International Journal of Biological Macromolecules 50 (2012) 263–269

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Antifungal property of quaternized chitosan and its derivatives

Warayuth Sajomsang[a,*], Pattarapond Gonil[a], Somsak Saesoo[a], Chitchamai Ovatlarnporn[b]

[a] Nanodelivery System Laboratory (NDS), National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani 12120, Thailand
[b] Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla 90112, Thailand

ARTICLE INFO

Article history:
Received 10 September 2011
Received in revised form 20 October 2011
Accepted 4 November 2011
Available online 15 November 2011

Keywords:
Chitosan
Quaternized chitosan
Antifugal activity
Dermatophytosis
Water-soluble chitosan derivatives

ABSTRACT

Five water-soluble chitosan derivatives were carried out by quaternizing either iodomethane or *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Quat188) as a quaternizing agent under basic condition. The degree of quaternization (DQ) ranged between 28 ± 2% and 90 ± 2%. The antifungal activity was evaluated by using disc diffusion method, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) methods against *Trichophyton rubrum* (*T. rubrum*), *Trichophyton mentagrophyte* (*T. mentagrophyte*), and *Microsporum gypseum* (*M. gypseum*) at pH 7.2. All quaternized chitosans and its derivatives showed more effective against *T. rubrum* than *M. gypseum* and *T. mentagrophyte*. The MIC and MFC values were found to range between 125–1000 μg/mL and 500–4000 μg/mL, respectively against all fungi. Our results indicated that the quaternized *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan chloride showed highest antifungal activity against *T. rubrum* and *M. gypseum* compared to other quaternized chitosan derivatives. The antifungal activity tended to increase with an increase in molecular weight, degree of quaternization and hydrophobic moiety against *T. rubrum*. However, the antifungal activity was depended on type of fungal as well as chemical structure of the quaternized chitosan derivatives.

1. Introduction

Dermatophytoses are an important public health problem [1,2]. It is caused by fungi in the genera *Microsporum*, *Trichophyton* and *Epidermophyton*. These organisms, called dermatophytes, are the pathogenic members of the keratinophilic soil fungi. *Microsporum* and *Trichophyton* are human and animal pathogens, while *Epidermophyton* is only a human pathogen. It is well known that fungal infection of skin is caused by *Microsporum canis* called ringworm, tinea or dermatomycosis. It is spread from person to person, from animal to person, or indirectly from contaminated objects or the soil. The associated spores can live for years in some conditions. Ringworm can infect three sites: scalp, body and nails. There are several organisms that cause ringworm including *M. canis*, *Microsporum gypseum*, *Trichophyton rubrum*, and *Trichophyton mentagrophyte* [3,4]. Up to the present time, there are several drugs that have been used to therapy ringworm such as griseofulvin, ketoconazole (KZ), miconazole, and salicylic acid. However, it has been shown in several studies that the compounds used in these fungicides cause strain resistance representing a potential risk for the environment and human health [5,6]. Therefore, the search of natural alternatives for the inhibition of fungi and bacteria has become challenging [7].

Currently, the natural compounds are the focus of some biotechnological companies looking for new antimicrobial agents [8]. Chitosan (Ch) is a kind of fine biomaterial. It is a linear polysaccharide obtained by deacetylation of naturally occurring polymer chitin. Ch consists of β-(1,4)-2-amino-2-deoxy-D-glucopyranose units (GlcN) and a small amount of 2-acetamido-2-deoxy-D-glucopyranose or *N*-acetyl-D-glucosamine (GlcNAc) residues. Ch is non-toxic and processes reactive amino groups under the acidic condition. Therefore, it has been proved to be useful in antimicrobial application. The antimicrobial activity of Ch has been shown against a wide variety of microorganisms including fungi, algae, and bacteria. However, its antimicrobial activity was prominent only in acidic medium because of its poor solubility in neutral and basic pH. To overcome this obstacle, quaternary ammonium salt of Ch has been synthesized. *N*,*N*,*N*-Trimethyl Ch chloride (TMChC) is one of water-soluble Ch derivatives that process a wide pH range. The TMChC can be prepared by reacting Ch with iodomethane in the presence of sodium hydroxide, sodium iodide, and *N*-methyl-2-pyrrolidone (NMP) in the basic condition [9,10]. Another Ch derivative is *N*-(2-hydroxypropyl) trimethylammonium Ch chloride (HPTChC). The HPTChC was performed by using either commercially available Quat188 or glycidyltrimethylammonium chloride (GTMAC) under basic/acidic condition [11,12]. The Quat188 is an aqueous solution of *N*-(3-chloro-2-hydroxypropyl)

* Corresponding author. Tel.: +66 2 564 7100x6558; fax: +66 2 564 6981.
E-mail address: warayuth@nanotec.or.th (W. Sajomsang).

0141-8130/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijbiomac.2011.11.004

trimethylammonium chloride. It is well-known as a quaternizing agent that introduce quaternary ammonium moiety into the polymer backbone such as the one in starch [13], cellulose [14], and Ch [15]. Previously, our research group focused on the synthesis of quaternary ammonium Ch containing aromatic moieties such as quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) Ch chloride (QDMBzChC), quaternized *N*-(4-*N*,*N*-dimethylaminocinnamyl) Ch chloride (QDMCmChC) and quaternized *N*-(4-pyridylmethyl) Ch chloride (QPyMeChC). We reported their antibacterial activity [16–18], and as well as drug, gene and antibody delivery [19–21]. However, the antifungal property of the quaternized Ch derivatives has not been investigated yet.

Up to the present time, there are many reports regarding antifungal properties of Ch and its quaternized derivatives, but the antifungal activities were investigated mainly in foods and agricultural products such as vegetables, fruits, and crops [22–24]. Much less attention has been paid on the antifungal activity of quaternized Ch derivatives against human and animal pathogens. In this study, five quaternized Ch derivatives with various chemical structures, degrees of quaternization (DQ) and molecular weights were synthesized. Their antifungal activities were evaluated by using disc diffusion method and minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) method against *T. rubrum*, *T. mentagrophyte*, and *M. gypseum* at pH 7.2. The effects of DQ, molecular weight and chemical structure will be discussed in this article.

