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# Comparative Studies on Enantiomer Resolution of α-Amino Acids and Their Esters Using (18-Crown-6)-tetracarboxylic acid as a Chiral Crown Ether Selector by NMR Spectroscopy and High-Performance Liquid Chromatography

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In a previous study, our group and the Machida group have developed covalently bonded and/or dynamically coated chiral stationary phases (CSPs) derived from (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA) for HPLC studies (Figure 1).[1-8] These CSPs have been effectively applied in resolving several amino acids and various primary amino compounds.[1-10] In addition, our group reported that 18-C-6-TA as an NMR chiral solvating agent is quite efficient to resolve some amino acid analytes in NMR spectroscopy.[11-13] Therefore, we reported that the chiral crown ether selector 18-C-6-TA is useful for the enantiodiscrimination of phenylglycine, alanine, and diphenylalanine by NMR spectroscopy. Also other groups have reported some results using 18-C-6-TA as a chiral NMR solvating agent.[14-16] Even though each analytical method by HPLC and NMR spectroscopy has been independently studied, the direct comparative study for enantiomer resolution of α-amino acids and their esters using 18-C-6-TA as a chiral selector by two analytical methods has not been published. Especially, the enantiomer resolution results of two α-amino acids esters by NMR spectroscopy were only reported in our group.[11,13] Therefore, in this study, we investigate the enantiomer resolution of various α-amino acids and their esters using (+)-18-C-6-TA as a chiral crown ether selector in NMR studies and compare these NMR results with liquid chromatographic resolution results obtained using 18-C-6-TA derived CSP (CSP 1, Figure 1).

Tables 1 and 2 show comparative results for the enantiomer resolution of several amino acids and their esters by NMR and HPLC. All investigated amino acids and esters were well resolved on CSP 1 derived from (+)-18-C-6-TA by HPLC (α = 1.18-2.74), except for leucine methyl ester and phenylalanine methyl ester (entries 2 and 3 in Table 2). However, all analytes including these two analytes showed chemical shift differences for the α-proton in the presence of 18-C-6-TA by NMR spectroscopy. It means that 18-C-6-TA successfully discriminated all tested amino acids and esters in this study by NMR. Typical $^1$H NMR spectra of valine (Val) and phenylalanine methyl ester (Phe-OMe) in the

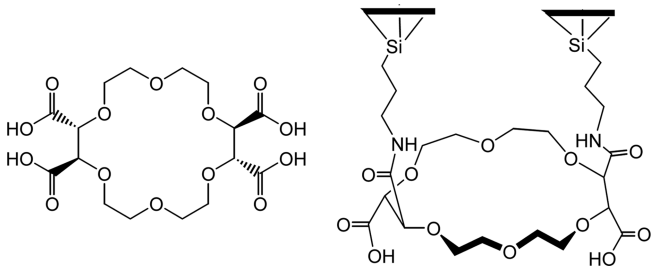

**Figure 1.** The structure of (+)-18-C-6-TA (1) and its derived CSP 1.

**Table 1.** NMR and HPLC results for the enantiomer discrimi-nations of several amino acids

| Entry | Analyte | ΔΔδ[a] (D-L) | Separation factor[b] | Conf.[c] |
|---|---|---|---|---|
| 1 | Alanine | 0.10 | 1.34 | D |
| 2 | Arginine | 0.25 | 1.73 | D |
| 3 | Diphenylalanine | 0.03 | 2.00 | D |
| 4 | Glutamic acid | 0.25 | 1.30 | D |
| 5 | Histidine | 0.29 | 1.49 | D |
| 6 | Isoleucine | 0.14 | 1.44 | D |
| 7 | Leucine | 0.10 | 1.62 | D |
| 8 | Lysine | 0.25[d] | 2.30 | D |
| 9 | Methionine | 0.21 | 1.66 | D |
| 10 | Phenylalanine | 0.21 | 1.60 | D |
| 11 | Phenylglycine | 0.21 | 2.74 | D |
| 12 | Serine | 0.15 | 2.10 | L |
| 13 | Threonine | 0.13[e] | 1.27 | L |
| 14 | Tyrosine | 0.17 | 1.55 | D |
| 15 | Valine | 0.32[d] | 1.56 | D |

[a]Chemical shift non-equivalences (ppm) were obtained by subtracting L-isomer values from D-isomer values of α-protons after complexation. Chemical shifts were determined from the spectra of racemic analytes (2 mM) in the presence of 1 equivalent of 18-C-6-TA. [b]Separation factor observed on CSP 1 by HPLC [Mobile phase: 80% methanol in water (V/V) containing 10 mM $H_2SO_4$; Flow rate = 1 mL/min; UV 210 nm; Temperature ambient (about 25 °C)]. [c]The configuration of the second eluted enantiomer by HPLC.[d,e] Two and four equivalents of 18-C-6-TA were used, respectively.

