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# Nuclear magnetic resonance studies for the chiral recognition of the novel chiral stationary phase derived from 18-crown-6 tetracarboxylic acid

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**Abstract**

Enantioselectivities observed in high-performance liquid chromatography (HPLC) with the novel chiral stationary phase (CSP-18C6I) derived from (+)-18-crown-6 tetracarboxylic acid ($18C6H_4$) were investigated by using nuclear magnetic resonance (NMR) spectrometry. The elution orders in CSP-18C6I, that is, the *S*-enantiomer of 1-(1-naphthyl)ethylamine (1-NEA) and the L-enantiomer (*S*-form) of alanine-β-naphthylamide (Ala-β-NA) eluted prior to each corresponding enantiomer, were successfully explained on the basis of the apparent binding constants ($K_a$) of the enantiomers to the CSP moiety which were calculated from $^1$H-NMR experiments. Detailed HPLC and NMR studies for the chiral recognition of racemic amino compounds with $18C6H_4$ hosts showed that $^1$H-NMR spectrometry is a useful technique for the investigation of the chiral recognition mechanism in HPLC. Additionally, it was found $18C6H_4$ can be recommended as a useful chiral shift reagent for the enantiomeric excess determination by $^1$H-NMR. 

*Keywords:* Enantiomer separation; Chiral stationary phases, LC; Nuclear magnetic resonance spectrometry; 18-Crown-6 tetracarboxylic acid; Amines

## 1. Introduction

Crown ethers, first introduced by Pedersen in 1967 [1,2], are synthetic macrocyclic polyethers that can form selective complexes with suitable cations. Recently crown ether derivatives have been widely used as chiral selectors for primary amines in analytical chemistry, particularly in capillary electrophoretic [3–5], gas chromatographic [6,7] and high-performance liquid chromatographic (HPLC) enantioseparations [8–12].

In the late 1970s, Cram and co-workers reported HPLC enantioseparation of amino acids and amino esters by using chiral stationary phases (CSPs) consisting of chiral crown ethers attached to polystyrene [8] or silica-gel [9]. Enantiopure crown ethers form complexes enantioselectively with chiral primary amines (in the form of ammonium cations). In 1987, Shinbo and co-workers [10,11] reported the separation of underivatized DL-amino acids by using a CSP in which a hydrophobic chiral crown ether was dynamically coated on an ODS column.

In our previous work [12], we reported the synthesis and evaluation of the novel CSP (CSP-18C6I, see Fig. 1) chemically immobilized (+)-18-crown-6 tetracarboxylic acid ($18C6H_4$). The CSP-18C6I

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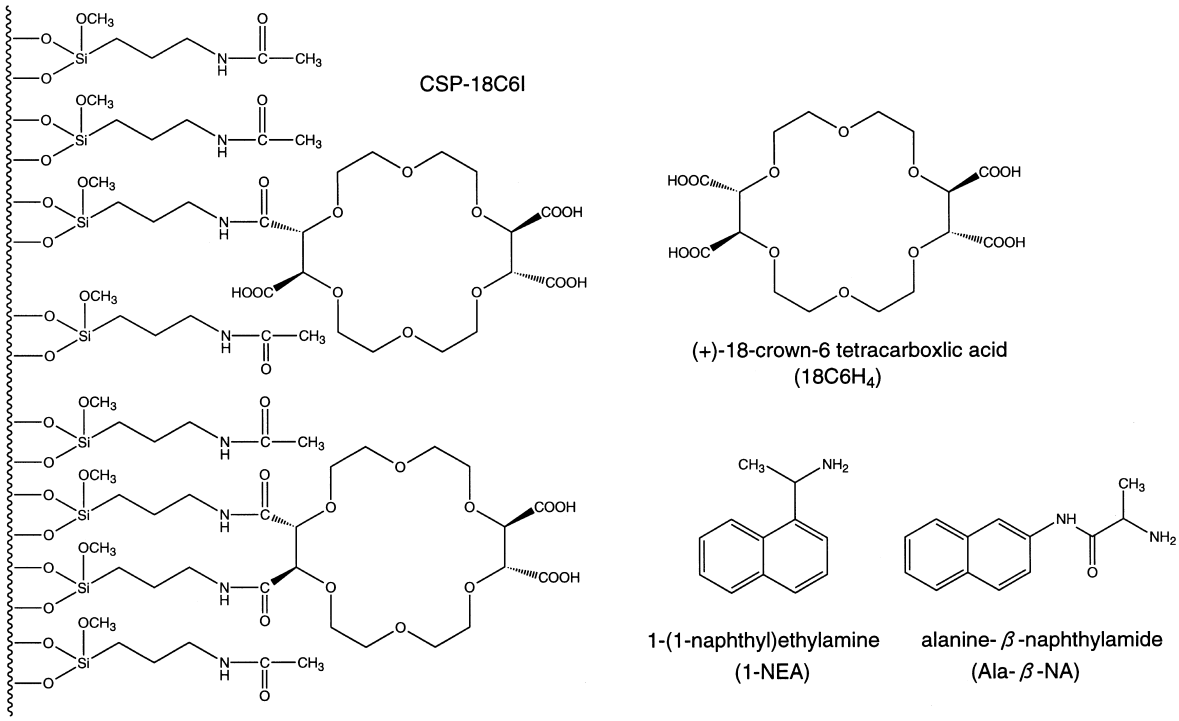


