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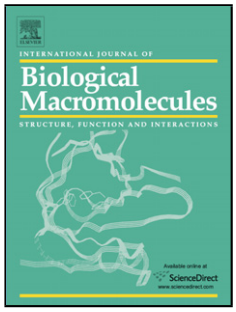

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Synthesis, characterization, and antifungal evaluation of novel 1,2,3-triazolium-functionalized starch derivative

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Abstract

1,2,3-Triazolium-functionalized starch derivative was obtained by straightforward quaternization of the synthesized starch derivative bearing 1,2,3-triazole with benzyl bromide by combining the robust attributes of cuprous-catalyzed azide-alkyne cycloaddition. These novel starch derivatives were characterized by FTIR, UV-vis, ^{1}H NMR, ^{13}C NMR, and elemental analysis. Their antifungal activities against *Colletotrichum lagenarium*, *Watermelon fusarium*, and *Phomopsis asparagi* were investigated by hypha measurement *in vitro*. The fungicidal assessment revealed that compared with starch and starch derivative bearing 1,2,3-triazole with inhibitory indices of below 15% at 1.0 mg/mL, 1,2,3-triazolium-functionalized starch derivative had superior antifungal activity with inhibitory rates of over 60%. Especially, the best inhibitory index of 1,2,3-triazolium-functionalized starch derivative against *Colletotrichum lagenarium* attained 90% above at 1.0 mg/mL. The results obviously showed that quaternization of 1,2,3-triazole with benzyl bromide could effectively enhance antifungal activity of the synthesized starch derivatives. The synthetic strategy described here could be utilized for the development of starch as novel antifungal biomaterial.

Keywords: Starch derivative; Functional; 1,2,3-Triazolium; Antifungal activity; Biomaterial; Benzyl bromide.

1. Introduction

As the main carbohydrate storage reserve for plants, starch is composed of anhydroglucose units (AGU) linked together by α-glucosidic bonds [1-3]. A number of interesting properties such as source universality, renewable, non-toxicity, biodegradable, and biocompatible facilitate a certain degree of applications of starch in biomedicine, biomaterials, and textile areas [4-7]. Rich in free available hydroxyl groups of anhydroglucose units, starch can be functionalized by chemical modification via introduction of the individual functional moieties to further improve the bioactivities and broaden application scopes of new valuable biomaterials based on starch [1, 5, 8-10].

1,2,3-triazole moiety formed by cuprous-catalyzed azide-alkyne cycloaddition (CuAAC) has emerged as attractive heterocyclic compounds in biochemistry and pharmaceutical research due to their wide range of biological properties, such as antimicrobial, anticancer, and antimalarial [11-14]. These interesting biological properties can facilitate the chemical modification of starch with 1,2,3-triazoles [15, 16]. Zhang et al. prepared a novel oral protein drug delivery made of starch nanoparticles as backbone and poly (L-glutamic acid) as graft chains by click reaction and these copolymer nanoparticles could protect the insulin against harmful gastric environment as well as controlling the drug release [17]. Tan et al. synthesized four different starch-linked-1,2,3-triazole derivatives bearing electron-withdrawing groups via 1,3-dipolar cycloaddition and further evaluated for *in vitro* antibacterial potential [18]. Besides, 1,2,3-triazolium cations have recently been developed by quaternization of 1,2,3-triazoles with halogenide [19, 20]. However, there are very few reports on synthesis of starch derivatives bearing 1,2,3-triazolium cations, and the effect of quaternization of 1,2,3-

triazole moieties with benzyl bromide on the bioactivity of starch derivative was still unknown.

In this paper, 1,2,3-triazolium-functionalized starch was prepared by quaternization of starch derivative bearing 1,2,3-triazole with benzyl bromide. The chemical structures of the derivatives were characterized by FTIR, UV-vis, ^{1}H NMR, ^{13}C NMR, and elemental analysis. Three plant-threatening fungi, *Colletotrichum lagenarium (C. lagenarium)*, *Watermelon fusarium (W. fusarium)*, and *Phomopsis asparagi (P. asparagi)* were selected to evaluate the antifungal property by hypha measurement *in vitro*.

2. Experimental

2.1 Material

Soluble starch from potato (granules) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). *N*-bromosuccinimide (NBS), triphenylphosphine (TPP), 3-butyn-1-ol, and benzyl bromide were purchased from the Sigma-Aldrich Chemical Corp (Shanghai, China). The other reagents were all analytical grade and used as received.

2.2 Analytical methods

2.2.1 Fourier transform infrared (FTIR) spectroscopy

All spectra were recorded on a Jasco-4100 Fourier Transform Infrared Spectrometer (JASCO Co., Ltd., Japan) at 25°C in the transmittance mode. About 1 mg of sample with 100 mg of KBr was fully grinded and mixed. The mixed samples were pressed into pills with a compressor and prepared pellets were used for studies. All spectra were scanned against a blank KBr pellet background in the range of 4000-400 cm^{-1} with resolution of 4.0 cm^{-1}.

2.2.2 Ultraviolet-visible (UV-vis) spectroscopy

The UV-vis spectra were carried out at 200-400 nm using a 3-5 mm quartz cuvette using a TU-1810 UV spectrometer (General Instrument Co., Ltd., China). For this analysis, 5 mL of 50 μg/mL aqueous solution of starch and starch derivatives were put in a cuvette for measurement. All the measurements were carried out at 25°C.

