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Carbohydrate Research 337 (2002) 1785–1789

CARBOHYDRATE RESEARCH

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Note

^{13}C NMR spectroscopic analysis on the chiral discrimination of *N*-acetylphenylalanine, catechin and propranolol induced by cyclic-(1→2)-β-D-glucans (cyclosophoraoses)

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Received 10 June 2002; accepted 13 August 2002

Abstract

Cyclosophoraoses (cyclic-(1→2)-β-D-glucans) produced by *Rhizobium meliloti* were used as a novel chiral NMR solvating agent. ^{13}C NMR spectroscopic analysis as an enantiodiscriminating tool was carried out where NMR signal splittings were observed on the interactions of cyclosophoraoses with the enantiomers of *N*-acetylphenylalanine, catechin and propranolol. The ^{13}C chemical shifts of cyclosophoraoses induced by the enantiomeric interactions predominantly occurred at the C-1 and C-2 carbons associated with the β-glycosidic linkage.

Keywords: Cyclosophoraoses; *Rhizobium meliloti*; Chiral NMR solvating agent; Enantiomers; Chiral recognition

Cyclosophoraoses are a family of unbranched cyclic-(1→2)-β-D-glucans produced as intra- or extraoligosaccharides by many strains of *Rhizobium* and *Agrobacterium* as a mixture of large-ring molecules consisting of a variable number of glucose residues (17–40) per ring.[1,7] The first report of cyclosophoraoses came in 1942 with their discovery in the extracellular media of *Agrobacterium tumefaciens* cultures.[2] They have also been reported to be involved in the symbiotic interaction between the *Rhizobiaceae* family and its specific symbiotic plant such as alfalfa, clover, and soybean.[1] Recently, cyclosophoraoses synthesized by *R. meliloti* have been applied as solubility enhancers for poorly soluble molecules.[3] Although their exact three-dimensional structures have not been elucidated, recent NMR[4a] or conformational reports[4] indicated that cyclosophoraoses seem to have flexible backbone structures and narrower cavity sizes than those expected from their bulky ring sizes (Fig. 1).

The increasing demand of the chemical and pharmaceutical industries for enantiomerically pure compounds has spurred the development of a range of so-called chiral technologies. The separation of the enantiomers of physiologically active chiral compounds and their individual determinations are of prime importance in the various areas such as pharmaceutical, clinical and toxicological studies. Various chiral resolving agents, including cyclomaltooligosaccharides (cyclodextrins, CDs), macrocyclic antibiotics and some proteins have currently been used for the discrimination and/or separation of enantiomers.[5] Among the abundance of those agents, CDs have been also the most widely employed as chiral NMR solvating agents.[6]

Here, we report for the first time that cyclosophoraoses can function as a novel NMR chiral solvating agent to resolve the enantiomers of an α-amino acid derivative, *N*-acetylphenylalanine, a plant isoflavonoid, catechin, and a β-adrenergic blocker, propranolol.

Neutral cyclosophoraoses were isolated and purified from the *R. meliloti* 2011 using several chromatographic techniques, and the exact structures were elucidated by NMR spectroscopic analysis.[3c–e] The ring distributions of the neutral cyclosophoraoses were confirmed through MALDI-MS analysis, ranging from DP 17 to 27.[3c] Based on the MALDI-MS analysis, the

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00286-0

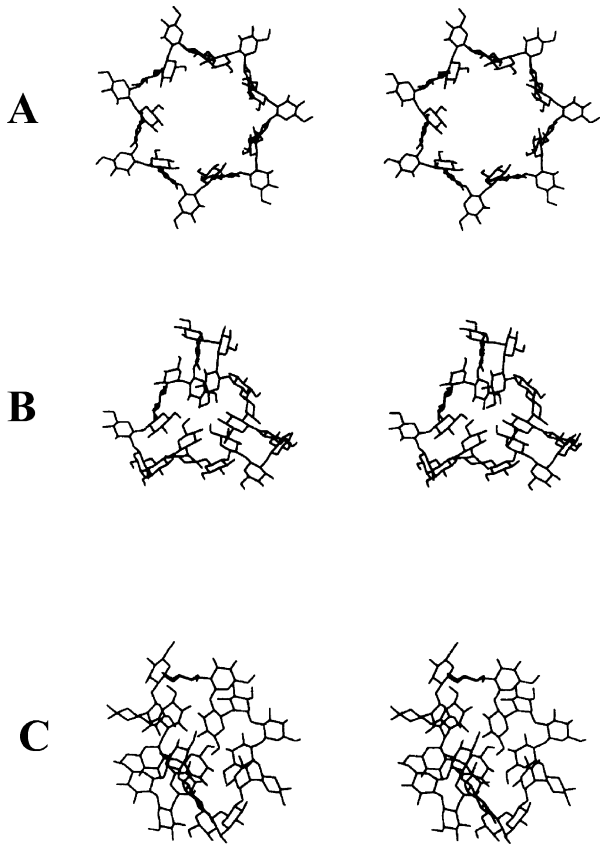

Fig. 1. Stereoviews of molecular models of neutral cyclosophoraoses proposed by (A) Palleschi and Crescenzi,[17] (B) York et al.,[18] and (C) Jung and co-workers.[4b]

number average molecular weight of a family of neutral cyclosophoraoses was determined as 3568.6.[3c]

