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Research paper

Synthesis and biological evaluation of novel phosphoramidate derivatives of coumarin as chitin synthase inhibitors and antifungal agents

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ABSTRACT

A series of novel phosphoramidate derivatives of coumarin have been designed and synthesized as chitin synthase (CHS) inhibitors. All the synthesized compounds have been screened for their chitin synthase inhibition activity and antimicrobial activity in vitro. The bioactive assay manifested that most of the target compounds exhibited good efficacy against CHS and a variety of clinically important fungal pathogens. In particular, compound **7t** with IC_{50} of 0.08 mM against CHS displayed stronger efficiency than the reference Polyoxin B with IC_{50} of 0.16 mM. In addition, the apparent K_i values of compound **7t** was 0.096 mM while the K_m of Chitin synthase prepared from *Candida tropicalis* was 3.86 mM for UDP-N-acetylglucosamine, and the result of the K_i showed that the compounds was a non-competitive inhibitor of the CHS. As far as the antifungal activity is concerned, compounds **7o**, **7r** and **7t** were highly active against *Aspergillus flavus* with MIC values in the range of 1 μg/mL to 2 μg/Ml while the results of antibacterial screening showed that these compounds have negligible actions to the tested bacteria. These results indicated that the design of these compounds as antifungal agents was rational.

1. Introduction

Invasive fungal infections have become alarming recently due to the high morbidity and mortality rates in patients who received stem cell transplantation, antineoplastic chemotherapy, organ transplant or suffered human immunodeficiency virus (HIV) infection [1]. Many fungal infections are caused by opportunistic pathogens from *Candida*, *Cryptococcus* and *Aspergillus* [2]. However, effective and safe antifungal agents are very limited. Representative antifungal agents used in Clinical therapy include triazoles (*i.e.* fluconazole, voriconazole, itraconazole and posaconazole), polyenes (*i.e.* amphotericin B, nystatin), glucan synthesis inhibitors (*i.e.* echinocandins, caspofungin, micafungin, anidulafungin), chitin synthesis inhibitors (*i.e.* nikkomycin, polyoxins) and flucytosine, but each of them had certain limitation [3–6]. Furthermore, as the increasing emergence of multidrug-resistant strains, intractable pathogenic microorganisms and newly arising pathogens [7], there is a continuous demand for the discovery of novel antifungal agents to treat fungal infections.

The fungal cell wall is a unique organelle and required for the growth and the maintenance of osmotic stability of the cell [8]. Chitin is an important structural component of the cell wall of many fungi, which is a linear β-(1–4)-linked polymer of N-acetyl-glucosamine (GlcNAc) and responsible for imparting shape, strength and rigidity to the cell wall. Chitin synthase (CHS) plays an important role in the process of biosynthesis of chitin that is absent in plant and human [9,10]. Thus the chitin synthase is a valuable and attractive target to design new fungicide [11]. The earliest inhibitors of chitin synthase are the naturally occurring polyoxins and nikkomycins (Fig. 1), which possess some of structural features of the natural substrate UDP-GlcNAc. They are the most potent chitin synthase inhibitors [12,13]. However, despite excellent in vitro results, clinical utility of these inhibitors is compromised by their metabolic instability and poor cellular uptake, resulting in a

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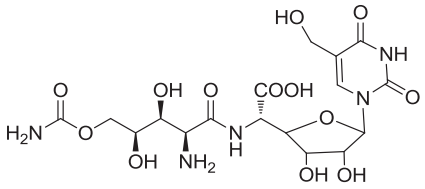
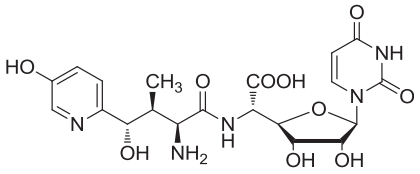


Polyoxin B



Nikkomycin Z

Fig. 1. The structures of chitin synthase inhibitor Polyoxin B and Nikkomycin Z.

decrease in efficacy. In addition, many derivatives of nikkomycins and polyoxins were designed and synthesized as natural substrate analogs, transition state mimesis or mechanistic inhibitors through changing some features of these molecules. These changes can be grouped into several categories, including changes of the terminal amino acid, the nucleoside moiety and the bridging unit of them. But none of these analogues has entered into clinical trial [14]. Therefore, there is a really perceived need to develop novel compounds with completely distinct skeleton structure as new CHS inhibitors with better antifungal activities, which are much desired.

Coumarin and its derivatives, a class of quite important lactones compounds containing a structure of benzene ring and α-pyrone, occupy an important position in medicinal chemistry, which have latent ability to exert noncovalent interactions (π-π, hydrophobic, electrostatic interactions, hydrogen bonds, metal coordination and van der Waals force *etc.*) with the various active sites in organisms [15]. As medicines, many of them display a wide range of bioactivities such as antibacterial, antifungal [16], anticoagulant [17], antioxidant [18], anti-inflammatory [19], analgesic [20], anticancer [21], anti-HIV [22] and antiviral [23] efficacies. For instance, the 7-substituted coumarin (**I**) showed a stronger efficacy against *Fusarium oxysporum* with MIC value of 19 μg/mL [24]; the 4-chloro-3-thiadiazole-imino coumarin (**II**) exhibited significant activity against *Candida albicans* with inhibitory zone diameter of 15 mm in 10 μg/mL [25]; and the 4-azidomethyl coumarin sulfonamides derivatives (**III**) showed excellent antifungal efficacies against *C. albicans* and *F. oxysporum* with MIC values of 1 μg/mL, which were 8 times more potent than fluconazole (MIC = 8 μg/mL) [26]. Hence, the diverse biological nature of coumarin derivatives has made it become a privileged structure in medicinal chemistry and much attention has been focused on the synthesis of numerous substituted coumarin derivatives.

The substituted phosphoramidates, as a class of orgnophosphorus compounds, possess relative low stability and rapid metabolic breakdown favorable properties in plants, animals, soil, and other components in environment, which have been used as impressive frameworks in drug and prodrug design in a number of fields [27,28]. Moreover, phosphoramidate moiety was widely used to enhance the water solubility of moleculars that had been proven to exhibit a wide range of biological activities such as anticancer [29], antiviral [30,31], anti-HIV [32], and antimicrobial [33,34] activities. For example, Bis(N,N-dimethylamino) pentachlorophenyl phosphate (**IV**) as organophosphorus fungicide showed moderate efficacy against *Blumeria graminis*; Phosphonamidate derivatives of 6-chloropurine (**V**) had good antifungal activity against *C. albicans* with inhibitory zone diameter of 16.5 mm in 100 μg/mL; N-aryl-*O*-ethyl phosphorodiamidates (**VI**) exhibited moderate antifungal activity against *Fusarium solani* and *Rhizoctonia solani*. (Fig. 2).

Keeping the biological importance of phosphonamidates and coumarin derivatives in mind and as part of our continuing research on the development of new chitin synthase inhibitors, we combined coumarin moiety and arylalkoxy-amino acid phosphoramide moiety with N-methylethanolamine which can afford a distance of 2–3 atoms between the two moieties to produce novel phosphoramidate derivatives of coumarin in hope of obtaining better chitin synthase inhibitors. Herein we report the screening results of chitin synthase inhibition activity and antifungal activity in vitro of the synthesized compounds.

2. Results and discussion

2.1. Chemistry

4-Hydroxycoumarin derivatives (**2a-e**) were synthesized from commercially available substituted phenol which combined with malonic acid in the presence of zinc chloride in phosphorus oxychloride at 65 °C in 41–66% yields [35]. Chlorination of compounds **2a-e** with phosphorus oxychloride gave 3-chloro-substitutedcoumarins (**3a-e**) in 60–80% yields in the presence of triethylamine at 65 °C [36]. Then the compounds **4a-e** were prepared by combining compounds **3a-e** with 2-(methylamino) ethanol in acetontrile at 65 °C in high yields ranging from 63% to 90% [37]. See in Scheme 1.

The amino acid ester salts (**5a-d**) were synthesized from the appropriate amino acid and thionyl chloride [38], which was showed in Scheme 2. The coupling with the appropriate amino acid ester salt (**5a-d**) and phenyl dichlorophosphate had been performed in the presence of Et_3N, giving the product (**6a-d**) as an oil. Finally the target compounds **7a-t** were obtained through coupling the compounds **4a-e** with **6a-d**, using *tert*-butylmagnesium chloride (^tBuMgCl) as a coupling reagent and THF as a solvent [39] (Scheme 3).

All the compounds were characterized by ^1H NMR, ^{13}C NMR and Mass spectra. In the ^1H NMR of **2a-e**, the singlet at δ12.30–12.60 ppm represented the OH group at C-4 which was disappeared after chlorination in the NMR spectrum of **3a-e**, while the singlet at δ5.50–5.65 ppm represents the CH group at C-3 which was shifted to δ6.45–6.60 ppm due to the substitution of OH group with Cl. In the ^1H NMR spectrum of **4a-e**, the singlet at δ2.00–2.11 ppm for one proton and singlet at δ3.00 ppm for three protons indicated presence of OH group and N–CH_3 group respectively. In the ^1H NMR spectrum of **7a-t**, the singlet at δ2.95–3.05 ppm and δ3.60–3.70 ppm for three protons each indicated presence of N–CH_3 group and $COOCH_3$ group respectively and the NH group was confirmed at δ2.01 ppm, which were further confirmed by their ^1H NMR,^{13}C NMR, ^{31}P NMR, HRMS spectra.

2.2. Biological activity

2.2.1. Chitin synthase inhibitory activity

All the newly synthesized compounds **7a-t** were evaluated for their in vitro chitin synthase inhibitory activities in comparison with commercially available polyoxin B as standard drug [40–42]. The CHS inhibitory activities were summarized in Table 1 and Fig. 3.