Fig. 1. Structures of CSP-18C6I, $18C6H_4$, 1-NEA and Ala-β-NA.

showed a wide enantioselectivity for a large number of primary amino compounds, and often afforded large separation factors ($\alpha$). For example, the CSP-18C6I separated the enantiomers of 1-(1-naphthyl)ethylamine (1-NEA) and alanine-β-naphthylamide (Ala-β-NA) with $\alpha$ values of 1.44 and 1.25, respectively. These properties coupled with its relatively simple $^1$H-NMR (nuclear magnetic resonance) spectra, suggested that 1-NEA and Ala-β-NA would be appropriate candidates for a spectroscopic investigation of the chiral recognition mechanism in the CSP-18C6I.

The measurement of the chemical shift of analytes in $^1$H-NMR spectrometry through the addition of chiral moiety, is one of the most commonly used methods for the investigation of the chiral recognition mechanism [13–18]. In fact, the quantitative analysis of the dependence of the chemical shifts on the substrate/chiral moiety, molar ratio affords a way to determine the stoichiometry [19] and the binding constants [20] of the complexes formed.

In this work, NMR spectroscopy is employed for the investigation of the interaction between analytes (1-NEA or Ala-β-NA) and $18C6H_4$ in an aqueous solution. The apparent binding constants ($K_a$) are determined to rationalize the enantioselective selector–solute interaction of both enantiomers with $18C6H_4$, which can be the basis of chiral discrimination phenomena observed.

## 2. Experimental

### 2.1. HPLC equipment

The HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-10AD high-pressure pump and an SPD-10A variable-wavelength UV detector, operating at 254 nm. Samples were applied to the column with a Rheodyne Model 7725i injector equipped with a 50-μl sample loop. Peak integration was carried

out with a Shimadzu Chromatopac C-R7A plus data processor.

Chromatographic runs were performed at a constant flow-rate of 0.3 ml/min and a constant temperature of 25°C. Typically, 2 μl of a 1% solution of racemate dissolved in the mobile phase was injected.

### 2.2. NMR equipment

$^1$H-NMR spectra were taken in $CD_3OD$ on Brucker DRX-500 FT-NMR spectrometer (Rheinstetten, Germany) operating at 500 MHz in the $^2$H lock mode. Chemical shifts were reported in parts per million (ppm) relative to tetramethylsilane (TMS), and coupling constants were reported in Hz. Typical acquisition parameters included a spectral width of 5000 Hz. Spinning tubes of 5 mm I.D. containing 600 μl of solution were employed. The pH of sample solutions were adjusted to around 1 by adding DCl for the protonation of tetracarboxylic acids and primary amines. The NMR data were plotted according to the molar ratio and the continuous variations method. The stoichiometry of the selector–solute complexes was determined by the continuous variation method [19]. The total concentration of the interacting species in the solution was kept constant at 10 m*M* and molar fraction of the selector was varied in the range of 0.2–0.8. The apparent binding constants of the enantiomers of 1-NEA or Ala-β-NA with $18C6H_4$ were calculated on the basis of Scott's modification [20] of the Benesi–Hildedrand equation [21]. In the measurements, the analyte was kept at 2 m*M*, while the concentration of $18C6H_4$ ranged from 0 to 8 m*M*.

### 2.3. Materials

Acetonitrile of HPLC grade and perchloric acid (70%) of analytical reagent grade were purchased from Katayama Kagaku Kogyo (Osaka, Japan). $18C6H_4$ was purchased from Aldrich (WI, USA). 1-NEA, *S*-(−)-1-NEA, *R*-(+)-1-NEA, Ala-β-NA and L-Ala-β-NA were purchased from Aldrich, Katayama Kagaku Kogyo, Nacalai Tesque (Kyoto, Japan), Tokyo Kasei Kogyo (Tokyo, Japan) and Wako (Tokyo, Japan). $CD_3OD$, DCl and TMS were purchased from Isotec (OH, USA).

