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Ligand-Exchange Chromatographic Resolution of DL-Amino Acids in the Presence of Nucleic Acids

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Summary. Nine representative DL-amino acids were resolved using ligand-exchange chromatography whose mobile phase contained Cu (II) and ribonucleic acids. It was also found that deoxyribonucleic acids could be substituted for RNAs. In the presence of 5′-GMP, 4XMP, (a mixture of 5′-GMP, -AMP, -CMP, and -UMP), and oligomer of RNA, L-amino acids, which seemed to give more stable complexes, were eluted more slowly than the opposite isomers.

Key words: Ligand-exchange chromatography — Resolution — DL-amino acid — Nucleic acid

Introduction

Because organisms are composed of carbon-containing compounds that are chiral (optically active), the origin of life must escapably be related to the establishment of an optically active molecular world. Over the years since Pasteur's discovery of the enantiomeric resolution of ammonium tartaric acid (Pasteur 1922), many proposals for the symmetry-breaking of existing racemic molecules have been made [e.g., physicochemically (Ulbricht 1959), biologically (Wald 1957)]. However, the origin of the chirality of live molecules (L-amino acid, D-nucleic acid, etc.) is as yet unsolved.

The discovery of RNA enzymes (ribozymes) (Cech and Bass 1986) gave rise to the interesting speculation that the first living molecule on the primitive earth might have been RNA (Gilbert 1986; Watson et al. 1987). If such an RNA world subsequently generated a ribonucleoprotein (as a more functional molecule) world, the preexisting RNAs might have selected L-amino acids from the racemic mixtures for the primordial ribonucleoproteins. It is here hypothesized that the symmetry breaking of DL-amino acids abiotically synthesized might have proceeded after the emergence of ribonucleic acids.

The chiral part of ribonucleic acids is constructed with D-ribose. It has already been demonstrated that D-glucose recognizes the amino acid chirality (Yuasa et al. 1980, 1984, 1985, 1986; Fukuhara and Yuasa 1986; Fukuhara et al. 1987). It is therefore possible that ribonucleic acids might also recognize the chirality of amino acids as well, although only a few related studies so far appear to have been undertaken (Profy and Usher 1984; Watanabe and Miura 1985; Weber 1987; Lacey et al. 1988).

In the present study, the authors report on novel ligand-exchange chromatographic resolution of DL-amino acids in the presence of nucleic acids by applying methods in which only amino acids are used as a chiral additive (Hare and Gil-Av 1979; Davankov et al. 1983).

Materials and Methods

Chromatographic System and Chemicals. An HPLC chromatographic system (Japan Spectroscopic Co., Ltd., Tokyo, Japan) was used for the experiment.

Amino acids, nucleic acids, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Wako Pure Chemical Co., Ltd. (Osaka, Japan), Oriental Yeast Co., Ltd. (Tokyo, Japan), and Yamasa Co., Ltd. (Chiba, Japan), respectively. Abbreviations for nucleic acids are 5′-CMP (cytidine 5′-monophosphate), 5′-UMP (uridine 5′-monophosphate), 5′-AMP (adenosine 5′-monophosphate), 5′-GMP (guanosine 5′-monophosphate), 5′-dAMP (deoxyadenosine 5′-monophosphate), and 5′-dGMP (deoxyguanosine 5′-monophosphate), respectively.

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RNA Oligomer. Two hundred and fifty micrograms of RNA (ribonucleic acid: lot R7125, Sigma) were suspended in 12.5 ml of 50 mM Tris-HCl (pH 7.6), to which 5 mg of ribonuclease A (lot R4875, Sigma) was added, and then the solution was incubated for 16 h at 37°C. The solution was treated 3 × with phenol. After removing phenol, the RNA solution was fractionated through a Sephadex G-25 column [bed volume: 60 cm × 1.6 cm internal diameter (i.d.)]. The oligonucleotide fraction (5–10 nucleotides) was collected and its amount estimated by using the absorbance at 260 nm. Cu(II) (0.5 mM) was then added to 0.5 mM RNA oligomer.

DNA Oligomer. Three hundred milligrams of DNA (lot D1626, Sigma) was suspended in 10 ml of 20 mM Tris-HCl (pH 7.6) and 10 mM $MgCl_2$, to which 2 mg of deoxyribonuclease I (lot D0876, Sigma) was added and then incubated for 16 h at 37°C. The other procedures were the same as those for the preparation of the RNA oligomer.

