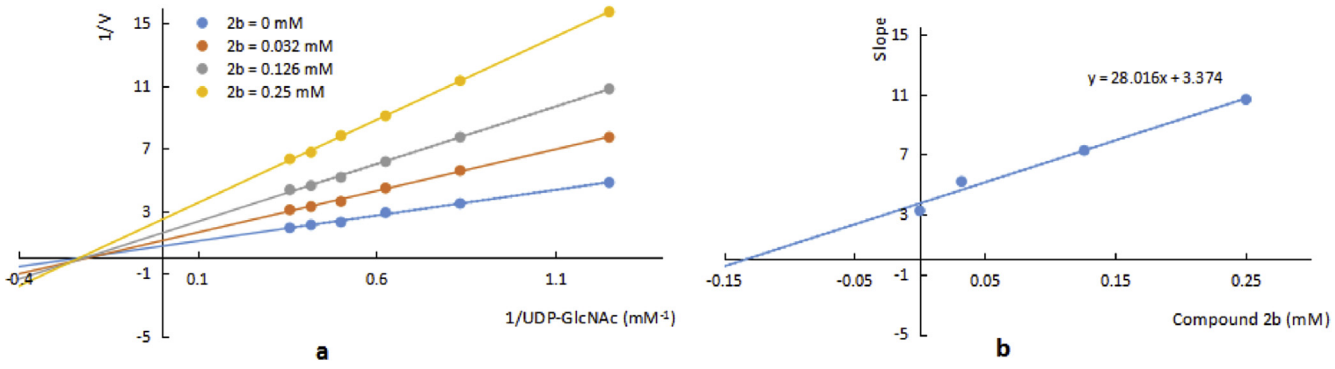


Fig. 6. Inhibition constant K_i of compound **2b** were determined by Lineweaver-Burk plot.

Table 2
The MIC value (μg/mL) of compounds **2a-s** against fungi *in vitro*.

Compounds	Fungi				
	C. albicans (ATCC 76615)	*A. fumigatus* (GIMCC 3.19)	*C. albicans* (ATCC 90023)	*C. neoformans* (ATCC 32719)	*A. flavus* (ATCC 16870)
2a	1	4	2	16	4
2b	2	4	1	64	2
2c	4	4	2	16	4
2d	1	4	1	8	2
2e	2	4	1	16	2
2f	4	8	2	4	4
2g	4	8	2	4	4
2h	8	4	2	8	2
2i	4	8	2	16	8
2j	4	8	2	4	4
2k	4	8	2	32	2
2l	2	4	1	32	4
2m	2	4	2	16	4
2n	8	1	4	16	8
2o	2	4	2	8	8
2p	4	8	2	16	8
2q	4	4	2	4	4
2r	2	4	2	4	8
2s	4	2	2	16	1
Fluconazole	1	2	1	2	2
Polyoxin B	2	2	4	4	4

compounds **2n** with MIC value of 1 μg/mL showed more excellent potency when compared with fluconazole. To *C. neoformans* (ATCC 32719), compounds **2f**, **2g**, **2j, 2q** and **2r** with MIC values of 4 μg/mL showed the equal potency of polyoxin B. To *A. flavus* (ATCC 16870), compound **2s** with MIC of 1 μg/mL showed good potency and others exhibited moderate activity.

2.2.4. Antibacterial activity

All target molecules were evaluated the antibacterial activity using three Gram-positive organisms and three Gram-negative organisms. Streptomycin and norfloxacin were selected as control drugs. Each of groups was tested triply. The minimum inhibition concentration (MIC) value of each compound was measured to evaluate the antimicrobial efficacy. As shown in Table 3, the MIC value of the strongest compound was several-fold compared with referenced drugs. It indicated that they were several-fold weaker than Streptomycin and Norfloxacin. Therefore, these compounds had no antibacterial activity against the selected strains.

2.3. Drug combination study

Studies have shown drug combination use had synergistic roles in many aspects, including expanding antibacterial spectrum, sterilizing, reducing toxicity, reducing and delaying drug resistance. These compounds of this work were designed to target the chitin synthase and destroy the cell wall of fungi, which are not same as fluconazole, so they can enhance the efficacy of antifungal activity by different modes of action [36] when combination use. Furthermore, when they were used with the polyoxin B, the result can provide the information that whether these compounds were competitive or non-competitive agents. The representative compounds **2b**, **2d** were chosen to combination use with fluconazole and polyoxin B respectively, using the *checkerboard method*. The results were depicted in Table 4 and Table 5.