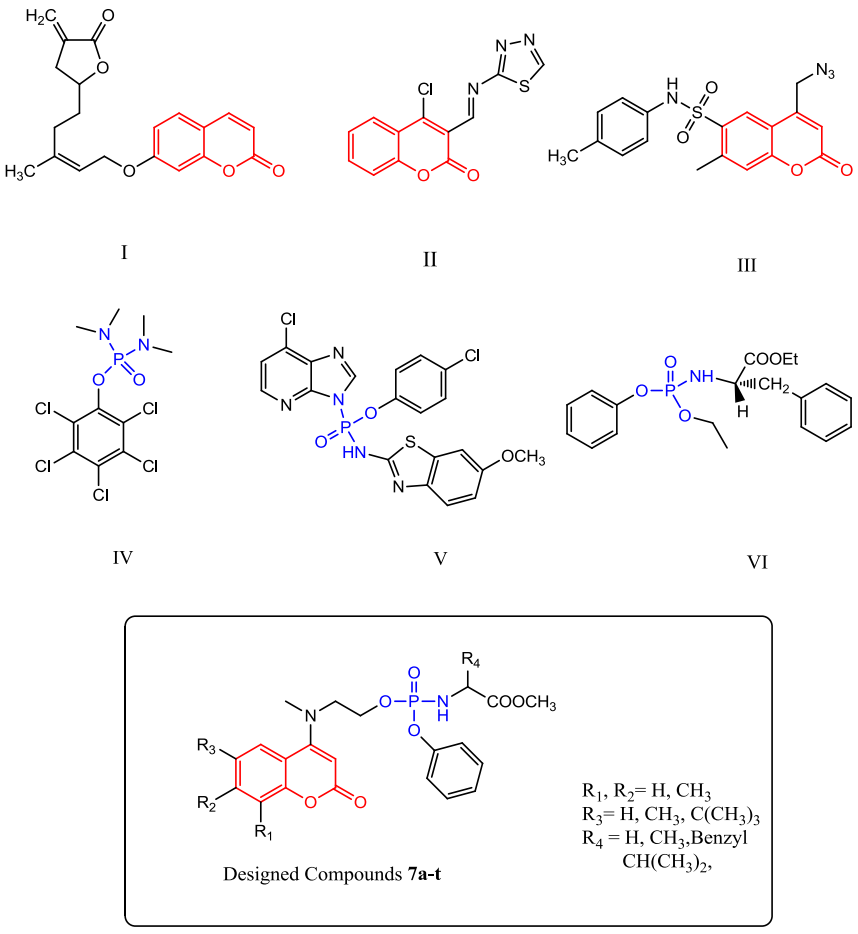
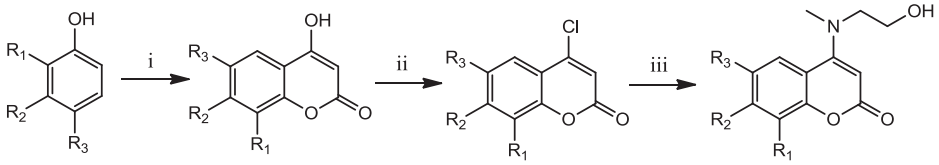
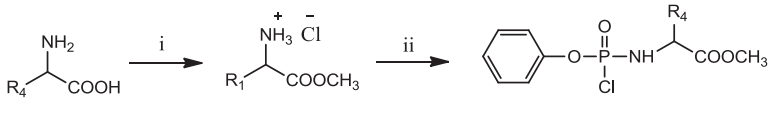


Fig. 2. Some reported antimicrobial agents containing coumarin or phosphoramidate moiety and the designed compounds **7a-t**.



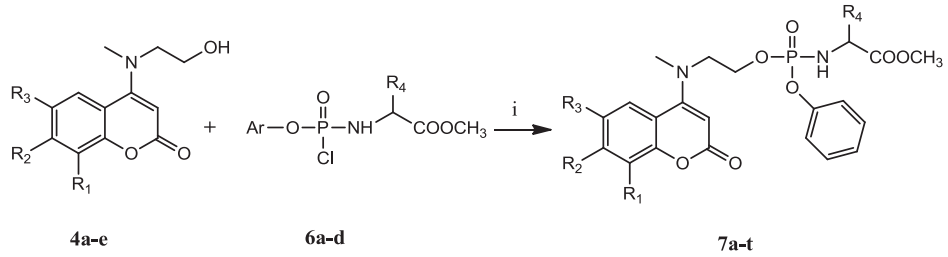
Reagents and conditions: (i) $ZnCl_2$, $POCl_3$, malonic acid, 65°C, 48h. (ii) $POCl_3$, triethylamine, 65°C. (iii) 2-(methylamino)ethanol, K_2CO_3, acetontrile, 65°C.

Scheme 1. Synthesis of compounds **4a-e**.



Reagents and conditions: (i) $SOCl_2$, CH_3OH, rt, 24h. (ii) phenyl dichiorophosphate, anhydrous TEA, anhydrous DCM,-78°C, 30 min to 1 h, then rt, 4-6 h.

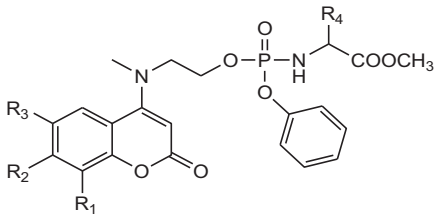
Scheme 2. Synthesis of compounds **6a-d**.



Reagents and conditions: (i) tert-BuMgCl, THF, rt, 16 h.

Scheme 3. Synthesis of compounds 7a-t.

Table 1
The inhibition ratio of all target compounds **7a-t** at 300 μg/mL.



Compound	R_1	R_2	R_3	R_4	Formula (Mw)	Inhibition%
7a	H	H	H	H	$C_{21}H_{23}N_2O_7P$(446.39)	49
7b	CH_3	H	H	H	$C_{22}H_{25}N_2O_7P$(460.41)	55
7c	H	CH_3	H	H	$C_{22}H_{25}N_2O_7P$(460.41)	59
7d	H	H	CH_3	H	$C_{22}H_{25}N_2O_7P$(460.41)	51
7e	H	H	$C(CH_3)_3$	H	$C_{25}H_{31}N_2O_7P$(502.50)	50
7f	H	H	H	CH_3	$C_{22}H_{25}N_2O_7P$(460.41)	77
7g	CH_3	H	H	CH_3	$C_{23}H_{27}N_2O_7P$(474.44)	64
7h	H	CH_3	H	CH_3	$C_{23}H_{27}N_2O_7P$(474.44)	45
7i	H	H	CH_3	CH_3	$C_{23}H_{27}N_2O_7P$(474.44)	61
7j	H	H	$C(CH_3)_3$	CH_3	$C_{26}H_{33}N_2O_7P$(516.52)	62
7k	H	H	H	$CH(CH_3)_2$	$C_{24}H_{29}N_2O_7P$(488.47)	54
7l	CH_3	H	H	$CH(CH_3)_2$	$C_{25}H_{31}N_2O_7P$(502.50)	45
7m	H	CH_3	H	$CH(CH_3)_2$	$C_{25}H_{31}N_2O_7P$(502.50)	38
7n	H	H	CH_3	$CH(CH_3)_2$	$C_{25}H_{31}N_2O_7P$(502.50)	41
7o	H	H	$C(CH_3)_3$	$CH(CH_3)_2$	$C_{28}H_{37}N_2O_7P$(544.58)	62
7p	H	H	H	Benzyl	$C_{28}H_{29}N_2O_7P$(536.51)	59
7q	CH_3	H	H	Benzyl	$C_{29}H_{31}N_2O_7P$(550.54)	48
7r	H	CH_3	H	Benzyl	$C_{29}H_{31}N_2O_7P$(550.54)	56
7s	H	H	CH_3	Benzyl	$C_{29}H_{31}N_2O_7P$(550.54)	43
7t	H	H	$C(CH_3)_3$	Benzyl	$C_{32}H_{37}N_2O_7P$(592.62)	90
Polyoxin B	–	–	–	–	$C_{17}H_{25}N_5O_{13}$(507.41)	82

The inhibition ratio in Table 1 revealed that compounds **7a-t** displayed moderate to high inhibitory activities on chitin biosynthesis at concentration of 300 μg/mL Compound **7t** ($R_3 = C(CH_3)_3$, R_4 = OBn) displayed highest CHS inhibitory activity (inhibition % = 90) among all the tested compounds. Compounds **7f**, **7g**, **7i**, **7j** and **7o** with inhibition ratio values over 60% showed significant

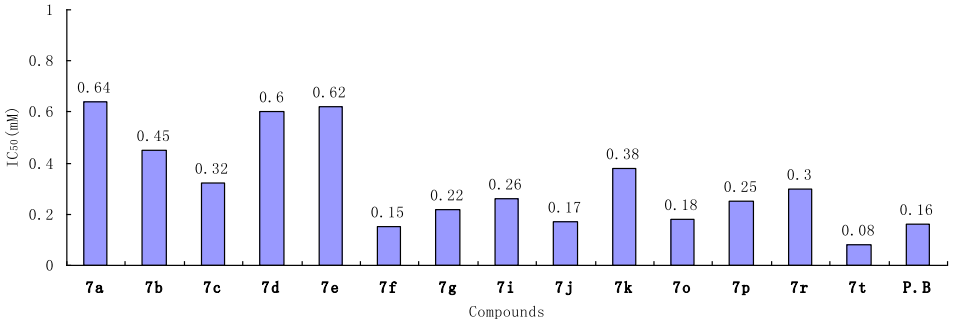


Fig. 3. The IC_{50} values of the some compounds against CHS.

