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Synthesis of some N-benzylphosphoryl chitosan derivatives and their fungicidal and insecticidal activity

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ABSTRACT

A series of some water-soluble N-benzylphosphoryl chitosan (NBPC) derivatives were synthesized by reaction of chitosan with aldehydes and phosphorus acid. Chemical identification was confirmed by 1H- and 31P-NMR spectroscopy. Their biological activities were tested against economic fungus of the grey mould, *Botrytis cinerea*, and insect of the cotton leafworm, *Spodoptera littoralis*. In a radial growth fungicidal bioassay, all of the synthetic compounds were about 6 times more potent than natural chitosan. Substitution with o-nitro group on the phenyl ring showed the highest activity (EC_{50} = 0.04%), whereas substitution with p-cyano was the lowest one (EC_{50} = 0.19%). In feeding bioassay, the insecticidal activity was screened against third-instar larvae of the cotton leafworm, *S. littoralis*, at 0.5 % (w/w) in the artificial diet and the most active compound was also N-(o-nitrobenzylphosphoryl) chitosan.

Keywords: N-benzylphosphoryl chitosan derivatives, 1H- and 31P-NMR spectroscopy, fungicidal activity, insecticidal activity, *Botrytis cinerea*, *Spodoptera littoralis*.

INTRODUCTION

Chitosan is a linear amino polysaccharide of glucosamine (deacetylated unit) and N-acetylglucosamine (acetylated unit) and can be derived by partial N-deacetylation of chitin from crustacean shells (Lee *et al.*, 1995; No and Meyers, 1997 and Yoksan *et al.*, 2001). Chitosan is used to describe a series of chitosan polymers with different molecular weights (50-2000 kDa), viscosities (1% chitosan in 1% acetic acid <2000 mPaS), and degrees of deacetylation (40-98%) (Illum, 1998 and Kurita, 2001). The free amino group in chitosans can offer great potential for further derivatization

(Kurita *et al.*, 2000, Sashiwa *et al.*, 2000, Rabea *et al.*, 2003 and Badawy *et al.*, 2004).

Chemical modification of chitosan depending on the degree of deacetylation to generate new bio-functional materials with new properties according to the nature of the group introduced.

Phosphate derivatives of chitosan have been proposed enhance biological and chemical properties of such compounds. For example, it could exhibit bactericidal (Tanigawa *et al.*, 1992); metal chelating properties. The introduction of group such as phosphonic acid or phosphonate onto chitosan increased the solubility in water (Nishi *et al.*, 1987). The introduction of α-amino methylphosphonic acid functions onto chitosan using the Kabachnik-Fields reactions was dealt by Heras *et al.*, (2001).

Botrytis cinerea, a common plant pathogenic fungus, is the causal agent of gray mould disease of several crops throughout the world in pre- and post-harvest. Many fruits, vegetables and ornamental crops were seriously effected by this disease (Debieu *et al.*, 2001). Failure to control this fungus can result in serious losses in worldwide agriculture products. The frequent development of *B. cinerea* being resistant to common fungicides and the desire to reduce pesticide applications have been developing alternatives (Yourman and Jeffers, 1999).

This work describes the synthesis of *N*-benzylphosphoryl chitosan (NBPC) derivatives and evaluating their fungicidal and insecticidal activities against the grey mould *B. cinerea* and the cotton leaf worm *S. littoralis*, respectively.

MATERIALS AND METHODS

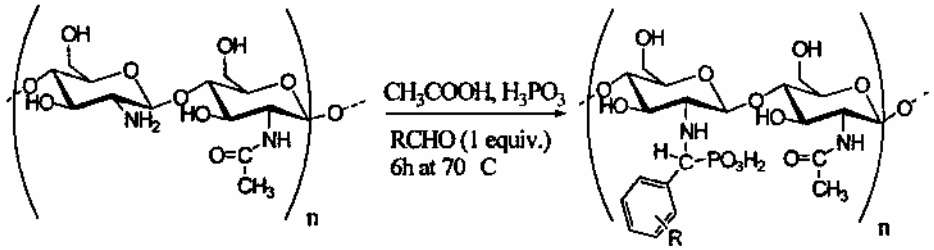
1. Materials

Chitosan of low molecular weight (made from coarse ground crab, 89% degree of deacetylation, DDA calculated from NMR spectrum) and aldehydes were purchased from Sigma-Aldrich Co. (Bornem, Belgium). All materials were used without further purification. For fungi test, Potato Dextrose Agar (PDA) was purchased from Oxoid Ltd. (Basingstoke,

Hampshire, England). For the insect bioassay, soybean-wheat germ insect artificial diet was purchased from Stonefly Ind. (Bryan, TX, USA).

