CHAPTER II

EXPERIMENTAL

2.1 Materials and Chemicals

Chitosan with a degree of deacetylation of 95.0% and M_w (viscosity) of 110,000 was purchased from Seafresh Chitosan (Lab), Co., Thailand. Irradiated chitosan was a gift from Dr. Chyagrit Siri-Upathum (Department of Nuclear Technology, Faculty of engineering, Chulalongkorn University, Thailand).

N,*N*′-Dimethyl formamide, dimethyl sulfoxide used in syntheses and spectroscopic techniques were reagent or analytical grades purchased from Labscan (Bangkok, Thailand). Methanol, ethyl acetate and dichloromethane were purified from commercial grade solvents prior to use by distillation.

1-Ethyl-3(3-dimethyaminopropyl) carbodiimide (EDCI), 4-methoxy cinnamic acid and 1-Hydroxy benzotriazole (HOBt) were purchased from Acros Organics (New Jersey, U.S.A.). Dicyclo-hexylcarbodiimide (DCC) and octyl methoxycinnamic acid were purchased from Merck Co. Ltd. (Darmstadt, Germany). Phthalic anhydride was purchased from Carlo Erba Reagent (Val de Reuil, France). Toluene-4-sulfonic acid monohydrate was purchased from Fluka Chemical Company (Buchs, Switzerland). Pyridine and piperidine were purchased from Sigma (Sigma Chemical Co. Ltd, USA.) Octyl methoxycinnamate (OMC, Eucolex 2292) was obtained from Merck Co. Ltd. (Darmstadt, Germany).

2.2 Instruments and Equipments

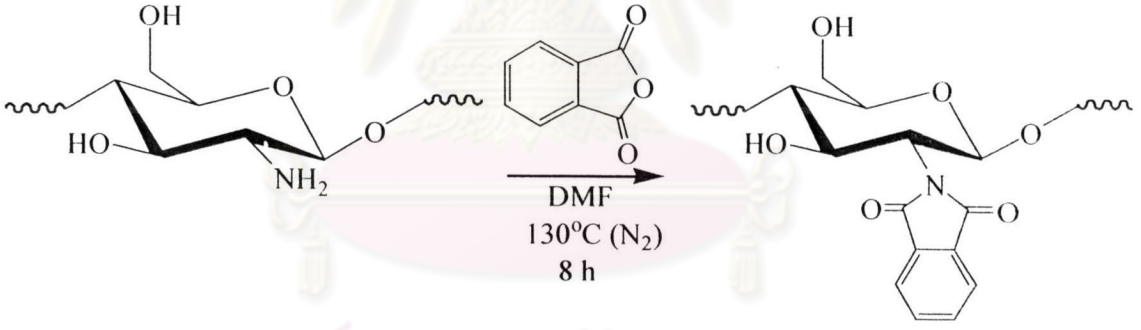
Thin layer chromatography (TLC) was performed on aluminum sheets precoated with silica gel (Merck Kieselgel 60 F_{254}) (Merck KgaA, Darmstadt, Germany). For UV radiation, broad band UVA (320-400 nm) was generated by FSX24T12/BL/HO (PUVA) lamp (National Biological Corporation, Twinsburg, Ohio, USA) and broad band UVB (280-320 nm) was generated by FSX24T12/BL/HO lamp (National Biological Corporation, Twinsburg, Ohio, USA). UV Irradiance was measured using UVA-400C and UVB-500C power meter (National Biological Corporation, Twinsburg, Ohio, USA).

The FT-IR spectra were recorded on a Nicolet Fourier Transform Infared spectrophotometer: Impact 410 (Nicolet Instruments Technologies, Inc. WI, U.S.A.). The ^{1}H-NMR and ^{13}C-NMR spectra were obtained in deuterated dimethylsulfoxide DMSO-d_6) with tetramethylsilane (TMS) as an internal reference using Varian Mercury spectrometer which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C (Varian Company. U.S.A.). Ultraviolet absorption spectra were obtained with the aid of HP 8453 UV/VIS spectrophotometer (Agilent Technologies, CA, U.S.A.). The UV absorbance was recorded using a sample in the 1 cm quartz call. Elemental Analysis (C,H,O,N) was performed with Perkin Elmer instruments.