2. Experimental

2.1. Materials

Ch with weight-average molecular weights (M_w) of 276 and 22 kDa were purchased from Seafresh Chitosan (lab) Co., Ltd. in Thailand. The degree of deacetylation (DDA) of this material was determined to be 94% by ^{1}H NMR spectroscopy. A dialysis membrane with M_w cut-off of 12,000–14,000 g/mol and 3500 g/mol from Cellu Sep T4, Membrane Filtration Products, Inc., (Segiun, TX, USA) was used to purify all modified Ch derivatives. 4-Dimethylaminobenzaldehyde, 4-diethylaminobenzaldehyde, 4-dimethylaminocinnamaldehyde, and sodium cyanoborohydride ($NaCNBH_3$) were purchased from Fluka (Deisenhofen, Germany). Iodomethane, and 1-methyl-2-pyrrolidone (NMP) were purchased from Acros Organics (Geel, Belgium). Sodium iodide was purchased from Carlo Erba Reagent (Italy). Iodine was purchased from Sigma–Aldrich (St. Louis, MO, USA). A 65% solution of *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Quat-188) was obtained from the Dow Chemical Company, Thailand, and all other reagents were used without further purification.

2.2. Characterization

All attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were collected with a Nicolet 6700 spectrometer (Thermo Company, USA) using the single-bounce ATR-FTIR spectroscopy (Smart Orbit accessory) with a diamond internal reflection element (IRE) at the ambient temperature (25 °C). These spectra were collected by using rapid-scan software in OMNIC 7.0 with 32 scans and a resolution of 4 cm^{-1}. The ^{1}H NMR spectra were measured on AVANCE AV 500 MHz spectrometer (Bruker, Switzerland). All measurements were performed at 300 K, using the pulse accumulation of 64 scans and LB parameter of 0.30 Hz. D_2O/CD_3COOD and D_2O were used as solvents for dissolving 5 mg of Ch and its quaternized derivatives, respectively.

2.3. Synthesis of chitosan Quat188

The Ch Quat188 (compound **2**) was carried out according to the previously reported procedure (Scheme 1) [15]. The regenerated Ch was quaternized by using *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Quat188) as a quaternizing agent under basic condition. The solution was stirred for 48 h at room temperature and then distilled water was added, after that temperature was raised to 50 °C for another 24 h. Finally, the solution was dialyzed with distilled water for 3 days to remove inorganic materials and then freeze-dried.

2.4. Synthesis of quaternized N-aryl chitosan derivatives

N-(4-*N*,*N*-Dimethylaminobenzyl) Ch (DMBzCh), *N*-(4-*N*,*N*-dimethylaminocinnamyl) Ch (DMCmCh) and *N*-(4-*N*,*N*-diethylaminobenzyl) Ch (DEBzCh) were prepared by reductive amination according to previously reported procedure (Scheme 1) [25]. Subsequently, the *N*-aryl Ch derivatives were quaternized by a single treatment with iodomethane in the presence of NMP, sodium hydroxide and sodium iodide yielded quaternized *N*-aryl Ch chloride (compounds **3**–**5**) [16–18]. Using the same technique described above, the Ch was used instead of *N*-aryl Ch derivatives in order to produce compound **1**.

DEBzCh. ^{1}H NMR (D_2O/CD_3COOD): δ (ppm) 7.5–7.3 (dd; 4H Ph), 4.7 (s; 1H H1), 4.5–3.5 (br. m; 9H $NHC\underline{H}_2$, $(C\underline{H}_2CH_3)_2NPh$, H3, H4, H5, H6 and H6′), 3.0 (br. s; 2H H2), 1.9 (s; 3H $NHCOC\underline{H}_3$), 0.9 (br. s; 6H $(CH_2C\underline{H}_3)_2NPh$).

5. ^{1}H NMR (D_2O): δ (ppm) 7.9–7.5 (br. s; 4H Ph), 5.4, 5.0 (s; 2H H1, H1′), 4.42–3.13 (br. m; 28H $-NHC\underline{H}_2$, H2, H3, H4, H5, H6 and H6′; br. s; $N^+(C\underline{H}_3)(C\underline{H}_2)_2$ Ph, $(C\underline{H}_2)_2NPh$: br. s; $N^+(C\underline{H}_3)_3$), 2.7 (br. m; 6H $N(C\underline{H}_3)_2$), 1.9 (s; 3H $NHCOC\underline{H}_3$), 0.9 (br. s; 6H $(C\underline{H}_3CH_2)_2NPh$).

2.5. Molecular weight determination

The weight average molecular weight (M_w), number average molecular weight (M_n), and M_w/M_n of Ch and its quaternized derivatives were determined by using the gel permeation chromatography (GPC). It consists of Waters 600E Series generic pump, injector, ultrahydrogel linear columns (M_w resolving range 1 kDa–20,000 kDa), guard column, pollulans as standard (M_w 5.9 kDa–788 kDa), and refractive index detector (RI). All samples were dissolved in acetate buffer pH 4 and then filtered through VertiPure nylon syringes filters 0.45 μm (Vertical chromatography Co., Ltd. Thailand). The mobile phases, 0.5 M AcOH and 0.5 M AcONa (acetate buffer pH 4), were used at a flow rate of 0.6 mL/min at 30 °C. Then the injection volume of 20 μL was used.