2.2.3 Nuclear magnetic resonance (NMR) spectroscopy

^{1}H Nuclear magnetic resonance (^{1}H NMR) and ^{13}C Nuclear magnetic resonance (^{13}C NMR) spectra were all recorded on a Bruker AVIII-500 Spectrometer (Bruker Tech. and Serv. Co., Ltd., Switzerland) at 25°C using DMSO-d_6 or D_2O as solvent. Chemical shifts (δ ppm) were referenced to tetramethylsilane (TMS).

2.2.4 Elemental analysis

The elemental analyses by combustion were used to quantitatively assess the extent of functionalization (degree of substitution) in starch derivatives. The analyses of elemental carbon, hydrogen, and nitrogen in starch derivatives were performed on a Vario EL III (Elementar, Germany). The degrees of substitution (DS) of starch derivatives were calculated on the basis of the percentages of carbon and nitrogen according to the following equations [21]:

$$DS_1 = \frac{M_C \times n_1}{M_N \times n_2 \times W_{C/N}} \quad (1)$$

$$DS_2 = \frac{M_N \times n_2 \times DS_1 \times W_{C/N} - M_C \times n_1}{n_3 \times M_C} \quad (2)$$

$$DS_3 = \frac{M_N \times n_2 \times DS_1 \times W_{C/N} - M_C \times n_1 - n_3 \times M_C \times DS_2}{n_4 \times M_C} \quad (3)$$

where DS_1, DS_2, and DS_3 represent the degrees of substitution of azido in 6-azido-6-deoxy starch, 1,2,3-triazole in starch derivative bearing 1,2,3-triazole, and 1,2,3-triazolium in starch derivative bearing 1,2,3-triazolium; M_C and M_N are the molar mass of

carbon and nitrogen, M_C = 12, M_N = 14; n_1 and n_2 are the number of carbon and nitrogen of 6-azido-6-deoxy starch, n_1 = 6, n_2 = 3; n_3 is the number of carbon of 3-butyn-1-ol, n_3 = 4; n_4 is the number of carbon of benzyl group, n_4 = 7; $W_{C/N}$ represents the mass ratio between carbon and nitrogen.

2.2.5 Thermogravimetric analysis (TGA) and differential thermogravimetry (DTG)

The thermal stability of samples was evaluated using a thermal analyzer (Mettler 5MP/PF7548, Mettler-Toledo, Switzerland) at a heating rate of 10°C/min with temperature range from 50°C to 800°C and nitrogen was used as the purge gas.

2.3 *Synthesis of starch derivatives*

2.3.1 Synthesis of 6-bromo-6-deoxy starch (A) and 6-azido-6-deoxy starch (B)

6-Bromo-6-deoxy starch (A) and 6-azido-6-deoxy starch (B) were prepared according to the previously reported procedure [22]. Briefly, soluble starch (3.24 g, 20 mmol AGU) was suspended in 80 mL of anhydrous DMF and stirred at 120°C for 1 h. After the slurry was allowed to cool to 90°C, anhydrous LiBr (3.47 g, 40 mmol) was added. After starch was dissolved completely and cooled to room temperature, *N*-bromosuccinimide (NBS, 14.24 g, 80 mmol) and triphenylphosphine (TPP, 20.99 g, 80 mmol) were added to this solution. The reaction mixture was heated to 80°C for 3 h under an argon atmosphere. The product was isolated by adding the reaction mixture slowly to 400 mL of absolute ethanol, followed by filtration. Then the precipitate was extracted in a Soxhlet apparatus with ethanol and acetone for 48 h, respectively. The 6-bromo-6-deoxy starch was obtained by freeze-drying overnight in vaccum. For the preparation of 6-azido-

6-deoxy starch, 6-bromo-6-deoxy starch (2.25 g, 10 mmol) was dissolved in 40 mL of DMSO, and NaN_3 (1.3 g, 20 mmol) was added to the solution. The solution was heated to 80°C and stirred for 24 h under an argon atmosphere. The product was isolated by pouring the reaction solution into 200 mL of absolute ethanol. The precipitate was collected by filtration, and washed with acetone. After being dialyzed against deionized water for 2 days to remove the probable remained sodium azide, the 6-azido-6-deoxy starch was obtained by freeze-drying. Yield: 76.52%. Elemental analysis: C(36.87%), N(18.34%), H(5.22%), C/N: 2.01. DS $_{azido}$: 0.85. FTIR: ν 3405, 2923, 2105, 1041 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ 5.72-3.30 (pyranose rings), 3.77 ($C\underline{H_2}N_3$) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ 100.22-70.26 (pyranose rings), 51.69 ($\underline{C}H_2N_3$) ppm.

2.3.2 Synthesis of starch derivative bearing 1,2,3-triazole (C)

6-Azido-6-deoxy starch (187 mg, 1 mmol) was dissolved in 20 mL of DMSO, cuprous iodide (19 mg, 0.1 mmol), triethylamine (0.14 mL, 1 mmol), and 3-butyn-1-ol (0.23 mL, 3 mmol) were added, and the solution was stirred at 75°C for 24 h under an argon atmosphere. The mixture was poured into 100 mL of acetone to precipitate the product. The precipitate was filtered and washed carefully with acetone for three times. The probable remained reagents were dialyzed against deionized water for 2 days, the starch derivative bearing 1,2,3-triazole was obtained by freeze-drying. Yield: 92.08%.

Elemental analysis: C(42.20%), N(13.03%), H(6.21%), C/N: 3.12. DS $_{triazole}$: 0.83. FTIR: ν 3401, 2923, 1554, 1045, 794 cm^{-1}. The UV λ_{max} was 220 nm. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.62 (triazole-5-H), 5.68-3.10 (pyranose rings), 4.69 (CH_2OH), 3.87 (NCH_2CH), 2.48 (CH_2CH_2OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ 144.58 (triazole-4-C), 123.99 (triazole-5-C), 99.98-69.76 (pyranose rings), 60.74 (CH_2OH), 50.44 (NCH_2CH), 29.21 (CH_2CH_2OH) ppm.