Membranes used for dialysis experiments were seamless cellulose tubing, molecular weight cut off (MWCO) 12,400 Dalton, size 36/32 100 ft (Viskase Companies, Inc., Japan).

A. Chitosan

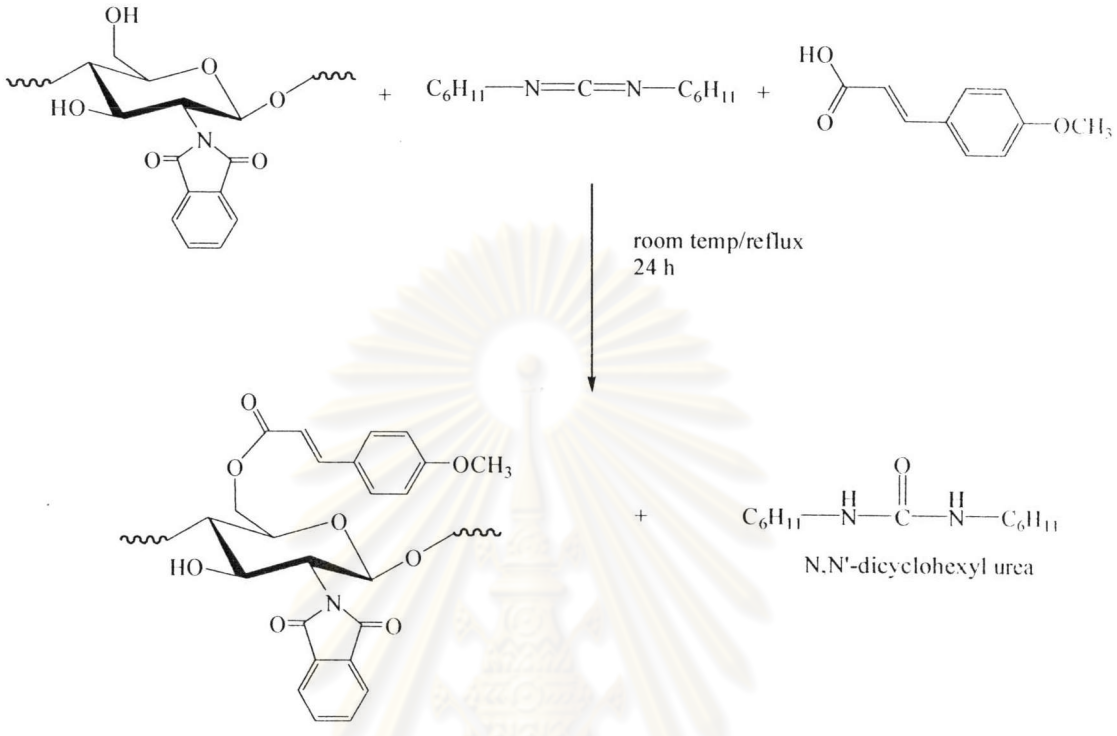
2.3 Phthaloylation of chitosan[19]



Chitosan (2.5 g, 8.6×10^{-3} mol) was added into a solution of 7 g (5.5×10^{-3} mol eq. of NH_2) phthalic anhydride in 10 mL DMF, and this mixture was heated to 130°C in N_2 atmosphere with stirring. After 8 h of reaction, the solution was filtered and the filtrated solution was poured into ice water. The precipitate was collected and excessively washed with methanol.