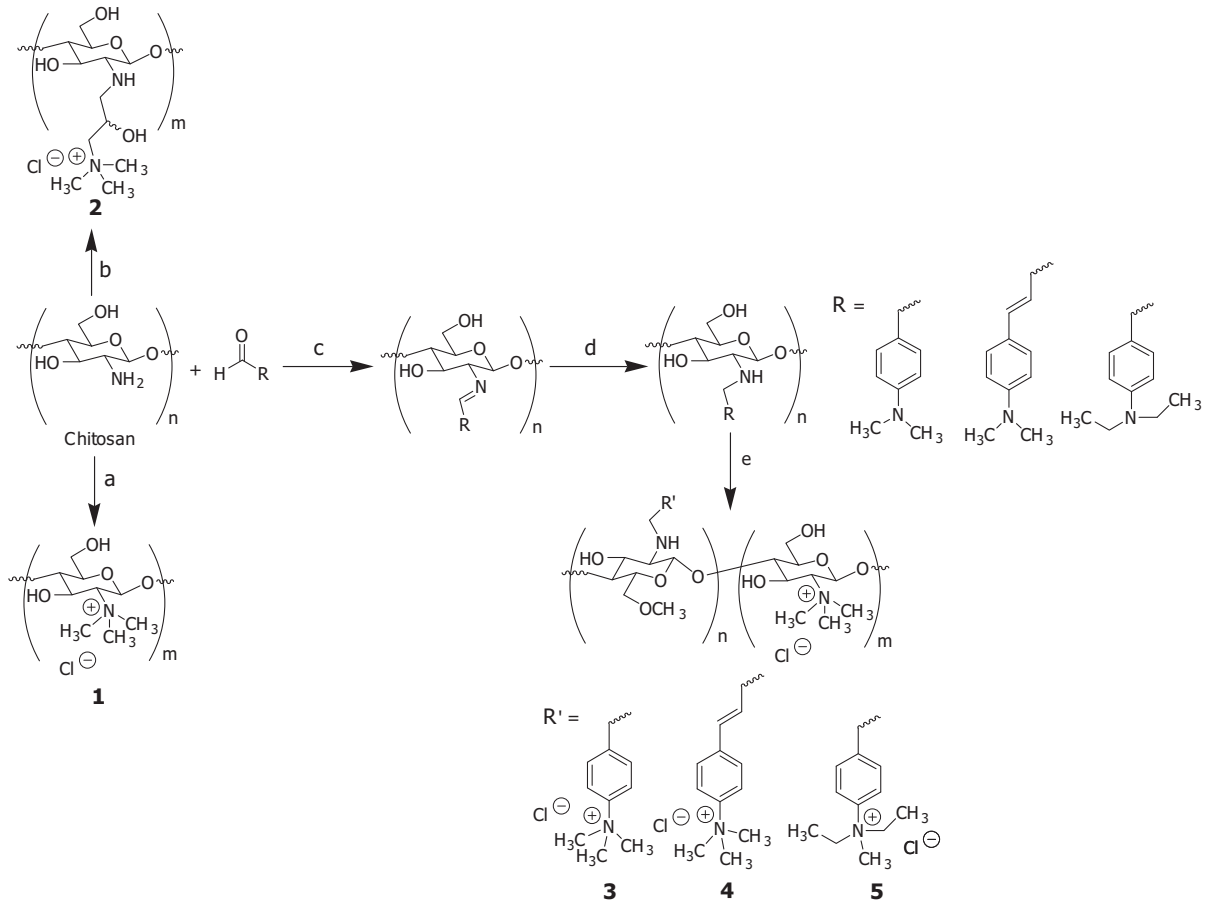
2.6. Antifungal assessments

2.6.1. Microorganisms

The fungi used for biological evaluation consisted of *Trichophyton rubrum* (SH-MU-2), *Trichophyton mentagrophytes* (SH-MU-3) and *Microsporum gypseum* (SH-MU-4) which were obtained from Songklanagarind Hospital.

2.6.2. Antifungal susceptibility test

The disc diffusion method [26] was used to screen for antifungal activity of the test samples. The tested fungi were taken to suspend in sterile 0.85% normal saline solution. The cell suspension was further diluted to achieve 0.5 McFarland standard suspensions (10^6 CFU/mL). One milliliter of standard suspensions (0.5 McFarland) of log phase growth cells of each fungus (*T. rubrum*, *T. mentagrophytes* and *M. gypseum*) was mixed with 25 mL melted (45 °C) SDA in 50 mL test tubes. The mixed medium was poured



Scheme 1. Synthesis of quaternized chitosan and its derivatives: (a) CH_3I/NMP/NaOH/NaI, (b) Quat 188/NaOH, (c) 1% CH_3COOH, (d) $NaCNBH_3$/pH 5, and (e) CH_3I/NMP/NaOH/NaI.

into Petri dishes (90 mm × 15 mm) and left in a clean conditioning room (27 °C + 2 °C) for cooling and setting. Sterile filter-paper discs (diameter 6 mm) containing 20 μL of 50 mg/mL of compound (each disc contains 1 mg of test compound) were then placed on the medium. The plates were incubated aerobically for 3 days at room temperature (27 °C + 2 °C). Any zone of inhibition occurring around the disc was then measured and compared with 25 μg/disc ketoconazole (KZ) as the positive control. The experiments were performed in triplicate.

2.6.3. Minimal inhibitory concentrations (MIC)

The MIC value was determined using micro agar dilution assay. A sequential two-fold dilution method according to the previous report [26,27] was used in MIC test with slightly modoification. Stock solution of the each tested sample (8 mg/mL) was prepared in 1% (v/v) acetic acid for Ch and in water for quaternized Ch. Equal volume of the each stock solution and broth culture media (SDB) were mixed to obtain the solution containing 4 mg/mL of each compound. The resulting solutions were further diluted with broth culture media by 2-fold serial dilution to obtain the final solutions having concentrations in a range of 0.5 to 4000 μg/mL and used for MIC determination.

Minimal inhibitory concentration (MIC) evaluation was performed by using 3 μL of fungal suspension (0.5 McFarland standard) aerobically incubated with 150 μL of test samples or positive control in each well of micro 96 well plate at room temperature for 7 days. The lowest concentration of each sample solution that inhibits fungal growth was used to determine the MIC. A series of KZ solution in SDB having concentrations in a range of 0.06 to 500 μg/mL were used as positive control. The experiments were performed in triplicate.

