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Preparation and application of a new ligand exchange chiral stationary phase for the liquid chromatographic resolution of α-amino acid enantiomers

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Abstract

A new liquid chromatographic ligand exchange CSP has been prepared by covalently bonding (*S*)-*N*,*N*-carboxymethyl undecyl leucinol monosodium salt onto silica gel and employed in resolving various α-amino acids. The new CSP was quite good in resolving various α-amino acids and the resolution results were dependent on the type and content of organic modifier in the mobile phase. From these results, a chiral recognition model using a lipophilic interaction between the tethering alkyl group of the CSP and the substituent at the chiral center of α-amino acids was proposed. The liquid chromatographic resolution of α-amino acids on the new CSP was also found to be dependent on the Cu(II) concentration in the mobile phase and the column temperature. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Amino acids

1. Introduction

Chiral ligand-exchange chromatography has been extensively studied and used in resolving various racemic α-amino acids since the pioneering work of Davankov and co-workers [1–3]. In general, Cu(II) complexes of α-amino acids and their derivatives have been successfully employed in resolving various racemic α-amino acids as chiral mobile phase additives [4,5] or chiral stationary phases (CSPs) after binding covalently [6,7] or hydrophobically [8–10] to solid column support. Very few other optically active materials have been used as chiral selectors for chiral ligand exchange chromatography [11,12].

In this area, our efforts have been focused on the use of Cu(II) complexes of optically active amino alcohol derivatives hydrophobically bound to octadecyl silica gel as dynamic CSPs in resolving various racemic α-amino acids. For example, Cu(II) complexes of (1*S*,2*R*)-norephedrine derivative and (*R*)-alaninol derivative hydrophobically adsorbed onto a commercial octadecyl silica gel column have been successfully used as dynamic chiral stationary phases in resolving various α-amino acids [13–15].

Based on the mechanistic evaluation of the resolution of α-amino acids on these two dynamic CSPs, more recently, we developed another improved dynamic CSP (CSP **1**; Fig. 1) by binding

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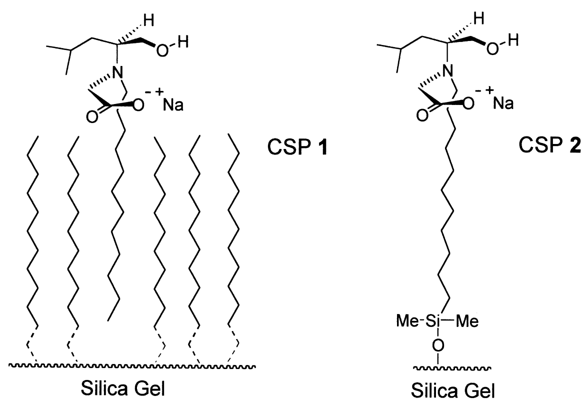


Fig. 1. The structures of CSP **1** and CSP **2**.

(*S*)-leucinol derivative, (*S*)-*N*,*N*-carboxymethyl dodecyl leucinol monosodium salt, onto a commercial reversed-phase octadecyl silica gel [16]. CSP **1** was quite effective in resolving various α-amino acids in the presence of Cu(II) ion in the mobile phase. However, the drawback of CSP **1** is that the chiral selector, which is hydrophobically bound on octadecyl silica gel, bleeds from the column when an aqueous mobile phase containing more than 20% acetonitrile is used.

In order to overcome the drawback of CSP **1**, in this study, we prepared a new covalently bonded CSP (CSP **2**; Fig. 1) starting from (*S*)-leucinol and applied it in resolving various α-amino acids. The covalent nature of the new CSP is expected to be useful under various mobile phase conditions.

2. Experimental

2.1. Instrumentation

^{1}H NMR spectra were recorded on a Varian Gemini 200 spectrometer using tetramethylsilane as an internal standard. IR spectra were recorded on a Jasco FT/IR-300E spectrometer.

Chromatography was performed with an HPLC system consisting of a Waters model 515 HPLC pump, a Rheodyne model 7725i injector with a 20-μl sample loop, a YoungLin M720 Absorbance detector (variable wavelength) and a YoungLin Autochro Data Module (Software:YoungLin Autochro-WIN 2.0 plus). The temperature of the chiral column was controlled using a Julabo F30 Ultratemp 2000 cooling circulator.

2.2. Preparation of CSP 2

CSP **2** was prepared starting from (*S*)-leucinol as shown in Fig. 2. The detailed procedures are as follows.