Table 2
The MIC values (μg/mL) of compounds **7a-t** against fungi in vitro.

Compound	Yield%	*Candida albicans*	*Aspergillus flavus*	*Aspergillums fumigatus*	*Cryptococcus neoformans*
7a	27	64	128	128	64
7b	31	64	64	128	64
7c	48	32	64	32	16
7d	42	128	64	64	64
7e	47	64	64	32	32
7f	70	32	64	32	8
7g	45	64	64	32	128
7h	37	128	64	128	128
7i	46	64	128	64	32
7j	37	64	16	64	64
7k	37	32	16	64	64
7l	30	128	128	128	128
7m	27	128	128	128	128
7n	33	64	128	128	32
7o	46	64	2	64	32
7p	24	64	8	128	64
7q	38	128	64	128	64
7r	28	64	1	64	32
7s	47	64	64	128	64
7t	76	8	2	32	8
Polyoxin B	–	32	64	64	8
Fluconazole	–	16	32	32	8

inhibition against CHS. Generally, the Phosphoramide substituted with alanine methyl ester and phenylalanine methyl ester exhibited the most potent inhibitory activity toward CHS such as **7t** and **7f**.

For further accurate evaluation of their inhibitions, the IC_{50} of these compounds whose inhibition ratios were greater than 50% at concentration of 300 μg/mL were measured and the results were deposited in Fig. 3. The results revealed that compound **7t** displayed highest chitin synthase inhibitory activity (IC_{50} = 0.08 mM) among all the tested compounds, which was more effective than polyoxins B which acts as control drug with IC_{50} of 0.16 mmol/L. Compounds **7f**, **7j** and **7o** had IC_{50} values of 0.15, 0.17, 0.18 mM respectively, which were almost equal to that of polyoxin B. Compounds **7c**, **7g**, **7i**, **7k**, **7p** and **7r** have slightly lower CHS inhibitory activity while the compounds **7a**, **7b**, **7d** and **7e** exerted lowest inhibition potency among these compounds.

2.2.2. In vitro antifungal activity

The in vitro antifungal activities of all the target coumarins **7a-t** were evaluated against four main pathogenic fungal species (*C. albicans* CMCC 76615, *Aspergillus fumigatus* GIMCC 3.19, *Cryptococcus neoformans* ATCC 32719 and *Aspergillus flavus* ATCC 16870) using two-fold broth dilution method in 96-well micro-test plates according to National Committee for Clinical Laboratory Standards (NCCLS) [43]. Clinical antifungal drugs polyoxin B and fluconazole are used as the positive control.

The results of antifungal activity in Table 2 showed that target compounds **7a-t** exhibited moderate to excellent antifungal efficacy against all the tested fungus strains. To *C. albicans*, compounds **7c**, **7f** and **7k** with MIC values of 32 μg/mL were comparative with that of polyoxin B. Compound **7t** whose MIC values were 8 μg/mL exhibited much higher inhibitory activities than those of fluconazole and polyoxin B whose values were 16 and 32 μg/mL, respectively. To *A. flavus*, compounds **7b**-**7h**, **7q** and **7s** with MIC values of 64 μg/mL were equal to that of polyoxin B. Compounds **7j** and **7k** with MIC value of 16 μg/mL had better inhibitory activities than those of the two controls. Compounds **7o**, **7p**, **7r** and **7t** which displayed MIC values of 2, 8, 1 and 2 μg/mL respectively were much stronger potency than those of fluconazole and polyoxin B whose MIC values were 32 and 64 μg/mL, respectively. To *A. fumigatus*, the MIC values of **7c**, **7e**, **7f**, **7g** and **7t** which were comparable with that of fluconazole whose MIC value was 32 mg/L, were better than that of polyoxin B. To *C. neoformans*, compounds **7f** and **7t** with MIC values of 8 μg/mL were equal to the two controls. Noticeably, compound **7t** displayed remarkable antifungal activities in comparison to polyoxin B and fluconazole, which indicated that compound **7t** should have large possibility as potent novel antifungal agent.

From these assays data, we can see that these target compounds have potency on chitin synthase and bioactivity against fungi. These results indicated that the design of these compounds as antifungal agents was rational. In general, the compounds which have good inhibitory activity against chitin synthase showed good antifungal activity. To some extent, the antifungal activity of these compounds has positive correlation with their inhibitory activity against chitin synthase.

2.3. Kinetic parameters of chitin synthase

Under a certain temperature, pH and constant of enzyme concentration conditions, the concentration of the substrate has a great influence on the rate of enzymatic reaction. When the substrate concentration is low, the speed of enzymatic reaction (v) increases rapidly with the increase of the concentration of substrate. With the increase of the substrate concentration, the increase of reaction rate starts slow down. When the substrate concentration adds to an extent, the reaction rate reaches a limit value (V_{max}). Michaelis constant Km is equal to the half of substrate concentration when the maximum reaction rate is reached.

2.3.1. Determination of Michaelis–Menten constant (K_m)

The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined using UDP-GlcNAc as substrate, which range from 0.8 to 4 mM. The effect of substrate concentration on the enzymic activities showed that it well fit to the Michaelis–Menten model as shown by Lineweaver-Burk plot (Fig. 4). The Km and V_{max} values were 3.86 mM and 1.15$\Delta OD_{450}\bullet h^{-1}$, respectively. The chitin synthase used in the assay was prepared from *Candida tropicalis* ATCC 750.

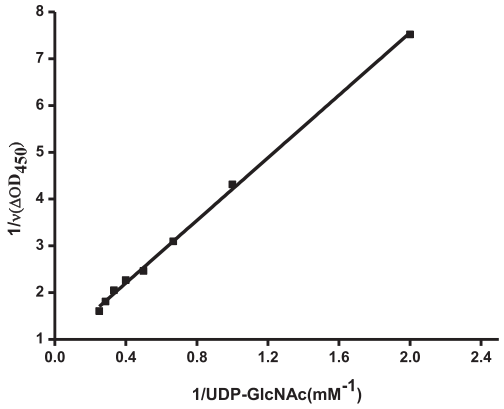


Fig. 4. The K_m of chitin synthetase determined by Lineweaver-Burk plot.

2.3.2. Determination of K_i

In order to determine K_i constants of compound **7t** in the media, concentrations of substrate UDP-GlcNAc were 0.5, 1.0, 1.5 and 2.0 mM, respectively. Different inhibitor concentrations graphs were drawn by the Lineweaver-Burk plot. The K_i value was 0.096 mM and the point of intersection of lines represents the value of K_i, which was drawn by using slope (obtained from Lineweaver-Burk plot) versus inhibitor concentrations values in Fig. 5. The Lineweaver-Burk plot proved that the compound **7t** was non-competitive inhibitor against chitin synthase.

3. Conclusion

In this study, new phosphoramidate derivatives of coumarin were synthesized and investigated for their anti-CHS and anti-fungal activity. The enzymatic assay results showed that all these target compounds have chitin synthase inhibitory activity. Among them, compounds **7f**, **7g**, **7i**, **7j**, **7o** and **6t** exhibited comparatively good activity against CHS; especially, **7t** with IC_{50} value of 0.08 mM is the strongest chitin synthase inhibitor in these compounds. The antifungal assay showed that most of these compounds exhibit moderate even excellent activity against the tested strains which are the common pathogen in clinic. Meanwhile, in these active compounds, bearing the tert-butyl group in coumarin ring and methyl, propyl, benzyl in phosphoramide moiety had higher inhibitory activities against CHS and fungal than other substituent group. Compounds substituted with methyl, propyl and benzyl in phosphoramide moiety evaluated potential inhibitory activities than hydrogen substituent in phosphoramide moiety. Moreover, the microbiological results revealed that these compounds exhibited more significant antifungal activity than activity against bacteria (see the Supplementary Material). This indicated that it is possible to develop new selective chitin synthase inhibitors from these compounds which may have potential for the treatment of fungal infections.

4. Experimental

All reactions were performed with commercially available reagents and they were used without further purification. Organic solvents were purified by standard methods. All reactions were monitored by TLC analysis which was done using pre-coated silica gel plates. The melting points were determined by X-6 melting point apparatus and are uncorrected. ^{1}H NMR ^{13}C NMR ^{31}P NMR spectra were recorded on Bruker AV 300 or 600 MHz spectrometer using CDCl3 and DMSO-*d6* as solvents and TMS as an internal standard. The chemical shifts are expressed in δ ppm. High-resolution mass spectra were determined by UHPLC Q-TOF HR-MS.

*4.1. General synthetic procedure for the 4-Hydroxycoumarin derivatives (**2a**-**e**)*

A mixture of phenol (19.8 g, 0.21 mol), anhydrous $ZnCl_2$ (84.8 g, 0.62 mol), $POC1_3$ (97 g, 0.63 mol), and malonic acid (22 g, 0.21 mol) was heated at 60–65 °C for 40 h, then the mixture was cooled. After the mixture was decomposed with water, the solid was filtered. The solid was dissolved with 10% aqueous Na_2CO_3, then acidified with diluted hydrochloric acid. The crystal was filtered to give the product **2a**. Others 4-Hydroxycoumarin derivatives **2b**-**e** were synthesized similarly. Physical constants and spectral data of compounds **2a**-**e** are summarized below.