The combination **2b**-fluconazole showed strongest synergism effects against *C.albicans* (ATCC 90023) (FIC = 0.5) and this combination displayed synergistic or additive effects against other strains. The combination **2d**-fluconazole displayed moderate to excellent synergistic effect for selected strains whose FIC values ranged from 0.375 to 1. The combinations **2b**-polyoxin B, **2d**-polyoxin B exhibited strongest synergistic effects against *C.albicans* (ATCC 90023) with FIC values of 0.375, 0.375 respectively. The **2d**-polyoxin B showed excellent synergistic effects against *A. fumigatus* (GIMCC 3.19) with FIC value 0.375. The **2d**-polyoxin B displayed good inhibition on other selected strains. The results suggested that these compounds were not competitive with polyoxin B for they can enhance the efficacy of polyoxin B.

Table 3
The MIC value (μg/mL) of compounds **2a-s** against bacterial *in vitro*.

Compounds	Gram-positive bacteria			Gram-negative bacteria		
	MRSA (N. 315)	*S. aureus* (ATCC 25923)	*B. subtilis* (ATCC 6633)	*C. coli* (JM 109)	*P. aeruginosa* (ATCC 9027)	*B. proteus* (ATCC 8427)
2a	256	128	512	256	512	256
2b	512	128	256	512	512	256
2c	256	128	512	256	512	256
2d	256	128	256	256	512	256
2e	256	128	256	512	512	256
2f	256	128	256	256	256	256
2g	256	128	256	128	512	256
2h	256	128	256	256	512	256
2i	512	128	512	256	256	512
2j	512	128	256	256	512	512
2k	256	128	512	512	512	512
2l	512	128	256	256	256	256
2m	256	128	512	512	512	512
2n	512	256	256	512	512	512
2o	512	128	512	256	256	256
2p	512	256	512	512	512	512
2q	256	256	256	512	256	256
2r	256	256	256	512	256	256
2s	512	128	256	256	256	512
Streptomycin	16	32	8	32	8	16
Norfloxacin	4	2	4	8	8	2

Table 4
Combination effects of **2b**, **2d** with fluconazole.

Fungi	MIC (μg/mL)[a]							FIC Index(2b)	FIC[b] Index(2d)
	Alone			In combination					
	2b	2d	FCA[c]	2b	2d	FCA (with 2b)	FCA (with 2d)		
ATCC 76615	2	1	1	2	0.5	0.5	0.25	1.5	0.75
GIMCC 3.19	4	4	2	2	1	0.5	0.5	0.75	0.375
ATCC 90023	1	1	1	0.25	0.25	0.25	0.25	0.5	0.5
ATCC 32719	64	8	2	32	2	2	0.5	1.5	0.5
ATCC 16870	2	2	2	2	1	1	1	1.5	1

[a] Each of groups was tested independently triply.
[b] FIC index = (MIC of compound A combined/MIC of compound A alone) + (MIC of compound B combined/MIC of compound B alone). FIC index >2 represents antagonistic effect, 1 < FIC index ≤2 represents additive effect, FIC index ≦ 1 represents synergistic effect.
[c] *C. albicans* (ATCC 76615), *A. fumigatus* (GIMCC 3.19), *C. albicans* (ATCC 90023), *C. neoformans* (ATCC 32719), *A. flavus* (ATCC 16870). FCA (fluconazole), P.B (Polyoxin B).

Table 5
Combination effects of **2b**, **2d** with polyoxin B.

Fungi	MIC (μg/mL)							FIC Index(2b)	FIC Index(2d)
	Alone			In combination					
	2b	2d	P.B[a]	2b	2d	P. B (with 2b)	P. B (with 2d)		
ATCC 76615	2	1	2	1	0.25	1	0.5	1	0.5
GIMCC 3.19	4	4	2	2	0.5	0.5	0.5	0.75	0.375
ATCC 90023	1	1	4	0.25	0.125	0.5	1	0.375	0.375
ATCC 32719	64	8	4	64	4	4	2	2	1
ATCC 16870	2	2	4	1	0.5	2	2	1	0.75

[a] *C. albicans* (ATCC 76615), *A. fumigatus* (GIMCC 3.19), *C. albicans* (ATCC 90023), *C. neoformans* (ATCC 32719), *A. flavus* (ATCC 16870). FCA (fluconazole), P.B (Polyoxin B).

