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Cyclosophoraose as a Novel Chiral Stationary Phase for Enantioseparation

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Abstract Cyclosophoraoses (Cys), cyclic β-(1→2)-D-glucans produced by *Rhizobium meliloti* 2011, were used as a novel chiral stationary phase for the enantiomeric separation. A novel Cys stationary phase, chemically immobilized onto porous silica via aminopropyltrimethoxysilane as a molecular linker, showed good separation for each racemate of bupivacain (separation factor, α=1.3), propranolol (α=1.3), and fenoprofen (α=2.9), respectively, under the mobile phase of water: methanol (80:20, v/v) at a constant flow rate of 0.9 ml/min at pH 7.

Key words: Chiral separation, cyclosophoraoses, HPLC, chiral stationary phases

Cyclosophoraoses (Cys) are a family of unbranched cyclic β-(1→2)-D-glucans produced as intra- or extra-oligosaccharides by many strains of *Rhizobium* and *Agrobacterium* as a mixture of molecules with various sizes in a neutral or anionic form [1, 7, 23, 30]. The recent reports showed that Cys had potential as a complexation host for the solubility enhancement of the poorly soluble guest molecules [19, 20] and was used as a novel chiral additive for separation of enantiomers in aqueous capillary electrophoresis (CE) [18] and NMR [17]. Chirality remains an important consideration for many compounds such as pharmaceuticals, food additives, and agrochemicals. In recent decades, the analysis of chiral compounds by gas and HPLC with chiral stationary phases (CPS) has become remarkably advanced and is now among the practical analytical methods. HPLC methods can also be used for large-scale industrial separation. Here, we describe for the first time a family of Cys functions as a chiral selector for enantiomeric separations. CSPs are key components for this technique. In order to prepare the chiral column with a Cys-bonded stationary phase, Cys was chemically immobilized onto porous silica via aminopropyltrimethoxysilane as a linker [12]. The enantioseparation of several chiral drugs including bupivacaine, propranolol, and fenoprofen was performed on the Cys-bonded stationary phase.

Preparation of CSPs for HPLC is of great interest since enantiomers have quite different pharmacological and toxicological effects in the human body. HPLC provides, in general, fast and accurate methods for the enantiomeric separation of various chemicals, where the indirect approach utilizes the derivatizing agents, such as 2,3,4,5,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [3], for the target enantiomers, and the direct one uses the chiral stationary phases or chiral mobile phase additives for the separation of enantiomers. We have focused our interest on the direct approach with the development of Cys-bonded chiral stationary phases. Although α-cyclic glucans such as cyclodextrins (CD) or their derivatives have been successfully used as chiral selectors on the stationary phases for the chromatographic separation of some enantiomers [8, 27], no β-cyclic glucans have been used for chiral selectors. Herein, we describe for the first time a family of cyclic-β-1,2-glucan (Cys) that functions as a novel chiral selector in HPLC for enantioseparation of (±) bupivacaine (Sigma, U.S.A.), (±) propranolol (Sigma, U.S.A.), and (±) fenoprofen (Sigma, U.S.A.). R-(+)-Bupivacaine is known to be more toxic to the central nervous and the cardiovascular systems than S-(–)-bupivacaine [21]. Propranolol, a racemic β-adrenergic blocking agent, is widely used in the treatment of cardiovascular diseases and prophylaxis of acute myocardial infarction. (S)-Propranolol is about 100 times more active than its (R)-antipode against b-receptors [10]. Fenoprofen is a nonsterodial anti-inflammatory drug, undergoing a fortuitous metabolic chiral inversion in which the inactive (–)-R enantiomer is converted into the active (+)-S form [9]. Though several attempts for the enantiomeric separation of these chiral drugs [5, 15, 22, 26] have been reported, no reports have been published with β-cyclic glucans such as

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Cys used as a chiral stationary phase for the enantiomeric separation.