Preparation of the Column Coated with Octyl 5′-AMP. One gram of 5′-AMP, 0.64 ml of epoxyoctane, and 0.18 ml of triethylamine were mixed with 4.9 ml of ethyl alcohol and 2.6 ml of water. The mixture was stirred with a magnetic stirrer and incubated for 10 h at 60°C. The solution was evaporated in vacuo, resuspended in ca. 250 ml of water, and purified by passing through an ion-exchange column (Dowex 1 × 8, bed volume: 20 cm × 1.6 cm i.d., elution: 0.01 M formic acid). The octyl 5′-AMP fraction collected was directly passed through a Lichrosorb RP-18 column to give a column coated with octyl 5′-AMP.

Ligand-Exchange Chromatographic Resolution of DL-Amino Acids in the Presence of a Chiral Additive. LiChrosorb RP-18, 5 μm (Merck Co., Darmstadt, FRG) was packed (400 kg/cm²) in a stainless steel column (250 mm × 4.6 mm i.d.) in our laboratory by a conventional slurry-packing technique. DL-amino acids were resolved under various conditions (details are given in the table of figure legends). In the case of DL-Trp resolution, 1–2 μg introduced into a column were eluted at ca. 100 kg/cm² with 50 mM sodium acetate buffer (pH 4.5) containing 1 mM cupric acetate [Cu(II)], either 1 mM nucleoside or nucleotide, and 3% acetonitrile. Chromatography was carried out after thoroughly equilibrating the packed column with the eluant. The eluant (flow rate: 1 ml/min) was monitored at 300–310 nm. As for other DL-amino acids, 50 mM sodium acetate buffer (pH 4.5) containing 1.0 mM nucleic acids and 1.0 mM Cu(II) was used. The flow rate was 0.5 ml/min (ca. 40 kg/cm²) and the eluant was monitored at either 440 nm (Pro) or 570 nm (other amino acids) after mixing with ninhydrin reagent (flow rate: 0.5 ml/min). Enantiomeric assignments for D- and L-amino acids were made by cochromatography of a different ratio of amino acid enantiomer (e.g., D:L = 1:3 or vice versa).

Results

Effects of Various Ribonucleotides on Resolutions

Table 1 shows the effects of four different 5′-ribonucleotides (5′-AMP, -GMP, -CMP, and -UMP) on the resolutions of DL-amino acids. The chromatograms by which DL-Leu was resolved under the same conditions as those given in Table 1 are also represented as an example in Fig. 1. Under the conditions employed, enantiomeric separations were obtained only using AMP and GMP, suggesting that both might recognize the chirality of amino acids. The data show that all the L-amino acids were eluted faster than the opposite isomers in the presence of AMP, whereas the elution order of enantiomers was completely inverted when substituting GMP for AMP. Using GMP gave better resolution results than those of AMP (see α values). Whereas GMP has a positive recognition effect on L-amino acids, AMP conversely has a negative one. Using the GMP system, the results of five representative resolutions of DL-amino acids (aromatic, Tyr; aliphatic, Leu; sulfur containing, Met; imino, Pro; and charged, Arg, respectively) are represented in Fig. 2.

On the other hand, none of the 5′-pyrimidine nucleotides showed any detectable recognition for amino acid chirality under these experimental conditions (Table 1 and Fig. 1). This may be due to a difference in their metal complex conformation compared with purine nucleotide.

Resolutions in the Presence of RNAs

The results of resolving nine proteinaceous DL-amino acids using either AMP, GMP, 4XMP, or RNA oligomer are summarized in Table 2. The GMP system gives larger α values than those in the case of the AMP system. As shown in Table 1, D-amino acids were eluted faster than the opposite isomers in the GMP system. Evidence that the ability of GMP to recognize L-amino acids is superior to that by AMP is also found in the mixed nucleotide system (the third experiment using 4XMP), suggesting that the effect of GMP on L-amino acid recognition might be predominant whatever nucleotides are employed for the resolution. The same tendency was also seen in the last experiment (RNA oligomer).