*4.1.1. 4-Hydroxycoumarin (**2a**)*

Yellow solid; yield 41%, mp 211–213 °C. ^{1}H NMR (300 MHz, DMSO) δ 12.57 (s, 1H, OH), 7.84 (d, J = 7.7 Hz, 1H, coumarin-5-H), 7.67 (dd, J = 11.4, 4.1 Hz, 1H, coumarin-7-H), 7.39–7.29 (m, 2H, coumarin-6, 8-H), 5.62 (s, 1H, coumarin-3-H).

*4.1.2. 8-Methyl-4-hydroxycoumarin (**2b**)*

Light yellow solid; yield 52%, mp 231–235 °C. ^{1}H NMR (600 MHz, DMSO-d_6): 12.42 (s, 1H, OH), 7.59 (d, J = 8.1 Hz, 1H, coumarin-5-H), 7.46 (d, J = 8.9 Hz, 1H, coumarin-7-H), 7.32 (m, 1H, coumarin-6-H), 5.56 (s, 1H, coumarin-3-H), 2.12 (s, 3H, CH_3).

*4.1.3. 7-Methyl-4-hydroxycoumarin (**2c**)*

Yellow solid; yield 65%, mp 242–244 °C. ^{1}H NMR (600 MHz, DMSO-d_6): 12.26 (s, 1H, OH), 7.67 (d, J = 7.5 Hz, 1H, coumarin-5-H), 7.33 (d, J = 7.5 Hz, 1H, coumarin-6-H), 7.22 (s, 1H, coumarin-8-H), 5.62 (s, 1H, coumarin-3-H), 2.29 (s, 3H, CH_3).

*4.1.4. 6-Methyl-4-hydroxycoumarin (**2d**)*

Light yellow solid; yield 66%, mp 256–258 °C. ^{1}H NMR

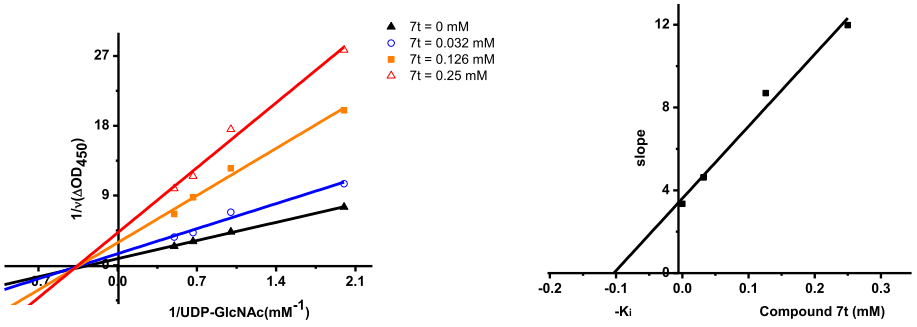


Fig. 5. Inhibition constant K_i of compound **7t** were determined by Lineweaver-Burk plot.

(600 MHz, DMSO-d_6): 12.38 (s, 1H, OH), 7.59 (s, 1H, coumarin-5-H), 7.40 (d, J = 8.7 Hz, 1H, coumarin-7-H), 7.21 (d, J = 8.7 Hz, 1H, coumarin-8-H), 5.55 (s, 1H, coumarin-3-H), 2.38 (s, 3H, CH_3).

4.1.5. 6-Tert-buty-4-hydroxycoumarin (**2e**)

White solid; yield 58%, mp 199–201 °C.^{1}H NMR (600 MHz, DMSO-d6) δ 12.48 (s, 1H, OH), 7.77 (s, 1H, coumarin-5-H), 7.71 (d, J = 8.1 Hz, 1H, coumarin-7-H), 7.31 (d, J = 8.6 Hz, 1H, coumarin-8-H), 5.59 (s, 1H, coumarin-3-H), 1.32 (s, 9H, CH_3).

4.2. General synthetic procedure for the 4-chlorocoumarin derivatives (**3a**-**e**)

A solution of 6-methyl-4-hydroxycoumarin (1.76 g, 10 mmol) and Phosphorus oxychloride (15 mL, 161 mmol was stirred at 0–5 °C for 10 min. Triethylamine (1.2 g, 12 mmol) was added dropwise over 5 min, and the resulting solution was stirred at 40 °C for 30 min, then heated at 100 °C for 4 h under N_2. After the solution was added to ice water and was stirred at room temperature for 1 h. The resulting black precipitate was filtered and extracted with EtOAc, The solution was dried over anhydrous Na_2SO_4. After being filtered, the solution was concentrated in vacuum to give 6-methyl-4-chlorocoumarin (1.38 g, yield, 71%) as a pale yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.56 (d, 1H, coumarin-5-H, J = 2.1 Hz), 7.26 (d, 1H, coumarin-7-H, J = 2.1 Hz, 8.7 Hz), 7.28 (d, 1H, coumarin-8-H, J = 8.7 Hz), 6.53 (s, 1H, coumarin-3-H), 2.39 (s, 3H, CH_3).

4.3. General synthetic procedure for 4-((2-hydroxyethyl) (methyl) amino)-coumarin derivatives (**4a**-**e**)

One intermediate of **3a**-**e** (13.6 mmol) was added to a mixture of N-methylethanolamine (1.07 g, 14.2 mmol), potassium carbonate (2.0 g, 14.7 mmol) and dry CH_3CN (60 mL). The mixture was reflux for 4–5 h. After cooling, the resulting mixture was filtrated. The filtrate was concentrated in vacuum to give a yellow solid **4a**-**e**.

4.3.1. 4-((2-Hydroxyethyl) (methyl)amino)-coumarin (**4a**)

Yellow solid; yield, 78%, mp 107–108 °C.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85 (d, J = 8.1 Hz, 1H, coumarin-5-H), 7.59 (t, J = 12.5, 5.2 Hz, 1H, coumarin-7-H), 7.42–7.27 (m, 2H, coumarin-6, 8-H), 5.67 (s, 1H, coumarin-3-H), 4.05 (t, J = 5.7 Hz, 2H, CH_2), 3.55 (t, J = 5.7 Hz, 2H, CH_2), 2.99 (s, 3H, NCH_3), 2.11 (s, 1H, OH).

4.3.2. 4-((2-Hydroxyethyl) (methyl)amino)-8-methylcoumarin (**4b**)

Yellow solid; yield, 90%, mp 111–112 °C..^{1}H NMR (600 MHz, $CDCl_3$) δ 7.72 (d, J = 8.1 Hz, 1H, coumarin-5-H), 7.33 (d, J = 7.3 Hz, 1H, coumarin-7-H), 7.13 (t, J = 7.7 Hz, 1H, coumarin-6-H), 5.72 (s, 1H, coumarin-3-H), 3.96 (t, J = 5.6 Hz, 2H, CH2), 3.53 (t, J = 5.6 Hz, 2H, CH_2), 3.03 (s, 3H, NCH_3), 2.43 (s, 3H, CH_3), 2.09 (s, 1H, OH).

4.3.3. 4-((2-Hydroxyethyl) (methyl)amino)-7-methylcoumarin (**4c**)

Yellow solid; yield, 63%, mp 110–112 °C..^{1}H NMR (600 MHz, $CDCl_3$): 7.69 (d, J = 7.8 Hz, 1H, coumarin-5-H), 7.39 (d, J = 7.8 Hz, 1H, coumarin-6-H), 7.25 (s, 1H, coumarin-8-H), 5.56 (s, 1H, coumarin-3-H), 3.85 (t, J = 5.5 Hz, 2H, CH2), 3.49 (t, J = 5.5 Hz, 2H, CH_2), 3.05 (s, 3H, NCH_3), 2.30 (s, 3H, CH_3), 2.06 (s, 1H, OH).

4.3.4. 4-((2-Hydroxyethyl) (methyl)amino)-6-methylcoumarin (**4d**)

Yellow solid; yield, 82%, mp 107–108 °C.^{1}H NMR (600 MHz, $CDCl_3$): 7.55 (s, 1H, coumarin-5-H), 7.42 (d, J = 8.8 Hz, 1H, coumarin-7-H), 7.23 (d, J = 8.8 Hz, 1H, coumarin-8-H), 5.57 (s, 1H, coumarin-3-H), 4.06 (t, J = 5.8 Hz, 2H, CH2), 3.51 (t, J = 5.8 Hz, 2H, CH_2), 3.06 (s, 3H, NCH_3), 2.39 (s, 3H, CH_3), 2.02 (s, 1H, OH).

4.3.5. 4-((2-Hydroxyethyl) (methyl)amino)-6-tert-buty-coumarin (**4e**)

Yellow solid; yield, 75%, mp 115–117 °C.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.79 (s, 1H, coumarin-5-H), 7.73 (d, J = 8.3 Hz, 1H, coumarin-7-H), 7.32 (d, J = 8.6 Hz, 1H, coumarin-8-H), 5.61 (s, 1H, coumarin-3-H), 3.89 (t, J = 5.2 Hz, 2H, CH2), 3.48 (t, J = 5.3 Hz, 2H, CH_2), 2.98 (s, 3H, NCH_3),1.32 (s, 9H, CH_3), 2.04 (s, 1H, OH).

4.4. General procedure for the preparation of amino acid methyl esters (**5a**-**d**)

To the solution of L-amino acid (100 mmol) and anhydrous methanol (100 mL), $SOCl_2$ (10 mL, 140 mmol) was added slowly at −5 °C for about 10–30 min until clear. And then the reaction mixture was stirred for about 24 h at room temperature. After the reaction was completed, the superfluous $SOCl_2$ and methanol were removed on the rotary evaporator to give a white solid. These were recrystallized from the solvent of methanol and diethyl ether to give the L-amino acid methyl ester hydrochloride in 85–95% yield.