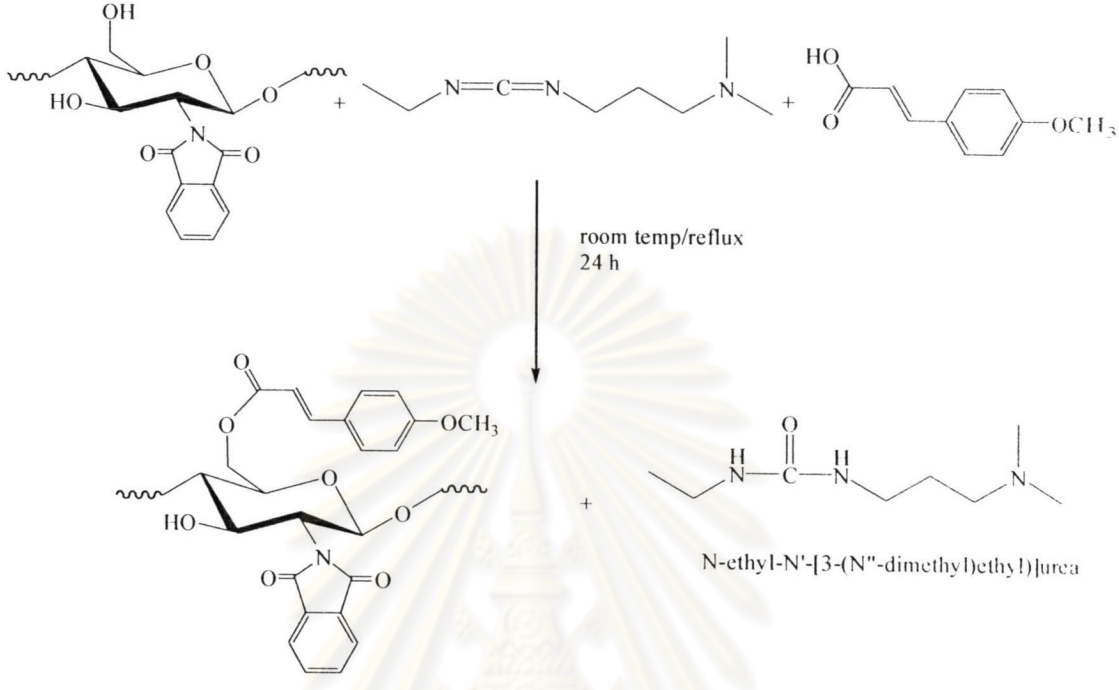
2.4 Grafting of 4-methoxycinnamic acid on Chitosan