2.2.1. (S)-N-(10-Undecenoly)leucinol 3

In a 250-ml round bottom flask were dissolved (*S*)-leucinol (2.30 g, 19.6 mmol) and triethylamine (3.48 ml, 25.0 mmol) in 100 ml of methylene chloride. To the stirred solution was added 10-undecenoyl chloride (4.30 ml, 20.0 mmol) slowly under nitrogen at room temperature. The reaction mixture was stirred at room temperature for 10 min and then washed successively with 0.5 N HCl, 0.5 N NaOH, and water. The organic solution was dried over anhydrous $MgSO_4$, filtered and evaporated. The residue was purified by column chromatography on silica gel to afford **3** (5.06 g, 91%) as a colorless oily material. ^{1}H NMR ($CDCl_3$) δ 0.91 (dd, 6H), 1.27

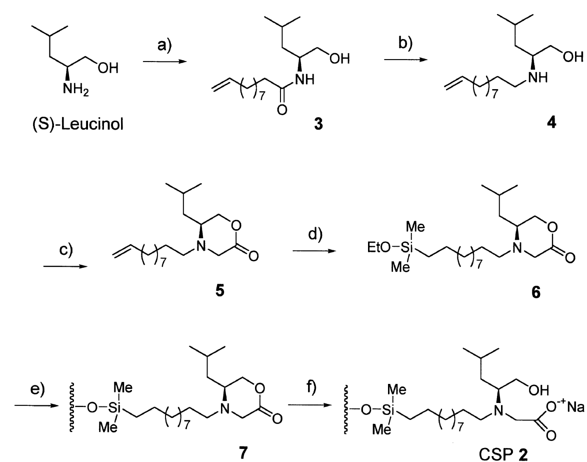


Fig. 2. Scheme for the preparation of CSP **2**. (a) 10-Undecenoyl chloride, triethylamine, methylene chloride, room temperature, 10 min, 91%. (b) $LiAlH_4$, tetrahydrofuran, 12 h, 97%. (c) ethyl bromoacetate, triethylamine, methylene chloride, room temperature, 24 h, 48%. (d) (1) chlorodimethylsilane, Pt/C (10 wt.%), methylene chloride, reflux, 18 h. (2) A mixture of absolute ethanol and triethylamine (1:1 mixture, v/v), methylene chloride, room temperature, 1 h, 38%; (e) 5 μm Rexchrom silica gel, Dean–Stark trap, toluene, reflux, 72 h. (f) 1 *M* NaOH solution, methanol, room temperature, 24 h.

(broad s, 13H), 1.58–1.64 (m, 2H), 2.01 (q, 2H), 2.19 (t, 2H), 3.33 (broad, 1H), 3.51 (dd, 1H), 3.66 (1H), 3.95–4.12 (m, 1H), 4.88–5.01 (m, 2H), 5.69–5.86 (m, 2H). IR (KBr) cm^{-1} 3292, 3078, 2927, 2858, 1641, 1546.

2.2.2. (S)-N-(10-Undeceny)leucinol **4**

A solution of **3** (5.06 g, 17.9 mmol) in 40 ml of dry tetrahydrofuran was slowly added to a stirred solution of $LiAlH_4$ (2.72 g, 72 mmol) in 40 ml of tetrahydrofuran through a dropping funnel at 0 °C. The whole mixture was refluxed for 12 h. The reaction mixture was cooled to 0 °C and then quenched by adding water. The whole mixture was passed through the bed of celite and then tetrahydrofuran was removed by rotary evaporator. The aqueous solution was extracted with ethyl acetate. The ethyl acetate solution was dried over anhydrous $MgSO_4$, filtered and then ethyl acetate was evaporated. The residue was purified by column chromatography on silica gel to afford **4** (4.67 g, 97%) as a colorless oily material. ^1H NMR ($CDCl_3$) δ 0.91 (d, 6H), 1.27 (broad s, 15H), 2.01 (q, 2H), 2.24–2.41 (m, 2H), 2.50–2.65 (m, 4H), 3.19 (dd, 1H), 3.61 (dd, 1H), 4.89–5.02 (m, H), 5.71–5.88 (m, 1H). IR (KBr) cm^{-1} 3302, 3074, 2925, 2856, 1643, 1461.