2. Synthesis of NBPC derivatives

Chitosan (18 mM, 3 g, calculated as glucosamine unit) was dissolved in 1% aqueous solution acetic acid. This solution was added dropwise under continuous stirring during 1h to phosphorous acid (18 mM (1.5g)) dissolved in water. The temperature of the reaction vessel was raised to 70°C and 1.0 equivalent of aldehyde derivative was added dropwise over 1 h under reflux. Heating was continued at the same temperature for 6 h. The solution was dialyzed with a cut-off value of 12,400 Da in demineralized water for 48 h or until the pH of the water was raised to 6.8. Finally, the solution was frozen and freeze-dried to afford the title compounds in a white, brown or yellow color (Scheme. 1) (Heras, *et al.* 2001).



Scheme 1. Synthesis of NBPC derivatives.
R = H or *(un)*-substituted phenyl

3. NMR spectroscopy

^{1}H- and ^{31}P-NMR measurements were performed on a JEOL A-300 NMR spectrometer under a static magnetic field of 300 MHz, at 25°C. For these measurements, 20 mg of sample was introduced into 5 mm Φ NMR tube, to which 0.5 ml 1% CD_3COOD/D_2O for chitosan and D_2O for derivatives was added, and finally the tube was kept at 25°C to dissolve the polymer.

4. Fungicidal activity against the gray mould *B. cinerea*

B. cinerea (isolate R16 that resulted from the cross SAS56 x SAS405) obtained from laboratory of Phytopathology, Faculty of Bioscience Engineering, Gent University, Belgium. Chitosan in 1% acetic acid/water and synthesized derivatives solutions were prepared by dissolving these

compounds in water, and the pH was adjusted to 5.5-6.0 with 1M NaOH in the case of chitosan only (Stossel and Leuba, 1984 and Badawy *et al.*, 2004). PDA media in radial growth technique containing different concentrations of chitosan or synthesized derivatives ranging between 0 and 0.5% (w/v) were seeded in sterile culture Petri dishes (9-cm diameter) and infected with 6-mm-diameter agar plugs taken from the margin of a 7-day old culture of *B. cinerea*. For each compound, four replicates were used per concentration tested. The plates were incubated in the dark at 26 ± 2°C (El-Ghaouth *et al.*, 1992). Growth measurements were determined when the hyphae in the control had grown up to the edge of the plate. EC_{50}'s and corresponding 95% CL were estimated by probit analysis (Finney, 1971).

5. Insecticidal feeding bioassay against *S. littoralis*

A susceptible cotton leaf worm *S. littoralis* strain was obtained from a laboratory colony under controlled conditions (Department of Agrozoology, Faculty of Bioscience Engineering, Gent University, Belgium). The colony was reared in laboratory on an artificial diet. In a standardized screening toxicity test, freshly molted third-instar larvae of *S. littoralis* were selected. Chitosan and the synthesized derivatives were tested at 0.5% (w/w) in an artificial diet. The compound was dissolved in 37.5 part of water at room temperature and then incorporated with 12.5 part of an artificial diet and mixed with a homogenizer (Heidolph DIAX 600). Treated diet was divided and placed in Petri dishes. Three replicates for each treatment and control and 10 larvae were introduced onto each replicate. The experiments were kept in a growth chamber, at 23 (±2) °C, 70(±5) % RH and a 16:8h (L:D) photoperiod. After 7 days of continuous feeding on treated diet, normal growth and mortality were scored and compared with control (Smagghe *et al.*, 2002).