2.4.1. A method using N,N'-dicyclohexylcabodiimide (DCC) coupling agent



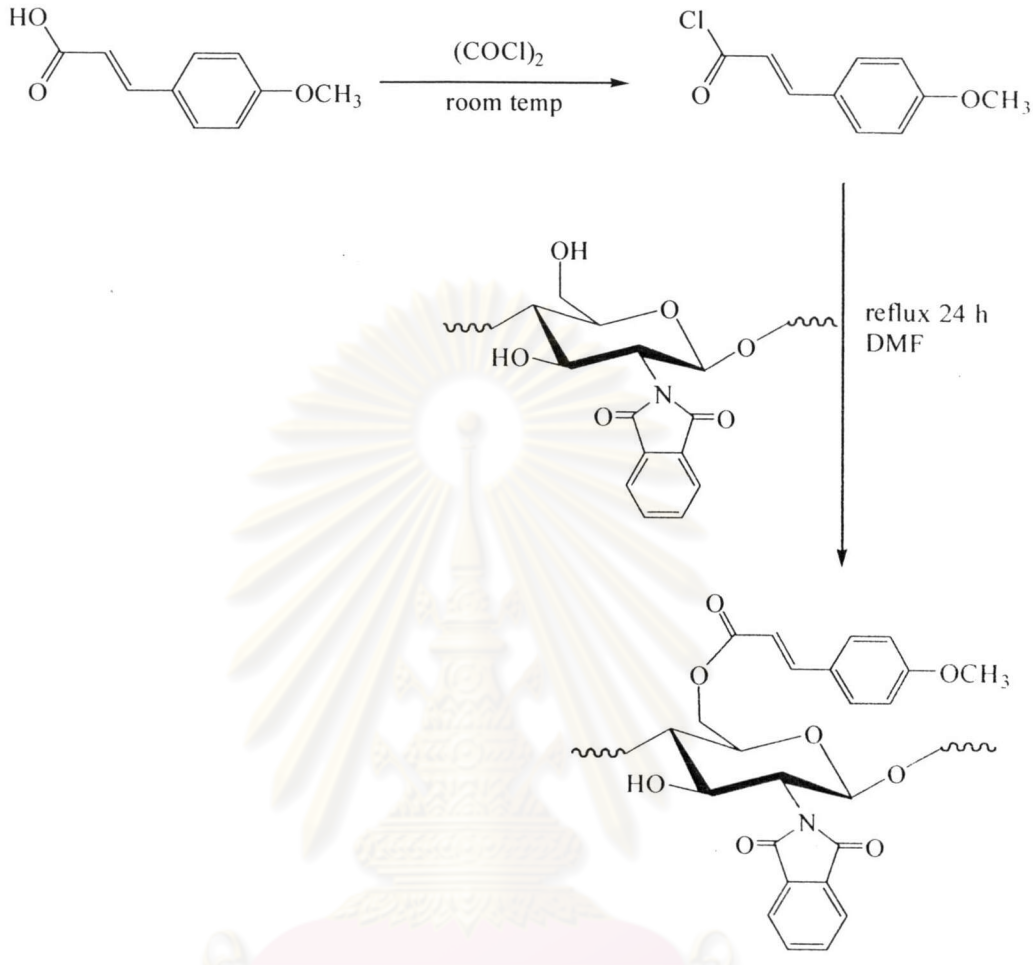
4-Methoxycinnamic acid (0.54 g, 3×10^{-3} mol) and 0.87 g (3×10^{-3} mol equivalent of hydroxyl group) phthaloylchitosan were dissolved in 20 mL DMF and DCC (0.62 g, 3×10^{-3} mol) was added. The mixture was stirred/refluxed at room temperature/130°C for 24 hours. Then the mixture was poured into 100 mL of 5% aqueous hydrochloric acid solution. The precipitate was washed with methanol and dried in descicator before subjected to ^{1}H-NMR analysis.

2.4.2 A method using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) coupling agent



4-Methoxycinnamic acid (0.54 g, 3×10^{-3} mol) and 0.87 g (3×10^{-3} mol equivalent of hydroxyl group) and phthaloylchitosan were dissolved in 20 mL DMF and EDCI (0.63 g, 4.38×10^{-3} mol) was added. The mixture was stirred/refluxed at room temperature/130°C for 24 hours. Then the mixture was poured into 100 mL of 5% aqueous hydrochloric acid solution. The precipitate was washed with methanol and dried in descicator before subjected to ^{1}H-NMR analysis.