2.2.3. (S)-4-(10-Undecenyl)-5-isobutyl-2,3,5,6-tetrahydro-4H-1,4-oxazin-2-one **5**

To a stirred solution of **4** (4.67 g, 17.3 mmol) in 50 ml of methylene chloride were added ethyl bromoacetate (3.80 ml, 34.7 mmol) and triethylamine (2.42 ml, 17.3 mmol). The whole mixture was stirred for 24 h at room temperature. The whole mixture was washed with 0.5 N HCl, dried over anhydrous $MgSO_4$ and then concentrated. The residue was purified by column chromatography on silica gel to afford **5** (2.57 g, 48%) as a colorless oily material. ^1H NMR ($CDCl_3$) δ 0.93 (dd, 6H), 1.27 (broad s, 15H), 1.59–1.68 (m, 2H), 2.02 (q, 2H), 2.36–2.49 (m, 2H), 2.53–2.80 (m, 1H), 3.42 (q, 2H), 4.13 (dd, 1H), 4.40 (dd, 1H), 4.89–5.04 (m, 2H), 5.71–5.88 (m, 1H). IR (KBr) cm^{-1} 3072, 2925, 2856, 1749, 1643, 1462.

2.2.4. (S)-N-(11-Dimethylethoxysilylundecyl)-5-isobutyl-2,3,5,6-tetrahydro-4H-1,4-oxazin-2-one **6**

In a 150-ml round bottom flask was dissolved **5** (2.57 g, 8.30 mmol) in 40 ml of methylene chloride. To the stirred solution was added a catalytic amount (about 30 mg) of Pt/C (10 wt.%) and chlorodimethylsilane (28.0 ml, 250 mmol). The whole mixture was heated at reflux for 18 h and then concentrated. The residue was dissolved in 10 ml of methylene chloride. A mixture of triethylamine and absolute ethanol (3 ml, 1:1 mixture, v/v) was then slowly added to the stirred solution, and the mixture was stirred at room temperature for 1 h. The mixture was concentrated and then purified by column chromatography on silica gel to afford **6** (1.3 g, 38%) as a yellow oily material. ^1H NMR ($CDCl_3$) δ 0.09 (s, 6H), 0.89 (q, 6H), 1.25 (broad s, 24H), 1.55–1.67 (m, 2H), 2.35–2.47 (m, 2H), 2.52–2.79 (m, 1H), 3.40 (q, 2H), 3.62 (q, 2H), 4.12 (dd, 1H), 4.42 (dd, 1H). IR (KBr) cm^{-1} 2925, 2856, 1748, 1461.

2.2.5. Modified silica gel **7**

A flask equipped with a Dean–Stark trap and a condenser was charged with Regis Rexchrom silica gel (4.5 g, particle size: 5 μm, surface area: 212 m^2/g) and toluene (100 ml). After heating the heterogeneous mixture at reflux until azeotropic removal of water was complete, compound **6** (1.3 g, 3.2 mmol) was added and then the whole mixture heated to reflux for 72 h. The silica gel was filtered and washed extensively with toluene, ethyl acetate, methanol, acetone, dimethylether and hexane to afford modified silica gel **7**. Elemental analysis of modified silica gel **7** (C 7.56%, N 0.41%, H 1.39%) showed a loading of 0.30 mmol (based on C) or 0.29 mmol (based on N) of compound **6** per g of modified silica gel. The surface concentration of the chiral selector calculated based on the known equation [17] was 1.60 $\mu mol/m^2$ (based on C) or 1.56 $\mu mol/m^2$ (based on N).

2.2.6. Preparation of CSP **2** and column packing

A mixture of modified silica gel **7** (4.2 g) suspended in 10 ml of methanol and NaOH solution (1 *M* in H_2O, 0.5 ml) in a 100-ml round bottom flask was stirred for 24 h at room temperature. The modified silica gel was filtered and washed with methanol. The modified silica gel was slurried in methanol and packed into a 4.6 mm×250 mm

stainless steel HPLC column using a conventional method with an Alltech HPLC Slurry Packer.

2.3. Chromatography

All analytes used in this study were available from previous study [16] or from Aldrich except for 5,5-dimethylthiazolidine-4-carboxylic acid, which was prepared by treating penicillamine with aqueous formaldehyde solution as reported previously [18].