NMR spectroscopic analysis enables the discrimination on the differential interactions of each enantiomer with a chiral solvating agent in liquid phases.[9] The NMR signals observed in binary selector-solute solutions are time-averaged signals of both the complexed and uncomplexed substances. These can give rise to the shift displacements coupled with shift nonequivalence.

These phenomena were observed on the ^{13}C NMR spectra when neutral cyclosophoraoses were mixed with the enantiomers. Fig. 2 is the spectra showing the chiral recognitions of *N*-acetylphenylalanine, catechin and propranolol by neutral cyclosophoraoses. Upon the complexation of cyclosophoraoses with the α-amino acid derivative, *N*-acetylphenylalanine, the resonance of the carbon signal (δ 54.39) in position-2 of the derivatized phenylalanine enantiomers was split (Fig. 2A) in D_2O containing 10% CD_3OD at pD 6.5, and a significant chemical shift ($\Delta\delta$ 1.20) was observed downfield. The signal of the split carbon was also broadened in the presence of cyclosophoraoses, which suggested that the enantiomers were bound to the cyclosophoraoses,[6g] and the interaction was thus enantioselective. Although no shift nonequivalence was observed for the other carbons, the amide and carboxyl carbonyl carbons of the derivatized phenylalanine showed more significant shift displacements ($\Delta\delta$ -1.71 and 3.02, respectively) than the aromatic carbons (data not shown). Chiral recognition of α-amino acid derivatives, including *N*-acetylphenylalanine, has also been reported for the polyprotonated CDs.[6g]

In the case of catechin, there were also splittings of the carbon signals (δ 145.53 and 117.11, respectively) in positions 4′ and 5′ due to the different interactions of each enantiomer with the cyclosophoraose (Fig. 2B) in D_2O containing 30% CD_3OD at pD 6.5. No definite results on the chiral separation of the catechin enantiomers using cyclic oligosaccharides have been reported. Only an enantiomeric separation with high-performance liquid chromatography based on a chiral amine-bonded silica gel has been reported.[16]

Propranolol is an important β-adrenergic blocking agent[14] as the basic drug (pK_a 9.5). In earlier studies, the organic molecule[8c,14] and the derivatized CDs[8a,b] were applied to recognize the propranolol enantiomers in aqueous or nonaqueous solution by using either ^1H NMR spectroscopy[8] or capillary electrophoresis (CE)[15] as an enantioseparation tool. Fig. 2C shows that the ^{13}C signal splittings occurred when neutral cyclosophoraoses were added to the propranolol enantiomers in the acidic solution. Among the aliphatic carbon signals of the propranolol enantiomers, the carbon signals (δ 66.75, 52.05 and 47.83, respectively) in positions 12, 15 and 13 were shifted to the higher magnetic fields, and the complexation-induced chemical shifts of the (*R*)-(+) enantiomer were more remarkable than those of the (*S*)-(−) enantiomer (Fig. 2C), and shift displacements were also observed upfield ($\Delta\delta$ -0.51, -0.26 and -0.15, respectively).

As for the chiral recognition by cyclosophoraoses, the observed chemical shifts ($\Delta\delta$) on the carbons signals of the complexed cyclosophoraoses were entirely comparable to those without the corresponding enantiomers (Fig. 3). The C-1 and C-2 carbons, which are responsible for the glycosidic linkage of cyclosophoraoses, were noticeably shifted to the higher mag-

Fig. 2. Partial ^{13}C NMR spectra showing chiral recognitions of (A) *N*-acetylphenylalanine, (B) catechin and (C) propranolol enantiomers by neutral cyclosophoraoses. The spectra were obtained in equimolar solutions of 15 mM cyclosophoraoses with non-racemic mixtures of *N*-acetylphenylalanine (D/L = 2/3), catechin ((+)/(−) = 2/1) and propranolol ($R(+)/S(-) = 2/3$), respectively. Each spectrum contains partial carbon signals of the enantiomers in the absence (lower trace) and presence (upper trace) of cyclosophoraoses. NMR solvents are composed as follows: D_2O containing 10% CD_3OD at pD 6.5, for *N*-acetylphenylalanine; D_2O containing 30% CD_3OD at pD 6.5, for catechin; 10 mM NaH_2PO_4–D_2O buffer at pD 3.4 ± 0.1, for propranolol.