In order to prepare Cys, *R. meliloti* 2011 was cultured in a 5-l jar fermenter containing GMS medium as previously reported [7]. After harvesting, cells were extracted with 75% (v/v) ethanol at 70°C, and the extracts were fractionated by gel filtration on a Sephadex G-50 column (1.51×10 cm) at a flow rate of 20 ml/h and then applied to a column (2×35 cm) of DEAE-cellulose to separate neutral and anionic Cys. The neutral Cys was then desalted on a Sephadex G-10 column. For preparation of a Cys-bonded stationary phase, the isolated neutral Cys was chemically immobilized onto porous silica via aminopropyltrimethoxysilane as a molecular linker. The Cys-bonded stationary phases were prepared from these amino silanes. The synthesis scheme is presented in Fig. 1. First, 4.4 mmol of Cys was dried in vacuum at 100°C for 18 h. After cooling, the Cys was dissolved in 50 ml of dry pyridine and then 8.8 mmol of 4-nitrophenylchloroformate was added and the mixture was stirred for 2 h under nitrogen at room temperature. 4-Nitrophenylchloroformate reacted with the hydroxyl group at the 3,4,6-positions of Cys to form ester bond ((1) in Fig. 1). Then, 5.3 mmol of 3-aminopropyltrimethoxysilane was added to form carbomates ((2) in Fig. 1) at room temperature for 2 h and 10 g of silica gel was added and stirred at room temperature for 18 h ((3) in Fig. 1). Prepared bonded-Cys silica gel was then filtered, and washed successively with pyridine, water, ethanol, and acetone. It was dried overnight in vacuum at 60°C for the next preparation. Structures of the synthesized products were analyzed by matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager-DE™ STR BioSpectrometry, Applied Biosystems Farmingham, MA, U.S.A.) and Elemental Analyzer (Flash EA 1112series/ CE Instruments). Mass spectra of Cys-linker ((2) in Fig. 1) were obtained with MALDI-TOF mass spectrometry in the positive ion mode using 2,5-dihydroxy benzoic acid as matrix. Approximately 0.5 μl of the sample/matrix mixture was applied to the MALDI probe, after which the solvent was removed by evaporation. Compositional analysis of Cys-linker ((2) in Fig. 1) by Elemental Analyzer gave carbon contents of 4.25%, which indicated the presence of Cys linked to silica, where BBOT (benzoxazole) (N 6.51%, H 6.09%, S 7.44%) and acetanilide (C 71.1%, H 6.7%, N 10.4%, O 11.8%) were used as standards. The combustion temperature is 1,100°C. A stainless-steel HPLC column (4.6×250 mm) was slurry packed with the prepared Cys CSP. The packing was done by the following

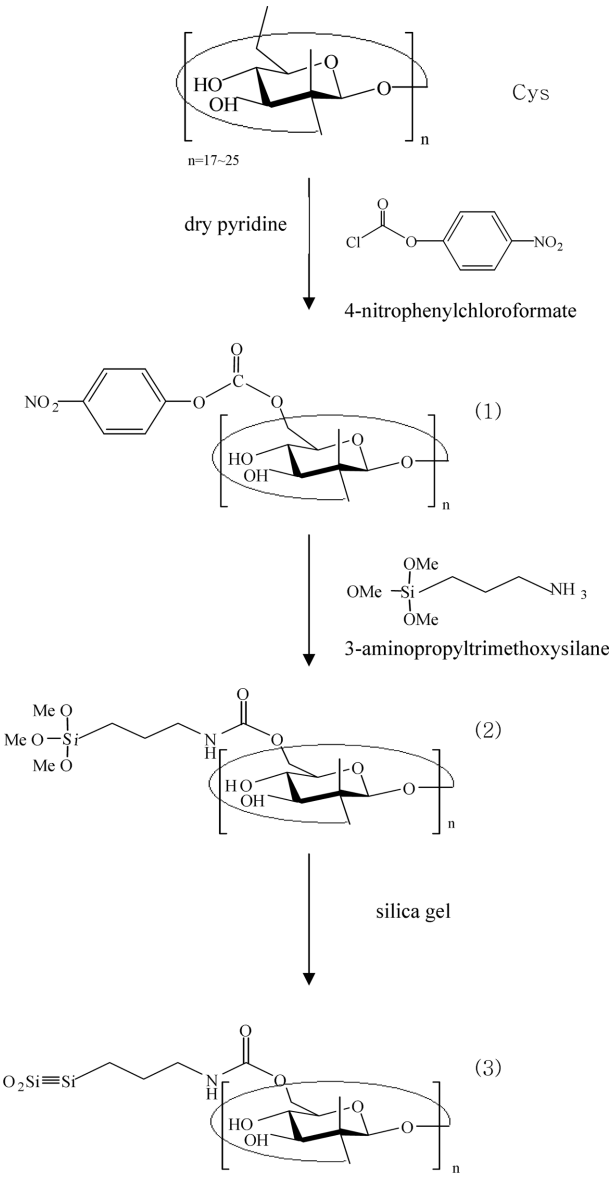


Fig. 1. Scheme for the synthesis of Cys-bonded phases.