2.3.3 Synthesis of starch derivative bearing 1,2,3-triazolium (D)

A solution of starch derivative (C) (257 mg, 1 mmol) and benzyl bromide (0.36 mL, 3 mmol) in 15 mL of DMSO was stirred at 60°C for 24 h. The reaction solution was poured into 100 mL of acetone to precipitate the product. The solid product was filtered and washed carefully for three times with acetone. After being dialyzed against deionized water for 48 h, the 1,2,3-triazolium-functionalized starch derivative was obtained by freeze drying. Yield: 72.95%. Elemental analysis: C(45.98%), N(9.37%), H(5.33%), C/N: 4.91. DS $_{triazolium}$: 0.76. FTIR: ν 3386, 2927, 1577, 1496, 1450, 1045, 821, 728 cm^{-1}. The UV λ_{max} were 203, 225, 258 nm. ^{1}H NMR (500 MHz, D_2O): δ 8.40 (triazolium-5-H), 7.51-7.25 (phenyl-H), 5.77 ($C_6H_5CH_2N^+$), 4.39 (NCH_2CH), 3.79 (CH_2OH), 3.09 (CH_2CH_2OH) ppm. ^{13}C NMR (125 MHz, D_2O): δ 142.62 (triazolium-4-C), 131.79-

128.23 (phenyl-C), 130.82 (triazolium-5-C), 62.54 ($C_6H_5CH_2N^+$), 58.32 (CH_2OH), 54.68 (NCH_2CH), 26.23 (CH_2CH_2OH) ppm.

2.4 Antifungal assay

The starch and starch derivatives were dissolved in distilled water at a concentration of 5 mg/mL. Then, each samples solution was added to sterilized PDA medium to give final concentrations of 0.1, 0.5, and 1.0 mg/mL. The final solutions were poured into sterilized Petri dishes (9 cm). After solidification, a mycelia disk (diameter: 5 mm) of active fungi was transferred to the center of the PDA Petri dishes and inoculated at 27°C. When the mycelium of fungi reached the edges of the control plate (without the presence of samples), the growth inhibition was calculated by the formula [23]:

$$\text{Inhibitory index (\%)} = (1 - D_a / D_b) \times 100 \quad (4)$$

where D_a is the diameter of the growth zone in the test plates and D_b is the diameter of the growth zone in the control plate.

2.5 Statistical analysis

Each experiment was performed three times. All data were expressed as means ± SD. Data were analyzed by an analysis of variance ($P < 0.05$) and the means were separated by Scheffe's multiple range test. The results were processed by the computer programs: Origin and SPSS.

3. Results and discussion

3.1 Chemical synthesis and characterization

The synthetic procedure for the preparation of 1,2,3-triazolium-functionalized starch is shown in Scheme 1. 1,2,3-Triazolium-functionalized starch was prepared via

regioselective bromination, azidation, cuprous-catalyzed azide-alkyne cycloaddition, and quaternization.

3.1.1 FTIR characterization

The FTIR spectrum for starch is recorded in Fig. 1(a) and it exhibits distinct absorption bonds at 3428, 2927, and 1072 cm^{-1}. The broad band at around 3428 cm^{-1} is attributed to –OH stretching vibration of starch molecules [5, 7, 24]. The characteristic band at 2927 cm^{-1} indicates the presence of methylene [5, 25]. Specific band denoting C–O–C stretching vibrations of the glucose ring can be seen at 1072 cm^{-1} [24, 25]. In FTIR spectrum of 6-azido-6-deoxy starch, a new peak at 2105 cm^{-1} is assigned to C6–N_3 group [26, 27]. Subsequently, the azide peak at 2105 cm^{-1} disappears completely when the C6–N_3 is transformed to 1,2,3-triazole and a new absorption band at about 1554 cm^{-1} appears [22]. After the quaternization, the new characteristic band at 1577 cm^{-1} is assigned to 1,2,3-triazolium groups [21]. Besides, two bands at 1496 and 728 cm^{-1} corresponding to the C=C stretching vibration and the =C–H deformation vibration mode of the phenyl group are newly appeared [28, 29]. In addition, the weak absorption band at nearly 1450 cm^{-1} is attributed to the C–H deformation vibration of the methylene of benzyl group in 1,2,3-triazolium-functionalized starch derivative (D).

3.1.2 UV-vis characterization

The UV-vis spectra of starch, starch derivatives (C) and (D) at a concentration of 50 μg/mL in deionized water are shown in Fig. 1(b). There are no absorption peaks appeared ranging from 200 to 400 nm because of absence of chromophore in starch. The UV-vis spectrum of starch derivative bearing 1,2,3-triazole (C) exhibits one peak centered at 220 nm corresponding to the 1,2,3-triazole ring [21]. The absorption peak of 1,2,3-triazolium is red-shifted from 220 to 225 nm because of the change in electron configuration of 1,2,3-triazole after the quaternization with benzyl bromide. Moreover, it is observed that the new peaks at 203 and 258 nm are assigned to the phenyl group in starch derivative bearing 1,2,3-triazolium (D) [30].