4.5. General procedure for the preparation of compounds **6a**-**d**

Anhydrous triethylamine (2.0 mol equiv) was added dropwise at −78 °C to a stirred solution of Phenyl phosphorodichloridate (1.0 mol equiv) and an appropriate amino acid ester (1.0 mol equiv) in anhydrous DCM. The mixture was then allowed to slowly warm to room temperature and stirred for 24 h. Formation of a desired compound was monitored by TLC (developing solvent methanol-chloroform 1:30). After the reaction was completed, the solvent was evaporated under reduced pressure and the resulting residue was redissolved in anhydrous Et_2O and filtered. The filtrate was reduced to dryness to give a crude product as oil, which was in some cases used without further purification in the next step.

4.6. General procedure for the preparation of compounds **7a**-**t**

To a solution of 4-((2-hydroxyethyl) (methyl)amino)-coumarin derivatives (**4a**-**e**) (1.0 mmol) in anhydrous THF (7 mL) was added dropwise under an argon atmosphere tert-BuMgCl (1.4 mmol), and the reaction mixture was stirred at room temperature for 30 min. Then, a solution of the appropriate phosphorochloridate (4.0 mmol) in anhydrous THF (6 mL) was added dropwise. The reaction mixture was stirred at room temperature overnight and then evaporated in vacuo to give a crude residue that was purified by column chromatography on silica gel, with EtOAc-P.E as a gradient (25%–50% EtOAc) to afford the products **7a**-**t** as yellow oil.

4.6.1. Methyl 2-((2-(methyl(2-oxo-2H-chromen-4-yl)amino) ethoxy) (phenoxy)phosphorylamino)acetate (**7a**)

Yellow oil; yield, 27%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.72 (d, J = 8.5 Hz, 1H, coumarin-5-H), 7.49 (t, J = 7.9 Hz, 1H, coumarin-7-H), 7.31 (d, J = 8.6 Hz, 1H, coumarin-6-H), 7.26 (d, J = 5.8 Hz, 1H, coumarin-8-H), 7.232–7.04 (m, 5H, benzene-H), 5.68 (s, 1H, coumarin-3-H), 4.40 (m, J = 12.2, 6.2 Hz, 2H, CH_2), 3.84–3.72 (m, 1H, $NHCH_2$), 3.69 (s, 3H, OCH_3), 3.67 (dd, J = 13.2, 5.5 Hz, 2H, CH_2), 3.01 (s, 3H, NCH_3), 2.05 (s, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.83, 162.71, 161.56, 152.72, 150.54, 129.70, 128.69, 125.10, 122.81, 121.56, 120.05, 117.16, 115.94, 96.37, 63.49, 54.27, 52.38, 42.69, 39.65. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.45–2.72. HRMS: calcd for $C_{21}H_{23}N_2O_7P$ $[M+H]^+$, 447.1316, found, 447.1319.

4.6.2. Methyl 2-((2-(methyl(8-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino)- acetate (**7b**)

Yellow oil; yield, 31%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.54 (d, J = 7.9 Hz, 1H, coumarin-5-H), 7.34 (d, J = 7.3 Hz, 1H, coumarin-7-

H), 7.29 (t, J = 7.9 Hz, 2H, coumarin-6-H, benzene-H), 7.15 (t, J = 8.9 Hz, 3H, benzene-H), 7.10 (t, J = 7.7 Hz, 1H, benzene-H), 5.70 (s, 1H, coumarin-3-H), 4.41 (m, J = 12.2, 6.2 Hz, 2H, CH_2), 3.87 (dd, J = 11.8, 6.1 Hz, 1H, $NHCH_2$), 3.75 (dd, J = 10.6, 6.2 Hz, 1H, $NHCH_2$), 3.72 (s, 3H, OCH_3), 3.66 (dd, J = 13.2, 5.5 Hz, 2H, CH_2), 2.99 (s, 3H, NCH_3), 2.45 (s, 3H, CH_3), 2.07 (s, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.80, 162.77, 161.25, 152.36, 150.45, 142.26, 129.71, 127.35, 125.89, 124.43, 121.66, 120.06, 115.69, 96.21, 63.57, 54.11, 52.38, 42.77, 39.69, 21.29. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.44–2.68. HRMS: calcd for $C_{22}H_{25}N_2O_7P$ $[M+H]^+$, 461.1472, found, 461.1471.

*4.6.3. Methyl 2-((2-(methyl(7-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino)- acetate (**7c**)*

Yellow oil; yield, 48%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.58 (d, J = 8.3 Hz, 1H, coumarin-5-H), 7.28 (dd, J = 9.4, 6.4 Hz, 2H, benzene-H), 7.18–7.10 (m, 4H, benzene-H, coumarin-8-H), 7.00 (d, J = 8.2 Hz, 1H, coumarin-6-H), 5.60 (s, 1H, coumarin-3-H), 4.40 (m, 2H, CH_2), 3.78–3.73 (m, 2H, $NHCH_2$), 3.71 (s, 3H, OCH_3), 3.68–3.62 (m, 2H, CH_2), 2.99 (s, 3H, NCH_3), 2.41 (s, 3H, CH_3), 2.05 (s, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$)δ 170.81, 162.65, 161.35, 152.53, 150.47, 132.66, 129.70, 127.16, 125.10, 122.92, 122.83, 120.04, 115.95, 96.25, 63.55, 54.15, 52.38, 42.76, 39.74, 15.95. ^{31}P NMR (243 MHz, $CDCl_3$) δ 2.45–2.69. HRMS: calcd for $C_{22}H_{25}N_2O_7P$ $[M+H]^+$, 461.1472, found, 461.1474.

*4.6.4. Methyl 2-((2-(methyl(6-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino) acetate (**7d**)*

Yellow oil; yield, 42%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.49 (s, 1H, coumarin-5-H), 7.30–7.23 (m, 3H, coumarin-7-H, benzene-H), 7.19 (d, J = 8.4 Hz, 1H, coumarin-8-H), 7.16–7.08 (m, 3H, benzene-H), 5.64 (s, 1H, coumarin-3-H), 4.40 (d, J = 5.0 Hz, 2H, CH_2), 3.94 (d, J = 5.3 Hz, 1H, $NHCH_2$), 3.74 (d, J = 6.1 Hz, 2H, $NHCH_2$), 3.68 (s, 3H, OCH_3), 3.66 (d, J = 4.9 Hz, 2H, CH_2), 2.98 (s, 3H, NCH_3), 2.37 (s, 3H, CH_3), 2.03 (d, J = 2.9 Hz, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.80, 162.78, 161.46, 152.29, 150.45, 135.59, 132.15, 129.70, 127.09, 121.79, 120.05, 117.16, 115.56, 96.18, 63.58, 54.26, 52.38, 42.79, 39.68, 21.68. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.45–2.69. HRMS: calcd for $C_{22}H_{25}N_2O_7P$ $[M+H]^+$, 461.1472, found, 461.1474.

*4.6.5. Methyl 2-((2-(methyl(6-tert-buty-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino) acetate (**7e**)*

Yellow oil; yield, 47%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.67 (s, 1H, coumarin-5-H), 7.54 (d, J = 8.7 Hz, 1H, coumarin-7-H), 7.27 (dd, J = 8.2, 5.5 Hz, 3H, coumarin-8-H, benzene-H), 7.17–7.11 (m, 3H, benzene-H), 5.68 (s, 1H, coumarin-3-H), 4.43 (dd, J = 12.2, 5.8 Hz, 2H, CH_2), 3.76–3.73 (m, 2H, $NHCH_2$), 3.71 (s, 3H, OCH_3), 3.70 (d, J = 5.0 Hz, 2H, CH_2), 3.03 (s, 3H, NCH_3), 2.05 (s, 1H, NH), 1.35 (s, 9H, $C(CH_3)_3$).^{13}C NMR (150 MHz, $CDCl_3$)δ 170.79, 162.84, 161.14, 152.19, 150.42, 146.31, 129.70, 129.04, 125.10, 121.47, 120.01, 117.39, 115.43, 96.16, 63.59, 54.03, 52.38, 42.78, 39.66, 34.62, 31.37. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.44–2.62. HRMS: calcd for $C_{25}H_{31}N_2O_7P$ $[M+H]^+$, 503.1942, found, 503.1944.

*4.6.6. Methyl 2-((2-(methyl(2-oxo-2H-chromen-4-yl)amino) ethoxy) (phenoxy)phosphorylamino)propanoate (**7f**)*

Yellow oil; yield, 70%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.69 (dd, J = 12.0, 8.2 Hz, 1H, coumarin-5-H), 7.47 (t, J = 7.7 Hz, 1H, coumarin-7-H), 7.31 (d, J = 8.3 Hz, 1H, coumarin-6-H), 7.28 (t, J = 4.6 Hz, 1H, coumarin-8-H), 7.22–7.07 (m, 5H, benzene-H), 5.65 (d, J = 11.2 Hz, 1H, coumarin-3-H), 4.42–4.30 (m, 2H, CH_2), 4.04–3.91 (m, 1H, CH), 3.92–3.72 (m, 1H, NH)3.67 (s, 3H, OCH_3), 3.66–3.60 (m, 2H, CH_2), 2.98 (d, J = 13.1 Hz, 3H, NCH_3), 1.33 (dd, J = 13.3, 7.0 Hz, 3H, $CHCH_3$).^{13}C NMR (150 MHz, $CDCl_3$) δ 173.79, 162.35, 160.71, 154.24, 150.60, 131.37, 129.66, 125.29, 124.99, 123.26, 120.06, 117.81, 116.24, 96.25, 63.36, 54.01, 52.42, 50.19, 39.64, 20.80. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.42–2.62. HRMS: calcd for $C_{22}H_{25}N_2O_7P$ $[M+H]^+$, 461.1472, found, 461.1473.