2.4.3 Acid chloride Method



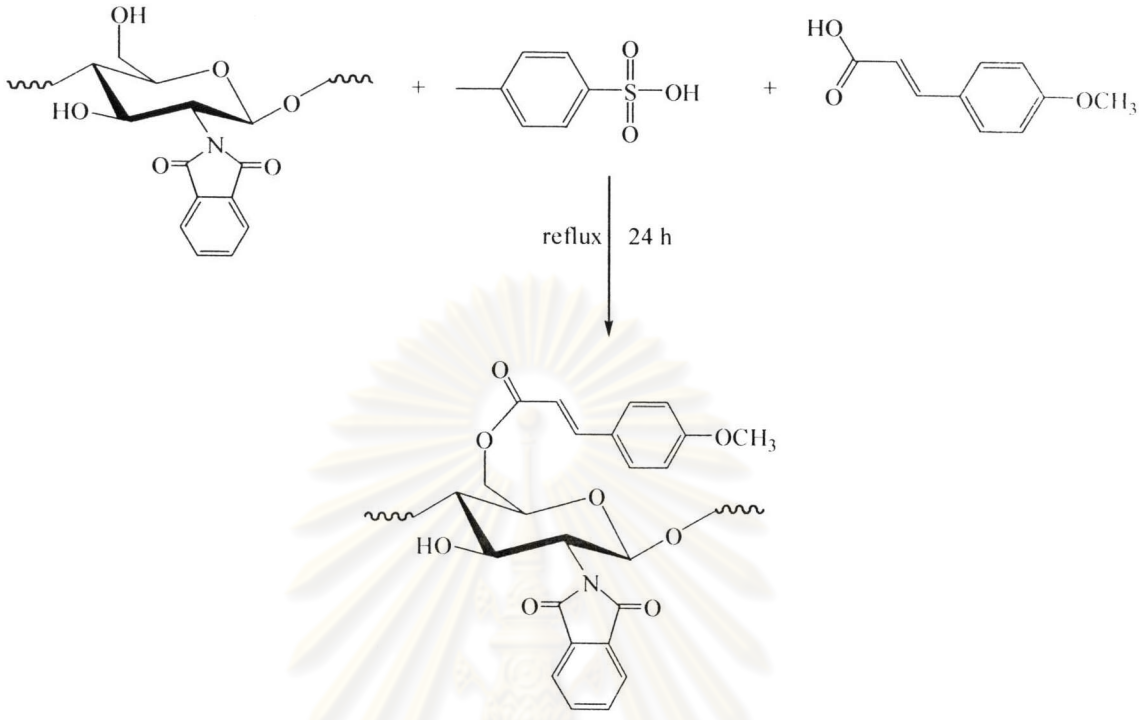
Preparation of 4-methoxycinnamoyl chloride

A mixture of 0.18 g (0.01 mol) 4-methoxycinnamic acid and 0.13 g (0.01 mol) oxalyl chloride in 20 mL dichloromethane was stirred at room temperature for 30 minutes in round bottom flask connected with a condenser with a gas absorption trap (K_2CO_3) at the top. A mixture was stirred until no further evolution of hydrogen chloride (60-90 minutes). The solvent was removed by rotary evaporator.

Reaction of 4-methoxycinnamoyl chloride with phthaloylchitosan

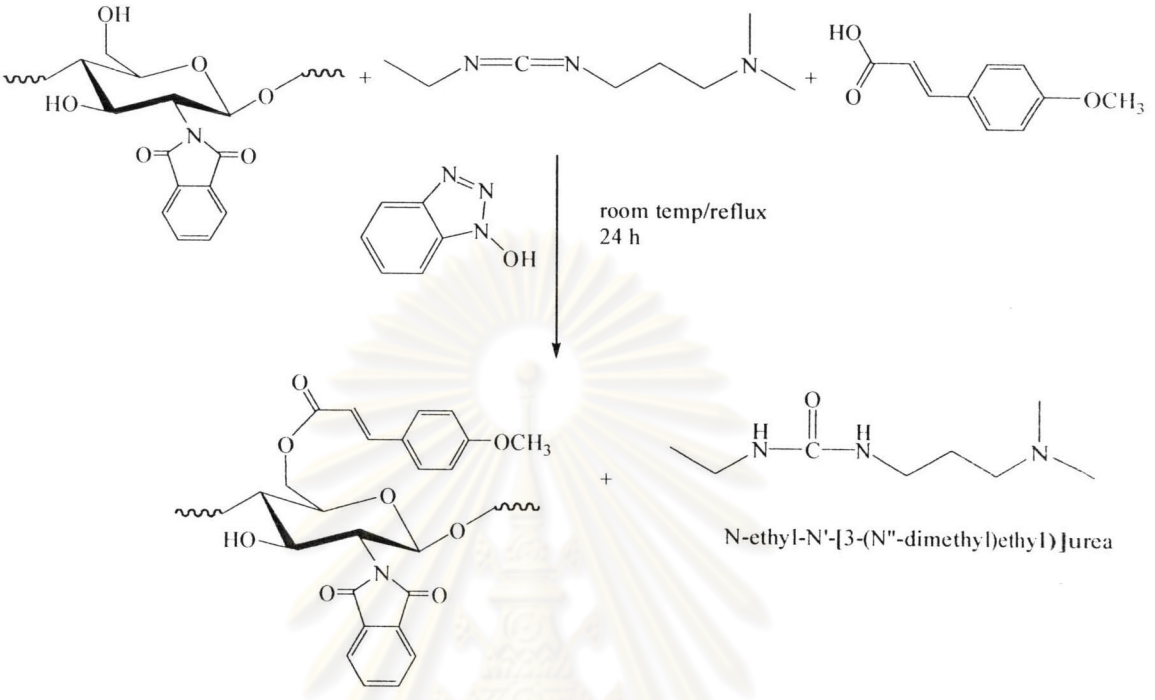
Under N_2 atmosphere, a solution containing 0.29 g phthaloylchitosan and 10 mL DMF was slowly dropped into freshly prepared 4-methoxycinnamoyl chloride with stirring. After completion, the mixture was refluxed for 24 hours (still under N_2 atmosphere) before it was poured into water. Precipitate formed was washed with 100 mL of 5% aqueous hydrochloric acid solution.

