Preparation of Amino-cyclosophoraoses from the Neutral Cyclosophoraoses Isolated from *Rhizobium leguminosarum bv. trifolii*

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Received May 30, 2006

Key Words : Cyclosophoraoses, Amino-cyclosophoraoses

Cyclosophoraoses are a class of unbranched cyclic oligosaccharides composed of β-1,2-D-glucans varying in size from 17 to 40 in a neutral or anionic form. They were originally found in fast growing soil bacteria, *Agrobacterium* and *Rhizobium* species, as *intra*- or *extra*-oligosaccharides.[1,2] Cyclosophoraoses are synthesized in the cytosol and transported to the periplasmic space where they play an important role in regulating the osmolarity in response to external osmotic shock.[3] They are also known to be involved in the initial stage of root-nodule formation of *Rhizobium* species during nitrogen fixation.[4] Throughout this interaction, cyclosophoraoses are suspected to be involved in complexation with various plant flavonoids.[5] Thus, much attention has been focused not only on their biological functions but also on their potential ability to form inclusion complexes with other molecules. Several reports have shown that neutral cyclosophoraoses or anionic cyclosophoraoses have good potential as a host molecule in various inclusion complexation technologies such as a solubility enhancer[6-10] and a chiral selector.[11] In addition, the investigations into the chemical modifications of neutral cyclosophoraoses have been concerned with modifying their binding behaviors, *e.g.*, carboxymethylated and sulfated cyclosophoraoses which were successfully used as a solubility enhancer[12] and chiral selector,[13,14] respectively. Their modifications are of particular importance for the investigation at the frontier of various research fields ranging from supramolecular chemistry to analytical techniques.

In this study, for further application of cyclosophoraoses, neutral cyclosophoraoses were modified with tosyl, azide and amino groups through the chemical derivatization, and their modified structures were confirmed by nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy.

Isolation, purification and structural analyses of neutral cyclosophoraoses were carried out as described previously.[12,15,16] Purified neutral cyclosophoraoses were separated with an R_f value of 0.125 on thin layer chromatography (TLC, ethanol : butanol : water = 5 : 5 : 4, v/v/v). Through matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, we confirmed that the ring sizes of the neutral cyclosophoraoses ranged from degree of polymerization (DP) 17 to 23 (data not shown).[4] Although the exact three-dimensional structure of cyclosophoraoses is not known, several NMR studies and molecular dynamics simulations have provided molecular models with flexible glycosidic linkage backbones.[17-20] The cyclosophoraoses seem to have narrower cavity sizes than those expected from their bulky ring sizes (Figure 1A).

The amino-cyclosophoraoses were obtained through three steps, tosylation (tosyl-cyclosophoraoses), azidation (azido-cyclosophoraoses) and amination (Figure 1B). First, the hydroxyl groups of neutral cyclosophoraoses were subjected to chemical modification with *p*-toluenesulfonyl chloride[21] and the reaction was monitored on TLC. The R_f value of the purified tosyl-cyclosophoraoses was 0.241 (ethanol : butanol : water = 5 : 5 : 4, v/v/v). The tosyl-cyclosophoraoses was synthesized in a 25 percent yield. Next, the azido-cyclosophoraoses were obtained from tosyl-cyclosophoraoses which were treated with NaN_3 in water (90 percent yield). And then the azido-cyclosophoraoses and triphenylphos-