To resolve racemic α-amino acids on the chiral column packed with CSP **2**, a mobile phase, which was prepared by dissolving a specified amount of $CuSO_4$ in deionized water or deionized water containing acetonitrile or methanol as an organic modifier, was passed through the column until the baseline (UV monitor, 254 nm) became stable to equilibrate the column and then, a methanolic solution (usually 3 μl) containing a racemic or optically enriched α-amino acid (usual concentration: 1.0 mg/ml) was injected. Column void volume (the elution time of an unretained solute) was measured by injecting aqueous $NaNO_3$ solution [19]. The chiral column prepared in this study was found to be equally effective during the period of its use for more than 12 months.

3. Results and discussion

A chiral column packed with CSP **2** was successfully employed in resolving various α-amino acids. The chromatographic results for the resolution of various α-amino acids are summarized in Table 1.

Table 1
Resolution of α-amino acids on CSP **2** with the mobile phase of water containing $CuSO_4$ (2.0×10^{-4} M) at 20 °C

Amino acid	k_1 [a]	k_2 [b]	α [c]	R_S [d]	Conf. [e]
Alanine	2.03	2.35	1.16	0.35	L
2-Aminoadipic acid	4.42	8.96	2.03	2.19	L
Asparagine	2.29		1.00		
Aspartic acid	1.61	2.72	1.69	0.88	L
Glutamic acid	2.95	6.57	2.23	1.74	L
Glutamine	2.65	3.33	1.26	0.75	L
Leucine	5.03	10.68	2.12	2.54	L
iso-Leucine	4.48	12.69	2.83	2.44	L
nor-leucine	5.24	14.96	2.85	3.45	L
tert.-Leucine	3.54	11.89	3.36	3.54	
Methionine	5.05	8.88	1.76	1.91	L
Phenylalanine	9.38	24.79	2.64	3.49	L
4-Chlorophenylalanine	22.85	84.34	3.69	4.45	
Phenylglycine	4.51	14.18	3.14	3.88	L
Proline	4.21	6.26	1.49	0.94	L
Serine	1.98	2.43	1.23	0.71	L
2-Thienylalanine	6.90	17.46	2.53	3.80	
Threonine	2.33	2.82	1.21	0.60	L
Tryptophan	24.88	71.00	2.85	3.35	
Tyrosine	5.10	15.40	3.02	3.60	L
α-Methyltyrosine	6.05	15.67	2.59	2.80	
Valine	3.07	6.81	2.22	2.38	L
nor-Valine	3.40	7.13	2.10	2.43	L
5,5-Dimethylthiazolidine -4-carboxylic acid	10.44	63.49	6.08	7.77	

Flow-rate: 0.8 ml/min. Detection: 254 nm UV.
[a] Retention factor of the first eluted enantiomer.
[b] Retention factor of the second eluted enantiomer.
[c] Separation factor.
[d] Resolution factor.
[e] Absolute configuration of the second eluted enantiomer. For blanks, the elution order has not been determined.

All data in Table 1 were obtained using water containing Cu(II) (2.0×10^{-4} M) as a mobile phase with a flow-rate of 0.8 ml/min at 20 °C. Most of the α-amino acids investigated were resolved with reasonable or good separation factors except asparagine as shown in Table 1. The elution orders were determined by injecting configurationally known samples and they were consistent, (L)-enantiomers being retained longer.

From the comparison of the chromatographic results for the resolution of some α-amino acids on CSP **2** with those on CSP **1** reported previously [16], we found that separation factors, α, were generally greater on CSP **1** than CSP **2** under the identical mobile phase condition. Even though CSP **1** was excellent in terms of separation factors in resolving α-amino acids, the long retention of the two enantiomers on the column, which might stem from the strong lipophilic interaction between the C_{18} octadecyl group of the CSP and the solute, was a severe problem. However, the retention factors are reduced very much on CSP **2** compared to those on CSP **1**. Consequently, CSP **2** is expected to be more useful in the analytical purpose of determining the enantiomeric composition of α-amino acids than CSP **1**.

In order to elucidate the effect of the content of organic modifier in the mobile phase on the resolution of α-amino acids on CSP **2**, we selected five α-amino acids and resolved them on CSP **2** with the variation of the content of organic modifier in the aqueous mobile phase at constant Cu(II) concentration (2.0×10^{-4} M). The chromatographic resolution results are summarized in Table 2. The chromatographic resolution trends are quite dependent on the content of organic modifier in the mobile phase and, as an example, the chromatograms for the resolution of phenylglycine on CSP **2** with the variation of the methanol content in the aqueous mobile phase are presented in Fig. 3.