RESULTS AND DISCUSSION

1. Characterization of chitosan derivatives

N-benzylphosphoryl chotosan (NBPC) derivatives were prepared at DS of 0.01 to 0.10 by treatment of chitosan with aromatic aldehydes and phosphorus acid at mole ratio of 1:1:1 in a solution of 1% aqueous acetic acid (Table 1). A white hydrogel was produced in case of benzaldehyde, *p*-methylthiobenzaldehyde, *p*-chlorobenzaldehyde and *p*-cyanobenzaldehyde, brown color in case of *p*-methylbenzaldehyde and a yellow hydrogel in that with 2,5-dimethoxybenzaldehyde, *o*-nitrobenzaldehyde and *p*-

nitrobenzaldehyde. As the results, the synthesized chitosan derivatives that isolated are 79-89% DDA and the high DS value is obtained with *N*-(*p*-chlorobenzylphosphoryl) chitosan (5). The chemical structure of the substituted chitosan was identified by ^{1}H- and ^{31}P-NMR. DDA was calculated to be 89% in chitosan from the integral ratio between proton on C-2 and the glucose unit protons of *N*-acetyl-glucosamine (GlcNAc) (Badawy *et al.*, 2004). The data demonstrated that the increase of DS value resulting in the decrease of the DDA which indicated that the reaction occurred on the amino group on C-2 of the glucosamine unit. The synthesized derivatives are highly water-soluble.

A water-soluble chitosan derivative carrying phosphonic group named as *N*-methylene phosphonic chitosan was synthesized by Heras, *et al.* (2001) using a one-step reaction that allowed homogeneous modifications without any sharp decrease in its properties, such as filmogenic capacity. The ^{1}H-NMR, ^{13}C-NMR assignments and ^{1}H-^{13}C NMR correlation permitted the identification of the structure by the subtituent distribution of the product, which is partly *N*-monophosphonomethylated (DS = 0.24) and *N,N*-diphosphonomethylated (DS = 0.14) and *N*-acetylated (DS = 0.16) without modification of the initial degree of acetylation (DA). The identity of the *N*-methylene phosphonic chitosan was also confirmed by FT-IR spectrometry, X-ray diffraction and elemental analysis (Heras, *et al.*, 2001).

1.1. ^{1}H- and ^{31}P-NMR spectral data

Spectral data for chitosan:

^{1}H-NMR δ 2.09 (s, 0.33H, NHAc), 3.24 (t, 0.89H, H-2 of glucosamine, GlcN residue), 3.57-4.14 (br m, 5.11H, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.62 (br s, H-1 of GlcNAc residue), 4.93 (d, H-1 of GlcN residue). MF of NH_2 (DDA, x) was estimated from δ 3.24 (x) vs. 3.57-4.14 (6-x). MF of NHAc (DA, y) was estimated from δ 2.09 (3y) vs. 3.24-4.14 (6H).

Spectral data for compound 1:

^{1}H-NMR: δ 2.09 (s, 0.33H, NHAc), 3.21 (br s, 0.88H, H-2 of GlcN residue), 3.41-4.18 (br m, 5.12H, H-3, 4, 5, 6 of GlcN, H-2 of GlcNAc), 4.45 (s, H-1 of GlcN), 7.46 - 7.74 (br s, 0.05H, Ph). MF of NH_2(DDA, x) was estimated from δ 3.21 (x) vs. 3.41-4.18 (6-x). MF of NHAc (DA, y) was estimated from δ 2.09 (3y) vs. 3.21-4.18 (6H). MF of NHR (DS, z) was estimated from δ 7.46-7.74 ((Ph, 5z) vs. 3.41-4.18 (6H). **^{31}P-NMR:** 2.58

Spectral data for compound 2:
^{1}H-NMR: δ 2.12 (s, 0.30H, NHAc), 3.20 (br s, 0.89H, H-2 of GlcN residue), 3.42-4.25 (br m, 5.11H, H-3, 4, 5, 6 of GlcN, H-2 of GlcNAc), 4.70 (s, H-1 of GlcN), 7.60-7.79 (br m, 0.02H, Ph), 8.15-8.38 (br m, 0.02, Ph). MF values were estimated in a manner similar to that used for compound 1. **^{31}P-NMR:** 3.28