Role of Ribose

In order to clarify the role of the 2′-hydroxyl residue of ribose on the formation of metal complexes with amino acids, the effects of 2′-deoxyribonucleotides on the resolutions were investigated (Table 3). The results are similar to those obtained from the ribonucleotide systems. The presence of dGMP gives the best result (second column). The resolutions in the presence of 4dXMP and DNA oligomers are qualitatively the same as that using dGMP. Thus there appears to be no difference between ribose and deoxyribose in the metal-mediated complex formation of amino and nucleic acid.

Assignment of Enantiomers and Complex Stability of Metal–Nucleic Acid–Amino Acid

In order to assign enantiomers eluted from a column, enantiomers in a different ratio (D:L = 1:3 or

Table 1. Effect of ribonucleoside 5′-monophosphate on the resolution of seven DL-amino acids

Chiral[a] additive: DL-amino acid	Control[b] retention time (min)	5′-CMP retention time (min)			5′-UMP retention time (min)			5′-AMP retention time (min)			5′-GMP retention time (min)		
	D (L)	D	L	α^c	D	L	α^c	D	L	α^c	D	L	α^c
Tyr	26.2	26.0	26.0	1.0	26.7	26.7	1.0	23.0	21.4	1.12	42.0	59.5	1.51
Met	15.5	14.4	14.4	1.0	15.5	15.5	1.0	18.4	17.2	1.13	27.0	31.0	1.21
Val	11.5	11.5	11.5	1.0	11.7	11.7	1.0	12.2	11.7	1.14	17.2	21.0	1.41
Leu	19.6	20.0	20.0	1.0	20.0	20.0	1.0	24.3	22.7	1.11	38.0	45.5	1.25
Ile	18.7	18.8	18.8	1.0	19.1	19.1	1.0	22.4	20.5	1.15	35.0	44.5	1.35
Pro	9.6	9.4	9.4	1.0	9.7	9.7	1.0	11.5	11.5	1.0	14.5	18.5	1.62
Arg	9.3	10.5	10.5	1.0	10.0	10.0	1.0	12.0	12.0	1.0	19.0	20.5	1.14

[a] A 50 mM sodium acetate buffer (pH 4.5) containing 1.0 mM Cu(II) and 1.0 mM 5′-ribonucleotide was used as eluant
[b] Without chiral additive
[c] $\alpha = k'_D/k'_L$ or k'_L/k'_D [$k' = t_D(t_L) - t_0$, where t_D and t_L are the retention times for the peaks of the D-enantiomer and L-enantiomer respectively, and t_0 (8 min) is the time for void volume]

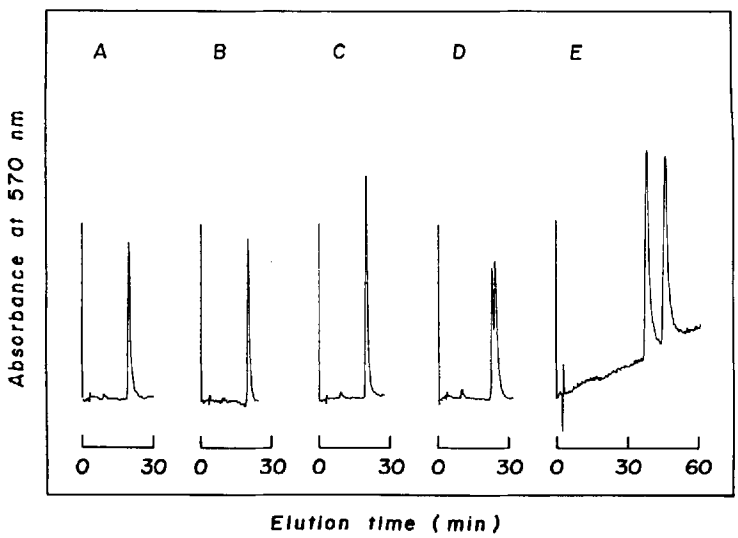

Fig. 1. Effect of ribonucleoside 5′-monophosphate on the resolution of DL-Leu. **A**, without ribonucleotide; **B**, 5′-CMP; **C**, 5′-UMP; **D**, 5′-AMP; and **E**, 5′-GMP, respectively.

vice versa) were analyzed as shown in Fig. 3 (B). Using this method, the resolution profile was discerned. Because all of the present results seem to arise from complex stability between nucleic acid, amino acid, and Cu(II) in the mobile phase, it is important to know whether the fast-eluted enantiomers giving the earlier peak on the chromatogram form an unstable metal complex or not, as compared with the slowly eluted isomers in the column. It is generally known that using a chiral stationary phase column, the enantiomers having a strong interaction with the phase are eluted more slowly than those interacting weakly.