**Table 2.** The enantiomer discriminations of several amino acid esters by NMR and HPLC

| Entry | Analyte | ΔΔδ[a] (D-L) | Separation factor[b] | Conf.[c] |
|---|---|---|---|---|
| 1 | Alanine methyl ester | 0.11 | 1.18 | D |
| 2 | Leucine methyl ester | 0.16 | 1.00 | - |
| 3 | Phenylalanine methyl ester | 0.29 | 1.00 | - |
| 4 | Phenylglycine methyl ester | 0.27 | 2.24 | D |
| 5 | Phenylglycine ethyl ester | 0.27 | 2.46 | D |
| 6 | Phenylglycine *n*-propyl ester | 0.27 | 2.34 | D |
| 7 | Phenylglycine *n*-butyl ester | 0.28 | 2.42 | D |
| 8 | Valine methyl ester | 0.23 | 1.21 | D |

[a]Chemical shift non-equivalences (ppm) were obtained by subtracting L-isomer values from D-isomer values of α-protons after complexation. Chemical shifts were determined from the spectra of racemic analytes (2 mM) in the presence of 1 equivalent of 18-C-6-TA. [b]Separation factor observed on CSP 1 by HPLC [Mobile phase: 80% methanol in water (V/V) containing 10 mM $H_2SO_4$; Flow rate = 1 mL/min; UV 210 nm; Temperature ambient (about 25 °C)]. [c]The configuration of the second eluted enantiomer by HPLC.

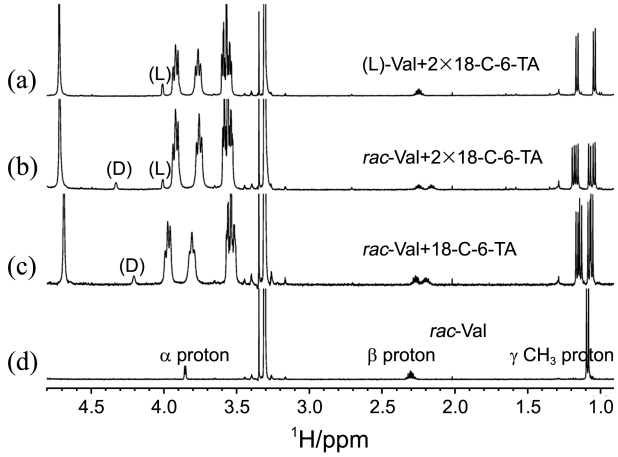


**Figure 2.** $^1$H NMR spectra of Val and Val/18-C-6-TA complex; (a) (L)-Val (2.0 mM) with 18-C-6-TA (4.0 mM), (b) *rac*-Val (2.0 mM) with 18-C-6-TA (4.0 mM), (c) *rac*-Val (2.0 mM) with 18-C-6-TA (2.0 mM), and (d) *rac*-Val (2.0 mM).

presence and absence of 18-C-6-TA at 30 °C are shown in Figures 2 and 3. All α-proton chemical shifts in the presence of 18-C-6-TA were moved in the downfield shift direction compared to the free state. On chiral complexation in the presence of 18-C-6-TA, chemical shift changes of α-protons were greater than those of other protons. The chemical shift differences (ΔΔδ) of the α-proton of these enantiomers in the presence of 18-C-6-TA were found to be 0.03-0.32 ppm for α-amino acids in methanol-$d_4$ containing 10 mM $H_2SO_4$ and for α-amino acid esters in methanol-$d_4$. Unlike all resolved analytes, the proline analyte, which does not contain a primary amino moiety, was not resolved by NMR or HPLC.[1] In case of lysine, threonine, and valine in Table 1, two or four equivalents of 18-C-6-TA were used, because their α-proton peaks in the presence of equimolecular amounts of 18-C-6-TA overlapped with other NMR peaks, as shown in Figure 2. As the stoichiometric ratio of 18-C-6-TA to analytes was increased, the chemical shift non-equivalences

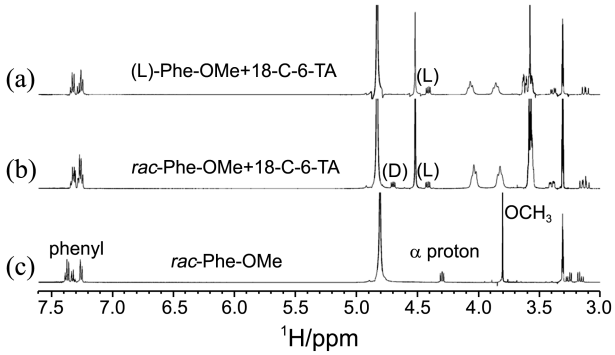


**Figure 3.** $^1$H NMR spectra of Phe-OMe and Phe-OMe/18-C-6-TA complex containing equimolar mixtures (20 mM each); (a) (L)-Phe-OMe with 18-C-6-TA, (b) *rac*-Phe-OMe with 18-C-6-TA, (c) *rac*-Phe-OMe.