2.6.4. The minimal fungicidal concentration (MFC)

The MFC test is the most common estimation of fungicidal activity and is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the initial inoculums after incubation [26,27]. In this experiment, the MFC was defined as the lowest concentration at which no turbidicity was visible after 7 days of aerobic incubation at room temperature. The MFC was determined by swabbing of broth from each clear well (from MIC determination) onto SDA. MFC of standard antibiotics, ketoconazole was performed similary. The experiments were performed in triplicate.

3. Results

3.1. Synthesis and characterization of quaternized chitosan and its derivatives

The formation of quaternary ammonium salt into the Ch backbone was carried out by quaternizing either iodomethane or *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Quat188) as a quaternizing agent under basic condition, leading to highly water-soluble Ch derivatives (compounds **1** and **2**) as shown in Scheme 1 [9–11,15–18]. To generate quaternary ammonium salt of the Ch derivatives, the Ch was performed into two steps: preparation of Ch derivatives and quaternization (methylation) of Ch derivatives. These two steps can be exchanged back and forth. The *N*-aryl Ch derivatives, *N*-(4-*N*,*N*-dimethylaminobenzyl)

Table 1
Quaternization of chitosan and its derivatives (mean ± SD, $n = 3$).

Sample	DS (%)	DQ_T (%)		$N(CH_3)_2$ (%)	$NHCH_3$ (%)	Total O-CH_3 (%)	Recovery (%)
		DQ_{Ar} (%)	DQ_{Ch} (%)				
1a	–	–	28 ± 2	50 ± 2	15 ± 2	16 ± 2	122
1d	–	–	64 ± 2	24 ± 2	5 ± 2	35 ± 2	74
2	–	–	90 ± 2	–	–	–	98
3	70 ± 2	70 ± 2	8 ± 2	10 ± 2	Trace	18 ± 2	120
4a	50 ± 2	50 ± 2	15 ± 2	24 ± 2	Trace	15 ± 2	90
4b	75 ± 2	75 ± 2	6 ± 2	10 ± 2	Trace	26 ± 2	109
5	62 ± 2	14 ± 2	32 ± 2	Trace	–	Trace	96

DS is the degree of *N*-substitution; DQ_{Ar} is degree of quaternization at aromatic substituents; DQ_{Ch} is degree of quaternization at primary amino group of chitosan; $N(CH_3)_2$ is *N*,*N*-dimethylation; $NHCH_3$ is *N*-methylation; total O-CH_3 is total degree of O-methylation of 3-O and 6-O at 3-hydroxyl and 6-hydroxyl positions of GlcN of Ch, respectively; recovery (%) is weight of product (g)/weight of starting reactant (g) × 100.

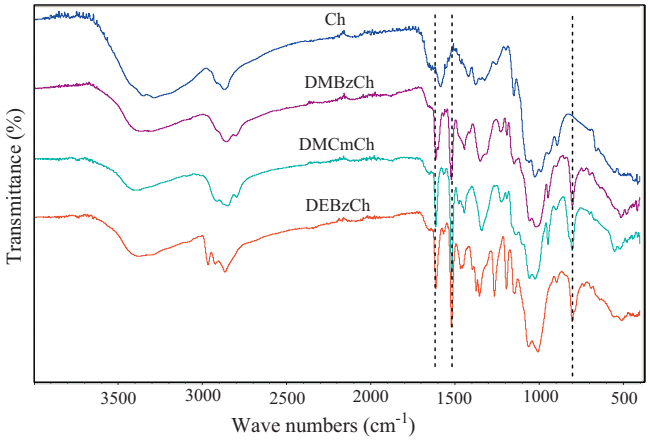


Fig. 1. ATR-FTIR spectra of chitosan (Ch), *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (DMBzCh), *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan (DMCmCh), and *N*-(4-*N*,*N*-diethylaminobenzyl) chitosan (DEBzCh).

Ch (DMBzCh), *N*-(4-*N*,*N*-dimethylaminocinnamyl) Ch (DMCmCh) and *N*-(4-*N*,*N*-diethylaminobenzyl) Ch (DEBzCh), were successfully synthesized by reductive amination (Scheme 1) [15–19]. The reaction was occured through the Schiff base intermediate, then it was reduced with sodium cyanoborohydride. The degree of *N*-substitution (DS) of *N*-aryl Ch derivatives, ranging from 50 ± 2% to 75 ± 2%, was determined by ^{1}H NMR spectroscopy [25] (Table 1). Subsequently, the *N*-aryl Ch derivatives were quaternized with iodomethane in the presence of NMP, sodium hydroxide and sodium iodide. Finally, they yielded compounds **3**–**5** (Scheme 1). The DQ of **3**–**5** was found to range between 65 ± 2% and 81 ± 2% using ^{1}H NMR spectroscopy. The chemical structures of Ch and its quaternized derivatives were characterized by ATR-FTIR and ^{1}H NMR spectroscopy. In this study, the solid samples of Ch and its derivatives were characterized by using the single-bounce ATR-FTIR spectroscopy (Smart Orbit accessory) with a diamond internal reflection element (IRE). Figs. 1 and 2 display the ATR-FTIR of Ch and its derivatives. The characteristic ATR-FTIR pattern of Ch exhibited the absorption band at wavenumber 3357 cm^{-1} due to the OH and NH_2 groups. The absorption band at wavenumbers 1639 and 1374 cm^{-1} were corresponded to the C=O and C–O stretching of amide group, respectively. The ATR-FTIR spectrum of the DMBzCh, DMCmCh and DEBzCh was similar to that of Ch except the additional absorption bands at wavenumbers 1605, 1518 and 801 or 799 cm^{-1}. These bands were assigned to the C=C stretching and C–H deformation (out of plane) of the aromatic group, respectively (Fig. 1). Fig. 2 shows the ATR-FTIR of Ch and its quaternized derivatives. The **1–5** exhibited the characteristic ATR-FTIR spectra at wavenumbers ranging from 1452 to 1475 cm^{-1} due to C–H symmetric bending of the methyl substituent of quaternary ammonium groups [15,18]. The ^{1}H NMR spectra of **3** and **4** were already reported in our previous work [16,18]. The ^{1}H NMR spectra of DEBzCh that showed proton signals at δ 0.9 ppm occurred due to the methyl protons of ethyl group, Ph-N($CH_2C\underline{H}_3$)$_2$. The signals at δ 3.4, 4.5, and 7.3–7.5 ppm were assigned to *N*,*N*-dimethylene protons of aromatic group, Ph-N($C\underline{H}_2CH_3$)$_2$, methylene protons, and aromatic protons, respectively. After quaternization of DEBzCh, the **5** was obtained. The additional signal at δ 3.7 ppm was observed due to the methyl protons at N position of aromatic substituent, Ph-N$^+$($C\underline{H}_3$)(CH_2CH_3)$_2$. The signals at δ 3.2 and 2.7 were assigned to *N*,*N*,*N*-trimethyl protons and *N*,*N*-dimethyl protons of the GlcN of Ch, respectively. In addition, the proton signals of H1 and H1′ of the GlcN of Ch and **5** at δ ranging from 5.1 to 5.6 ppm were also detected. In comparison to **3** and **4**, the DQ of **5** was quite low (Table 2). This could be attributed to the bulkiness of *N*,*N*-diethylamino group compared to *N*,*N*-dimethylamino group, causing steric hindrance to the *N*-methylation of aromatic substituent.