3.1.3 ^{1}H NMR characterization

The ^{1}H NMR spectra of starch and starch derivatives are shown in Fig. 2 for comparison. The ^{1}H NMR spectrum of starch exhibits the proton resonances of anhydroglucose units at 3.0-5.7 ppm [8, 9]. After regioselective bromination of starch, the absorption peak at 3.44 ppm is attributed to hydrogens of –C$\underline{H_2}$Br. The effective functionalization of 6-azido-6-deoxy starch is further confirmed by the presence of the protons of –C$\underline{H_2}N_3$ at 3.77 ppm [22]. The new signal at high frequency (7.62 ppm) is produced by the hydrogen at 5-H position of 1,2,3-triazole, which demonstrates that the 1,2,3-triazole group has been successfully introduced to starch backbone [13, 31].

Besides, the appearance of the additional signals of alkyl protons linked to 1,2,3-triaole at 4.69 and 2.46 ppm further proves the successful CuAAC reaction [21]. Moreover, the ^{1}H NMR spectrum of starch derivative (D) corroborates the quaternization through the disappearance of the peak corresponding to the proton of the 1,2,3-triazole group at 7.62 ppm and the appearance of new signal for the proton of the 1,2,3-triazolium at 8.40 ppm [19, 32]. In addition, completion of the quaternization is also corroborated by the appearance of new signals at 5.77 and 7.20-7.50 ppm for the methylene and phenyl groups of the pendant benzyl moiety, respectively [12, 33]. And all signals of adjacent methylene groups are shifted compared to those initially neighboring the 1,2,3-triazole.

3.1.4 ^{13}C NMR characterization

Moreover, the chemical structures of starch and the synthesized starch derivatives are further confirmed by ^{13}C NMR spectra. The ^{13}C NMR spectrum of starch reveals the carbons in the pyranose ring at around 60-100 ppm [34]. After the reaction of starch with NBS and TPP, the structure of the obtained 6-bromo-6-deoxy starch may be easily confirmed by the observation of the –$\underline{C}H_2Br$ appearing at 34.78 ppm [35]. After azidation, it is noteworthy that the signal at 51.69 ppm is attributed to the resonance of the –$\underline{C}H_2N_3$ moiety [36]. The starch derivative bearing 1,2,3-triazole, obtained by cuprous-catalyzed azide-alkyne cycloaddition of 6-azido-6-deoxy starch and 3-butyn-1-ol, may be also

straightforwardly detected by the new peaks of the 1,2,3-triazole linker at 144.58 and 123.99 ppm [22]. Meanwhile, the alkyl carbons linked to both the 1,2,3-triazole and hydroxyl resonate at 29.21 and 60.74 ppm, respectively [21]. Finally, the quantitative formation of 1,2,3-triazolium unit is also confirmed in the starch derivative bearing 1,2,3-triazolium by ^{13}C NMR spectroscopy as there are no distinguishable signals in the 1,2,3-triazole region at 144.58 and 123.99 ppm. Instead, new signals for the 1,2,3-triazolium carbons appear at 142.62 and 130.82 ppm [21, 37]. Completion of the quaternization reactions is also corroborated by the quantitative appearance of signals characteristic of the pendant benzyl group at 131.79-128.23 and 62.54 ppm [38]. Notice that most other carbon signals bound to 1,2,3-triazole are slightly shifted due to the positive global charge density deshielding effect of the cationic macromolecules.

3.1.5 TGA and DTG characterization

The TGA and DTG curves of the synthesized products in the reactions are compared in Fig. 4. The thermal properties of starch and the modified starch derivatives reveal two distinct stages in their thermal degradation. In all the samples, an initial weight loss of 7-10% at approximately 100°C is observed, and this can be attributed to the loss of adsorbed and bound water. In the second stage of decomposition, the weight loss of starch happens in the temperature range of 270-360°C, within which the weight loss percentage is 75%

and the highest decomposition rate is at 326°C. In contrast with starch, decomposition of starch derivative bearing 1,2,3-triazole commences at 250°C and attains maximum decomposition at 321°C with weight loss of 60%. After quaternization, the sample of compound (D) shows a significant decrease in thermal stability. The decomposition temperature starts at 200°C and the highest decomposition rate is observed at 254°C, and the total weight loss in this stage is 65%. It is quite conspicuous that further modification might lead to lower thermal stability [39, 40]. The introduction of functional groups has a significant influence on its thermal stability, and it is due to the changed crystalline structure of starch, especially through the loss of hydrogen bonding between starch chains [18].

3.2 Antifungal activity

The capabilities of 1,2,3-triazolium-functionalized starch at 0.1, 0.5, and 1.0 mg/mL to inhibit the growth of the tested three plant-threatening fungi, including *C. lagenarium*, *W. fusarium*, and *P. asparagi*, are shown in Fig. 5-7, respectively. Significant differences ($P < 0.05$) are confirmed for the antifungal property of starch derivatives against the tested three plant-threatening fungal strains.

As can be inferred from Fig. 5-7, starch derivatives are active against three plant-threatening fungi at the tested concentration to various extents. The inhibitory indices of all the samples are concentration-dependent ($P < 0.05$), and the strongest antifungal

activity is observed at 1.0 mg/mL. It is found that starch has less antifungal activity with the inhibitory index of below 5%. Whereas, starch derivative bearing 1,2,3-triazole (C) show slightly higher antifungal property than starch because of the introduction of 1,2,3-triazole group ($P < 0.05$). This slightly stronger antifungal activity should be ascribed to the hydrogen bond interaction formed by 1,2,3-triazole and biomolecular targets. It could inhibit synthesis of the cell membrane and cell wall to exhibit antimicrobial activity [41].