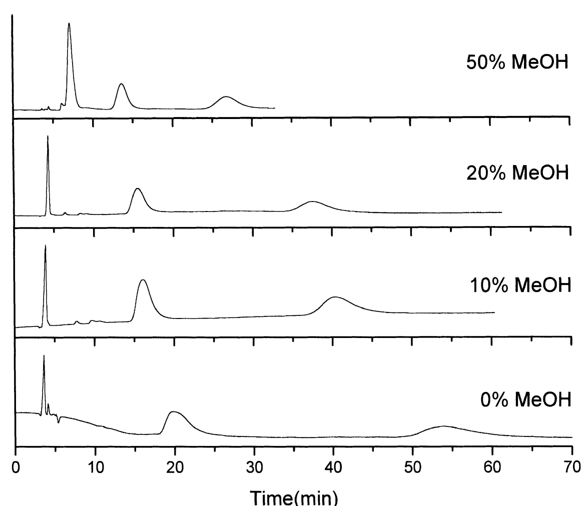


Fig. 3. Chromatograms for the resolution of phenylglycine on CSP **2** with the variation of the methanol content in the aqueous mobile phase at a constant Cu(II) concentration (2.0×10^{-4} M) at 20 °C. Flow-rate: 0.8 ml/min. Detection: 254 nm UV.

As shown in Table 2, an increase in the content of organic modifier such as methanol or acetonitrile in the aqueous mobile phase diminishes the retention of the two enantiomers as denoted by the retention factors (k). As an example, the trends of retention factors for the resolution of 2-aminoadipic acid on

Table 2
Resolution of five amino acids on CSP **2** with variation of methanol and acetonitrile content in the aqueous mobile phase at constant Cu(II) concentration (2.0×10^{-4} M)

Mobile phase	2-Aminoadipic acid			α-Methyltyrosine			Leucine			Phenylglycine			5,5-Dimethylthiazolidine-4-carboxylic acid		
	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S
10% MeOH	3.36	1.88	2.21	4.46	2.51	2.52	3.47	2.08	2.66	3.24	2.98	4.18	7.15	5.46	6.71
20% MeOH	2.92	1.83	2.12	3.27	2.42	2.68	3.19	1.99	2.60	2.95	2.89	4.47	6.32	4.94	5.64
50% MeOH	2.29	1.55	1.40	2.37	2.01	1.88	2.69	1.69	1.79	2.43	2.38	3.30	5.24	3.09	4.55
10% CH_3CN	3.22	1.68	1.95	3.74	2.10	2.32	3.60	1.84	2.43	3.31	2.61	4.26	6.38	4.55	7.41
20% CH_3CN	2.26	1.57	1.44	2.46	1.84	2.00	2.82	1.73	2.09	2.53	2.46	4.37	4.67	3.68	6.16
50% CH_3CN	1.85	1.38	0.84	1.66	1.58	1.18	2.38	1.48	1.20	2.05	1.99	2.68	3.53	2.65	3.57

Flow-rate: 0.8 ml/min. Detection: 254 nm UV. Temperature: 20 °C; k_1, retention factor of the first eluted enantiomer; a, separation factor; R_S, resolution factor.

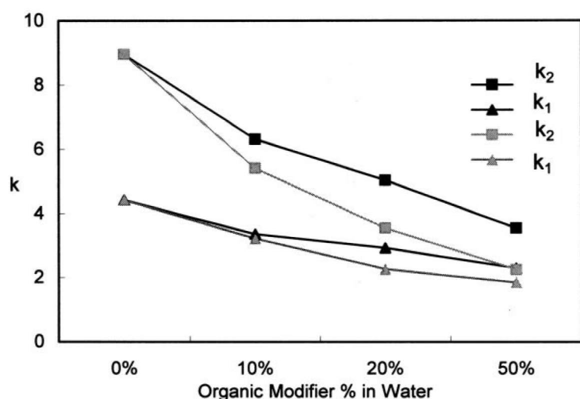


Fig. 4. The trends of the retention factors (k_1 and k_2) for the resolution of 2-aminoadipic acid on CSP **2** with the variation of methanol content (black lines) or with the variation of acetonitrile content (gray lines) in the aqueous mobile phase at constant Cu(II) concentration (2.0×10^{-4} *M*) at 20 °C. Flow-rate: 0.8 ml/min. Detection: 254 nm UV.

