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

**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. They 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. They are also known to be involved in the initial stage of root-nodule formation of *Rhizobium* species during nitrogen fixation. Throughout this interaction, cyclosophoraoses are suspected to be involved in complexation with various plant flavonoids. 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 and a chiral selector. 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 and a chiral selector 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. Purified neutral cyclosophoraoses were separated with an *Rf* 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). 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. 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 and the reaction was monitored on TLC. The *Rf* 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 NaN₃ in water (90 percent yield). And then the azido-cyclosophoraoses and triphenylphosphine...
Phenyl groups were dissolved in dimethylformamide, to which aqueous NH₃ was added. Finally, the amino-cyclosphorases were acquired in an 85 percent yield.

The structures of three cyclosphorases derivatives were characterized with NMR and FTIR spectroscopy. The ¹H NMR spectra of these cyclosphorases derivatives are shown in the Figure 2. In the ¹H NMR spectra, each resonance of H-1 to H-6 protons of the cyclosphorases 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-cyclosphorases was estimated from the area ratio of H-7 (H-8 or H-9) to H-1 signals in the ¹H NMR spectrum recorded in deuterium oxide. As a result, we deduced its degree of substitution to be approximately 0.014-0.019. In the ¹H NMR spectrum of azido-cyclosphorases, the protons for the tosyl group were disappeared at 2-3 ppm and 7-8 ppm (Figure 2B). The peaks of unmodified C-6 (CH₂OH) and modified C-6' (CH₂N₃) carbons were observed at 63.5 and 62.2 ppm in the ¹³C NMR spectrum of azido-cyclosphorases (data not shown). In the spectrum of amino-cyclosphorases, 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 (δ 3.13, 3.11, 2.84, 2.82, 2.81 and 2.79 ppm).

Figure 3 shows the FTIR spectra of neutral cyclosphorases (A) and their derivatives. Tosyl-cyclosphorases (B), azido-cyclosphorases (C), amino-cyclosphorases (D). Spectra were acquired between 4000 and 400 cm⁻¹.

In this study, we investigated the synthesis of amino-cyclosphorases from the isolated neutral cyclosphorases through the three steps of modification (tosylation, azidation and amination) and performed the structural analysis for synthesized cyclosphorases derivatives with NMR and FTIR spectroscopy. The amino-cyclosphorases were confirmed to have DS values ranging from 0.014-0.019. The amino groups were substituted on the hydroxyl portions of neutral cyclosphorases at the position 6. The introduction of amino group is interesting for increased association with anionic molecules relative to the neutral or anionic cyclosphorases. The amino groups may provide the additional ability for electrostatic interaction and hydrogen bonding of amino-cyclosphorases and guest molecules. A few papers related to the synthesis and application of cyclosphorases derivatives modified with anionic functional groups were reported. In particular, it has been known that the carboxymethylated cyclosphorases showed a conformational change because of the way that the charge distribution of the weak acidic carboxymethyl group varied according to the aqueous pH conditions. The three-dimensional structures of amino-cyclosphorases 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 1H, 125 MHz for 13C) at 25 °C. The purified neutral cyclosophoraoses and their derivatives were dissolved in deuterated water (D2O, 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 3H, 22, 26-dimethyl-2, 4, 6-trimethyl-4H-pyran-3-yl 3-methyl-3-butenyl-2, 6-diol (DSS) at 0.00 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 trifolii was used to produce cyclosophoraoses. Precultures formed with 0.7 mL samples in 5 mm NMR tubes. (150 rpm) for 12 days at 25 °C

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 x 27 cm) packed with Sephadex G-10. The above tosyl-cyclosophoraoses were added to a solution of 10 equivalents of sodium azide (NaN3) 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 (PPh3, 30 mg) were dissolved in 0.5 mL of dimethylformamide (DMF), to which aqueous NH3 (24 equivalents) was added, and the solution was stirred at room temperature for 5 h. The amino-cyclosophoraoses precipitated by addition of acetone and filtered and precipitated with ion-exchange column chromatography (CM Sephadex C25) using 0.1 M NH4HCO3 as the eluent. The fractions containing amino-cyclosophoraoses were desalted and dried.22 To identify three cyclosophoraoses derivatives, we used NMR and FTIR spectroscopy.

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References