After one-step alkylation with benzyl bromide, 1,2,3-triazolium-functionalized starch derivative (D) exhibits evidently stronger ability of inhibiting the growth of tested strains than pristine starch and starch derivative (C), which indicates that 1,2,3-triazolium should be the efficient antifungal function group. Compared with starch and starch derivative (C) with inhibitory indices of below 10%, starch derivative (D) shows superior antifungal activity with inhibitory rates of over 80% at 1.0 mg/mL ($P < 0.05$). Among the pathogenic fungi species, *C. lagenarium* is the most susceptible yeast to the compound (D), and the best inhibitory index of starch derivative (D) against *C. lagenarium* attained 90% above at 1.0 mg/mL ($P < 0.05$). Notably, starch derivative (D) is still active against tested fungi even when the dosage is lowered to 0.5 mg/mL with inhibitory values of over 40% ($P < 0.05$), and the inhibitory indices of it at 0.1 mg/mL are even higher than those

of starch derivative (C) at 1.0 mg/mL, which suggest that the quaternization of 1,2,3-triazole with benzyl bromide can enhance antifungal property by one order of magnitude.

The powerful disruptive effect of 1,2,3-triazolium-functionalized starch derivative on the microorganism was probably based on the adsorption of the amphiphile molecules on the outer cellular membranes [38]. The interaction between positive charged moieties of the cationic molecules and the negative charged components on fungal cell outer membranes could make these cations clung tightly to the microbial cell surface and hinder the transport of essential nutrients into the cell [42]. Once this electrostatic contact was accomplished by the hydrophilic region, the hydrophobic region proceeded to penetrate the hydrophobic bilayer to cause cell leakage and lysis and end up in the death of fungi [43]. And these electrostatic and hydrophobic interactions could exhibit greater impact on antifungal activity than the hydrogen bond interaction [44] and cause the higher inhibitory indices of 1,2,3-triazolium-functionalized starch derivative compared with starch derivative bearing 1,2,3-triazole.

4. Conclusion

In summary, 1,2,3-triazolium-functionalized starch derivative was prepared and the effect of quaternization of 1,2,3-triazole moiety with benzyl bromide on the antifungal property of starch derivative was estimated by observing the percentage inhibition of mycelial growth. The 1,2,3-triazolium-functionalized starch derivative exhibited

significant improved antifungal activity than starch and starch derivative bearing 1,2,3-triazole. The results indicated that 1,2,3-triazolium should be high-efficiency antifungal function group. This 1,2,3-triazolium-functionalized starch derivative exhibited outstanding antifungal performance and should be potentially used as antimicrobial biomaterial.

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Scheme 1. Synthetic routes for starch derivatives.

Fig. 1. FTIR (a) and UV-vis (b) spectra of starch and starch derivatives.

Fig. 2. ^{1}H NMR spectra of starch and starch derivatives.

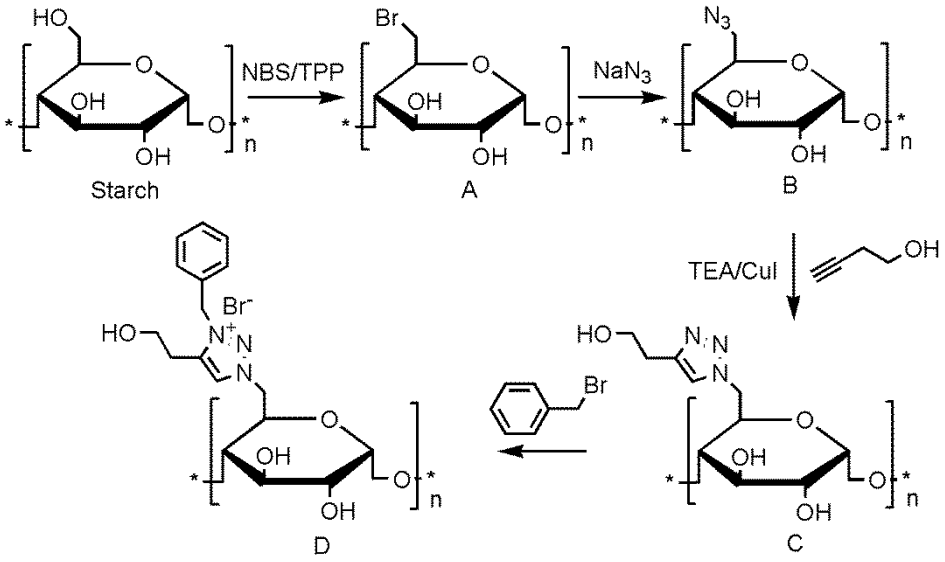
Fig. 3. ^{13}C NMR spectra of starch and starch derivatives.

Fig. 4. TGA (a) and DTG (b) curves of starch and starch derivatives.

Fig. 5. The antifungal activity of starch and starch derivatives against *C. lagenarium*.

Fig. 6. The antifungal activity of starch and starch derivatives against *W. fusarium*.

Fig. 7. The antifungal activity of starch and starch derivatives against *P. asparagi*.