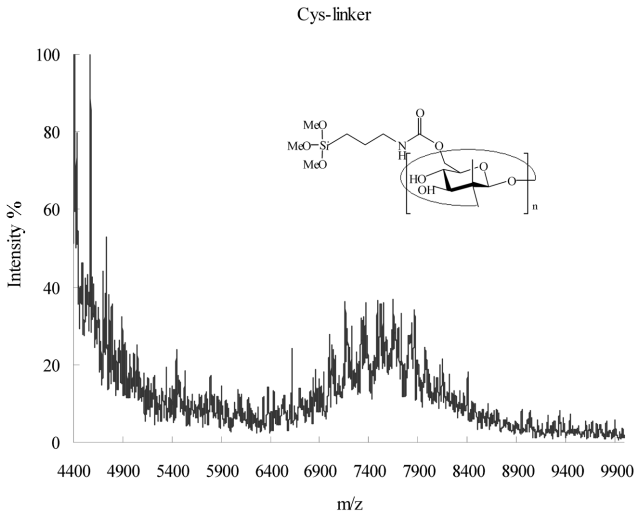


Fig. 2. MALDI-TOF mass spectrum of Cys-linkers.
MALDI-TOF mass spectrum using 2,5-dihydroxy benzoic acid as matrix.

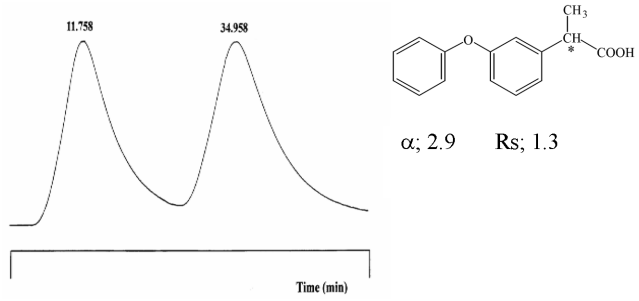


Fig. 3. Separation of fenoprofen racemates by HPLC with Cys-bonded chiral phases.
Chromatographic conditions: Column size, 4.6×250 mm; temperature, 30°C; flow rate, 0.9 ml/min; detection, 288 nm; mobile phase, phosphate buffer:MeOH=8:2, pH 7.

procedure. First, 2.67 g Cys-silica was prepared with 20 ml ethanol and was sonicated for 15 min. Then, this mixture was poured into a reservoir column and was packed with 40 ml ethanol.

Table 1 shows the measured and calculated mass data of the Cys-linker ((2) in Fig. 1) analyzed by MALDI-TOF mass spectrometry in the positive ion mode. It indicates the presence of $[M]^+$, $[M+H]^+$ $[M+Na]^+$, and $[M+K]^+$. The measured mass units showed the DS (degree of substitution of molecular linkers attached to Cys) ranging from 0.17 to 0.5. Major peaks appeared in DP (degree of polymerization) 22 of Cys with 21 linker substituents, which indicated about one linker substituent per one glucose unit of Cys. HPLC analysis was performed in reverse mode using various compositions of water:methanol (90:10, 80:20, 70:30, v/v) as mobile phase at various constant flow rates of 1, 0.9, and 0.8 ml/min, respectively, with pH 3 or pH 7. The optimum condition for chiral separation was obtained in water:methanol (80:20 v/v) at 0.9 ml/min at pH 7. Typically, 10 μl (10 μg/ml) of chiral racemates was injected at 30°C of column temperature.

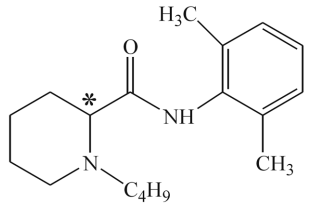
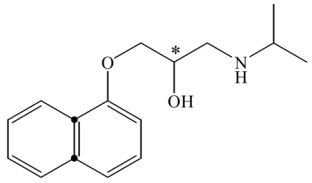
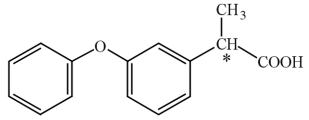
Figure 3 shows a chromatogram of the resolution of the racemate, fenoprofen, on the column packed with Cys-immobilized silica gel. Bupivacaine and propranolol

Table 1. Calculated mass units of Cys-linkers from the MALDI mass spectrum.