A LiChrosorb RP-18 column coated with octyl-5′-AMP was prepared and DL-amino acids were then resolved using an achiral eluant. One example is represented in Fig. 3A. In both cases (Fig. 3A and B), L-Phe was eluted faster than the opposite enantiomer. L-Phe might be weakly interacting with the phase (Fig. 3B) and the complex containing L-Phe might be more unstable than that of the opposite enantiomers (Fig. 3A). It is therefore suggested that the fast-eluted enantiomers might result from an unstable complex formation in the column.

Conditions for Resolution

Resolutions were initially carried out at different pHs. The most satisfactory resolutions giving the biggest α values were obtained at pH 4.5 (data not shown). All resolutions in the experiments were therefore carried out at this pH.

Table 4 indicates the effects of the concentrations of GMP and Cu(II) on the resolution. In the absence of either GMP or Cu(II) in the mobile phase, no resolution was obtained (first and second columns). The remaining columns show the effects of different concentrations of GMP and Cu(II). The results sug-

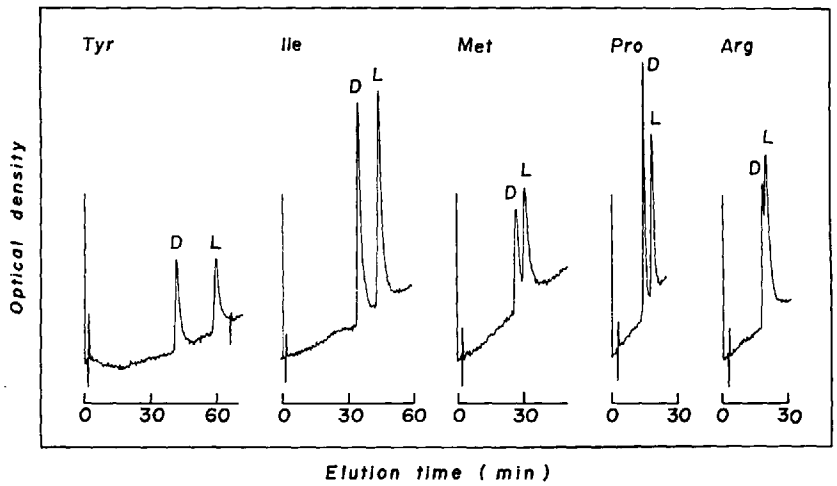

Fig. 2. Representative resolutions of DL-amino acids by using a 5'-GMP–Cu(II) complex.

Table 2. Resolutions of nine proteinaceous DL-amino acids in the presence of either AMP, GMP, 4×MP, or RNA oligomer

Chiral additive: DL-amino acid	5'-AMP[a] retention time (min)			5'-GMP[a] retention time (min)			4XMP[b] retention time (min)			RNA oligomer retention time (min)		
	D	L	α	D	L	α	D	L	α	D	L	α
Trp	(54.5)[c]	(48.3)	(1.13)	(46.7)	(54.5)	(1.17)	(50.5)	(50.5)	(1.0)	ut	ut	
Phe	82.0	72.0	1.15	95.0	129.0	1.39	80.5	85.5	1.07	95.0	101.0	1.07
Tyr	23.0	21.4	1.12	42.0	59.5	1.51	37.0	40.5	1.12	41.5	45.0	1.10
Met	18.4	17.2	1.13	27.0	31.0	1.21	24.8	24.8	1.0	23.5	23.5	1.0
Val	12.2	11.7	1.14	17.2	21.0	1.41	16.6	16.6	1.0	21.6	22.8	1.09
Leu	24.3	22.7	1.11	38.0	45.5	1.25	34.0	36.0	1.08	45.0	47.0	1.05
Ile	22.4	20.5	1.15	35.0	44.5	1.35	32.7	35.0	1.09	33.0	36.0	1.12
Pro	11.5	11.5	1.0	14.5	18.5	1.62	13.0	14.0	1.20	17.3	19.2	1.20
Arg	12.0	12.0	1.0	19.0	20.5	1.14	20.2	20.2	1.0	54.0	54.0	1.0

[a] A 1 mM 5'-AMP or 5'-GMP was used
[b] 4XMP contained 0.25 mM each of 5'-AMP, -GMP, -CMP, and -UMP
[c] ( ) indicates data from different experiment; ut: untested.