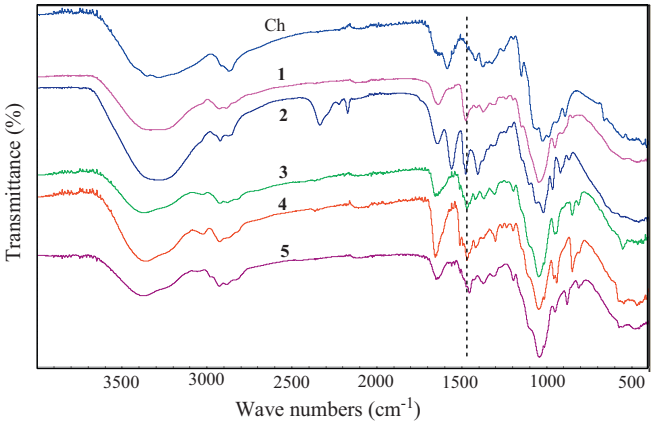


Fig. 2. ATR-FTIR spectra of chitosan (Ch), *N*,*N*,*N*-trimethyl chitosan chloride (1), chitosan Quat188 (2), quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan chloride (3), quaternized *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan chloride (4), and quaternized *N*-(4-*N*,*N*-diethylaminobenzyl) chitosan chloride (5).

Table 2
Weight average molecular weight (M_w), number average molecular weight (M_n) and M_w/M_n of chitosan before and after quaternization.

Sample	DS (%)	DQ_T (%)	M_n (kDa)	M_w (kDa)	M_w/M_n
Ch	–	–	48	276	5.6
1a	–	28 ± 2	40	204	4.9
1b	–	28 ± 2	19	78	4.0
1c	–	64 ± 2	22	120	5.2
1d	–	64 ± 2	16	55	3.3
2	–	90 ± 2	44	70	1.6
3	70 ± 2	78 ± 2	18	63	3.5
4a	50 ± 2	65 ± 2	65	92	1.4
4b	75 ± 2	81 ± 2	26	40	1.5
5	62 ± 2	46 ± 2	24	48	2.0

Table 3
Antifungal activity of quaternized chitosan and its derivatives.

Compound	M_w (kDa)	DQ_T (%)	*T. rubrum*		*T. mentagrophyte*		*M. gypseum*	
			MIC (μg/mL)	MFC (μg/mL)	MIC (μg/mL)	MFC (μg/mL)	MIC (μg/mL)	MFC (μg/mL)
Ch	276	–	–	–	–	–	–	–
1a	204	28 ± 2	250	1000	500	2000	500	2000
1b	78	28 ± 2	250	1000	500	2000	500	2000
1c	120	64 ± 2	250	1000	1000	4000	500	2000
1d	55	64 ± 2	500	1000	1000	4000	500	2000
2	70	90 ± 2	250	1000	500	2000	250	1000
3	63	78 ± 2	500	1000	1000	4000	1000	2000
4a	92	65 ± 2	250	1000	1000	4000	500	2000
4b	40	82 ± 2	125	500	1000	4000	250	1000
5	48	46 ± 2	500	1000	1000	2000	500	1000
KZ	–	–	0.97	7.8	7.8	31.3	7.8	15.6

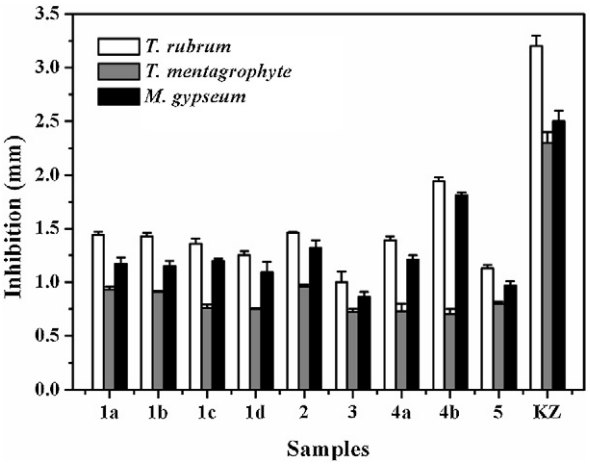


Fig. 3. Antifungal activity of chitosan and its quaternized derivatives by using disc diffusion method.