3. Conclusion

A series of N^1-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl)-N^4-phenylfumaramide derivatives were designed and synthesized. Their inhibitory potency of chitin synthase and antimicrobial activity were evaluated *in vitro* as well. The results showed that all compounds exhibited moderate to excellent inhibitory potency against CHS. Compounds **2b**, **2d**, **2e** and **2s** displayed excellent potency with IP over 70% and their IC_{50} values are 0.09, 0.16, 0.21 and 0.19 mM respectively. **2b** was the strongest among them whose IC_{50} value was 0.09 mM which was equal to that of polyoxin B whose IC_{50} value was 0.09 mM. Kinetic parameters indicated compound **2b** had stronger affinity binding with chitin synthase than UDP-GlcNAc and was non-competitive inhibitor. The results of antifungal evaluation revealed that these compounds had good potency against the selected fungi (except *C. neoformans*) and their MIC values were between 1 and 4 μg/mL. Compounds **2a**, **2b**, **2d**, **2e**, **2l**, **2m, 2o**, **2r** showed excellent potency against *C. albicans* (ATCC 76615), and their MIC values were equal to these of polyoxin B and fluconazole. Compound **2n** was strongest agent against *A. fumigatus* (GIMCC 3.19) with MIC value 1 μg/mL. All compounds exhibited excellent antifungal activities against *C. albicans* (ATCC 90023), and the MIC values of them exceeded polyoxin B (MIC = 4 μg/mL). Compounds **2b**, **2d**, **2e**, **2h**, and **2k** with MIC values of 2 μg/mL

respectively, had similar potency to fluconazole against *A. flavus* (ATCC 16870). In addition, drug combination provided that FIC values were ranging from 0.375 to 1, showing that these compounds had synergistic or additive effects with fluconazole or polyoxin B against these selected strains. The result indicated that these compounds had different antifungal action mode from reference drugs. Antibacterial experiments confirmed that these compounds had little bactericidal potency against the selected bacteria. These results suggested that these designed compounds were chitin synthase inhibitors and had selective antifungal activities.

4. Experimental protocols

4.1. Chemistry

All chemicals were commercial grade and no further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV 400 or 600 spectrometer (Bruker Company, Germany) using TMS as an internal standard and DMSO-d_6, $CDCl_3$ or D_2O as solvent. Chemical shift values were given in ppm (δ values), coupling constants were given in Hz (J values). The following abbreviations were used to set multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. The mass spectra were acquired in positive mode, scanning over the mass range, it were recorded on Shimadzu LC-MS-2010A/EV and the ESI-HRMS spectra were recorded on Bruker impact II. Melting point (m.p.) was obtained on amicroscopic melting point apparatus(x-4 type). Reaction process was monitored by TLC and UV detection was monitored at 254 nm.

4.1.1. General method for preparation **1a-s**. [37]

Substituted aniline (10 mmol) was added to a solution of maleic anhydride (0.98 g, 10 mmol) in methane dichloride. The reaction was stirred at room temperature for approximately 2 h. The process of the reaction was monitored by TLC until the reaction was completed. The resulting suspension was filtered and then the corresponding amide was dried at 50 °C, yielding 96–99% of the desired products **1a-s**.

4.1.2. General method for preparation **2a-s**. [38]

Iodines (2.54 g, 10 mmol) were added to a solution of triethyl phosphite (1.66 g, 10 mmol) in methane dichloride (50 mL) cooled with ice bath. One compound of **1a-s** (10 mmol) was added to the solution after the iodine dissolved completely. The triethylamine (6.96 mL, 50 mmol) was added to the mixture and stirred for 15 min at a cooling bath. 3-Amino-3,4-dihydroquinolin-2(1*H*)-one (1.95 g, 12 mmol) was added and the mixture was stirred at room temperature to complete the reaction (reaction monitored by TLC). The solution was washed with saturated aqueous sodium bicarbonate (3 × 15 mL), 1 M HCl solution (3 × 15 mL), water (3 × 15 mL), brine (3 × 15 mL). The combined organic layer was dried with Na_2SO_4, filtered and concentrated. The crude product was purified by silica column chromatography (hexanes/EtOAc).

*4.1.2.1. N^1-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl)-N^4-(o-tolyl) fumaramide (**2a**).* White powder, yield 80%, m.p. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1H), 10.41 (s, 1H), 9.23 (d, J = 7.7 Hz, 1H), 7.62 (d, J = 7.8 Hz, 1H), 7.24–7.14 (m, 4H), 7.08 (t, J = 7.2 Hz, 1H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.41 (s, 2H), 4.62–4.51 (m, 1H), 3.16–3.07 (m, 1H), 2.99–2.87 (m, 1H), 2.25 (s, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 168.58, 165.13, 163.72, 137.90, 136.49, 134.80, 131.39, 130.79, 130.40, 128.62, 128.09, 126.41, 125.51, 124.62, 122.79, 122.67, 115.64, 48.66, 31.61, 18.40. HRMS (ESI): calcd for $C_{20}H_{20}N_3O_3$ $[M+H]^+$, 350.1499, found, 350.1499. $C_{20}H_{19}N_3NaO_3$ $[M+Na]^+$, 372.1319, found, 372.1317.