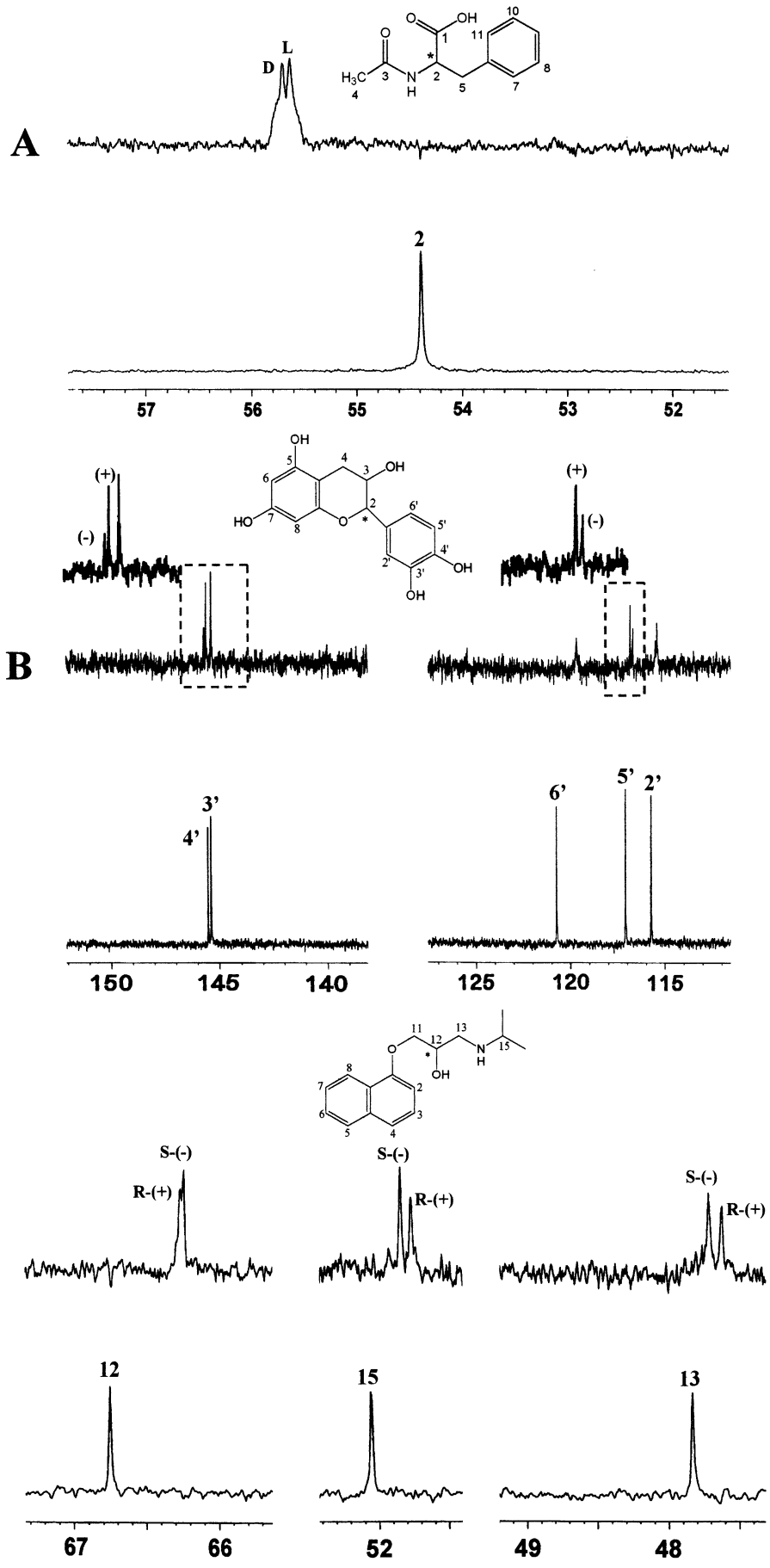
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Fig. 2.