3.2. The disc diffusion method

The antifungal activity of all quaternized Ch and its derivatives was analyzed by disc diffusion method against *T. rubrum*, *T. mentagrophyte*, and *M. gypseum* at pH 7.2. In this study, KZ was used as a positive control. It was found that all quaternized Ch derivatives (compounds **1**–**5**) yield antifungal activity against *T. rubrum*, *T. mentagrophyte*, and *M. gypseum* at neutral pH indicated by diameters of inhibition in centimeter scale. Importantly, they showed antifungal activity against *T. rubrum* better than *M. gypseum* and *T. mentagrophyte*. The compounds **1a**–**1d** are the same chemical structures containing trimethyl ammonium moieties at primary amino group of Ch backbone, but they have different molecular weights and the total degrees of quaternization (DQ_T). We found that the antifungal activity of compound **1a** and **1b** ($DQ_T = 28 \pm 2\%$) against all fungal was not significantly different at concentration 1 mg/disc even though the molecular weight was different (Fig. 3 and Table 2). However, **1a** showed higher antifungal activity against *T. rubrum* than **1b** at concentration 0.5 mg/disc (data not shown). In comparison to **1c** ($DQ_T = 64 \pm 2\%$), the antifungal activity of **1d** was slightly decreased against *T. rubrum* while antifungal activities of **1c** and **1d** were not significantly different against *M. gypseum* and *T. mentagrophyte* at concentration 1.0 mg/disc. The results revealed that the antifungal activity of **1a**–**1d** tended to increase with decreased DQ_T from 28 ± 2% to 64 ± 2% against *T. rubrum* and *T. mentagrophyte* while the antifungal activity against *M. gypseum* was not different. Moreover, the antifungal activity of higher molecular weight of compound **1** tended to increase compared to lower molecular weight at similar the DQ_T. The compound **2** contained longer trimethyl ammonium moiety, 2-hydroxypropyltrimethyl ammonium moiety, with high DQ_T, showed inhibition zone 1.46 ± 0.01 mm, 0.96 ± 0.02 mm, and 1.32 ± 0.07 mm against *T. rubrum*, *T. mentagrophyte*, and *M. gypseum*, respectively. The antifungal activity of compound **2** was slightly increased compared to compounds **1a**–**1d**. In contrast, the antifungal activity of the compounds **3** and **5** was slightly decreased when trimethyl ammonium moiety was changed to the *p*-trimethylbenzyl ammonium and *p*-diethylmethylbenzyl ammonium moieties. The inhibition zone of **3** and **5** ranged from 1.00 ± 0.10 to 1.13 ± 0.03 mm, 0.72 ± 0.03 to 0.80 ± 0.02 mm, and 0.86 ± 0.05 mm against all fungal. When chain length between Ch backbone and *p*-trimethylbenzyl ammonium moiety changed to *p*-trimethylcinnamyl ammonium moiety (compound **4**), the antifungal activity was increased against *T. rubrum* and *M. gypseum*. However, the antifungal activity of **4** against *T. mentagrophyte* was decreased compared to **1** and **2**. Compound **4b** showed higher antifungal activity than **4a** except *T. mentagrophyte* due to higher DQ_T. The **4b** showed the highest antifungal activity with inhibition zone of 1.94 ± 0.04 mm and 1.81 ± 0.03 mm against both *T. rubrum* and *M. gypseum* except *T. mentagrophyte* with inhibition zone of 0.70 ± 0.05 mm.

3.3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) test

The antifungal activity of all quaternized Ch and its derivatives was determined by minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) procedure against *T. rubrum*, *T. mentagrophyte*, and *M. gypseum* as shown in Table 3. The MIC and MFC were determined at the 7th day since the growth of *T. rubrum*, *T. mentagrophyte*, and *M. gypseum* was insufficient at the 4th day. All quaternized Ch and its derivatives had MIC and MFC ranging between 125–1000 μg/mL and 500–4000 μg/mL against all *T. rubrum*, *T. mentagrophyte*, and *M. gypseum*, respectively. The KZ, which is the positive control drug for fungal, exhibited strong antifungal activity with the MIC and MFC values of 0.1–7.8 μg/mL and 7.8–31.3 μg/mL, respectively. Furthermore, the quaternized Ch derivatives showed higher antifungal activity especially against *T. rubrum* than *M. gypseum* and *T. mentagrophyte*. It was noted that compound **4b** showed the highest antifungal activity, particularly against *T. rubrum* and *M. gypseum* with MIC and MFC 125 and 500 μg/mL and 250 and 1000 μg/mL, respectively. The result is consistent with disc diffusion method.

4. Discussions

The Ch has been shown to have antifungal activity against several fungi. The MIC of Ch against fungi was ranging from 10 to 5000 ppm depending mainly on the concentration, molecular weight, DDA, chemical modification, degree of substitution, type of

fungi and pH [22,28–31]. Generally, the Ch has stronger antimicrobial activity against bacteria than fungi [30]. In addition, the activity against bacteria does not ensure activity against fungi. Since the majority of polyquaternary amines is effective by reducing fungal growth such as Ch [32–34], therefore, the activity of this material is thought to be related to its polycationic nature. Several reports have suggested that surface bound polycations are capable of killing various microbes, including yeasts by disrupting the integrity of the cell membrane [35–37]. One of the mechanistic hypothesis that has been reported by Lin et al., who explained the wide range of cells, is susceptible to polyquaternary amines including recruitment of membrane lipids into membrane blebs causing disruption of functional and direct insertion of the polymer into the membrane [36]. Other possible mechanisms are that Ch may enter the fungal cell, interact with DNA, alter its conformation, chelate with metals, spore elements, and essential nutrients, leading to inhibition of the synthesis of mRNA and proteins [38,39]. In our case, the Ch and its derivatives were quaternized at both primary amino groups and *N*,*N*-dimethylaminobenzyl, *N*,*N*-diethylaminobenzyl and *N*,*N*-dimethylaminocinnamyl groups to generate the polymeric quaternary amines, leading to high water-soluble Ch derivatives. The exact mechanism of quaternary salts of Ch against fungi remains debatable, but generally it is attributed to a formation of polyelectrolyte complex between positively charged of quaternary ammonium salts and negatively charged of cell walls of fungi. However, it is possible that the water-soluble quaternized Ch derivatives are more likely to be able to penetrate cell walls and membranes than the ion exchange mechanism of cell kill [35].