## 3. Results and discussion

### 3.1. Enantiomer separations on the CSP-18C6I

The novel CSP-18C6I [12] was designed, and prepared through immobilizing $18C6H_4$ to the silicagel. This CSP-18C6I was found to be able to resolve the enantiomers of a large number of primary amino compounds. For example, the CSP-18C6I separated 25 enantiomers out of 32 compounds. Typical chromatograms of enantiomers of 1-NEA and Ala-β-NA are shown in Fig. 2. The mobile phase used was a mixture of 10 m*M* perchloric acid–methanol (50:50), which is not permitted in the commercially available Crownpak CR(+) (Daicel, Tokyo, Japan) column. The *S*-enantiomer of 1-NEA, and the L-enantiomer (*S*-form) of Ala-β-NA eluted prior to the corresponding enantiomers.

### 3.2. NMR studies of selector–solute interactions

The comparison of the $^1$H-NMR spectra of 1-NEA (Fig. 3) and Ala-β-NA (Fig. 4) in the presence and in the absence of an equimolar amount of $18C6H_4$ revealed that in the former, two sets of resonances were observed for almost each proton or group of equivalent proton of 1-NEA and Ala-β-NA. These peaks were simply superposition of spectra from the individual diastereomeric inclusion complexes that have formed. The chemical shifts and these assignments of 1-NEA and Ala-β-NA are summarized in Table 1 (1-NEA) and Table 2 (Ala-β-NA). Discussion was based on the best resolved doublets ($CH_3$) centered at 1.77 ppm ($J$=6.8 Hz, 1-NEA) or 1.66 ppm ($J$=7.0 Hz, Ala-β-NA), and qualtets (CH) centered at 5.39 ppm ($J$=6.8 Hz, 1-NEA) or 4.17 ppm ($J$=7.0 Hz, Ala-β-NA). The continuous variation method [19] (Job plot, total concentration 10 m*M*, molar fraction of $18C6H_4$ varying from 0.2 to 0.8) applied to $CH_3$ and CH proton of 1-NEA and Ala-β-NA, gave symmetrical bell-curves, supporting the 1:1 complex formation (Fig. 5).

The association constants $K_a$ for the 1:1 complexes between 1-NEA or Ala-β-NA and $18C6H_4$ were evaluated by using Scott's modification [20] of the Benesi–Hildebrand equation [21]:

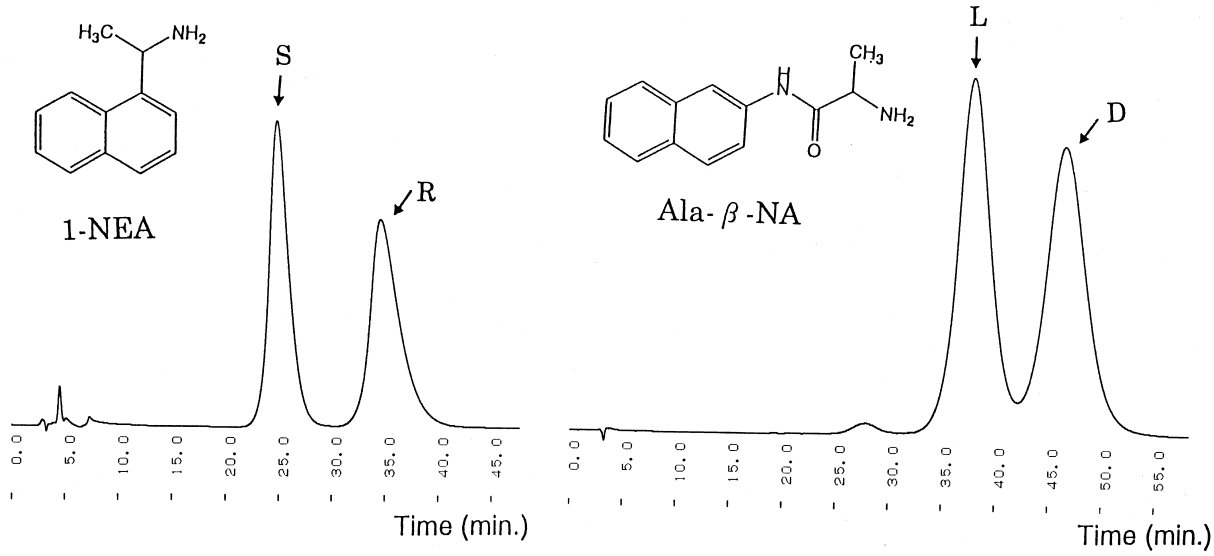


Fig. 2. Enantiomer separations of 1-NEA and Ala-β-NA by CSP-18C6I. Mobile phase: methanol–10 m*M* perchloric acid (50:50); column temperature: 25°C; detection: 254 nm.