*4.6.7. Methyl 2-((2-(methyl(8-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy)phosphorylamino) propanoate (**7g**)*

Yellow oil; yield, 45%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.56–7.51 (t, 1H, coumarin-6-H), 7.34 (d, J = 7.3 Hz, 1H, coumarin-5-H), 7.29 (d, J = 7.8, 2.4 Hz, 2H, coumarin-7-H, benzene-2-H), 7.16 (d, J = 8.6 Hz, 1H, benzene-6-H), 7.13 (t, J = 7.0 Hz, 2H, benzene-3,5-H), 7.10 (t, J = 7.8, 2.3 Hz, 1H, benzene-4-H), 5.68 (d, J = 10.0 Hz, 1H, coumarin-3-H), 4.42–4.30 (m, 2H, CH_2), 4.04–3.93 (m, 1H, CH), 3.68 (s, 3H, OCH_3), 3.63 (dd, J = 12.3, 7.0 Hz, 2H, CH_2), 3.57 (s, 1H, NH), 2.98 (d, J = 11.8 Hz, 3H, NCH_3), 2.45 (s, 3H, CH_3), 1.34 (dd, J = 18.2, 7.1 Hz, 3H, $CHCH_3$). ^{13}C NMR (150 MHz, $CDCl_3$)δ 173.74, 162.54, 160.80, 152.79, 150.56, 131.98, 129.67, 127.44, 125.36, 121.77, 120.01, 118.45, 115.88, 96.31, 63.39, 54.05, 52.41, 50.17, 39.68, 20.85, 16.77. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.47–2.65. HRMS: calcd for $C_{23}H_{27}N_2O_7P$ $[M+H]^+$, 475.1629, found, 475.1633.

*4.6.8. Methyl 2-((2-(methyl(7-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy)phosphorylamino) propanoate (**7h**)*

Yellow oil; yield, 37%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.57 (dd, J = 11.4, 8.3 Hz, 1H, coumarin-5-H), 7.31–7.24 (m, 2H, coumarin-6-H, benzene-H), 7.19–7.09 (m, 4H, benzene-H), 7.00 (d, J = 8.2 Hz, 1H, coumarin-8-H), 5.61 (d, J = 11.2 Hz, 1H, coumarin-3-H), 4.42–4.31 (m, 2H, CH_2), 3.98 (dd, J = 19.2, 8.4 Hz, 1H, CH), 3.85 (d, J = 10.5 Hz, 1H, NH), 3.68 (s, 3H, OCH_3), 3.63 (t, J = 5.4 Hz, 2H, CH_2), 2.99 (d, J = 12.8 Hz, 3H, NCH_3), 2.42 (s, 3H, CH_3), 1.35 (dd, J = 15.3, 6.8 Hz, 3H, $CHCH_3$). ^{13}C NMR (150 MHz, $CDCl_3$)δ 173.77, 162.48, 160.69, 152.56, 150.59, 142.25, 129.62, 125.41, 124.40, 121.59, 120.04, 117.46, 115.67, 96.48, 63.29, 54.10, 52.36, 50.17, 39.62, 21.55, 20.21. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.47–2.68. HRMS: calcd for $C_{23}H_{27}N_2O_7P$ $[M+H]^+$, 475.1629, found, 475.1630.

*4.6.9. Methyl 2-((2-(methyl(6-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy)phosphorylamino) propanoate (**7i**)*

Yellow oil; yield, 46%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.49 (d, J = 9.1 Hz, 1H, coumarin-8-H), 7.29–7.25 (m, 3H, coumarin-5-H, benzene-H), 7.21 (d, J = 8.4 Hz, 1H, coumarin-7-H), 7.18–7.09 (m, 3H, benzene-H), 5.66 (d, J = 9.6 Hz, 1H, coumarin-3-H), 4.41–4.30 (m, 2H, CH_2), 4.04–3.93 (m, 1H, CH), 3.86–3.76 (t, J = 10.3 Hz, 1H, NH), 3.67 (s, 3H, OCH_3), 3.64 (t, J = 5.4 Hz, 1H, CH_2), 2.99 (d, J = 11.4 Hz, 3H, NCH_3), 2.38 (s, 3H, CH_3), 1.33 (dd, J = 17.6, 7.1 Hz, 3H, $CHCH_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.76, 162.66, 160.84, 152.33, 150.55, 132.91, 132.40, 129.66, 125.04, 121.69, 120.02, 117.56, 115.90, 96.37, 63.41, 54.03, 52.44, 50.18, 39.70, 20.77, 21.02. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.46–2.69. HRMS: calcd for $C_{23}H_{27}N_2O_7P$ $[M+H]^+$, 475.1629, found, 475.1633.

*4.6.10. Methyl 2-((2-(methyl(6-tert-buty-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino) propanoate (**7j**)*

Yellow oil; yield, 39%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.65 (dd, J = 7.3, 2.2 Hz, 1H, coumarin-5-H), 7.53 (dd, J = 8.7, 1.1 Hz, 1H, coumarin-7-H), 7.33–7.23 (m, 3H, coumarin-8-H, benzene-H), 7.19–7.09 (m, 3H, benzene-H), 5.66 (d, J = 9.9 Hz, 1H, coumarin-3-H), 4.45–4.31 (m, 2H, CH_2), 4.02–3.93 (m, 1H, CH), 3.76 (t, J = 10.2 Hz, 1H, NH), 3.69 (d, J = 5.0 Hz, 1H, CH_2), 3.67 (s, 3H, OCH_3), 3.65 (d, J = 6.5 Hz, 1H, CH_2), 3.01 (d, J = 11.3 Hz, 3H, NCH_3), 1.35 (s, 10H, $C(CH_3)_3$, $CHCH_3$), 1.32 (d, J = 7.1 Hz, 2H, $CHCH_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.71, 162.69, 160.74, 152.56, 150.49, 145.26, 129.68, 125.16, 123.40, 121.21, 120.04, 116.98, 115.58, 96.43, 63.52, 54.09, 52.52, 50.21, 39.59, 31.25, 21.85. ^{31}P NMR (243 MHz, $CDCl_3$) δ 2.47–2.70. HRMS: calcd for $C_{26}H_{33}N_2O_7P$ $[M+H]^+$, 517.2098, found, 517.2104.

*4.6.11. Methyl 3-methyl-2-((2-(methyl(2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino) butanoate (**7k**)*

Yellow oil; yield, 37%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.69 (t, J = 8.7 Hz, 1H, coumarin-7-H), 7.48 (t, J = 7.7 Hz, 1H, coumarin-6-H), 7.32 (d, J = 8.3 Hz, 1H, benzene-2-H), 7.28 (d, J = 8.7 Hz, 2H, coumarin-5,8-H), 7.20 (t, J = 7.7 Hz, 1H, benzene-4-H), 7.14 (m, J = 15.0, 8.0 Hz, 3H, benzene-3,5,6-H), 5.67 (s, 1H, coumarin-3-H), 4.38 (m, J = 11.1, 5.1 Hz, 2H, CH_2), 3.81–3.70 (m, 2H, CH_2), 3.66 (s, 1H, CHNH), 3.65 (s, 3H, OCH_3), 2.99 (d, J = 11.5 Hz, 3H, NCH_3), 2.06 (s, 1H, NH), 2.05–1.99 (m, 1H, CH_3CHCH_3), 0.89 (dd, J = 14.8, 6.8 Hz, 3H, CH_3), 0.84 (t, J = 6.5 Hz, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.98, 162.55, 160.43, 152.56, 150.72, 129.61, 128.61, 125.52, 122.58, 121.66, 120.05, 117.36, 115.91, 96.58, 63.46, 59.97, 54.81, 52.38, 39.71, 32.21, 18.79, 17.30. ^{31}P NMR (243 MHz, $CDCl_3$) δ 2.41–2.65. HRMS: calcd for $C_{24}H_{29}N_2O_7P$ $[M+H]^+$, 489.1785, found, 489.1789.

*4.6.12. Methyl 3-methyl-2-((2-(methyl(8-methyl-2-oxo-2H-chromen-4-yl)amino)ethoxy) (phenoxy) phosphorylamino) butanoate (**7l**)*

Yellow oil; yield, 30%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.52 (t, J = 8.3 Hz, 1H, coumarin-6-H), 7.33 (d, J = 7.3 Hz, 1H, coumarin-5-H), 7.28 (d, J = 7.0 Hz, 2H, coumarin-7-H, benzene-H), 7.18–7.07 (m, 4H, benzene-H), 5.67 (d, J = 7.5 Hz, 1H, coumarin-3-H), 4.39–4.28 (m, 2H, CH_2), 3.79–3.72 (m, 1H, CH_2), 3.64 (s, 3H, OCH_3), 3.60 (dd, J = 9.2, 3.1 Hz, 1H, CH_2), 3.52 (t, J = 10.4 Hz, 1H, NHCH), 2.97 (d, J = 10.7 Hz, 3H, NCH_3), 2.44 (s, 3H, CH_3), 2.04 (s, 1H, NH), 2.01 (dd, J = 13.2, 6.6 Hz, 1H, CH_3CHCH_3), 0.89 (dd, J = 15.8, 6.8 Hz, 3H, $CHCH_3$), 0.84 (t, J = 6.6 Hz, 3H, $CHCH_3$).^{13}C NMR (150 MHz, $CDCl_3$) δ 173.01, 162.48, 161.23, 152.55, 150.65, 132.63, 129.61, 127.12, 124.94, 122.91, 122.71, 120.03, 115.95, 96.32, 63.49, 59.98, 54.13, 52.06, 39.78, 32.10, 18.78, 17.31, 15.98. ^{31}P NMR (243 MHz, $CDCl_3$) δ 2.40–2.65. HRMS: calcd for $C_{25}H_{31}N_2O_7P$ $[M+H]^+$, 503.1942, found, 503.1943.