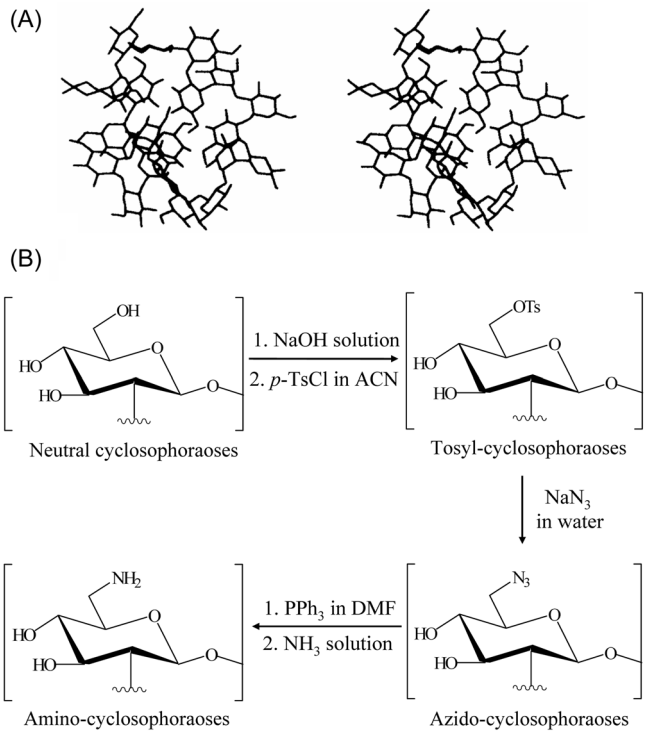

Figure 1. (A) Stereoview of molecular model of neutral cyclosophoraoses.[18] (B) Three-step synthesis of amino-cyclosophoraoses from neutral cyclosophoraoses.

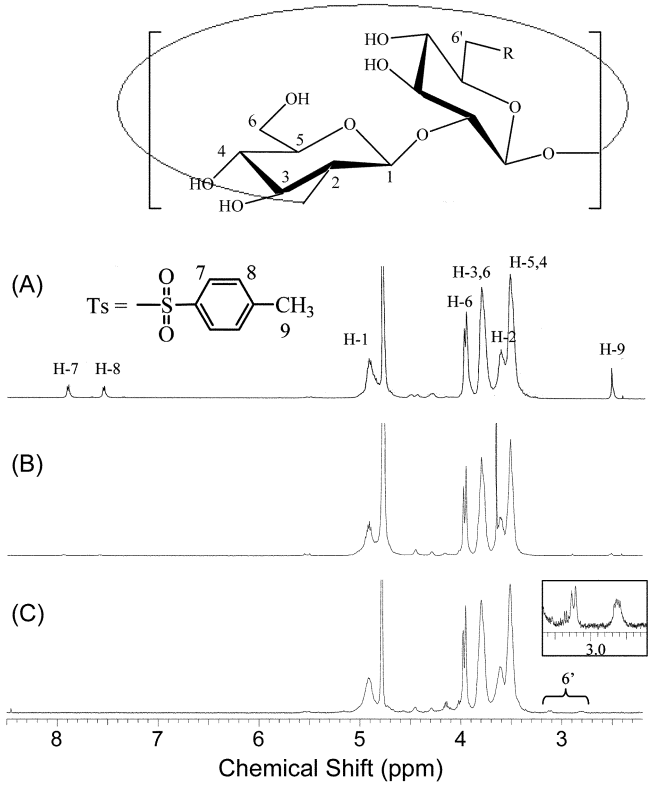

Figure 2. ^{1}H NMR spectra of cyclosophorases derivatives. Tosyl-cyclosophorases (A), azido-cyclosophorases (B), amino-cyclosophorases (C).

phine were dissolved in dimethylformamide, to which aqueous NH_3 was added. Finally, the amino-cyclosophoraoses were acquired in an 85 percent yield.[22]

The structures of three cyclosophoraoses derivatives were characterized with NMR and FTIR spectroscopy. The ^{1}H NMR spectra of these cyclosophoraoses derivatives are shown in the Figure 2. In the ^{1}H NMR spectra, each resonance of H-1 to H-6 protons of the cyclosophoraoses derivatives was assigned in the 4.96-3.52 ppm region. The aromatic proton peaks of toluenesulfonyl groups as substituents appeared at 7.95 and 7.59 ppm and the absorption of methyl hydrogens was also seen at 2.52 ppm. (Figure 2A). The degree of substitution (DS) of the tosyl-cyclosophoraoses was estimated from the area ratio of H-7 (H-8 or H-9) to H-1 signals in the ^{1}H NMR spectrum recorded in deuterium oxide. As a result, we deduced its degree of substitution to be approximately 0.014-0.019. In the ^{1}H NMR spectrum of azido-cyclosophoraoses, the protons for the tosyl group were disappeared at 2-3 ppm and 7-8 ppm (Figure 2B). The peaks of unmodified C-6 (CH_2OH) and modified C-6' (CH_2N_3) carbons were observed at 63.5 and 62.2 ppm in the ^{13}C NMR spectrum of azido-cyclosophoraoses (data not shown). In the spectrum of amino-cyclosophoraoses, the signals (H-6') of the H-6 protons with an amino group were observed at about 0.8 ppm lower fields than those of H-6 protons of other rings (Figure 2C), and appeared as a ddd signal due to an AB pattern (δ3.13, 3.11, 2.84, 2.82, 2.81 and 2.79 ppm).[22]