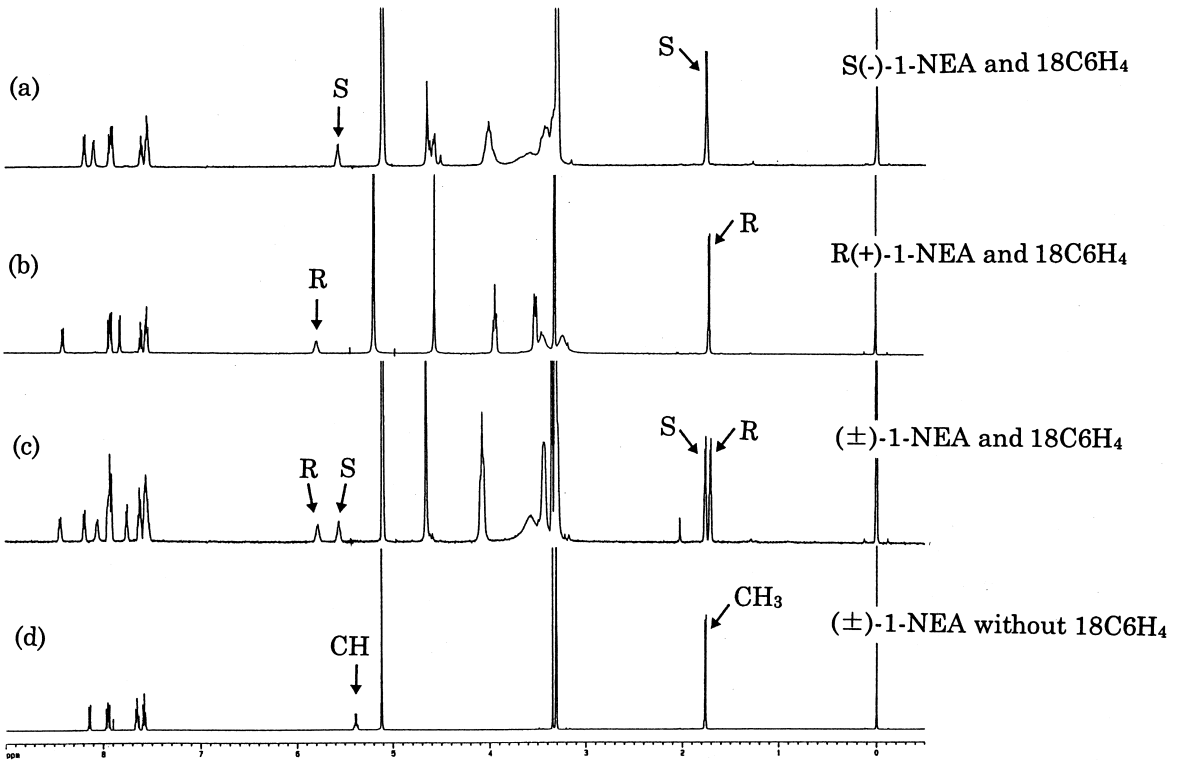


Fig. 3. $^{1}$H-NMR spectra of 1-NEA and equimolar mixtures (5 m*M* each) of 1-NEA/18C6H$_4$. (a) *S*-(−)-1-NEA and 18C6H$_4$; (b) *R*-(+)-1-NEA and 18C6H$_4$; (c) (±)-1-NEA and 18C6H$_4$; (d) (±)-1-NEA without 18C6H$_4$.