Figr-1

Scheme 1

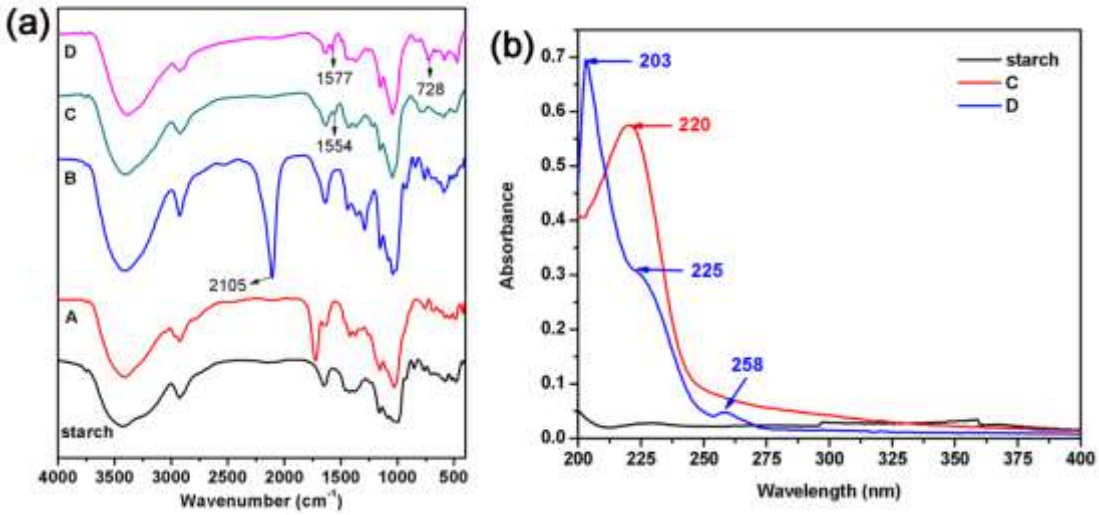


Fig. 1

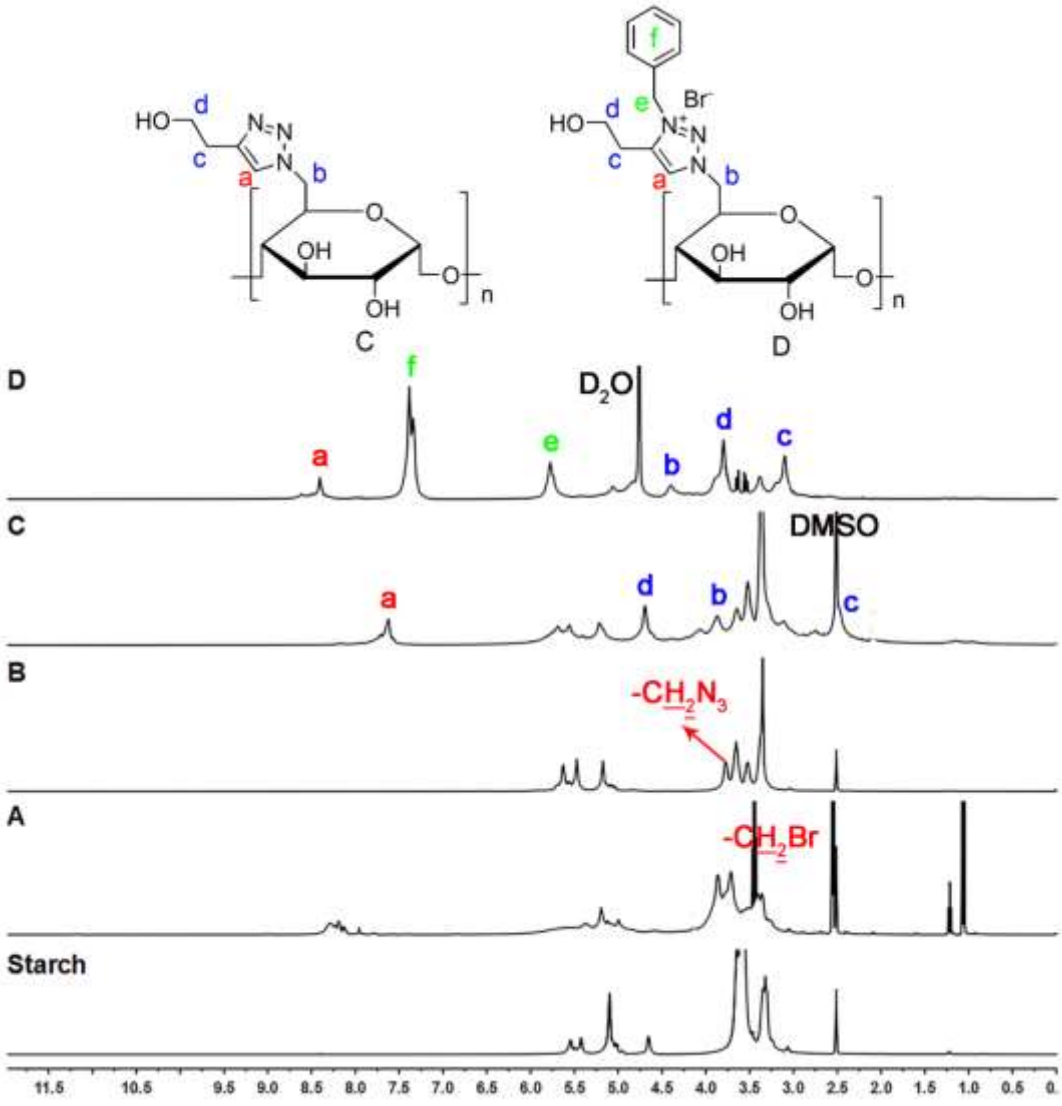


Fig. 2