Table 3. Effect of deoxyribonucleotides on resolutions

Chiral additive: DL-amino acid	5'-dAMP[a] retention time (min)			5'-dGMP[a] retention time (min)			4dXMP[b] retention time (min)			DNA oligomer retention time (min)		
	D	L	α	D	L	α	D	L	α	D	L	α
Tyr	26.8	25.5	1.07	41.8	56.9	1.45	28.8	33.2	1.21	46.0	51.4	1.14
Met	16.8	16.8	1.0	24.7	28.4	1.22	18.4	18.4	1.0	37.8	37.8	1.0
Val	11.5	11.5	1.0	15.0	18.0	1.43	12.3	13.1	1.19	18.3	19.0	1.07
Leu	21.3	21.3	1.0	34.5	41.5	1.26	23.4	26.6	1.21	54.6	54.6	1.0
Ile	20.9	20.0	1.08	34.5	47.7	1.50	22.6	25.5	1.20	67.5	70.7	1.05
Pro	10.1	10.1	1.0	13.8	17.5	1.64	10.7	11.3	1.22	15.5	18.1	1.35
Arg	13.0	13.0	1.0	16.0	16.0	1.0	13.8	13.8	1.0	47.8	47.8	1.0

[a] A 1.0 mM 5'-dAMP and 5'-dGMP was used
[b] 4dXMP contained 0.25 mM each of 5'-dAMP, -dGMP, -dCMP, and -dTMP

gest that the 0.5 mM system may be adequate, as the α values are mostly similar in both the 0.5 mM and 1 mM systems.

Because DL-amino acids were resolved by means of the mediation of Cu(II), other metal ions were also tested (Table 5). In the first column, it can be seen that the system in which metal is absent gave no resolution, which is the same as in Table 4. How-

ever, no resolution occurred either in the presence of Co(II) or Ni(II), suggesting that only Cu(II) may be effective for resolution under the conditions employed.

Discussion

Structure of the Ligand Complex

The roles of metal ions in the prebiotic synthesis of oligonucleotides have been reported (Sawai 1976; Lohrmann et al. 1980; Kanaya and Yanagawa 1986). Because the Cu(II) ion exists abundantly on the earth (Egami 1974), it might have played a role as a metal ion agent for chiral interaction between amino and nucleic acids. The present results suggest that the difference in ligand complex formation between D- and L-amino acid enantiomers may result in enantiomeric separation. Preliminary observation suggests that the N(7) of purines may play a role in complex formation with Cu(II). The inversion of elution order of enantiomers, which occurred using either AMP or GMP as a chiral additive (Table 1), suggests that $NH_2(6)$ of AMP and O(6) of GMP may affect complex formation considerably. In order to elucidate the role of phosphate, a number of nucleotides were compared. The effects of 5′-purine nucleotides on the resolutions are shown in Tables 1–5 and Fig. 1. Experiments using 3′-purine nucleotides were also carried out (data not shown), and the results showed that 5′-A(G)MP = 5′-dA(G)MP > 3′-A(G)MP. Namely, the oxygen atom attached to the 5′-phosphate might play an important role in metal complex formation. Because there is no difference between ribose and deoxyribose in resolution (Table 3), the 2′-hydroxyl group of ribose is not necessarily involved in complex formation.

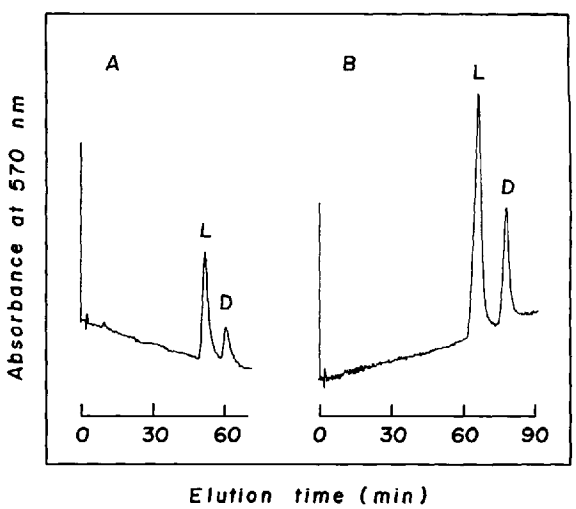

Fig. 3. Resolution of DL-Phe by using an octyl-5′-AMP-coated column (**A**) and eluant containing 1.0 mM 5′-AMP–Cu(II) complex (**B**)