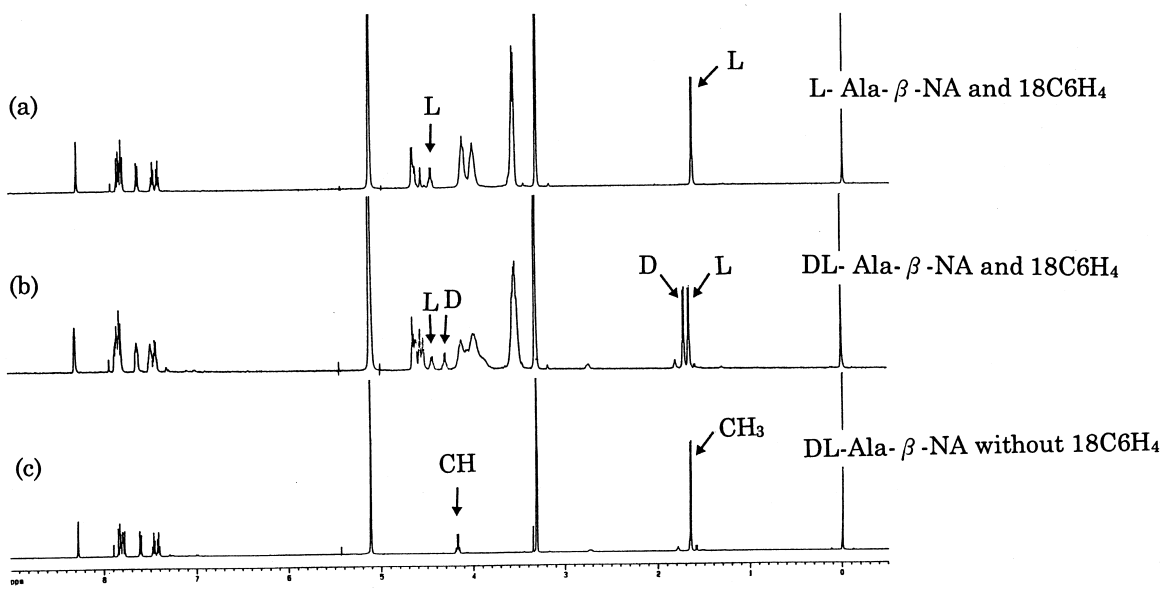


Fig. 4. $^1$H-NMR spectra of Ala-β-NA and equimolar mixtures (5 m*M* each) of Ala-β-NA/18C6H$_4$. (a) L-Ala-β-NA and 18C6H$_4$; (b) DL-Ala-β-NA and 18C6H$_4$; (c) DL-Ala-β-NA without 18C6H$_4$.

Table 1
Observed $^1$H chemical shifts of 1-NEA in the presence of 18C6H$_4$

| | 18C6H$_4$ | CH$_3$ | CH | 3H | 6H | 7H | 2H | 4H | 5H | 8H |
|---|---|---|---|---|---|---|---|---|---|---|
| *RS*-form | Absence | 1.765 | 5.388 | 7.580 | 7.583 | 7.651 | 7.664 | 7.943 | 7.963 | 8.141 |
| *S*-(−)-form | Presence | 1.799 | 5.624 | 7.537 | 7.552 | 7.610 | 8.116 | 7.909 | 7.927 | 8.254 |
| Δδ | | 0.034 | 0.236 | −0.043 | −0.031 | −0.041 | 0.452 | −0.034 | −0.036 | 0.113 |
| *R*-(+)-form | Presence | 1.719 | 5.783 | 7.537 | 7.545 | 7.602 | 7.814 | 7.904 | 7.928 | 8.397 |
| Δδ | | −0.045 | 0.394 | −0.043 | −0.038 | −0.049 | 0.150 | −0.039 | −0.035 | 0.257 |

All chemical shifts reported in ppm relative to TMS in CD$_3$OD at 25°C.
Concentration of all components is 5 m*M*.

Table 2
Observed $^1$H chemical shifts of Ala-β-NA in the presence of 18C6H$_4$

| | 18C6H$_4$ | CH$_3$ | CH | 7H | 6H | 3H | 5H | 8H | 4H | 1H |
|---|---|---|---|---|---|---|---|---|---|---|
| DL-form | Absence | 1.653 | 4.173 | 7.414 | 7.468 | 7.617 | 7.786 | 7.811 | 7.839 | 8.272 |
| L-form | Presence | 1.626 | 4.456 | 7.407 | 7.466 | 7.630 | 7.791 | 7.809 | 7.840 | 8.272 |
| Δδ | | −0.027 | 0.283 | −0.007 | −0.002 | 0.013 | 0.005 | −0.002 | 0.001 | 0.000 |
| D-form | Presence | 1.699 | 4.288 | N.A. | N.A. | N.A. | N.A. | N.A. | N.A. | 8.278 |
| Δδ | | 0.045 | 0.116 | | | | | | | 0.006 |

All chemical shifts reported in ppm relative to TMS in CD$_3$OD at 25°C.
Concentration of all components is 5 m*M*.
N.A. = Not assigned.