Spectral data for compound 3:
^{1}H-NMR: δ 1.33 (s, 0.06H, CH_3), 2.09 (s, 0.30H, NHAc), 3.19 (br s, 0.88H, H-2 of GlcN residue), 3.40-4.43 (br m, 5.12H, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.86 (br s, H-1 of GlcN residue), 7.59 (br, 0.08H, Ph). MF values were estimated in a manner similar to that used for compound 1. **^{31}P-NMR:** 3.25

Spectral data for compound 4:
^{1}H-NMR: δ 2.09 (s, 0.45H, NHAc), 3.19 (br s, 0.80H, H-2 of GlcN residue), 3.48-4.25 (br m, 5.20H, H-3, 4, 5, 6 of GlcN, H-2 of GlcNAc and $O\underline{CH_3}$ group), 4.90 (s, H-1 of GlcN), 6.87-7.49 (br m, 0.15H, Ph). MF values were estimated in a manner similar to that used for compound 1. **^{31}P-NMR:** 2.83

Spectral data for compound 5:
^{1}H-NMR: δ 2.10 (s, 0.33H, NHAc), 3.20 (br s, 0.79H, H-2 of GlcN residue), 3.37-4.20 (br m, 5.21 H, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.61 (br s, H-1 of GlcN residue), 8.08-8.68 (br m, 0.40H, Ph). MF values were estimated in a manner similar to that used for compound 1. **^{31}P-NMR:** 2.86

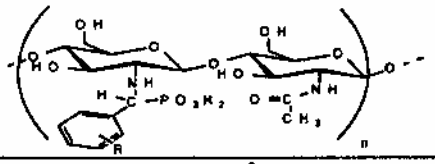
Spectral data for compound 6:
^{1}H-NMR: δ 2.10 (s, 0.33H, NHAc), 3.20 (br s, 0.87H, H-2 of GlcN residue), 3.49-4.18 (br m, 5.13 H, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.61 (br s, H-1 of GlcN residue), 7.06 (br s, 0.04H, Ph), 7.40-7.73 (br s, 0.04H, Ph). MF values were estimated in a manner similar to that used for compound 1. **^{31}P-NMR:** 2.89

Spectral data for compound 7:
^{1}H-NMR: δ 2.11 (s, 0.33H, NHAc), 3.19 (br s, 0.87H, H-2 of GlcN residue), 3.43-4.21 (br m, 5.13 H, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.61 (br s, H-1 of GlcN residue), 6.95-7.28 (br m, 0.08H, Ph). MF

values were estimated in a manner similar to that used for compound 1. **^{31}P-NMR:** 3.24

Spectral data for compound 8:
^{1}H-NMR: δ 2.09 (s, 0.27H, NHAc), 3.20 (br s, 0.89H, H-2 of GlcN residue), 3.49-4.18 (br m, 5.11 H, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.61 (br s, H-1 of GlcN residue), 8.21-8.47 (br m, 0.08H, Ph). MF values were estimated in a manner similar to that used for compound 1. **^{31}P-NMR:** 3.28

Table 1. Chemical structures of NBPC derivatives:



Entry	R	MF of NH_2[a] (DDA, x)	MF of NHAc[b] (DA, y)	DS of NHR[c] (z)
Chitosan	-	0.89	0.11	-
1	H	0.88	0.11	0.01
2	*p*-CH_3	0.89	0.10	0.01
3	*p*-SCH_3	0.88	0.10	0.02
4	2,5-di-OCH_3	0.80	0.15	0.05
5	*p*-Cl	0.79	0.11	0.10
6	*p*-CN	0.87	0.11	0.02
7	*o*-NO_2	0.87	0.11	0.02
8	*p*-NO_2	0.89	0.09	0.02

[a] MF of NH_2 (DDA, x) was calculated from this equation: A/B = X/(6-X)
[b] MF of NHAc (DA, y) = 1 - (x + z)
[c] MF of NHR (DS, z) calculated from C/(A+B) = nDS/6,
where **A** the peak area of H-2 of GlcN unit, **B** the peak area of H-2 of GlcNAc and H-3,4,5,6 of GlcN and **C** the peak area of the substituent, where **n** is the number of hydrogen atoms per substituent.