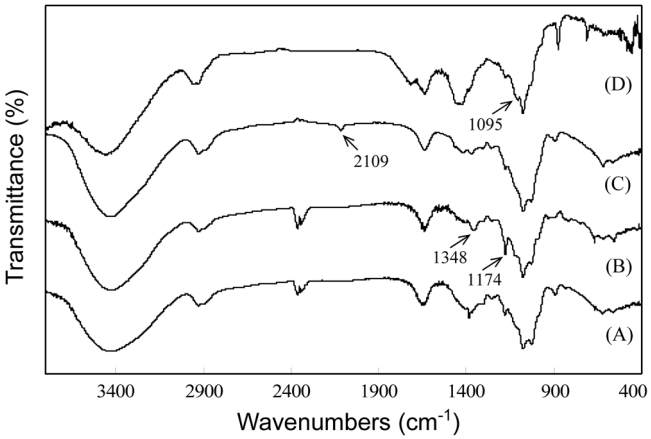

Figure 3. FTIR spectra of neutral cyclosophorases (A) and their derivatives. Tosyl-cyclosophoraoses (B), azido-cyclosophoraoses (C), amino-cyclosophoraoses (D). Spectra were acquired between 4000 and 400 cm^{-1}.

Figure 3 shows the FTIR spectra of neutral cyclosophoraoses and their derivatives. In the spectrum of tosyl-cyclosophoraoses, the 1348 and 1174 cm^{-1} bands resulted from the S=O asymmetric and symmetric stretching vibrations, respectively. The several bands (985, 925, 835 and 748 cm^{-1}) for S-O stretching also occurred in the range 1000-750 cm^{-1}, indicating that the sulfonates were successfully formed as the *p*-toluenesulfonyl groups were substituted at the hydroxyl positions of neutral cyclosophoraoses. These stretching modes of tosyl groups were not detected but azide (N_3) band was observed at 2109 cm^{-1} in the spectrum of azido-cyclosophoraoses. The disappearance of azide band at 2109 cm^{-1} was shown and the C-N stretching absorption was newly observed at 1095 cm^{-1} in the spectrum of amino-cyclosophoraoses.

In this study, we investigated the synthesis of amino-cyclosophoraoses from the isolated neutral cyclosophoraoses through the three steps of modification (tosylation, azidation and amination) and performed the structural analysis for synthesized cyclosophoraoses derivatives with NMR and FTIR spectroscopy. The amino-cyclosophoraoses were confirmed to have DS values ranging from 0.014-0.019. The amino groups were substituted on the hydroxyl portions of neutral cyclosophoraoses at the position 6. The introduction of amino group is interesting for increased association with anionic molecules relative to the neutral or anionic cyclosophoraoses. The amino groups may provide the additional ability for electrostatic interaction and hydrogen bonding of amino-cyclosophoraoses and guest molecules.[23] A few papers related to the synthesis and application of cyclosophoraoses derivatives modified with anionic functional groups were reported.[12-14,23] In particular, it has been known that the carboxymethylated cyclosophoraoses showed a conformational change because of the way the charge distribution of the weak acidic carboxymethyl group varies according to the aqueous pH conditions.[24,25] The three-dimensional structures of amino-cyclosophoraoses can be also regulated corresponding to the external pH

change and its conformational changes will affect the efficiency of the interaction behavior with guest molecules. Therefore, the amino-cyclosophoraoses may have potential for the development of solubility enhancers or chiral selectors based on the external pH, especially for anionic molecules. Further study on amino-cyclosophoraoses as pH-dependent chiral selectors will be performed for various anionic enantiomers, compared with the neutral or anionic cyclosophoraoses.