*4.1.2.2. N^1-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl)-N^4-(m-tolyl) fumaramide (**2b**).* White powder, yield 82%, m.p. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.01 (s, 1H), 10.41 (s, 1H), 9.12 (s, 1H), 7.47–7.38 (m, 2H), 7.25–7.14 (m, 3H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.7 Hz, 2H), 6.37 (s, 2H), 4.62–4.50 (m, 1H), 3.18–3.08 (m, 1H), 2.97–2.86 (m, 1H), 2.28 (s, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 168.64, 165.06, 163.79, 139.16, 138.42, 137.90, 134.30, 130.42, 129.07, 128.63, 128.07, 124.76, 122.79, 122.70, 120.34, 117.09, 115.63, 48.63, 31.66, 21.64. HRMS (ESI): calcd for $C_{20}H_{19}N_3NaO_3$ $[M+Na]^+$, 372.1319, found, 372.1318.

*4.1.2.3. N^1-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl)-N^4-(p-tolyl) fumaramide (**2c**).* White powder, yield 80%, m.p. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.99 (s, 1H), 10.40 (s, 1H), 9.16 (s, 1H), 7.51 (d, J = 8.1 Hz, 2H), 7.24–7.17 (m, 2H), 7.12 (d, J = 8.1 Hz, 2H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.37 (s, 2H), 4.59–4.50 (m, 1H), 3.17–3.08 (m, 1H), 2.97–2.87 (m, 1H), 2.26 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.65, 165.08, 163.59, 137.90, 136.70, 134.11, 133.04, 130.62, 129.61, 128.62, 128.06, 122.78, 122.71, 119.88, 115.63, 48.66, 31.65, 20.95. HRMS (ESI): calcd for $C_{20}H_{19}N_3NaO_3$ $[M+Na]^+$, 372.1319, found, 372.1317.

*4.1.2.4. N^1-(2-methoxyphenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2d**).* White powder, yield 81%, m.p. 189–191 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.99 (s, 1H), 10.42 (s, 1H), 9.21 (d, J = 6.8 Hz, 1H), 8.10 (d, J = 7.5 Hz, 1H), 7.24–7.16 (m, 2H), 7.13–7.06 (m, 1H), 7.04 (d, J = 7.7 Hz, 1H), 6.99–6.86 (m, 3H), 6.40 (s, 2H), 4.63–4.52 (m, 1H), 3.81 (s, 3H), 3.17–3.09 (m, 1H), 2.99–2.88 (m, 1H). ^{13}C NMR(101 MHz, DMSO-d_6) δ 168.59, 165.20, 163.30, 150.11, 137.92, 134.59, 130.93, 128.60, 128.08, 127.63, 125.13, 122.79, 122.69, 122.08, 120.73, 115.64, 111.72, 56.24, 48.62, 31.59. HRMS (ESI): calcd for $C_{20}H_{20}N_3O_4$ $[M+H]^+$, 366.1448, found, 366.1449. $C_{20}H_{19}N_3NaO_4$ $[M+Na]^+$, 388.1268, found, 388.1267.

*4.1.2.5. N^1-(4-methoxyphenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2e**).* White powder, yield 81%, m.p. 189–191 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.92 (s, 1H), 10.40 (s, 1H), 9.21 (d, J = 7.3 Hz, 1H), 7.54 (d, J = 8.5 Hz, 2H), 7.25–7.15 (m, 2H), 6.99–6.85 (m, 4H), 6.42–6.31 (m, 2H), 4.59–4.50 (m, 1H), 3.72 (s, 3H), 3.16–3.09 (m, 1H), 2.97–2.86 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.66, 165.08, 163.33, 155.98, 137.91, 133.98, 132.32, 128.62, 128.07, 122.77, 121.43, 115.63, 114.39, 55.65, 48.66, 31.65. HRMS (ESI): calcd for $C_{20}H_{19}N_3NaO_4$ $[M+Na]^+$, 388.1268, found, 388.1269.