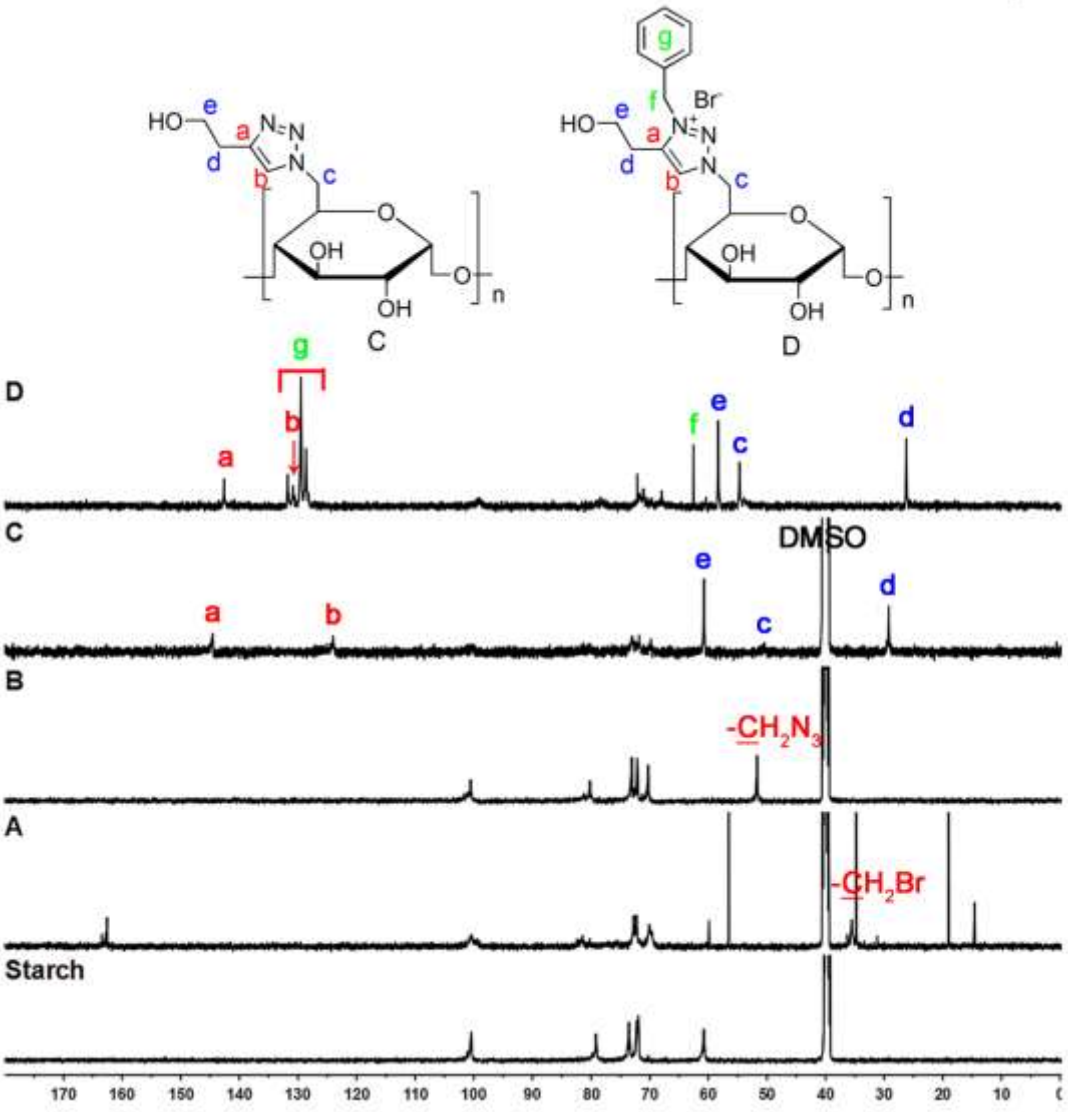


Fig. 3

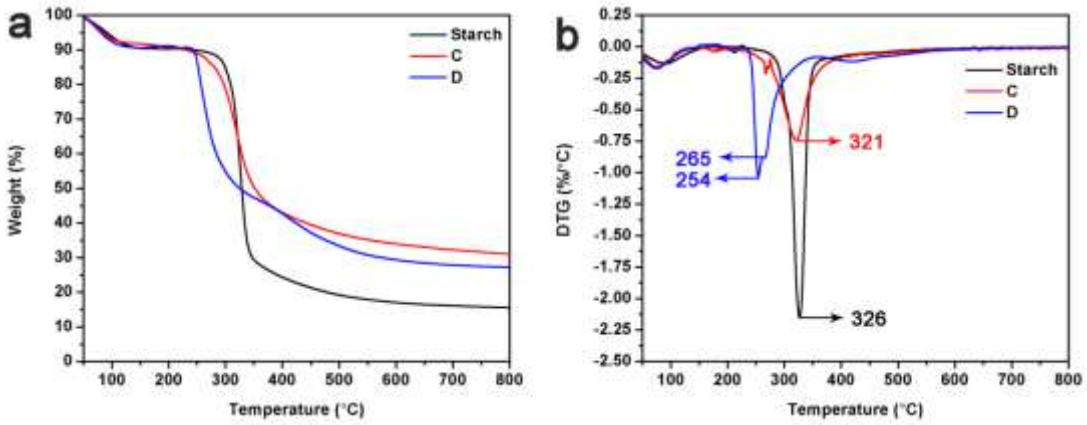


Fig. 4

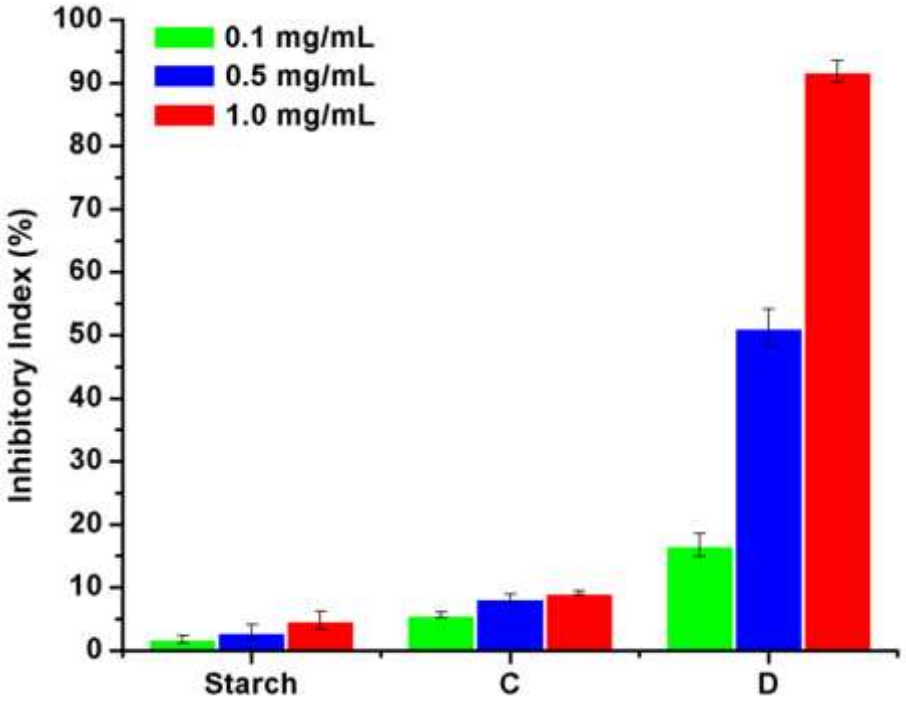