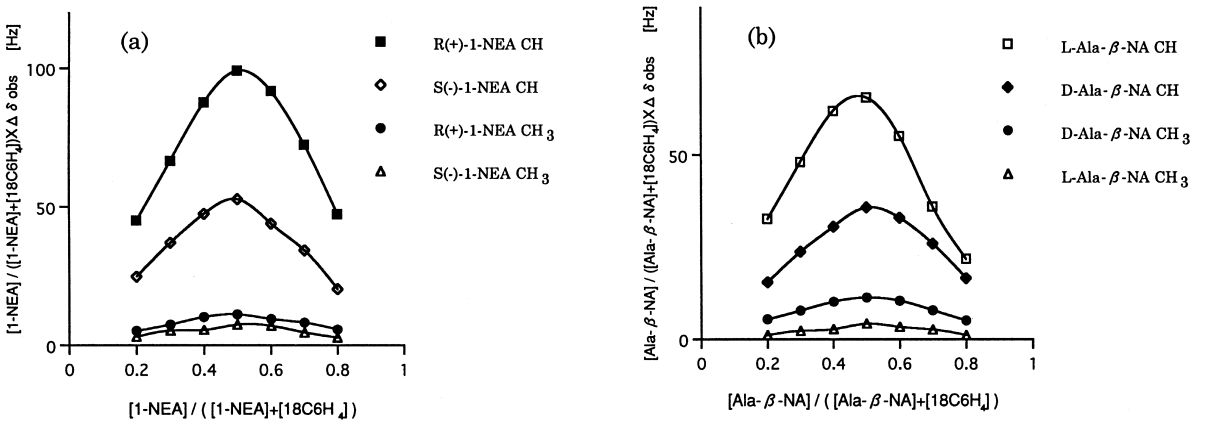


Fig. 5. Job plots for 1-NEA and Ala-β-NA in solution with $18C6H_4$. (a) 1-NEA in solution with $18C6H_4$; (b) Ala-β-NA in solution with $18C6H_4$.

$$\frac{[\text{selector}]_t}{\Delta\delta_{obs}} = \frac{[\text{selector}]_t}{\Delta\delta_c} + \frac{1}{K_a \Delta\delta_c} \quad (1)$$

where $[\text{selector}]_t$ is the molar concentration of the chiral selector, $\Delta\delta_{obs}$ is the observed chemical shift difference for a given $[\text{selector}]_t$ concentration, $\Delta\delta_c$ is the chemical shift difference between a pure sample of complex and the free component at the saturation. In the experiments, the analyte was kept at 2 m*M*, while the concentration of $18C6H_4$ ranged from 0 to 8 m*M*. The change of the chemical shift of the $CH_3$ and CH proton in analyte was measured at 25°C and the ratio of the $[\text{selector}]_t$ and the $\Delta\delta_{obs}$ was plotted as a function of $[\text{selector}]_t$ for both the complexes. Scott plots of the complexes of analytes (1-NEA or Ala-β-NA) with $18C6H_4$ are given in Fig. 6. The slope of the plot of $[\text{selector}]_t/\Delta\delta_{obs}$ against $[\text{selector}]_t$ is thus equal to $1/\Delta\delta_c$ and the intercept with the vertical axis to $1/K_a\Delta\delta_c$, allows the estimation of $K_a$. The values of the complexation-induced chemicals shifts at saturation ($\Delta\delta_c$) and the apparent binding constants ($K_a$) calculated for two different $^1$H-NMR signals are given in Table 3.

The values of $\Delta\delta$ ($CH_3$ and CH proton) of the *R*-enantiomer (*R*-form) were larger than those of the *S*-enantiomer (*S*-form) in 1-NEA. The complexation-

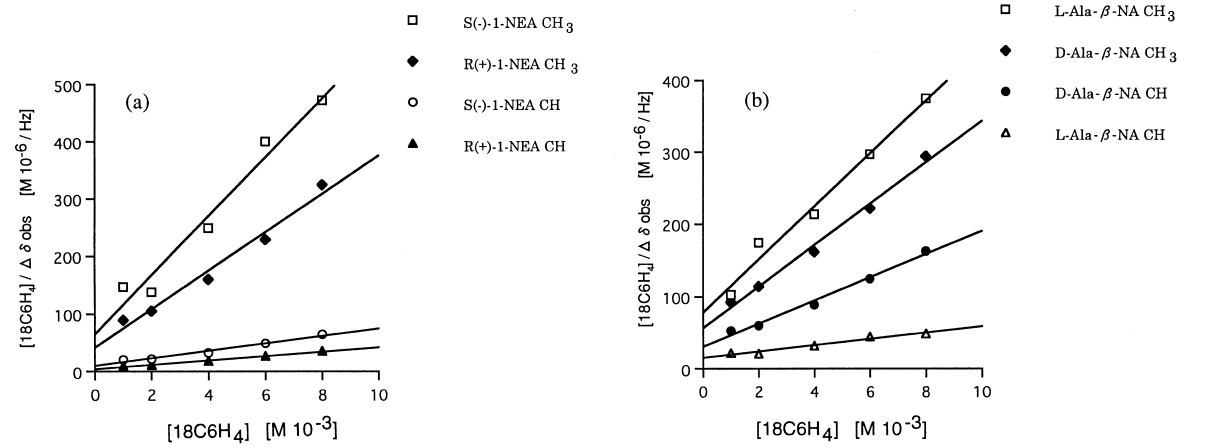


Fig. 6. Scott plots for 1-NEA and Ala-β-NA in solution with $18C6H_4$. (a) 1-NEA in solution with $18C6H_4$; (b) Ala-β-NA in solution with $18C6H_4$.