*4.1.2.6. N^1-(2-nitrophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2f**).* White powder, yield 84%, m.p. 190–193 °C. ^{1}H NMR (400 MHz, DMSO-d_6) ^{1}H NMR (400 MHz, DMSO) δ 11.20 (s, 1H), 10.42 (s, 1H), 8.98 (d, J = 7.7 Hz, 1H), 7.98 (dd, J = 6.6, 5.6 Hz, 1H), 7.78 (d, J = 8.0 Hz, 1H), 7.75–7.70 (m, 1H), 7.42–7.36 (m, 1H), 7.25–7.15 (m, 2H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.50–6.41 (m, 2H), 4.59–4.48 (m, 1H), 3.15–3.05 (m, 1H), 2.97–2.86 (m, 1H). ^{13}C NMR(101 MHz,DMSO-d_6)δ168.59, 164.74,164.52, 142.50, 137.88, 134.56, 133.39, 131.50, 130.92, 128.59, 128.07, 125.85, 125.80, 125.40, 122.80, 122.67, 115.64, 48.62, 31.60. HRMS (ESI): calcd for $C_{20}H_{19}N_3NaO_3$ $[M+Na]^+$, 403.1013, found, 403.1014.

*4.1.2.7. N^1-(3-nitrophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2g**).* White powder, yield 83%, m.p. 190–193 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.19 (s, 1H), 10.42 (s, 1H), 8.98 (d, J = 7.6 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.80–7.69 (m, 2H), 7.39 (t, J = 7.5 Hz, 1H), 7.24–7.13 (m, 2H), 6.95 (t, J = 7.4 Hz, 1H), 6.88 (d,

J = 7.8 Hz, 1H), 6.51–6.41 (m, 2H), 4.58–4.48 (m, 1H), 3.15–3.07 (m, 1H), 2.96–2.85 (m, 1H). ^{13}C NMR(101 MHz, DMSO-d_6) δ 168.59, 164.73, 164.52, 142.51, 137.87, 134.56, 133.39, 131.50, 130.91, 128.59, 128.08, 125.86, 125.81, 125.40, 122.80, 122.67, 115.64, 48.62, 31.60.

*4.1.2.8. N^1-(4-nitrophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2h**).* White powder, yield 83%, m.p. 190–193 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.38 (s, 1H), 10.41 (s, 1H), 8.93 (d, J = 4.9 Hz, 1H), 8.23 (d, J = 8.7 Hz, 2H), 7.87 (d, J = 8.8 Hz, 2H), 7.24–7.14 (m, 2H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.7 Hz, 1H), 6.52–6.41 (m, 2H), 4.58–4.49 (m, 1H), 3.15–3.07 (m, 1H), 2.97–2.87 (m, 1H).^{13}C NMR (101 MHz, DMSO-d_6) δ 168.65, 167.02, 165.43, 164.66, 145.61, 142.72, 137.87, 133.83, 132.73, 130.13, 128.62, 128.07, 125.49, 125.46, 122.79, 122.67, 119.47, 119.42, 48.58, 31.68.

*4.1.2.9. N^1-(2-chlorophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2i**).* White powder, yield 81%, m.p. 189–191 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.27 (s, 1H), 10.44 (s, 1H), 9.24 (d, J = 7.1 Hz, 1H), 7.92 (d, J = 7.7 Hz, 1H), 7.50 (d, J = 7.8 Hz, 1H), 7.38–7.32 (m, 1H), 7.23–7.14 (m, 3H), 6.98–6.87 (m, 2H), 6.54–6.38 (m, 2H), 4.63–4.49 (m, 1H), 3.15–3.07 (m, 1H), 3.00–2.89 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.49, 165.13, 163.86, 137.90, 135.22, 134.26, 131.13, 129.95, 128.60, 128.07, 127.85, 126.65, 126.19, 125.89, 122.78, 122.63, 115.66, 48.64, 31.54. HRMS (ESI): calcd for $C_{19}H_{16}ClN_3NaO_3$ $[M+Na]^+$, 392.0772, found, 392.0772.

*4.1.2.10. N^1-(4-chlorophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2j**).* White powder, yield 80%, m.p. 189–191 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.57 (s, 1H), 10.43 (s, 1H), 9.21 (s, 1H), 8.15–8.05 (m, 1H), 7.27–7.13 (m, 5H), 6.96 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.50–6.36 (m, 2H), 4.62–4.53 (m, 1H), 3.18–3.08 (m, 1H), 3.00–2.88 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.66, 164.90, 164.27, 138.26, 137.89, 135.23, 133.94, 130.31, 129.38, 129.13, 128.87, 128.62, 128.07, 127.51, 122.79, 122.69, 121.39, 115.63, 48.62, 31.67. HRMS (ESI): calcd for $C_{19}H_{17}ClN_3O_3$ $[M+H]^+$, 370.0953, found, 370.0950. $C_{19}H_{16}ClN_3NaO_3$ $[M+Na]^+$, 392.0772, found, 392.0770.