2. Fungicidal activity against *B. cinerea*

Although chitosan has been studied in several areas for its fungicidal and bactericidal activity against several fungi and bacteria (Rabea *et al.*, 2003), information on the fungicidal activity of chitosan derivatives is limited in the literature. The fungicidal activity of NBPC derivatives against

B. cinerea is presented in Table 2 as EC_{50} value with the corresponding 95% confidence limits (CL). In general, all of the synthesized derivatives were more active than chitosan and exhibited good fungicidal activity. The result shows that *N*-(*o*-nitrobenzylphosphoryl) chitosan (7) (Fig. 1) and *N*-(*p*-methylbenzylphosphoryl) chitosan (2) are the most active compounds against *B. cinerea* with EC_{50} values of 0.04 and 0.05%, respectively. It is very clear that substitution with nitro group at *ortho* position on the phenyl ring led to an increase in the activity than that with substitution at *para* position (see compound 7 versus 8). The results also showed that the un-substituted phenyl ring (1) is the lowest active one against the tested fungus.

Chitosan showed low activity against *B. cinerea* because of the lower solubility of chitosan in neutral water compared with the synthesized derivatives.

Chitosan is already known to interfere with the growth of several phytopathogenic fungi including *B. cinerea* (Du *et al.*, 1997; El Ghaouth *et al.*, 1994 and Oh *et al.*, 1998). It is believed that chitosan interferes with the negatively charged residues of macromolecules on the fungal cell surface, and thereby changing the permeability of the plasma membrane (Benhamou, 1996).

Table 2. Fungicidal activity of chitosan and NBPC derivatives against *B. cinerea*

Compound	EC_{50} (95% CL) (%, w/v)
Chitosan	0.56 (0.40-0.77)
1	0.13 (0.12-0.14)
2	0.05 (0.04-0.06)
3	0.12 (0.11-0.14)
4	0.08 (0.07-0.10)
5	0.08 (0.07-0.09)
6	0.19 (0.17-0.21)
7	0.04 (0.03-0.05)
8	0.08 (0.07-0.09)

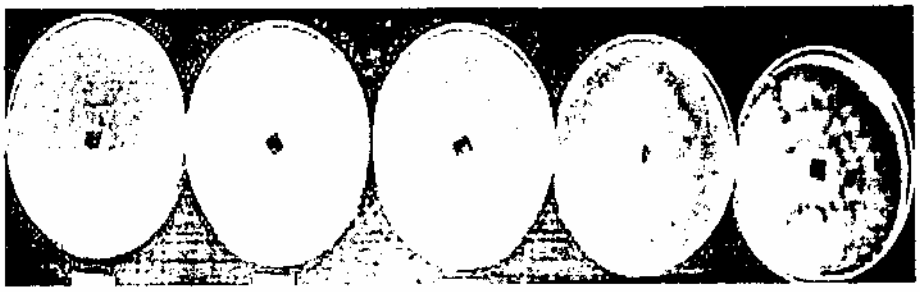


Fig. 1. The effect of *N*-(*o*-nitrobenzylphosphoryl) chitosan (7) on hyphal growth of *B. cinerea* (0.2, 0.1, 0.075, 0.05% and control from left to right).

3. Insecticidal activity against larvae of *S. littoralis*

We examined the insecticidal activity of chitosan and NBPC derivatives against the cotton leaf worm, *S. littoralis* and the results are shown in Table 3 and presented as mortality (%) determined at 0.5%. The results indicated that all of these derivatives were low active against the tested insect, which may be referred to the low of DS values. However, slight mortality was found with *N*-(*o*-nitrobenzylphosphoryl) chitosan (7).