Experimental Section

Materials and apparatus. All the chemicals (Figure 1B) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). NMR spectroscopic analysis was performed on a Bruker AMX spectrometer (operated at 500 MHz for ^{1}H, 125 MHz for ^{13}C) at 25 °C. The purified neutral cyclosophoraoses and their derivatives were dissolved in deuterated water (D_2O, 99.96%). All NMR measurements were performed with 0.7 mL samples in 5 mm NMR tubes. Chemical shifts were reported relative to a trace of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 0.000 ppm, with an accuracy of ± 0.002 ppm. Fourier-transform infrared spectra were obtained on a JASCO FTIR-300E spectrometer (REV, USA). The samples were dried under vacuum for 1 h and the 1.5-2.0 mg of the purified samples was mixed with a KBr pellet.

Bacterial cultures and conditions. *R. leguminosarum bv. trifolii* was used to produce cyclosophoraoses. Precultures were prepared by inoculating the organism into standard GMS media.[15] Cells from the late exponential phase were inoculated into 1-L Erlenmeyer flasks containing 500 mL of 3 × GMS media. The cells were incubated on a rotary shaker (150 rpm) for 12 days at 25 °C and pH 7.0.

Isolation of cyclosophoraoses. After 10 days, extracellular polysaccharides (EPS) and cells in the culture were precipitated by the addition of 3 volumes of ethanol. After centrifugation (8000 rpm, 10 min), the 75 percent alcoholic supernatant was concentrated by rotatory evaporation under vacuum to about 1/10 of the original volume. Up to 8 volumes of ethanol was then added to the remaining solution. While this solution stood overnight at 4 °C, a white precipitate of cyclosophoraoses was formed and collected by centrifugation (8000 rpm, 10 min). The precipitate was resuspended in 2 mL of a mixture of ethanol, butanol and water (5 : 5 : 4, v/v/v). The sample was loaded onto a flash column[16] packed with Silica Gel 60 (4 × 25 cm, 400-230 mesh, E. Merck) and eluted with the same solvent using air pressure to maintain a 1.5 mL/min flow rate. The fractions containing cyclosophoraoses were desalted on a column (2 × 27 cm) packed with Sephadex G-10. The purified cyclosophoraoses were confirmed on TLC (E. Merck), and by NMR spectroscopy and MALDI-TOF mass spectrometry.

Preparation of amino-cyclosophoraoses from neutral cyclosophoraoses. As shown in Figure 1B, the amino-cyclosophoraoses were prepared by reduction of azido-cyclosophoraoses, this in turn being obtained from the tosyl-cyclosophoraoses. The neutral cyclosophoraoses (100 mg) were suspended in 0.8 mL of water, and NaOH (13 mg) in 0.2 mL of water was added. The suspension became homogeneous and slightly yellow before the addition was complete. *p*-toluenesulfonyl chloride (*p*-TsCl, 110 mg) in 0.1 mL of acetonitrile (ACN) was added, causing immediate formation of a white precipitate.[21] After 4 h of stirring at 25 °C the precipitate was removed by filtration and the filtrate was treated by 8 volumes of acetone. The resulting white precipitate dissolved in water was purified on a flash column packed with Silica Gel 60 (4 × 15 cm, 400-230 mesh, E. Merck). The tosyl-cyclosophoraoses and neutral cyclosophoraoses were separated with the mixture of ethyl acetate, isopropyl alcohol and water (7 : 7 : 5, v/v/v). The flow rate was 40 mL/min. The fractions of tosyl-cyclosophoraoses were concentrated and desalted on a column (2 × 27 cm) packed with Sephadex G-10. The above tosyl-cyclosophoraoses were added to a solution of 10 equivalents of sodium azide (NaN_3) in water (0.5 mL). The mixture was stirred for 5 h at 80 °C. The azido-cyclosophoraoses were precipitated by addition of acetone and filtered. Next, azido-cyclosophoraoses (20 mg) and triphenylphosphine (PPh_3, 30 mg) were dissolved in 0.5 mL of dimethylformamide (DMF), to which aqueous NH_3 (24 equivalents) was added, and the solution was stirred at room temperature for 5 h. The amino-cyclosophoraoses precipitated by addition of acetone were filtered and purified with ion-exchange column chromatography (CM Sephadex C25) using 0.1 M NH_4HCO_3 as the eluent. The fractions containing amino-cyclosophoraoses were desalted and dried.[22] To identify three cyclosophoraoses derivatives, we used NMR and FTIR spectroscopy.

Acknowledgement. This research was supported by the National R&D Project for Biodiscovery in MOST 2004 and partially supported by the Korea Research Foundation (KRF-2006-005-J03402). SDG.

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