*4.1.2.11. N^1-(2-bromophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2k**).* White powder, yield 82%, m.p. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.00 (s, 1H), 10.42 (s, 1H), 9.19 (d, J = 6.0 Hz, 1H), 7.79 (d, J = 7.7 Hz, 1H), 7.66 (d, J = 7.9 Hz, 1H), 7.39 (t, J = 7.6 Hz, 1H), 7.26–7.10 (m, 3H), 7.00–6.84 (m, 2H), 6.53–6.38 (m, 2H), 4.62–4.51 (m, 1H), 3.16–3.06 (m, 1H), 3.00–2.85(m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.54, 165.05, 163.90, 137.89, 136.46, 134.06, 133.15, 131.20, 128.61, 128.42, 128.08, 127.40, 126.93, 122.79, 122.65, 115.64, 48.65, 31.58. HRMS (ESI): calcd for $C_{19}H_{16}BrN_3NaO_3$ $[M+Na]^+$, 436.0267, found, 436.0263.

*4.1.2.12. N^1-(3-bromophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2l**).* White powder, yield 83%, m.p. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.99 (s, 1H), 10.41 (s, 1H), 8.96 (d, J = 7.6 Hz, 1H), 7.98 (s, 1H), 7.50 (d, J = 7.4 Hz, 1H), 7.34–7.15 (m, 4H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.47–6.36 (m, 2H), 4.62–4.48 (m, 1H), 3.16–3.07 (m, 1H), 2.96–2.86 (m, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.62, 164.85, 164.52, 140.89, 137.91, 133.89, 131.20, 130.34, 128.61, 128.06, 126.56, 122.79, 122.69, 122.21, 122.01, 118.68, 115.65, 48.66, 31.72. HRMS (ESI): calcd for $C_{19}H_{17}BrN_3O_3$ $[M+H]^+$,414.0448, found, 414.0451. $C_{19}H_{16}BrN_3NaO_3$ $[M+Na]^+$, 436.0267, found, 436.0265.

*4.1.2.13. N^1-(4-bromophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2m**).* White powder, yield 84%, m.p. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.04 (s, 1H), 10.41 (s, 1H), 9.01 (d, J = 7.5 Hz, 1H), 7.60 (d, J = 8.6 Hz, 2H), 7.50 (d, J = 8.6 Hz, 2H), 7.25–7.13 (m, 2H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.46–6.35 (m, 2H), 4.59–4.48(m, 1H), 3.16–3.06 (m, 1H), 2.96–2.85 (m, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.60, 164.95, 164.19, 138.65, 137.91, 133.89, 132.03, 130.51, 128.61, 128.05, 122.78, 122.69, 121.83, 115.65, 115.59, 48.68, 31.69. HRMS (ESI): calcd for $C_{19}H_{17}BrN_3O_3$ $[M+H]^+$,414.0448, found, 414.0443. $C_{19}H_{16}BrN_3NaO_3$ $[M+Na]^+$, 436.0267, found, 436.0265.

*4.1.2.14. N^1-(2-fluorophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2n**).* White powder, yield 81%, m.p.187–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.04 (s, 1H), 10.41 (s, 1H), 9.02 (d, J = 7.6 Hz, 1H), 7.65 (d, J = 8.8 Hz, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.24–7.15 (m, 2H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.45–6.34 (m, 2H), 4.59–4.49 (m, 1H), 3.16–3.07 (m, 1H), 2.96–2.87 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.49, 165.28, 163.67, 137.88, 134.53, 130.94, 128.63, 128.09, 124.86, 124.83, 123.87, 122.80, 122.62, 115.94, 115.75, 115.64, 48.67, 31.54. HRMS (ESI): calcd for $C_{19}H_{16}FN_3NaO_3$ $[M+Na]^+$, 376.1068, found, 376.1067.

*4.1.2.15. N^1-(4-fluorophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2o**).* White powder, yield 80%, m.p. 187–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.98 (s, 1H), 10.41 (s, 1H), 9.06 (d, J = 7.5 Hz, 1H), 7.64 (dd, J = 8.9, 5.0 Hz, 2H), 7.24–7.13 (m, 4H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.43–6.34 (m, 2H), 4.59–4.48 (m, 1H), 3.16–3.07 (m, 1H), 2.97–2.85 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.66, 164.94, 163.99, 137.90, 135.69, 133.90, 130.42, 128.62, 128.06, 122.78, 122.71, 121.68, 121.61, 115.91, 115.69, 115.63, 48.62, 31.67. HRMS (ESI): calcd for $C_{19}H_{16}FN_3NaO_3$ $[M+Na]^+$, 376.1068, found, 376.1069.