Generally, chitosan showed negligible insecticidal activity against *S. littoralis, in vitro*. However, Zhang *et al.*, (2003) reported the *in vivo* activity of chitosan and oligo-chitosan (degree of polymerization, DP = 20) on several plant insects. They found that the insecticidal activity of chitosan at concentration of 3000 mg/l against *Plutella xylostella* was higher than against *Helicoverpa armigera*. The activity of chitosan against *H. armigera* was 38.4 and 40% after 24 and 72 h, respectively. However, the mortality was 62 and 72% after 48 and 72 h, respectively against *P. xylostella*. They also found different insecticidal activities of chitosan to various aphids at a concentrations range from 600 to 6000 mg/l. For example, chitosan had a very high insecticidal activity for *H. pruni* on flowers; the mortality was between 93 and 99%. It had 70-80% insecticidal activity against *R. padi*, *M. dirhodum* and *A. gossypii*, while *S. avenae* and *M. persicae* showed a lower susceptibility for chitosan.

Table 3. Insecticidal activity of chitosan and NBPC derivatives (0.5 %) against larvae of the cotton leaf worm, *S. littoralis*.

Compound	Mortality (%) ± SEM[a]
chitosan	13.0 ± 0.33
1	39.0 ± 0.88
2	7.00 ± 0.33
3	4.00 ± 0.33
4	21.0 ± 0.88
5	7.00 ± 0.33
6	14.0 ± 0.58
7	60.5 ± 0.67
8	16.5 ± 0.33

[a]Data are expressed as mean % with n = 30

CONCLUSION

The goal of this work is to modify chitosan and to use as a biologically active compound against important economic pests. *N*-benzylphosphoryl chitosan derivatives (NBPC) were synthesized with degrees of substitution ranged from 0.01 to 0.10 calculated by ^{1}H-NMR spectra. The biological data against the grey mould *B. cinerea* reported that *N*-(*o*-nitrobenzylphosphoryl) chitosan and *N*-(*p*-methylbenzylphosphoryl) chitosan were the most active derivatives. The promising antifungal activity against *B. cinerea* makes this research field worthy of further investigations including defining the spectrum of its antifungal activity and improving its biological activity by a suitable structural formulation. The activity of chitosan and its synthetic derivatives against larvae of a major insect pest, the cotton leaf worm *S. littoralis* indicated that there is no clear activity except with a compound of *N*-(*o*-nitrobenzylphosphoryl) chitosan.

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تحضير بعض مشتقات من ن_بنزيل فوسفوريل كيتوزان وتقييم الفعل الابادى الفطرى والحشرى لها

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تم تحضير سلسلة مركبات جديدة من البنزيل فوسفوريل كيتوزان والجيدة الذوبان فى الماء وذلك بواسطة التفاعل الكيميائى بين الكيتوزان وحامض الفوسفورس ومشتقات الألديهيد الاروماتى المختلفة. الخصائص الكيميائية لهذة المركبات تم التعرف عليها بواسطة جهاز الرنين النووى المغناطيسى بقياس بروتون الهيدروجين والفوسفور. تم تقييم النشاط الحيوي ضد فطر العفن البنى الذى يصيب معظم النباتات وثمار الفاكهة والخضروات وايضا ضد يرقات دودة ورق القطن. النشاط الابادى الفطرى ضد فطر البوتريتس سيناريا بواسطة التقييم الحيوى بقياس قطر نمو الميسيليوم اوضح ان كل المركبات المحضرة كانت أكثر فعالية حوالى ٦ مرات من الكيتوزان. وقد وجد ان الاستبدال بالاورثو_نيترو على حلقة الفينايل كان الاعلى نشاط ضد الفطر المختبر بينما الاستبدال مع البارا_سيانو كان الاقل فى النشاط الابادى الفطرى. فى التقييم الحيوى لمعرفة النشاط الابادى الحشرى ضد يرقات العمر الثالث لحشرة دودة ورق القطن (سبودوبترا ليتوراليس) على تركيز قدرة ٠,٥% (وزن/وزن) في البيئة الغذائية الإصطناعية، اوضحت النتائج ان مركب الاورثو نيتروبنزيل فوسفوريل كيتوزان كان الاعلى فى التاثير الابادى الحشرى.