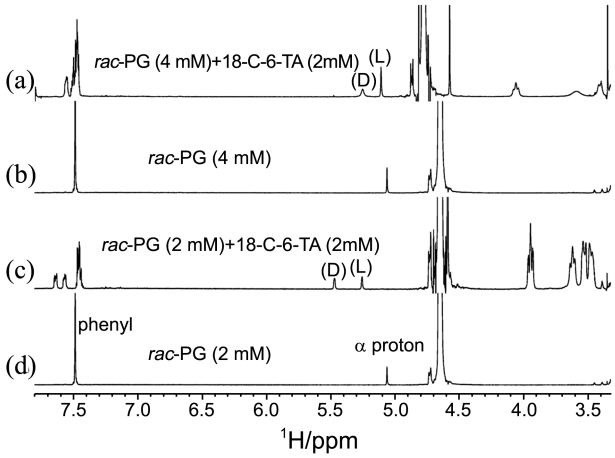


**Figure 4.** $^1$H NMR spectra of PG and PG/18-C-6-TA complex; (a) *rac*-PG (4 mM) with 18-C-6-TA (2 mM), (b) *rac*-PG (4 mM), (c) *rac*-PG (2 mM) with 18-C-6-TA (2 mM), and (d) *rac*-PG (2 mM).

[ΔΔδ(D-L)] of α-protons of analytes gradually increased. For example, the chemical shift differences of the α-protons of alanine methyl ester (Ala-OMe) and phenylglycine methyl ester (PG-OMe) in the presence of 1 and 5 equivalents of 18-C-6-TA were 0.11 and 0.16, respectively, for Ala-OMe, and 0.27 and 0.31 for PG-OMe. Even in the presence of the half equimolecular solution (2 mM) of 18-C-6-TA, the chemical shift non-equivalence of α-proton of the PG analyte (4 mM) was observed in solution NMR (Figure 4).

In addition, all α-proton chemical shift changes of D-isomers on complexation were consistently greater than those of L-isomers in NMR, although serine and threonine show the reversed elution order in HPLC (entries 12 and 13 in Table 1). Except for serine and threonine, D-enantiomers of the resolved α-amino acids and their esters were preferentially retained on (+)-18-C-6-TA derived CSP 1 in HPLC (Figure 5).[1,5,17,18] Owing to free environment of the chiral selector (+)-18-C-6-TA in solution NMR unlike the stationary phase environment of CSP 1 in HPLC, presumably, all D-enantiomers including serine and threonine would form more stable complexes with the chiral selector than L-enantiomers in NMR. On the other hand, there is a hydroxy

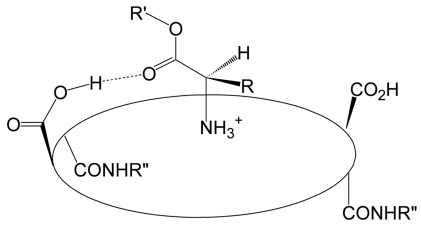


**Figure 5.** Proposed chiral recognition between (+)-18-C-6-TA derived CSP 1 and the second eluted enantiomer of the D-amino acids or esters (except serine and threonine), showing intermolecular hydrogen bonding (dotted line) between the chiral selector of the CSP 1 and the carbonyl oxygen of the D-amino acid or its ester.[4,11,17]

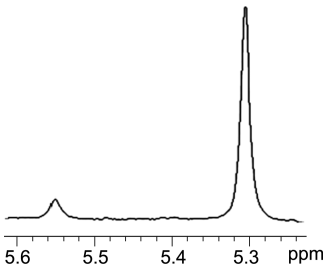


**Figure 7.** $^1$H NMR spectrum of the α-proton of an enantiomerically enriched phenylglycine sample (D:L = 1:10) containing 3 equivalents (3 mM) of (+)-18-C-6-TA (left peak D-isomer, right peak L-isomer).

group on the β-carbon of both serine and threonine, and it is considered that this might directly influence the chiral recognition interaction with 18-C-6-TA of stationary phase in HPLC. Unlike our previously proposed chiral recognition mechanism in Figure 5,[4,11,17] therefore, we suggest a much stronger intermolecular hydrogen bonding between the β-hydroxy group of serine (or threonine) and the COOH of 18-C-6-TA, instead of the intermolecular hydrogen bonding between the carbonyl oxygen of D-enantiomers and the COOH of 18-C-6-TA. Figure 6 shows a schematic representation of the proposed chiral recognition between the CSP 1 derived from (+)-18-C-6-TA and the strongly eluted enantiomer of L-serine (or L-threonine). In terms of spatial orientations during the formation of diastereomeric complex between L-serine (or L-threonine) and (+)-18-C-6-TA, it is expected that this intermolecular hydrogen bonding interaction by the β-hydroxy group of L-serine (or L-threonine) affords a favorable interaction, and that this results in a reversal of the HPLC elution orders (entries 12 and 13 in Table 1).