*4.1.2.16. N^1-(3,4-difluorophenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2p**).* White powder, yield 79%, m.p.186–189 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.01 (s, 1H), 10.40 (s, 1H), 9.03 (s, 1H), 7.85–7.77 (m, 1H), 7.44–7.35 (m, 1H), 7.30 (d, J = 8.9 Hz, 1H), 7.23–7.15 (m, 2H), 6.98–6.92 (m, 1H), 6.89 (d, J = 7.7 Hz, 1H), 6.44–6.36 (m, 2H), 4.57–4.45 (m, 1H), 3.15–3.05 (m, 1H), 2.97–2.84 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.68, 164.76, 164.61, 137.89, 136.52, 133.88, 130.19, 128.62, 128.06, 122.78, 122.70, 118.00, 117.83, 116.18, 115.62, 108.91, 108.70, 48.58, 31.69. HRMS (ESI): calcd for $C_{19}H_{15}F_2N_3NaO_3$ $[M+Na]^+$, 394.0974, found, 394.0972.

*4.1.2.17. N^1-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl)-N^4-phenylfumaramide (**2q**).* White powder, yield 81%, m.p. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.12 (s, 1H), 10.44 (s, 1H), 9.19 (d, J = 6.1 Hz, 1H), 7.63 (d, J = 7.9 Hz, 2H), 7.31 (t, J = 7.7 Hz, 2H), 7.24–7.15 (m, 2H), 7.07 (t, J = 7.3 Hz, 1H), 6.99–6.86 (m, 2H), 6.45–6.34 (q, J = 12.6 Hz, 2H), 4.60–4.49 (m, 1H), 3.16–3.07 (m, 1H), 2.98–2.87 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.65, 165.07, 163.94, 139.28, 137.92, 133.92, 130.71, 129.20, 128.61, 128.05, 124.02, 122.76, 122.72, 119.90, 115.65, 48.64, 31.64. HRMS (ESI): calcd for $C_{19}H_{17}N_3NaO_3$ $[M+Na]^+$, 358.1162, found, 358.1163.

*4.1.2.18. N^1-(naphthalen-1-yl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2r**).* White powder, yield 84%, m.p. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.38 (s, 1H), 10.43 (s, 1H), 9.23 (d, J = 7.5 Hz, 1H), 8.21 (d, J = 5.8 Hz, 1H), 7.98–7.91 (m, 1H), 7.87 (d, J = 7.3 Hz, 1H), 7.77 (d, J = 8.1 Hz, 1H), 7.59–7.46 (m, 3H), 7.26–7.13 (m, 2H), 7.00–6.83 (m, 2H), 6.60–6.43 (m, 2H), 4.68–4.56 (m, 1H), 3.19–3.09 (m, 1H), 3.01–2.88 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.60, 165.25, 164.46, 137.91, 134.83, 134.16, 133.67, 130.36, 128.61, 128.09, 127.90, 126.49, 126.46, 126.05, 125.75, 123.13, 122.80, 122.67, 121.47, 115.64, 48.67, 31.63. HRMS (ESI): calcd for $C_{23}H_{19}FN_3NaO_3$ $[M+Na]^+$, 408.1319, found, 408.1313.

*4.1.2.19. N^1-(3,5-dimethylphenyl)-N^4-(2-oxo-1,2,3,4-tetrahydroquinolin-3-yl) fumaramide (**2s**).* White powder, yield 78%, m.p. 189–192 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.02 (s, 1H), 10.41 (s, 1H), 9.14 (d, J = 7.7 Hz, 1H), 7.27–7.16 (m, 4H), 6.95 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.72 (s, 1H), 6.36 (s, 2H), 4.61–4.52 (m, 1H), 3.16–3.08 (m, 1H), 2.98–2.88 (m, 1H), 2.23 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.60, 165.16, 163.58, 139.02, 138.22, 137.90, 134.38, 130.58, 128.62, 128.07, 125.67, 122.80, 122.69, 117.69, 115.66, 48.69, 31.67, 21.52. HRMS (ESI): calcd for $C_{21}H_{21}N_3NaO_3$ $[M+Na]^+$, 386.1475, found, 386.1475.