CSP **2** with the variation of the content of organic modifier in the aqueous mobile phase is graphically illustrated in Fig. 4. The retention of the more retained enantiomers is diminished more rapidly than that of the less retained enantiomers as the content of organic modifier in the mobile phase increases. As a consequence, the separation factors, α, decrease as the content of organic modifier in the mobile phase increases. When acetonitrile was used as an organic modifier, the retention of the more retained enantiomers decreases even more significantly than when methanol was used as the content of organic modifier increases as shown in Table 2 and Fig. 4. Consequently, the separation factors, α, decrease more rapidly with the use of acetonitrile than with the use of methanol as an organic modifier.

The chromatographic resolution trends with the variation of the content of organic modifier in the mobile phase shown in Table 2 and Fig. 4 are exactly consistent with those observed on dynamic CSP **1** [16]. In this instance, the chiral recognition mechanism for the resolution of α-amino acids on CSP **2** is assumed to be quite similar to that on dynamic CSP **1**. Previously, we proposed a chiral recognition mechanism for the resolution of α-amino acids on dynamic CSP **1**, using the formation of two energetically different ternary complexes formed from the fixed ligand, (D)- or (L)-α-amino acid and Cu(II) [16]. In that chiral recognition mechanism, the favorable lipophilic interaction between the octadecyl chains of CSP **1** and the alkyl group at the chiral center of both (L)- and (D)-enantiomers was proposed to play important roles. However, the octadecyl chains are no longer present in CSP **2** and consequently, the chiral recognition mechanism for the resolution of α-amino acids on CSP **2** might be slightly different from that for the resolution of α-amino acids on CSP **1**.

The chiral recognition mechanism proposed for the resolution of α-amino acids on CSP **2** is shown in Fig. 5. As shown in Fig. 5a, the mechanism for the retention of (L)-enantiomers on CSP **2** is identical to that on dynamic CSP **1**. Under the reverse mobile phase condition, the lipophilic interaction between the tethering undecyl group of the CSP and the alkyl group at the chiral center of (L)-enantiomers seems to be quite favorable. However, the similar lipophilic interaction between the tethering undecyl group of the CSP and the alkyl group at the chiral center of (D)-enantiomers requires the inversion of the configuration of the tertiary amino group of the CSP as proposed in the previous report [16]. This process might be possible with dynamic CSP **1** because of the strong lipophilic interaction between the octadecyl chains of the CSP and the alkyl group at the chiral center of (D)-enantiomers but does not seem to be possible with CSP **2**. In contrast, we propose that the alkyl group at the chiral center of (D)-enantiomer is directed toward the bulk mobile phase as shown in

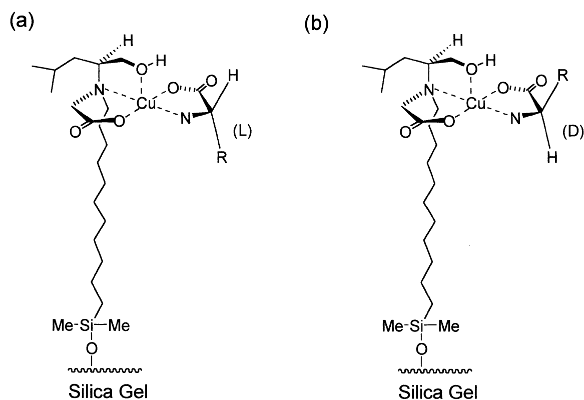


Fig. 5. The proposed structures of the ternary complexes formed from the fixed ligand of CSP **2**, Cu(II) and (a) L- and (b) D-amino acid.

Fig. 5b and consequently the lipophilic interaction between (D)-enantiomers and the CSP is not so favorable. In this instance, the diastereomeric complex shown in Fig. 5a is energetically more stable than that shown in Fig. 5b and the (L)-enantiomers are retained longer on the column.

The use of organic modifier in the aqueous mobile phase reduces the lipophilic interaction and consequently diminishes the retention of the two enantiomers. However, the retention of the more retained enantiomers is more significantly diminished than that of the less retained enantiomers because the lipophilic interaction is more effective in the more stable ternary complex. Consequently, the separation factors, α, decrease as the content of organic modifier increases as shown in Table 2.

The effect of organic modifier on the lipophilic interaction should be more significant with less polar organic modifier (acetonitrile in this case) than with more polar organic modifier (methanol). In this instance, the retention of the two enantiomers on CSP **2** and the enantioseparation decrease more significantly with the mobile phase containing acetonitrile as an organic modifier than with the mobile phase containing more polar methanol as an organic modifier as shown in Table 2 and Fig. 4.