The antifungal activity of quaternized Ch and its derivatives against *T. rubrum*, *T. mentagrophyte*, and *M. gypseum* is rather less reported at neutral pH. Recently, the MIC value of Ch was found to be 2200 ppm against *T. mentagrophyte* in acidic medium [29]. Our result revealed that all quaternized Ch derivatives showed MIC values less than or equal to 1000 ppm in neutral pH. Moreover, they were most effective, particularly against *T. rubrum* compared to *T. mentagrophyte*, and *M. gypseum*. This could be explained in terms of adaptation and defense mechanisms of the fungus due to stress, affecting the structural integrity of the cell wall, or inducing the synthesis of defense compounds [40]. The chemical structure of compound **1** contained quaternary ammonium moiety at primary amino group of the Ch backbone. The effects of molecular weight and DQ_T on antifungal activity were evaluated in **1a**–**1d**. We found that the antifungal activity of **1a**–**1d** is more likely to increase when we decreased DQ_T from $28 \pm 2\%$ to $64 \pm 2\%$ against *T. rubrum* and *T. mentagrophyte* while the effect of DQ_T on the antifungal activity against *M. gypseum* was not different (Fig. 3). Even though the antifungal activity of quaternized Ch derivatives could be increased by the quaternary ammonium ion density on the molecule, the hydrophobic moiety (*N*,*N*-dimethylamino group in **1**) is an important factor affecting the antifungal activity. The result was in agreement with the results obtained by Badawy, who found that the antifungal activity of Ch and its quaternized derivatives against the plant pathogenic fungi of grey mold *Botrytis cinerea*, root rot disease *Fusarium oxysporum*, and damping off disease *Pythium debaryanum* was increased with an increase in the chain length of alkyl substituent [41]. It was noted that the antifungal activity of **1** was based on different molecular weights. The higher molecular weight showed the lower DQ_T (Table 2) due to repeated quaternization, leading to reduction of molecular weight. Moreover, the antifungal activity of higher molecular weight of compound **1** tended to increase compared to lower molecular weight at similar DQ_T (Fig. 3 and Table 3). This was consistent with Guo et al., who found that the quaternized Ch derivatives with high molecular weight showed stronger antifungal activities against *B. cinerea* Pers. and *Colletotrichum lagenarium* (Pass) Ell.ethalst than those with low molecular weight [42]. When increasing spacing of quaternary ammonium moiety as shown in **2**, the antifungal activity was slightly increased when compared to **1**. This could be due to increasing opportunity to bind on the fungi cell walls. However, the antifungal activity was slightly decreased when spacing was changed to the *p*-trimethylbenzyl ammonium and *p*-diethylmethylbenzyl ammonium moieties in the compounds **3** and **5**. Previously, we found that the presence of the *p*-trimethylbenzyl ammonium substituent on Ch backbone did not enhance the antibacterial activity against *Staphylococcus aureus*, but it slightly increased antibacterial activity against *Escherichia coli* when DQ_{Ch} was 17% and DQ_{Ar} was 16–30% [18]. Although the antifungal activity of quaternized Ch and its derivatives have not been clearly elucidated, the effects of chemical structure, molecular weight, DQ_T fungal type on antifungal activity were discussed in this point. Interestingly, **4b**, which was a similar head group of quaternary ammonium moiety to **3** with longer chain length component, showed the highest antifungal activity. The **4b** showed more effective against *T. rubrum* and *M. gypseum* than *T. mentagrophyte*. In this study, we demonstrated that there were many factors such as chemical structure, molecular weight, DQ_T, hydrophobic moiety, and fungal type that affected on antifungal activity mechanism.

5. Conclusion

All quaternized chitosans and its derivatives showed antifungal activity *T. rubrum*, *T. mentagrophyte*, and *M. gypseum* at neutral pH. We found that all quaternized chitosan derivatives are more effective against *T. rubrum* than *T. mentagrophyte*, and *M. gypseum*. The quaternized *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan chloride (**4b**) showed higher antifungal activity against *T. rubrum* and *M. gypseum* than other quaternized chitosan derivatives. This suggested that the chemical structure, the degree of quaternization, and hydrophobic/hydrophilic balance played important roles on antifungal activity. However, these factors were dependent on types of fungal and environmental condition. Our results provide proved explanation of proper kinds of quaternary ammonium substituents on Ch backbone for antifungal activity against specific fungi. It is useful for further modification in order to improve antifungal activity in many applications.

Acknowledgements

The author is grateful to Siremorn for her support throughout the time and the Dow Chemical Company in Thailand for providing *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Quat 188). This work is financially supported by National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Thailand (Project No. NN-B-22-EN4-94-51-10).