4.2. Biological activity assay

4.2.1. Inhibition against yeast CHS

The procedure of evaluating the inhibition potency against chitin synthase which was described by Lucero [39] was adopted with little modifications. In this experiment, the chitin synthase was extracted from the tropical yeast *C. tropicalis ATCC* 750 by low-speed and high-speed centrifugation. All tested compounds were prepared as stock solutions by dissolving in DMSO at a concentration of 10 mg/mL and then it were diluted to 1200, 600, 300, 150, 75 μg/mL with 50 mM of pH 7.5 Tris-HCl buffer, and the test concentration of the enzyme solution were diluted to 300, 150, 75.0, 37.5, 18.75, 9.375 μg/mL.

There were several simple steps in the experiment. Firstly, the biosynthesis of chitin was catalyzed by chitin synthase in 96 well plates coated with WGA. Secondly, the WGA-HRP which was conjugated WGA with HRP was added to bind to the immobilized chitin. Lastly, the 96 well plates were washed by distilled water to eliminate unbound components. TMB was oxidized by hydrogen peroxide and it was monitored the HRP activity. The reaction was stopped by adding 50 μL 2 M H_2SO_4 to each well. Polyoxin B was selected as a positive control. The OD values were measured by Biotek ELx808 microplate reader. Each group was tested in parallel for two groups. The calculation formula of Inhibition percentage: Inhibition percentage = $(B_0 - B_n)/(B_0 - OD_0)$.

4.2.2. Kinetic parameters of chitin synthase

In this experiment, UDP-GlcNAc was used as the substrate which ranged from 0.8 to 4.0 mM to measure the K_m and the V_{max}. The values of $1/K_m$ and V_{max} of UDP-GlcNAc were the intersection of the cross axle and vertical axis, respectively. The K_i value was determined by intersection of the straight lines whose abscissa and ordinate were fixed by concentrations of substrate UDP-GlcNAc and 1/v, respectively. The concentrations of UDP-GlcNAc were 0.5, 1.0, 1.5 and 2.0 mM, respectively and the concentrations of **2b** were 0, 0.032, 0.126 and 0.25 mM, respectively. Graphs of different inhibitor's concentrations were drawn by the Lineweaver-Burk plot.

4.2.3. Antibacterial and antifungal assays [40,41]

According to the National Committee's clinical trial standards, the target compound **2a-s** were used to carry on antimicrobial experiments in 96-well plate by *the standard two folds serial dilution method*, and the antimicrobial activity were measured. The MIC(-minimal inhibition concentration) is an indicator for assessing antibacterial activity of a drug. The time of incubating microorganisms in a specific environment is18–24 h.

4.2.3.1. Antifungal activity assay. In this experiment, five strains were tested, *Cryptococcus neoformans ATCC 32719*, *Candida albicans ATCC 76615, Candida albicans ATCC 90023, Aspergillus fumigatus GIMCC 3.19 and Aspergillus flavus ATCC 16870* were included. Firstly, stock solution was prepared by dissolving the test compounds and standard drugs in DMSO. Secondly, stock solution was diluted to the desired concentration (0.25–512 μg/mL). Fungi were incubated with modified Sabouraud chloramphenicol agar containing 1% peptone, 2% glucose and solid medium and 15% agar. Incubating for 48 h at 37 °C, inhibition was tested and the minimum inhibitory concentration (MIC, μg/mL) was recorded. Polyoxin B and fluconazole were used as positive controls.

4.2.3.2. Antibacterial assay. In this experiment, six strains were tested, three Gram-positive bacteria and three Gram-negative bacteria, such as *Methicillin-resistant staphylococcus aureus N 3.15, Staphylococcus aureus ATCC 25923, Bacillus subtilis ATCC 6633, Escherichia coli JM 109, Pseudomonas aeruginosa ATCC 9027, Bacillus proteus ATCC 8427*. The test compounds were prepared at different concentrations. The medium used for this assay was beef extract peptone medium containing 1% peptone, 0.3% beef extract, 0.5% sodium chloride, solid medium and 15% agar in distilled water.

4.2.4. Drug combination assay

The checkerboard method and the two-fold broth dilution method were used to do drug combination studies that combined **2b**, **2d** with fluconazole and polyoxin B, respectively. Each set of experiments was repeated three times independently. The effects of drug combination were evaluated by the fractional inhibitory concentration (FIC), which were calculated by formula as following: FIC index = (MIC of compound A combined/MIC of compound A alone) + (MIC of compound B combined/MIC of compound B alone). FIC index >2 represents antagonistic effect, 1 < FIC index ≤2 represents additive effect, FIC index ≦ 1 represents synergistic effect.

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Declaration of competing interest

I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. I declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112278.

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