Table 3
Complexation-induced chemical shifts at saturation ($\Delta\delta_c$) and apparent binding constants ($K_a$) for 1-NEA and Ala-β-NA with $18C6H_4$

| | $\Delta\delta_c$ (*R*) | | $\Delta\delta_c$ (*S*) | | $K_a$(*R*) | | $K_a$(*S*) | | $\alpha$ [$K_a$(*R*)/$K_a$(*S*)] | | $\alpha$ (*R*/*S*)[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $CH_3$ | CH | $CH_3$ | CH | $CH_3$ | CH | $CH_3$ | CH | $CH_3$ | CH | |
| 1-NEA | 27.6 | 260.7 | 18.7 | 151.5 | 1330.3 | 929.7 | 1074.0 | 661.6 | 1.24 | 1.41 | 1.48 |
| Ala-β-NA | 33.2 | 62.4 | 27.2 | 181.2 | 634.6 | 525.7 | 470.5 | 446.3 | 1.35 | 1.18 | 1.48 |

All chemical shifts reported in Hz relative to TMS in $CD_3OD$ at 25°C.
[a] Separation factors in CSP-18C6I.
D-Form of Ala-β-NA corresponds to the *R*-form in the Table. Similarly, L-form corresponds to the *S*-form.

induced chemical shifts of 1-NEA for $CH_3$ and CH proton significantly shifted for the *R*-enantiomer compared with the *S*-enantiomer. The apparent binding constants ($K_a$) calculated on the basis of $CH_3$ proton were 1330.3 $M^{-1}$ and 1074.0 $M^{-1}$, on the basis of CH proton were 929.7 $M^{-1}$ and 661.6 $M^{-1}$, respectively. These results (the values of $K_a$) were in good agreement with the enantiomer elution order of 1-NEA in CSP-18C6I. That is, in the chromatographic study of 1-NEA, the *S*-enantiomer eluted prior to the *R*-enantiomer.

The $\Delta\delta$ value of CH proton of the L-enantiomer (L-form) (181.2 Hz) was larger than the D-enantiomer (D-form) (62.4 Hz), on the other hand, the $\Delta\delta_c$ value of $CH_3$ proton of the L-enantiomer (27.2 Hz) was smaller than the D-enantiomer (33.2 Hz) in Ala-β-NA. These results have been also observed in the chiral recognition of amide-derivative of naproxen [14] and atropisomeric binaphthyl derivatives [17]. The complexation-induced chemical shifts of Ala-β-NA for $CH_3$ proton significantly shifted for the D-enantiomer compared with the L-enantiomer, on the other hand, CH proton significantly shifted for the L-enantiomer than the D-enantiomer. The apparent binding constants given from $CH_3$ and CH proton for the D-enantiomer were larger than the L-enantiomer.

### *3.3. Chiral discrimination and stereochemistry*

As shown in Tables 1 and 3, in the chromatographic study, *R*-(+)-1-NEA with $18C6H_4$ complex seems to be more stable, judging from the elution behavior of 1-NEA on CSP-18C6I. The complexation-induced chemical shift of 1-NEA for CH proton significantly shifted for the *R*-enantiomer (0.394 ppm) than for the *S*-enantiomer (0.236 ppm). Because the CH proton is adjacent to the ammonium cation, the CH proton must be significantly influenced by the chiral moiety (carboxylic acids) and shifted. These proved that the polyether ring forms the shape of a cavity which is able to form stable complexes, particularly ammonium or primary amines held inside the cavity of $18C6H_4$, are bound by three $+NH-O$ hydrogen bonds in a tripod arrangement. In the aromatic protons, the 2,8-position protons of the aromatic ring in 1-NEA, were significantly shifted. The reason for the shifting field of 2,8-position protons seems to be the steric interaction. Molecular models of the 1-NEA/$18C6H_4$ complex indicated that chiral recognition occurs merely by the steric barrier mechanism. Proposed stereoscopic images of 1-NEA are shown in Fig. 7. The chiral recognition is probably based on hydrogen bonding between the oxygen of polyether ring and ammonium ion, with steric interaction between the 2,8-position protons of aromatic ring and substituents (tetracarboxylic acids) or polyether ring of $18C6H_4$.