*4.6.13. Methyl 3-methyl-2-((2-(methyl(7-methyl-2-oxo-2H-chromen-4-yl)amino)ethoxy) (phenoxy) phosphorylamino) butanoate (**7m**)*

Yellow oil; yield, 27%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.60 (d, J = 8.5 Hz, 1H, coumarin-5-H), 7.29 (dd, J = 9.4, 6.4 Hz, 2H, benzene-H), 7.18–7.10 (m, 4H, benzene-H, coumarin-8-H), 7.02 (d, J = 8.6 Hz, 1H, coumarin-6-H), 5.69 (d, J = 7.8 Hz, 1H, coumarin-3-H), 4.41–4.30 (m, 2H, CH_2), 3.75–3.68 (m, 2H, CH_2), 3.66 (s, 3H, OCH_3), 3.56 (t, J = 11.5 Hz, 1H, NHCH), 3.02 (d, J = 9.9 Hz, 3H, NCH_3), 2.39 (s, 3H, CH_3), 2.05 (s, 1H, NH), 2.01–1.97 (m, 1H, CH_3CHCH_3), 0.89 (dd, J = 16.1, 6.9 Hz, 3H, $CHCH_3$), 0.84 (t, J = 6.7 Hz, 3H, $CHCH_3$).^{13}C NMR (150 MHz, $CDCl_3$) δ 173.25, 162.54, 160.89, 152.38, 150.58, 142.61, 129.69, 126.01, 125.34, 121.56, 120.03, 117.72, 115.86, 96.47, 63.52, 59.99, 54.55, 52.38, 39.69, 32.18, 21.55, 18.78, 17.30. ^{31}P NMR (243 MHz, $CDCl_3$) δ 2.42–2.64. HRMS: calcd for $C_{25}H_{31}N_2O_7P$ $[M+H]^+$, 503.1942, found, 503.1942.

*4.6.14. Methyl 3-methyl-2-((2-(methyl(6-methyl-2-oxo-2H-chromen-4-yl)amino)ethoxy) (phenoxy) phosphorylamino) butanoate (**7n**)*

Yellow oil; yield, 33%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.57 (t, J = 8.6 Hz, 1H, coumarin-5-H), 7.29 (t, J = 7.8 Hz, 2H, benzene-H), 7.20–7.12 (m, 4H, benzene-H, coumarin-8-H), 7.01 (d, J = 8.2 Hz, 1H, coumarin-7-H), 5.61 (d, J = 7.8 Hz, 1H, coumarin-3-H), 4.36 (m, 2H, CH_2), 3.77 (dd, J = 4.7, 3.2 Hz, 1H, NHCH), 3.66 (s, 3H, OCH_3), 3.65–3.60 (m, 2H, CH_2), 3.49 (m, 1H, CH_3CHCH_3), 2.99 (d, J = 10.5 Hz, 3H, NCH_3), 2.43 (s, 3H, CH_3), 2.03 (s, 1H, NH), 0.80–0.95 (m, 6H, $CHCH_3$).^{13}C NMR (150 MHz, $CDCl_3$) δ 173.55, 162.98, 160.89, 152.35, 150.77, 135.61, 132.01, 129.61, 127.55, 121.71, 120.03, 117.11, 115.88, 96.45, 63.59, 59.93, 54.75, 52.12, 39.65, 32.13, 21.75, 18.79, 17.29. ^{31}P NMR (243 MHz, $CDCl_3$) δ 2.42–2.64. HRMS: calcd for $C_{25}H_{31}N_2O_7P$ $[M+H]^+$, 503.1942, found, 503.1943.

*4.6.15. Methyl 3-methyl-2-((2-(methyl(6-tert-buty-2-oxo-2H-chromen-4-yl)amino)ethoxy) (phenoxy)phosphorylamino) butanoate (**7o**)*

Yellow oil; yield, 46%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.65 (d, J = 10.3 Hz, 1H, coumarin-5-H), 7.55 (d, J = 8.7 Hz, 1H, coumarin-7-H), 7.31–7.24 (m, 3H, coumarin-8-H, benzene-H), 7.19–7.11 (m, 3H, benzene-H), 5.67 (d, J = 6.4 Hz, 1H, coumarin-3-H), 4.46–4.29 (m, 2H, CH_2), 3.80–3.73 (m, 1H, CH_2), 3.69 (dd, J = 9.7, 4.7 Hz, 1H, CH_2), 3.66 (s, 3H, OCH_3), 3.50 (m, J = 29.3, 10.5 Hz, 1H NHCH), 3.02 (d, J = 9.5 Hz, 3H, NCH_3), 2.08–1.99 (m, 1H, CH_3CHCH_3), 1.36 (s, 9H, $C(CH_3)_3$), 0.90 (dd, J = 16.6, 6.8 Hz, 3H, $CHCH_3$), 0.85 (t, J = 6.8 Hz, 3H, $CHCH_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.94, 162.75, 161.03, 152.23, 146.26, 129.63, 129.05, 124.99, 121.43, 120.01, 117.42, 115.42, 96.19, 63.74, 63.59, 59.97, 54.05, 52.08, 39.77, 34.63, 32.12, 31.38, 18.78, 17.30. ^{31}P NMR (243 MHz, $CDCl_3$) δ 2.45–2.65. HRMS: calcd for $C_{28}H_{37}N_2O_7P$ $[M+H]^+$, 545.2411, found, 545.2413.

*4.6.16. Methyl 2-((2-(methyl(2-oxo-2H-chromen-4-yl)amino) ethoxy) (phenoxy)phosphorylamino) -3-phenyl propanoate (**7p**)*

Yellow oil; yield, 24%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.31 (d, J = 3.7 Hz, 3H, benzene, coumarin-H), 7.30–7.26 (m, 4H, benzene, coumarin-H), 7.15 (dd, J = 15.1, 7.2 Hz, 6H, benzene), 7.04 (dd, J = 15.8, 7.3 Hz, 1H, benzene), 5.81 (s, 1H, coumarin-3-H), 4.24 (t, J = 15.6, 11.1 Hz, 2H, OCH_2), 3.84–3.74 (m, 1H, CH), 3.68 (d, J = 3.6 Hz, 3H, OCH_3), 3.66 (d, J = 5.7 Hz, 2H, NCH_2), 3.58 (d, J = 11.5 Hz, 2H, $CHCH_2$), 3.01 (dd, J = 16.5, 9.7 Hz, 3H, NCH_3), 2.07 (s, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.58, 162.59, 160.95, 154.62, 150.25, 135.78, 131.50, 129.68, 129.32, 128.52, 126.15, 125.27, 124.32, 121.15, 120.06, 117.85, 115.84, 95.26, 63.38, 54.95, 52.85, 50.23, 40.54, 39.65. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.46–2.72. HRMS: calcd for $C_{28}H_{29}N_2O_7P$ $[M+H]^+$, 537.1785, found, 537.1790.

*4.6.17. Methyl 2-((2-(methyl(8-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy)phosphorylamino) -3-phenylpropanoate (**7q**)*

Yellow oil; yield, 38%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.52 (d, J = 8.3 Hz, 1H, coumarin-5-H), 7.26–7.17 (m, 6H, coumarin-8-H, benzene-H), 7.14–7.05 (m, 6H, benzene-H), 6.98 (d, J = 7.9 Hz, 1H, coumarin-6-H), 5.57 (s, 1H, coumarin-3-H), 4.25–4.15 (m, 2H, CH_2), 4.12 (d, J = 7.0 Hz, 1H, CH_2), 3.72–3.64 (m, 1H, CH_2), 3.63 (s, 3H, OCH_3), 3.53 (t, J = 5.4 Hz, 1H, NHCH), 3.02–2.95 (m, 2H, CH_2), 2.90 (d, J = 19.6 Hz, 3H, NCH_3), 2.41 (s, 3H, CH_3), 2.05 (s, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$)δ 173.71, 162.68, 160.81, 153.14, 150.54, 135.68, 131.98, 129.67, 129.18, 128.56, 127.41, 125.56, 125.11, 121.53, 120.04, 119.45, 115.63, 95.99, 63.38, 54.89, 52.98, 50.45, 40.54, 39.64, 16.77. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.48–2.69. HRMS: calcd for $C_{29}H_{31}N_2O_7P$ $[M+H]^+$, 551.1942, found, 551.1946.

*4.6.18. Methyl 2-((2-(methyl(7-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino) -3-phenylpropanoate (**7r**)*

Yellow oil; yield, 28%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.71 (d, 1H, coumarin-5-H), 7.34–7.21 (m, 7H, coumarin-H, benzene-H), 7.18–7.07 (m, 5H, benzene-H), 5.70 (s, 1H, coumarin-3-H), 4.43–4.28 (m, 2H, CH_2), 4.01–3.90 (m, 1H, CH), 3.68 (s, 3H, OCH_3), 3.61 (m, 2H, CH_2), 3.49 (d, J = 10.9 Hz, 2H, $CHCH_2$), 3.02 (d, J = 11.8 Hz, 3H, NCH_3), 2.48 (s, 3H, CH_3), 2.07 (s, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.55, 162.78, 160.76, 154.34, 150.54, 142.50, 135.56, 129.66, 129.40, 128.56, 127.15, 125.00, 124.48, 121.56, 120.04, 117.93, 113.69, 95.10, 63.42, 55.55, 53.90, 52.26, 40.34, 39.76, 21.34. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.44–2.71. HRMS: calcd for $C_{29}H_{31}N_2O_7P$ $[M+H]^+$, 551.1942, found, 551.1945.