Complex Stability and Resolution

In Table 4, either GMP or Cu(II) alone gave a short retention time and no resolution was seen. It seems that a Cu(II) ion-mediating coordinate bond of nucleic and amino acids is necessary for resolution. The stability of the ligand complex of amino acid and nucleic acid in a column seems to determine the resolution capability. The result using the chiral stationary phase (Fig. 3A) was the same as that in the chiral mobile phase (Fig. 3B), indicating that strong interaction (stable complex) gave a long retention time of enantiomers, which are eluted after the opposite isomer. The present results are compatible with reports (Grushka et al. 1983) that slow elution means the formation of a more stable complex in a column.

It has been reported that L-amino acids are strongly adsorbed to cellulose (D-glucose polymer) whereas D-isomers are not (Yuasa et al. 1980, 1984, 1985, 1986; Fukuhara and Yuasa 1986; Fukuhara et al. 1987), suggesting that L-enantiomers may interact with cellulose more strongly than the D-isomers. In the present experiments, using GMP, 4XMP, and RNA oligomer, D-enantiomers were eluted faster than the L-isomers, suggesting that the complexes composed of L-amino acids with GMP might be more stable than those composed of the

Table 4. Effect of concentrations of GMP and Cu(II) on resolutions

Chiral additive: DL-amino acid	0.5 mM Cu(II) retention time (min)	0.5 mM 5′-GMP retention time (min)	0.2 mM 5′-GMP, 0.2 mM Cu(II) retention time (min)			0.5 mM 5′-GMP, 0.5 mM Cu(II) retention time (min)			1.0 mM 5′-GMP, 1.0 mM Cu(II) retention time (min)		
	D (L)	D (L)	D	L	α	D	L	α	D	L	α
Tyr	19.5	14.1	23.4	27.3	1.25	22.1	30.0	1.56	42.0	59.5	1.51
Met	11.6	10.1	14.1	14.1	1.0	14.8	16.8	1.29	27.0	31.0	1.21
Val	9.6	8.3	10.8	10.8	1.0	11.2	12.3	1.34	17.2	21.0	1.41
Leu	15.4	11.6	18.0	19.6	1.16	18.4	21.3	1.29	38.0	45.5	1.25
Ile	14.5	11.2	16.1	18.0	1.23	17.7	21.4	1.38	35.0	44.5	1.35

Table 5. Effects of various metal ions on resolutions

Chiral additive: DL-amino acid	No metal ions, 0.5 mM 5'-GMP retention time (min)			0.5 mM Co(II), 0.5 mM 5'-GMP retention time (min)			0.5 mM Ni(II), 0.5 mM 5'-GMP retention time (min)			0.5 mM Cu(II), 0.5 mM 5'-GMP retention time (min)		
	D	L	α	D	L	α	D	L	α	D	L	α
Tyr	14.1	14.1	1.0	18.7	18.7	1.0	19.8	19.8	1.0	22.1	30.0	1.56
Met	10.1	10.1	1.0	11.4	11.4	1.0	12.1	12.1	1.0	14.8	16.8	1.29
Val	8.3	8.3	1.0	9.3	9.3	1.0	9.5	9.5	1.0	11.2	12.3	1.34
Leu	11.6	11.6	1.0	13.6	13.6	1.0	14.6	14.6	1.0	18.4	21.3	1.29
Ile	11.2	11.2	1.0	12.8	12.8	1.0	13.2	13.2	1.0	17.7	21.4	1.38

D-enantiomers. Although the inversion of the elution order of enantiomers was seen using AMP, the capability of chiral recognition seemed to be small and did not affect the resolution using 4XMP and RNA oligomer. Thus, it is likely that GMP plays an important role in the selection of L-amino acids using the present ligand-exchange chromatography system. Such a sterically significant complex would not be constructed with pyrimidine nucleotides under the present conditions (Table 1 and Fig. 1), suggesting that pyrimidine nucleotides might contribute less to the chiral recognition of amino acids.

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