It was observed that the enantioselectivities of amino acids observed by HPLC (Table 1) were greater than those of the corresponding amino acid methyl esters (Table 2). However, the chemical shift non-equivalences of amino acid methyl esters observed by NMR in Table 2 are greater than those of the corresponding amino acids in Table 1. The magnitude of chemical shift non-equivalences observed in NMR was not directly related to the enantioselectivities in HPLC. For example, diphenylalanine enantiomers with the smallest chemical shift non-equivalence (0.03 ppm) in this study had a fairly large separation factor (α = 2.00) (entry 3 in Table 1). In particular, although the enantiomers of leucine methyl ester and phenylalanine methyl ester were not resolved on CSP 1, they showed considerable chemical shift non-equivalences (0.16, 0.29 ppm) of α-protons in equimolecular solutions of 18-C-6-TA by NMR (entries 1 and 3 in Table 2). These results indicate that the NMR analytical method based on the use of 18-C-6-TA as a chiral solvating agent may be complementary to the HPLC analytical technique. Also, they indicate that 18-C-6-TA as a chiral solvating agent with amino acids and their esters in NMR affords meaningful chemical shift non-equivalences, and that it could be usefully applied to the determinations of the enantiomeric purities of these analytes. A typical $^1$H NMR spectrum of the α-proton of an enantiomerically enriched phenylglycine sample (D:L = 1:10) containing 3 equivalents (3 mM) of (+)-18-C-6-TA is shown in Figure 7. In order to determine the enantiomeric compositions of amino acids and their esters, generally, the NMR technique using 18-C-6-TA as a chiral solvating agent shows lower precision and accuracy than HPLC technique using chiral columns.[19] However, because of the simplicity of NMR-based analytical processes using a chiral solvating agent, this NMR analytical method may be practically useful for determinations of the enantiomeric purities of chiral analytes like α-amino acids and their esters.

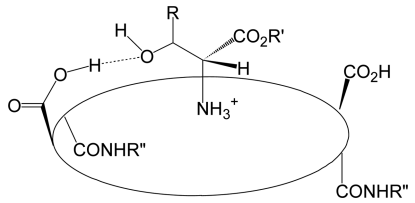


**Figure 6.** Proposed chiral recognition between (+)-18-C-6-TA derived CSP 1 and the second eluted enantiomer of L-serine (R=H) [or L-threonine (R = methyl)], showing intermolecular hydrogen bonding (dotted line) between the chiral selector of the CSP 1 and the oxygen of L-serine (or L-threonine) of the analyte.

In conclusion, NMR and HPLC enantiomer discrimination studies were performed based on the use of (+)-18-C-6-TA as a chiral crown ether selector for α-amino acids and their esters. NMR resolution results were compared with chromatographic resolution results obtained using CSP 1. Except for leucine methyl ester and phenylalanine methyl ester, all investigated analytes were well resolved on CSP 1 by HPLC. On the other hand, the chiral crown ether selector (+)-18-C-6-TA successfully discriminated enantiomers of all α-amino acids and their esters in the NMR study. Leucine methyl ester and phenylalanine methyl ester showed considerable chemical shift differences of α-protons in the presence of 18-C-6-TA by NMR. It is confident that 18-C-6-TA is a highly efficient NMR chiral solvating agent for the determinations of the enantiomeric compositions of α-amino acids and their esters. Therefore, we believe that NMR technique using the chiral solvating agent of 18-C-6-TA complements HPLC technique using 18-C-6-TA derived CSP 1.

## Experimental

All chemicals were of analytical-reagent grade and they were purchased from Aldrich (Milwaukee, WI) or Fluka (Switzerland). High-performance liquid chromatography was carried out using an HPLC Breeze system (Waters, Milford, MA) equipped with a Waters model 1525 binary pump, an autosampler, and a dual absorbance detector (Waters 2487 detector). ChiroSil RCA (CSP 1) (250 × 4.6 mm I.D., 5 μm) based on (+)-18-C-6-TA was purchased from RS Tech (Daejon, Korea). DRX500 (Bruker, Germany) in $^2$H lock mode was used for all $^1$H NMR experiments at 30 °C, and all NMR chemical shifts were referenced to tetramethylsilane (TMS). Methanol-$d_4$ containing 10 mM $H_2SO_4$ and methanol-$d_4$ as NMR solvents were used for α-amino acids and α-amino acid esters, respectively.

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