2.4.4 A method using toluene-4-sulphonic acid as catalyst



A mixture of 0.2 g (1×10^{-3} mol) 4-methoxycinnamic acid, 0.3 g (1×10^{-3} mol) phthloylchitosan and 0.02 g toluene-4-sulphonic acid monohydrate in 20 mL DMF was refluxed at about 130°C. After the reaction was completed (checked by TLC, about 24 h), the reaction was cooled to room temperature. Precipitate in the reaction mixture was filtered, washed with 5% sodium bicarbonate solution, dried and subjected to ^{1}H-NMR analysis. The liquid was then poured into ice-cold water to induce more precipitate (but no precipitate was formed).

2.4.5 A method using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 1-hydroxy benzotriazole (HOBt) coupling agent



A mixture containing 4-methoxycinnamic acid (0.26 g, 0.4 mol equivalent to phthaloylchitosan), phthaloylchitosan 1.08 g (3.71×10^{-3} mol), HOBt (0.60 g, 3 mol) and EDCI (0.92 g, 3 mol) in 20 mL DMF was stirred at room temperature overnight. Then the mixture was poured into 5% aqueous hydrochloric acid solution and washed thoroughly with methanol to obtain yellow precipitate. The precipitate was then dried and analyzed by ^{1}H-NMR and IR spectroscopy.

B. Irradiated Chitosan

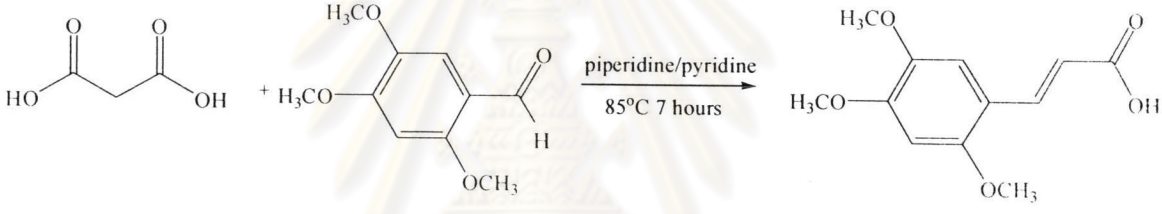
2.5 Phthaloylation of irradiated chitosan

Phthaloylation of irradiated chitosan was prepared using similar procedure to that described previously for non irradiated chitosan (2.3).