*4.6.19. Methyl 2-((2-(methyl(6-methyl-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino) -3-phenylpropanoate (**7s**)*

Yellow oil; yield, 47%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.53 (d, J = 9.5 Hz, 1H, coumarin-8-H), 7.38–7.24 (m, 5H, coumarin-H, benzene-H), 7.19–7.05 (m, 7H, benzene-H), 5.59 (s, coumarin-3-H), 4.38–4.29 (m, 2H, CH_2), 3.98 (m, 1H, NHCH), 3.68 (s, 3H, OCH_3), 3.64 (t, J = 5.4 Hz, 1H, CH_2), 3.45 (m, 2H, $CHCH_2$), 2.99 (d, J = 11.4 Hz, 3H, NCH_3), 2.41 (s, 3H, CH_3), 2.01 (s, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.66, 162.68, 160.76, 152.87, 150.61, 136.11, 135.66, 132.91, 129.85, 129.67, 128.52, 126.36, 125.04, 121.61, 120.04, 117.01, 114.26, 96.41, 63.68, 55.12, 53.23, 51.69, 39.46, 21.18. ^{31}P NMR (243 MHz, $CDCl_3$)δ 2.48–2.68. HRMS: calcd for $C_{29}H_{31}N_2O_7P$ $[M+H]^+$, 551.1942, found, 551.1946.

*4.6.20. Methyl 2-((2-(methyl(6-tert-buty-2-oxo-2H-chromen-4-yl) amino)ethoxy) (phenoxy) phosphorylamino) -3-phenylpropanoate (**7t**)*

Yellow oil; yield, 76%.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.60 (d, J = 5.1 Hz, 1H, coumarin-5-H), 7.52 (d, J = 8.5 Hz, 1H, coumarin-7-H), 7.23 (m, 6H, coumarin8-H, benzene-H, coumarin-8-H), 7.08 (m, 5H, benzene-H), 5.62 (d, J = 10.3 Hz, 1H, coumarin-3-H), 4.20 (d, J = 18.2 Hz, 2H, CH_2), 3.63 (d, J = 14.3 Hz, 3H, OCH_3), 3.59–3.51 (m, 2H, CH_2), 3.51–3.46 (m, 1H, NHCH), 3.00–2.96 (m, 2H, $CHCH_2$), 2.93 (d, J = 12.9 Hz, 3H, NCH_3), 2.04 (d, J = 6.5 Hz, 1H, NH), 1.33 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.51, 162.66, 160.96, 152.24, 146.23, 135.71, 135.48, 129.64, 129.44, 129.01, 128.57, 127.17, 125.00, 121.44, 120.00, 117.39, 115.43, 96.26, 63.48, 55.49, 53.95, 52.26, 40.35, 39.74, 34.62, 31.38. ^{31}P NMR (243 MHz, $CDCl_3$) δ 2.45–2.72. HRMS: calcd for $C_{32}H_{37}N_2O_7P$ $[M+H]^+$, 593.2411, found, 593.2413.

4.7. Biological activity assay

4.7.1. Inhibition on chitin synthase assay

4.7.1.1. Pretreatment of the enzyme. The protocol used for chitin synthase preparation is based on the procedure of Lucero [40] with some modifications. The principal of this method is that the wheat germ agglutinin (WGA) can bind some sites of chitin specifically with high affinity. Fluorescent derivatives and enzyme-tagged versions of WGA are widely used as primary reagents to identify cellular components. The horseradish perosidase-WGA conjugate (HRP-WGA) binds the synthesized chitin and detected at 600 nm. The absorbance values are converted to amounts of chitin using acid solubilized chitin as a standard.

Yeast cells (*C. tropicalis* ATCC 750) were grown in 1000 mL YPD medium (containing per liter: 10 g yeast extract, 20 g peptone, 20 g glucose). Culture media were incubated at 30 °C for 24 h with shaking at 130 rpm. Mycelium were collected through centrifugation at 1500 g for 15 min at 4 °C and washed with sterile water. The precipitates were suspended in 20 mL 100 mM Tris–HCl culture media at pH 7.0 which contained 40 μL fungal protease inhibitor cocktail and 50 μL solution of 200 mM Phenylmethanesulfonyl fluoride in DMSO, and were lysed by sonication treatment which worked for 5 s interval of 3 s at 4 °C for 50 min. Cell wall and fragment were removed by centrifugation at 1500 g for 15 min at 4 °C. 1 vol of supernatant was placed on top of 2 vol of 10% w/w sucrose in 100 mM Tris–HCl (w/w) at pH 7.5 buffer, and centrifuged at 55,000 g for 2 h at 4 °C. After centrifugation, the supernatant was discarded and the pellet was re-suspended in 5 mL 50 mM Tris–HCl at pH 7.5 and 33% glycerol to serve as CHS sample, and stored at −80 °C.

4.7.1.2. Chitin synthase assay. 200 μL 30 μg/mL WGA stock solutions in 50 mM Tris–HCl at pH 7.5 was added to each well of the microtiter plate and was incubated at 37 °C for 2 h. The WGA solution was removed by vigorous shaking of the plate content. The plates were washed at least three times by 200 μL of 50 mM Tris–HCl buffer at pH 7.5 and added 300 μL of 10 mg/mL bovine serum albumin in 50 mM Tris–HCl at pH 7.5 buffer and incubated for 2 h at 37 °C. At this stage plates can be covered and stored at −20 °C for over 6 months without noticeable loss of chitin binding activity.

Plates stored at −20 °C in blocking buffer were thawed at room temperature, emptied by shaking and washed at least three times by 200 μL of 50 mM Tris–HCl at pH 7.5 buffer. 100 μL of reaction mixture (80 mM L GlcNAc, 4 mM UDP–GlcNAc in 50 mM Tris-HCl buffer, pH 7.5) were added to the appropriate wells followed by the addition of candidate compound buffer (50 μL) and pretreated enzyme (50 μL) to a final volume of 200 μL. Microplates were incubated at 37 °C for 60 min. After, unbound components were removed and wells were washed at least three times by 200 μL of 50 mM Tris–HCl at pH 7.5 buffer. To each well, 200 μL solution of 1 μg/mL WGA-HRP in 50 mM Tris–HCl at pH 7.5 was added. After being gently shaken for 10 min, microplates were further kept at 37 °C for 15 min, and then washed five times with 50 mM Tris–HCl at pH 7.5 buffer. Finally, 150 μL peroxidase substrate buffer solutions (0.8 mM TMB, 2 mM H_2O_2, 50 mM Na_2HPO_4- Citric acid, pH3.7) was added for lucifuge reaction time of 30 min at 37 °C. The reaction was stopped with 50 μL 2 M H_2SO_4 and measured with Biotek ELX 800 Microplate reader at 450 nm.

4.7.1.3. Determination of chitin synthase biological activity. Plates stored at −20 °C in blocking buffer were thawed at room temperature, emptied by shaking and washed at least three times by 200 μL of 50 mM Tris–HCl buffer at pH 7.5. 100 μL of reaction mixture (80 mM L GlcNAc, 4 mM UDP–GlcNAc in 50 mM Tris-HCl buffer, pH 7.5) were added to the appropriate wells. Enzyme liquid was diluted by half-and-half to get a series of enzyme diluent (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64). Every well was added 50 μL enzyme diluent of different concentration and 50 μL of 50 mM Tris-HCl buffer, pH 7.5 to a final volume of 200 μL. Other steps were the same with chitin synthase assay. The most appropriate chitin synthase concentration was that the OD value ranged 0.5 to 1.0, which was prepared for all chitin synthase assays.

4.7.1.4. Determination of inhibitors IC_{50} assay. All target compounds were dissolved in DMSO at a concentration of 20 mg/mL. The solution of candidate compounds were diluted to 1.2, 0.6, 0.3, 0.15, 0.075 mg/mL with 50 mM Tris–HCl buffer solution at pH 7.5. Inhibition properties were tested at a final concentration of 300, 150, 75, 37.5, 18.75 μg/ml. The enzyme activity was measured and an experiment in the absence of inhibitor was used as control (100% activity). IC_{50} values were obtained from plots displaying the fractional activity (%) versus the concentration of the molecules.

4.7.2. Antimicrobial assays

Minimal inhibitory concentration (MIC, mg/mL) is defined as the lowest concentration of target compounds that completely inhibited the growth of microorganism. All the synthesized compounds **7a-t** were tested for activity in vitro by the standard two folds serial dilution method in 96-well microplates according to the National Committee for Clinical Laboratory Standards (NCCLS). DMSO was used as a solvent control to ensure that the solvent had no effect on microorganism growth. All the fungi and bacteria growth was monitored visually and spectrophotometrically, and these experiments were duplicated in three times.

4.7.2.1. Antifungal activity assays. Antifungal activity was screened against four main pathogenic fungal species (*C. albicans* CMCC 76615, *A. fumigatus* GIMCC 3.19, *C. neoformans* ATCC 32719 and *A.*

flavus ATCC 16870) in clinic. Fluconazole and polyoxin B used as standard antifungal drugs. DMSO was used as a solvent control. A spore suspension in sterile distilled water was prepared from 1-day old culture of the fungi growing on the media containing 1% peptone, 2% Glucose and solid media as well as 15% agar. The final spore concentration was $1–5 \times 10^3$ spore mL^{-1} All target compounds were dissolved in DMSO to prepare the stock solutions. The tests were made resulting in twelve wanted concentrations (0.25–512 μg/mL). These dilutions were incubated at 37 °C for 24 h. The MIC values of antifungal activitty in μg/mL were summarized in Table 2.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.11.027.

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