		DP									
TNS		17	18	19	20	21	22	23	24	25	26
0		2754	2916	3078	3240	3402	3564	3726	3888	4050	4212
14	Expected	5628	n.d	5948	6130a	6273	6435	6597	6757	6942	7084
	(Observed)	(5624)	(5786)	(5948)	(6110)	(6272)	(6434)	(6596)	(6758)	(6920)	(7082)
15	Expected	n.d	5991	6175	6314.9	6477	6662	6805	6963	7144	7326
	(Observed)	(5829)	(5991)	(6153)	(6315)	(6477)	(6639)	(6801)	(6963)	(7125)	(7287)
16	Expected	6034	6221	6377	6520	6700	6843	7025	7167	7350	n.d
	(Observed)	(6034)	(6196)	(6358)	(6520)	(6682)	(6844)	(7006)	(7168)	(7330)	(7492)
17	Expected	6240	6425	6563	6725	6915	7089	7229	7372	7535	n.d
	(Observed)	(6239)	(6401)	(6563)	(6725)	(6887)	(7049)	(7211)	(7373)	(7535)	(7697)
18	Expected	n.d	6610	6785	6942	7133	n.d	7451	7581	7762	7904
	(Observed)	(6444)	(6606)	(6768)	(6930)	(7092)	(7254)	(7416)	(7578)	(7740)	(7902)
19	Expected	6690	n.d	6975	7144	7300	7462	7639	7808	7949	n.d
	(Observed)	(6649)	(6811)	(6973)	(7135)	(7297)	(7459)	(7621)	(7783)	(7945)	(8107)
20	Expected	6897	n.d	7177	n.d	7523	n.d	7827	7987	8171	n.d
	(Observed)	(6854)	(7016)	(7178)	(7340)	(7502)	(7664)	(7826)	(7988)	(8150)	(8312)
21	Expected	n.d	7243	7425	7550	7709	7868	8051	8220	8377	8539
	(Observed)	(7059)	(7221)	(7383)	(7545)	(7707)	(7869)	(8031)	(8193)	(8355)	(8517)
22	Expected	7281	7444	7588	n.d	n.d	8097	8236	8423	8563	8721
	(Observed)	(7264)	(7426)	(7588)	(7750)	(7912)	(8074)	(8236)	(8398)	(8560)	(8722)
23	Expected	7711	7670	7796	7975	8136	8297				
	(Observed)	(7469)	(7631)	(7793)	(7955)	(8117)	(8279)	(8441)	(8603)	(8765)	(8927)
24	Expected	n.d	7847	8002	8199	8347	8484				
	(Observed)	(7674)	(7836)	(7998)	(8160)	(8322)	(8484)	(8646)	(8808)	(8970)	(9132)
25	Expected	7917	8041	n.d	8369						
	(Observed)	(7879)	(8041)	(8203)	(8365)	(8527)	(8689)	(8851)	(9013)	(9175)	(9337)
26	Expected	n.d	8250								
	(Observed)	(8084)	(8246)	(8408)	(8570)	(8732)	(8894)	(9056)	(9218)	(9380)	(9542)
27	Expected	8328	8451								
	(Observed)	(8289)	(8451)	(8613)	(8775)	(8937)	(9099)	(9261)	(9423)	(9585)	(9747)

a, $[M+Na]^+$; b, $[M+K]^+$; n.d, not detected; TNS, total number of substituents.

Table 2. Separation factor (α) and resolution (Rs) of racemates.

Racemic drug	α	Rs
 Bupivacaine	1.3	0.9
 Propranolol	1.3	0.7
 Fenoprofen	2.9	1.3

as pharmaceutically active drug enantiomers were also successfully separated on the Cys-bonded stationary phase for HPLC. Separation factors (α) and resolution (Rs) were obtained for bupivacain (α=1.3, Rs=0.9), propranolol (α=1.3, Rs=0.7), and fenoprofen (α=2.9, Rs=1.3), as shown in Table 2.

In study, we show for the first time that the Cys-bonded stationary phase for HPLC has good potential for application to enantioseparation for various chiral chemicals. Cys with its characteristic cyclic scaffolds will provide the differential binding capacity for the enantiomers with its inclusive complex-forming ability. Since Cys has a cavity capable of inclusion like CDs-phases [13], as well as flexibility of being capable of complexation like polysaccharides, it would be more potent as a chiral stationary phase. Further research on the chemical modification of Cys will be undertaken.

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