2.6 Grafting of 4-methoxycinnamic acid on irradiated chitosan

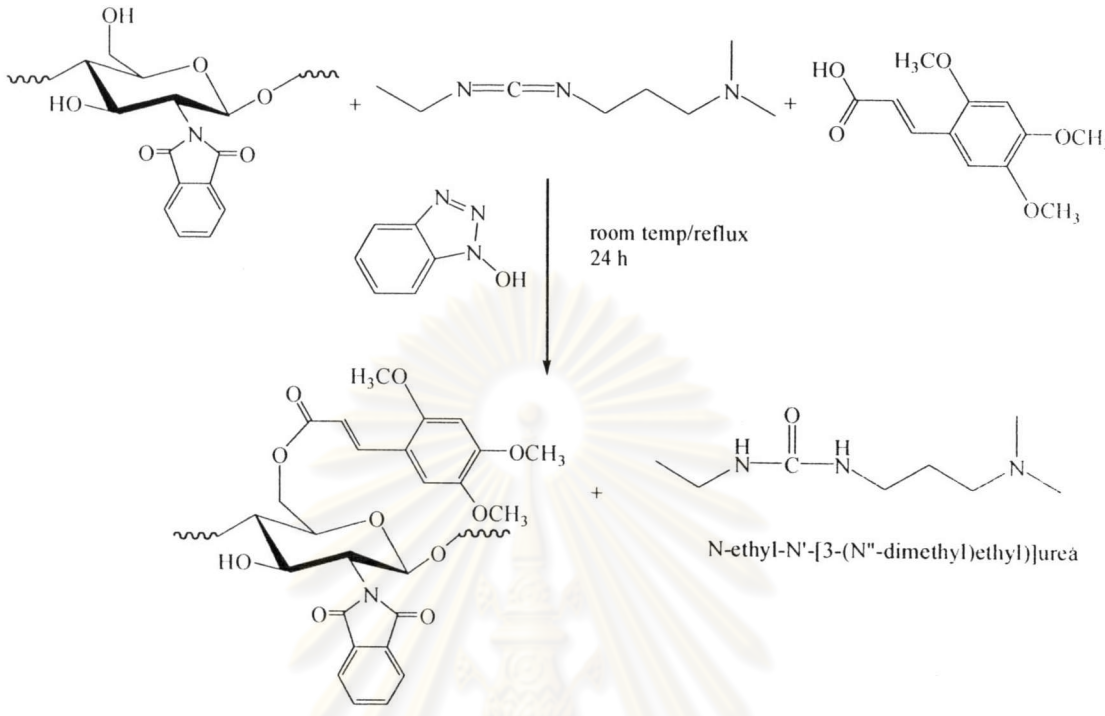
EDCI and HOBt coupling method was chosen for grafting 4-methoxy cinnamic acid onto irradiated chitosan. The procedure used for grafting 4-methoxycinnamic acid onto non-irradiated chitosan (described in 2.4.5) was also used for grafting the irradiated chitosan.