References

[1] B.E. Elewski, M.A. Charif, Arch. Dermatol. 133 (1997) 1172–1173.
[2] M. Ghannoum, N. Isham, R. Hajjrh, M. Cano, F. Al-Hasawi, D. Yearick, J. Warner, L. Long, C. Jessup, B. Elewski, J. Am. Acad. Dermatol. 48 (2003) 189–193.
[3] CFSPH Technical Fact Sheets, Dermatophytes, Available from: http://www.cfsph.iastate.edu/DiseaseInfo/default.htm.
[4] CDC website, Ringworm, Available from: http://www.cdc.gov/healthypets/diseases/ring worm.htm.
[5] J.E. Adaskaveg, H. Forster, N.F. Sommer, in: A. Kader (Ed.), Postharvest Technology of Horticultural Crops, DANR Publication, Oakland, 2002, pp. 163–195.
[6] J. Northover, T. Zhou, Can. J. Plant Pathol. 24 (2002) 144–153.
[7] B. Schachter, Nat. Biotechnol. 2 (2003) 361–365.
[8] P. Tripathi, N.K. Dubey, Postharvest Biol. Technol. 32 (2004) 235–245.
[9] R.A.A. Muzzarelli, F. Tanfani, Carbohydr. Polym. 5 (1985) 297–307.
[10] A. Domard, M. Rinaudo, C. Terrassin, Int. J. Biol. Macromol. 8 (1986) 105–107.

[11] W.H. Daly, M.A. Manuszak-Guerrini, Biocidal chitosan derivatives for cosmetics and pharmaceuticals, US Patent, No. 6,306,835 (2001).
[12] E. Loubaki, M. Ourevitch, S. Sicsic, Eur. Polym. J. 27 (1991) 311–317.
[13] T. Heinze, V. Haack, S. Rensing, Starch (Staerke) 56 (2004) 288–296.
[14] M. Hashem, P. Hauser, B. Smith, Textile Res. J. 73 (2003) 1017–1023.
[15] W. Sajomsang, S. Tantayanon, V. Tangpasuthadol, W.H. Daly, Carbohydr. Res. 344 (2009) 2502–2511.
[16] W. Sajomsang, P. Gonil, S. Saesoo, Polym. Eur. J. 45 (2009) 2319–2328.
[17] W. Sajomsang, Carbohydr. Polym. 80 (2010) 631–647.
[18] W. Sajomsang, S. Tantayanon, V. Tangpasuthadol, W.H. Daly, Carbohydr. Polym. 72 (2008) 740–750.
[19] W. Sajomsang, U. Ruktanonchai, P. Gonil, V. Mayen, P. Opanasopit, Carbohydr. Polym. 78 (2009) 743–752.
[20] M. Petchsangsai, W. Sajomsang, P. Gonil, O. Nuchuchua, B. Sutapunb, S. Puttipipatkhachorn, U. Rungsardthong Ruktanonchai, Carbohydr. Polym. 83 (2011) 1263–1273.
[21] P. Vongchan, Y. Wutti-In, W. Sajomsang, P. Gonil, S. Kothan, R.J. Linhardt, Carbohydr. Polym. 85 (2011) 215–220.
[22] E.I. Rabea, M.E.T. Badawy, C.V. Stevens, G. Smagghe, W. Steurnaut, Biomacromolecules 4 (6) (2003) 1457–1465.
[23] A.N. Hernandez-Lauzardo, S. Bautista-Banos, M.G. Velazquez-del Valle, M.G. Mendez-Montealvo, M.M. Sanchez-Rivera, L.A. Bello-Perez, Carbohydr. Polym. 73 (2008) 541–547.
[24] Y. Peng, B. Han, W. Liua, X. Xu, Carbohydr. Res. 340 (2005) 1846–1851.
[25] W. Sajomsang, S. Tantayanon, V. Tangpasuthadol, M. Thatte, W.H. Daly, Int. J. Biol. Macromol. 43 (2008) 79–87.
[26] V. Lorian, Antibiotics in laboratory medicine, fourth ed., Williams and Wilkins, Baltimore, 1996.
[27] N. Garcia, A. Gonzalez, M. Fuentes, M. Aviles, M.Y. Rios, G. Zepeda, M.G. Rojas, J. Ethnopharmacol. 87 (2003) 85–88.
[28] X.F. Liu, Y.L. Guan, D.Z. Yang, Z. Li, K.D. Yao, J. Appl. Polym. Sci. 79 (2001) 1324–1335.
[29] R.C. Goy, D. Britto, O.B.G. Assis, Polímeros: Ciênc. Tecnol. 19 (2009) 1–7.
[30] T. Yu, H.Y. Li, X.D. Zheng, Int. J. Food Microbiol. 714 (2007) 261–266.
[31] E.I. Rabea, M.E.I. Badawy, W. Steurbaut, C.V. Stevens, Polym. Eur. J. 45 (2009) 237–245.
[32] J. Rhoades, S. Roller, Appl. Environ. Microbiol. 2000 (66) (2000) 80–86.
[33] M. Plascencia-Jatomea, G. Viniegra, R. Olayo, M.M. Castillo-Ortega, K. Shirai, Macromol. Biosci. 3 (2003) 582–586.
[34] T. Ravikumar, H. Murata, R.R. Koepsel, A.J. Russell, Biomacromolecules 7 (2006) 2762–2769.
[35] S.B. Lee, R.R. Koepsel, S.W. Morley, K. Matyjaszewski, Y. Sun, A.J. Russell, Biomacromolecules 5 (2004) 877–882.
[36] J. Lin, S. Qiu, K. Lewis, A.M. Klibanov, Biotechnol. Bioeng. 83 (2003) 168–172.
[37] R. Kugler, O. Bouloussa, F. Rondelez, Microbiology 151 (2005) 1341–1348.
[38] L.A. Hadwiger, D.C. Loschke, Phytopathology 71 (1981) 756–862.
[39] R.G. Cuero, G. Osuji, A. Washington, Biotechnol. Lett. 13 (1991) 441–444.
[40] O. Cota-Arriola, M.O. Cortez-Rocha, E.C. Rosas-Burgos, A. Burgos-Hernández, Y.L. López-Francob, M. Plascencia-Jatomea, Polym. Int. 60 (2011) 937–944.
[41] M.E.I. Badawy, J. Appl. Polym. Sci. 117 (2010) 960–969.
[42] Z. Guo, R. Xing, S. Liu, Z. Zhong, X. Ji, L. Wang, P. Li, Carbohydr. Polym. 71 (2008) 694–697.