Fig. 5

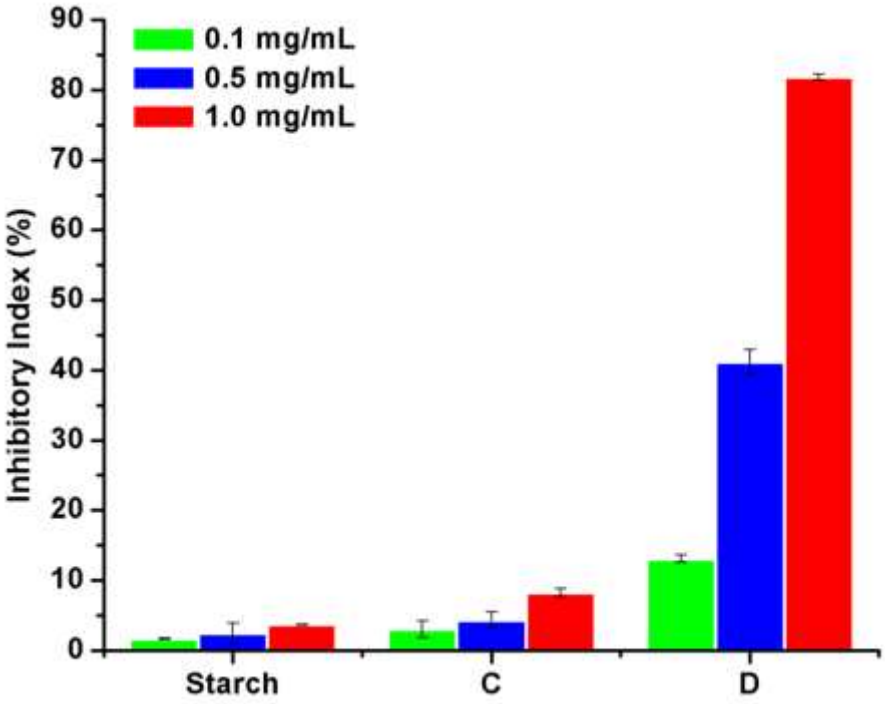


Fig. 6

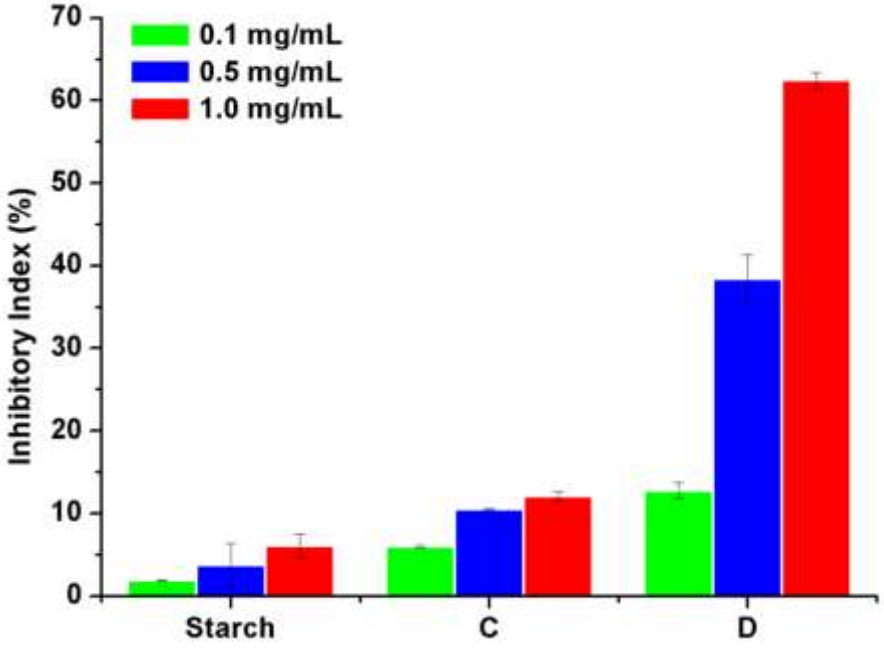


Fig. 7