2.7 Synthesis of 2,4,5-trimethoxycinnamic acid[42]



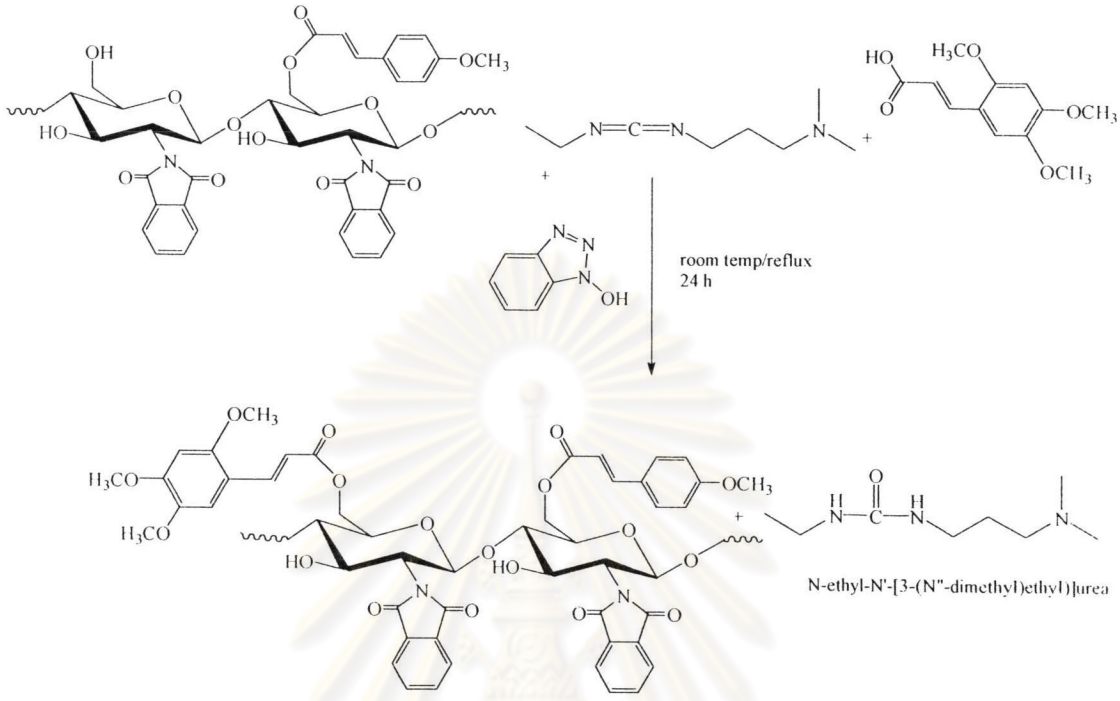
Malonic acid (2.08, 0.02 mol) was dissolved in 5 mL of pyridine and 0.01 mol 2,4,5-trimethoxybenzaldehyde and 0.15 mL piperidine were added. The mixture was refluxed for 4.5 hours at 70-75°C. After being cooled, the reaction mixture was poured into a beaker cantaining 40 mL of cold water. The mixture was acidified by slowly adding with 5 mL of concentrated hydrochloric acid. The solid was separated by suction filtration, washed with cold water and recrystallized with ethanol before subjected to ^{1}H-NMR analysis.

2.8 Grafting of 2,4,5-trimethoxycinnamic acid on chitosan



EDCI and HOBt coupling method was chosen for grafting 2,4,5-trimethoxycinnamic acid onto irradiated chitosan. Similar procedure to that used for grafting 4-methoxycinnamic acid (see 2.6) was used.

2.9 Grafting of 2,4,5-trimethoxycinnamic acid on 4-methoxycinamoyl-phthaloylchitosan



EDCI and HOBt coupling method was chosen for grafting 2,4,5-trimethoxycinnamic acid onto the grafted 4-methoxycinnamoyl-phthaloylchitosan. Similar procedure to that used for grafting 4-methoxycinnamic acid (see 2.6) was used.

2.10 General Procedure for Molar Absorptivity Measurements[43,44]

Tested compounds were dissolved in DMSO to concentration of 1 g/L. The resulting stock solution was then diluted to selected concentrations using corresponding solvents. The UV absorbance of each final dilution was recorded by scanning wavelength between 200 and 800 nm. The molar absorptivity (ε) at the wavelength of maximum absorbance (λ_{max}) was calculated using Beer's law:

$$A = \varepsilon bc$$

Where A is absorbance

b is the cell path length (1 cm)

c is the concentration of the adsorbing species in mol per litre

2.11 General Procedure for Photostability Test[44]

The photostability tests were performed in DMSO. Stock solution of each compound was prepared in a 100 mL volumetric flask. The resulting solutions were divided into two parts. One part was kept away from light (covered with foil) at room temperature (dark sample) while the other part was irradiated by artificial UV lamp which UVA at 5.8 mW/cm^2 and UVB at 0.47 mW/cm^2 (irradiated sample). Then UV absorption profile of each sample was acquired using UV/VIS spectrometer. The absorbances of irradiated sample at various irradiant times were compared to those of dark samples.

The calculation of percent relative absorbance of each irradiated sample is given by:

$$\text{Percent relative absorbance} = \left[\frac{\text{Absorbance of irradiated sample at time X}}{\text{Absorbance of dark sample (starting time)}}\right] \times 100$$