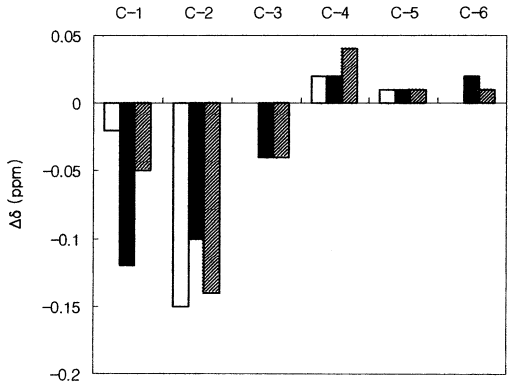

Fig. 3. Changes ($\Delta\delta$, ppm) in the ^{13}C NMR chemical shifts of neutral cyclosophoraoses upon interaction with the enantiomers. (□), *N*-acetylphenylalanine; (■), catechin; (▨), propranolol. NMR solvents for measurements are the same as in Fig. 2.

netic fields than the other carbons. The magnitude of the shifts was pronounced on C-2 carbon, followed by the C-1 carbon. This result suggested that the C-1 and C-2 carbons composed of glycosidic linkages actively participated in the enantiomeric interactions, which gave a possible molecular basis for the chiral discrimination induced by cyclosophoraoses.

A number of studies on chiral recognition by CDs and their derivatives, which are due to their easy availabilities, have been reported,[6] since the recognition of central chirality by β-CD was reported for the first time by Cramer and Dietsche.[10] In this study, we showed that cyclosophoraoses that belonged to a *Cn* point group recognized the chiralities of the three enantiomers among the several chiral drugs of which we investigated with cyclosophoraoses. Although the exact molecular mechanism of chiral recognition by cyclosophoraoses remains to be determined at present, our results suggest that the chiral recognition is likely to be induced by the active participation of the C-1 and C-2 carbons of the β-glycosidic linkages of cyclosophoraoses. Further research will be continued in this viewpoint.

1. Experimental

Preparation of neutral cyclosophoraoses.—*R. meliloti* 2011 was cultured in a 5-L jar fermenter containing GMS medium[11] at 30 °C for 72 h. Cells were separated from the culture supernatant by centrifugation at 3,000*g* for 20 min at 4 °C. Cell pellets were extracted in 75% ethanol at 70 °C for 30 min as described by Breedveld et al.[11] After centrifugation, the supernatant was concentrated and loaded onto a Sephadex G-50 column (1.5 × 110 cm). Fractions were assayed for total carbohydrate by the phenol–sulfuric acid method.[12] The glucan fraction was pooled, concentrated, and desalted by using a Sephadex G-15 column (2 × 27 cm). Neutral and charged cyclosophoraoses were separated on a DEAE-cellulose column (2 × 35 cm) by using a gradient of 0 to 200 mM KCl in 10 mM Tris–HCl (pH 8.4). A Sephadex G-15 column was used to desalt fractions corresponding to neutral cyclosophoraoses.

NMR spectroscopic analyses.—NMR spectroscopic analyses were carried out on a Bruker AVANCE 500 spectrometer for neutral cyclosophoraoses, *N*-acetylphenylalanine, catechin, and their complexes, or Bruker AVANCE 600 spectrometer for neutral cyclosophoraoses, propranolol, and their complexes, equipped with a *z* gradient probe head at 25 °C. All spectra were acquired with a 5-mm probehead. Proton-decoupled ^{13}C NMR experiments were carried out at 150.9 MHz ^{13}C frequency for propranolol and 125.7 MHz for *N*-acetylphenylalanine and catechin, respectively. Spinning tubes of 5 mm i.d. containing 0.7 mL of analytes were employed. Tetramethylsilane (TMS, Me_4Si) was used as an external reference, and chemical shifts were calibrated with an accuracy of 0.05 ppm.

Chemicals and reagents.—All chemicals, including enantiomers or racemates of *N*-acetylphenylalanine, catechin and propranolol, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). D_2O (99.9 atom% D) and CD_3OD (99.8 atom% D) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Preparation of the binary cyclosophoraose–enantiomer solutions.—*N*-Acetylphenylalanine, 15 mM, neutral cyclosophoraoses, 15 mM, and the non-racemic mixtures (D/L = 2/3) of the equimolar ratio were prepared in D_2O containing 10% CD_3OD at pD 6.5. Catechin 15 mM, neutral cyclosophoraoses, 15 mM, and the non-racemic mixtures((+)/(−)) = 2/1) of the equimolar ratio were prepared in D_2O containing 30% CD_3OD at pD 6.5. In case of propranolol, neutral cyclosophoraoses, 15 mM, racemic propranolol, 15 mM, and non-racemic mixture ($R(+)/S(-) = 2/3$) of the equimolar ratio were prepared in 10 mM NaH_2PO_4–D_2O buffer at pD 3.4 ± 0.1 and pD 7.4 ± 0.1 adjusted with DCl or NaOD. Each mixture was stirred for 12 h under darkness, followed by degassing before NMR measurements. Listed pD values were obtained by adding 0.4 to the pH meter reading, in accordance with the work of Glasoe and Long.[13]

Acknowledgements

This work was supported by grants from Bioproducts and Biotechnology Research Group (01-J-BP-01-B-59) in Ministry of Science and Technology in South Korea. SDG.

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