As shown in Table 2, the complexation-induced chemical shift of Ala-β-NA for the aromatic proton did not significantly shift, compared with 1-NEA. It seems that the aromatic ring of Ala-β-NA is not involved in the chiral recognition, because the distance between the $NH^{3+}$ and aromatic ring is long, naphthalene cannot interact with the polyether ring or substituents (tetracarboxylic acids).

Finally, in the HPLC study [12] with CSP-18C6I, basic DL-amino acids (arginine, histidine) were resolved, but acidic DL-amino acids (asparagine, aspartic acid) were not resolved on the CSP-18C6I. Furthermore, DL-amino acids (phenylalanine, phenylglycine etc.) having one bulky substituent group at the β-carbon atom showed a large enantioselectivity, but DL-amino acids (isoleucine, threonine etc) having

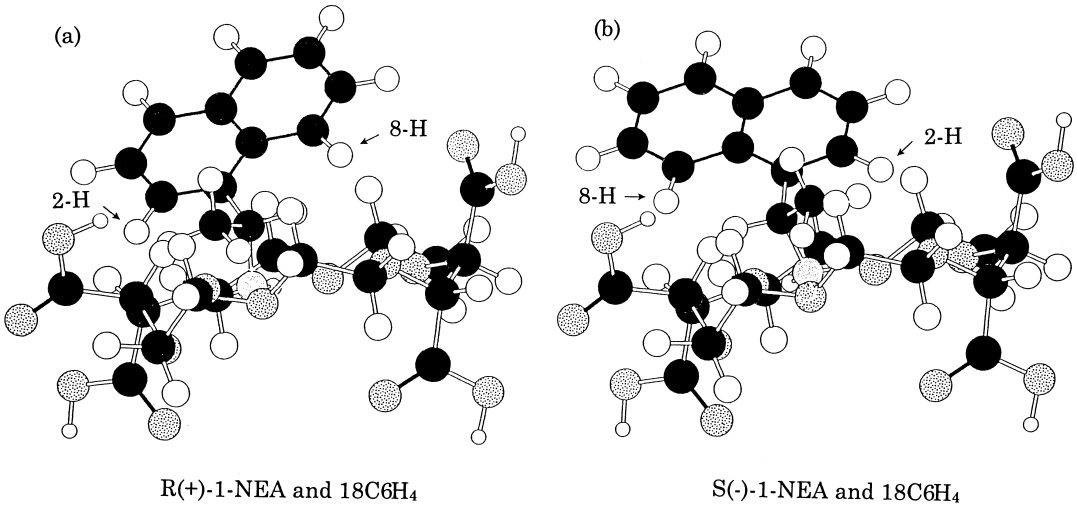


Fig. 7. Stereoscopic image of host-guest complexes of $18C6H_4$ with 1-NEA. Key for symbols: hydrogens are white, oxygens light, carbons black and nitrogens dark.

more than one substituent group at the β-carbon atom did not give an enantioselectivity. In the NMR study with $18C6H_4$, aromatic protons of 1-NEA significantly shifted, but those of Ala-β-NA were not significantly shifted. In the comparative HPLC and NMR studies of the chiral recognition of primary amino compounds with $18C6H_4$, it seems to be two interaction. First, the chiral recognition is caused by the electrostatic interaction between analytes and substituents (tetracarboxylic acids) or polyether ring in $18C6H_4$. On the other hand, the steric interaction between analytes and substituents (tetracarboxylic acids) or polyether ring of $18C6H_4$, seems to be the another major interaction.

## 4. Conclusions

Enantioselectivities of 1-NEA and Ala-β-NA observed in CSP-18C6I, i.e., the *S*-enantiomer of 1-NEA, and the L-enantiomer (*S*-form) of Ala-β-NA eluted prior to each corresponding enantiomer, were in good agreement with the apparent binding constants ($K_a$) of the enantiomers which were calculated using $^1$H-NMR spectrometry. Job plots gave symmetrical bell-curves, supporting the 1:1 complex formation. Scott plots gave the apparent binding constants ($K_a$) of the enantiomers. The chiral recognition mechanism seems to be based on hydrogen bonding between the oxygen of polyether ring and ammonium ion, with the steric interaction between the 2,8-position protons of aromatic ring and substituents (tetracarboxylic acids) or polyether ring of $18C6H_4$.

Additionally, $18C6H_4$ can be recommended as a useful chiral shift reagent for the enantiomeric excess determination by $^1$H-NMR spectrometry.

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