The effect of the variation of the Cu(II) concentration in the mobile phase of constant composition (methanol–water, 20:80, v/v) on the resolution of five selected α-amino acids on CSP **2** was also investigated. The chromatographic resolution results on CSP **2** with the variation of Cu(II) concentration in the mobile phase are summarized in Table 3 and the representative chromatograms are shown in Fig. 6.

As shown in Table 3, the retention of the two

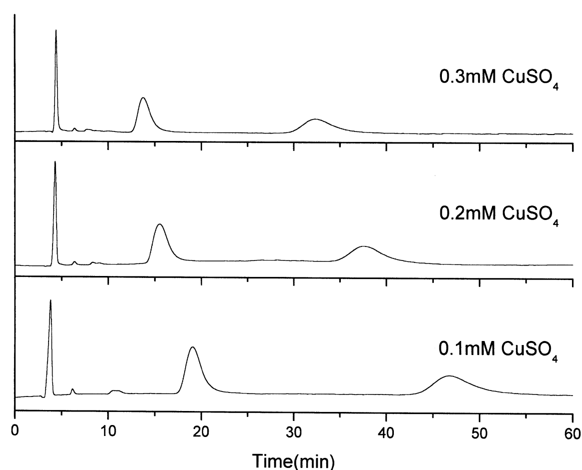
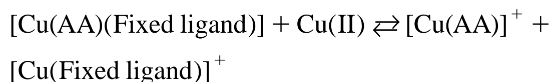


Fig. 6. Chromatograms for the resolution of phenylglycine on CSP **2** with the variation of Cu(II) concentration in the mobile phase of 20% methanol in water at 20 °C. Flow-rate: 0.8 ml/min. Detection: 254 nm UV.

enantiomers and the resolution factors, R_S, decrease as the Cu(II) concentration in the mobile phase increases. However, the separation factors, α, do not show any noticeable trends. These results are consistent with those for the corresponding dynamic CSP (CSP **1**) [16]. In the mobile phase, it is supposed that at least four species including complexes and Cu(II) ion are in equilibrium as shown in the following equation, where AA means α-amino acids [8]:



$$[\mathrm{Cu(AA)(Fixed\ ligand)}] + \mathrm{Cu(II)} \rightleftarrows [\mathrm{Cu(AA)}]^{+} + [\mathrm{Cu(Fixed\ ligand)}]^{+}$$

As the Cu(II) concentration in the mobile phase increases, formation of the mobile binary complex

Table 3
Resolution of five amino acids on CSP **2** with variation of Cu(II) concentration in the mobile phase of 20% methanol in water

Cu(II) (*M*)	2-Aminoadipic acid			α-Methyltyrosine			Leucine			Phenylglycine			5,5-Di-methylthiazolidine-4-carboxylic acid		
	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S
1.0×10^{-4}	4.04	1.81	2.48	5.03	2.38	2.75	4.40	1.95	3.14	3.94	2.84	4.72	8.85	4.92	7.40
2.0×10^{-4}	2.92	1.83	2.12	3.27	2.42	2.68	3.19	1.99	2.60	2.95	2.89	4.47	6.32	4.94	5.64
3.0×10^{-4}	2.50	1.83	2.00	3.18	2.41	2.43	2.74	1.69	1.82	2.44	2.99	4.26	5.06	4.98	5.54

Flow-rate: 0.8 ml/min. Detection: 254 nm UV. Temperature, 20 °C; k_1, retention factor of the first eluted enantiomer; *a*, separation factor; R_S, resolution factor.

from Cu(II) and α-amino acids improves as described previously to explain the resolution trends for the dynamic CSP [14–16] and, consequently, the retention of the two enantiomers decreases. In this instance, the difference in the retention times of the two enantiomers also decreases as the Cu(II) concentration in the mobile phase increases while the widths of the peaks corresponding to the two enantiomers are not significantly affected and, consequently, the resolution factors, R_S, decrease as the Cu(II) concentration in the mobile phase increases.

The equilibrium for the formation of ternary complex is expected to be dependent on column temperature. Based on this expectation, we investigated the resolution of five selected α-amino acids on CSP **2** with the variation of the column temperature and summarized the chromatographic results in Table 4. In addition, the dependence of the resolution on the column temperature is illustrated in Fig. 7. As shown in Table 4, retention factors, k, increase as the temperature decreases. Similarly, separation factors, α, increase slightly as the temperature decreases. However, the resolution factors, R_S, decrease as the temperature decreases. At lower temperature, the formation of the two transient diastereomeric ternary complexes formed from the two enantiomers, fixed chiral ligand and Cu(II) is expected to be more favorable. Consequently, retention times improve at lower temperature. In addition, the formation of more stable ternary complex is expected to be more favorable than that of the less stable ternary complex at lower temperature. As a consequence, the difference in the stability of the two diastereomeric ternary complexes increases and the separation factors, α, increase as the temperature decreases.

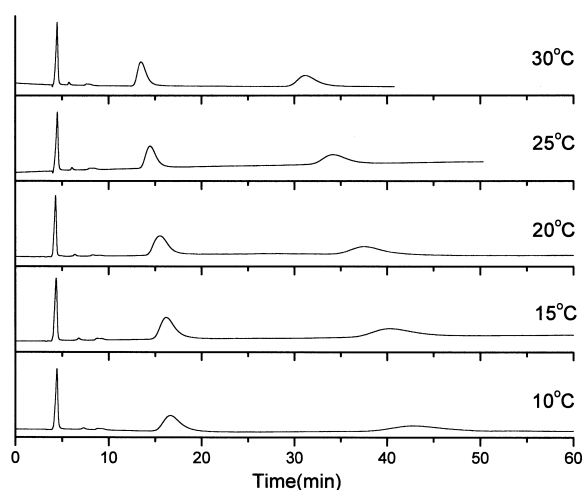


Fig. 7. Chromatograms for the resolution of phenylglycine on CSP **2** with variation of column temperature with the mobile phase of 20% methanol in water at constant Cu(II) concentration (2.0×10^{-4} M). Flow-rate: 0.8 ml/min. Detection: 254 nm UV.

The rate of equilibrium for the formation of ternary complexes might be slow at lower temperature. In this instance, the life time of the transient diastereomeric ternary complex is quite long at lower temperature and, consequently, the chromatographic peaks corresponding to the two enantiomers broaden. However, at higher temperature, the rate of equilibrium becomes quite fast, the life time of the transient diastereomeric ternary complex is quite short and the chromatographic peaks corresponding to the two enantiomers sharpen. All of these phenomena result

Table 4
Resolution of five amino acids on CSP **2** with the variation of column temperature with the mobile phase of 20% methanol in water at the constant Cu(II) concentration (2.0×10^{-4} M)

Temperature (°C)	2-Aminoadipic acid			α-Methyltyrosine			Leucine			Phenylglycine			5,5-Di-methylthiazolidine-4-carboxylic acid		
	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S
10	3.28	1.91	1.71	4.08	2.66	2.10	3.31	2.06	2.00	3.23	3.06	3.56	7.13	5.44	4.79
15	3.09	1.87	2.00	3.89	2.52	2.32	3.25	2.02	2.48	3.09	2.98	3.91	6.65	5.23	5.33
20	2.92	1.83	2.12	3.27	2.42	2.68	3.19	1.99	2.60	2.95	2.89	4.47	6.32	4.94	5.64
25	2.51	1.83	2.82	3.21	2.37	3.02	2.78	2.03	3.13	2.64	2.88	5.41	5.71	4.95	7.67
30	2.33	1.81	3.00	2.98	2.38	3.47	2.61	1.95	3.65	2.41	2.84	5.94	5.32	4.92	9.03

Flow-rate: 0.8 ml/min. Detection: 254 nm UV. Temperature: 20 °C; k_1, retention factor of the first eluted enantiomer; a, separation factor; R_S, resolution factor.

in a large resolution factor, R_S, at higher temperature, but in a small resolution factor, R_S, at lower temperature.

In summary, in this study, we developed a new ligand exchange CSP (CSP **2**) by covalently bonding (*S*)-*N*,*N*-carboxymethyl undecyl leucinol monosodium salt onto silica gel and employed the new CSP in resolving various α-amino acids. The chromatographic resolution of α-amino acids on the new CSP was quite good and was dependent on the type and content of organic modifier in the mobile phase. From these results, a chiral recognition mechanism using a lipophilic interaction between the tethering alkyl group of the CSP and the alkyl group at the chiral center of α-amino acids was proposed. The chromatographic resolution of α-amino acids on the new CSP was also dependent on the Cu(II) concentration in the mobile phase and the temperature of the column.

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