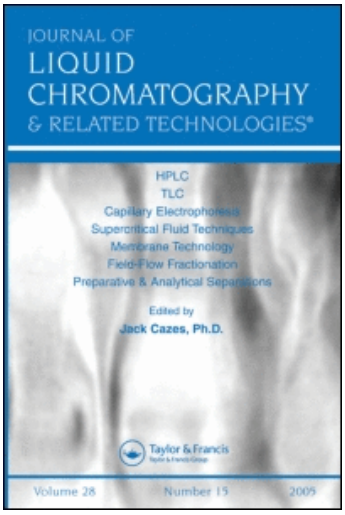


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# Evaluation of the Enantiomeric Separation of Dipeptides Using a Chiral Crown Ether LC Column

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# EVALUATION OF THE ENANTIOMERIC SEPARATION OF DIPEPTIDES USING A CHIRAL CROWN ETHER LC COLUMN

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## ABSTRACT

The direct optical resolution of six dipeptides into four stereoisomers each was achieved on an enantioselective crown ether column. An inclusion complex is formed between the stationary phase and the solute when using an acidic mobile phase. The acidic mobile phase serves to protonate the requisite primary amine of the dipeptide thereby allowing an attractive interaction between the ammonium functional group and the oxygens of the crown ether. Due to the differences in stability of the complexes formed, the four optical isomers elute at different times allowing the stereoisomeric separation. One of the factors affecting enantioselectivity is the distance between the primary amine functional group and the stereogenic center of the chiral moiety. Dipeptides are particularly useful molecules for the studying this "distance effect" since the bonding order of the two amino acids can be reversed. In addition to the enantiomeric separations of dipeptides possessing two stereogenic centers, the behavior of dipeptide separations possessing only one chiral center (*i.e.*, with achiral glycine as one of the residues) is examined to gain additional insight into the mechanism and the effect of the proximity of the primary amine group to the chiral center.

## INTRODUCTION

Due to the technological advances in enantiomeric separations and the recent emphasis on examining chiral antipodes as separate chemical entities, interest in chiral separations dealing with biological systems has escalated.[1] Obviously amino acids and dipeptides are basic, essential components in biological systems either as individual compounds or as constituents of proteins.

Direct enantiomeric separation of racemic amino acids has been successfully achieved by a variety of methods like membrane transport,[2,3] and liquid-liquid chromatography.[4] Successful HPLC separation methods include ligand exchange chromatography,[5-9] by conversion to N-3,5-dinitrobenzoyl or related derivatives followed by separation on a Pirkle type columns,[10] chiral inclusion mechanisms with cyclodextrin stationary phases[1,11] and by the interaction between a chiral crown ether stationary phase and the primary amine of the amino acid.[12-14] The present paper describes the optical resolution of dipeptides by a "host-guest" mechanism with a chiral crown ether stationary phase. Also, an investigation into the effect of the location of the interactive amine group in relation to the stereogenic center, as well as steric effects on enantioselectivity is considered.



## EXPERIMENTAL

High performance liquid chromatography was performed with a Waters (Milford, MA) model 590 solvent delivery module, a Shimadzu (Kyoto, Japan) SPD-2AM ultraviolet spectrometric detector and a Shimadzu C-R6A chromotopac recorder. Temperature was controlled by circulating ethylene glycol/water through a jacket surrounding the column using a Haake model FK heating-cooling circulator (Saddle Brook, NJ).

The mobile phase was a 0.01*M* perchloric acid solution (pH =2) in HPLC grade water. Solvents and chiral analytes were obtained from the following companies Aldrich (St. Louis, MO), Sigma (St. Louis, MO) and Fisher (Pittsburgh, PA).

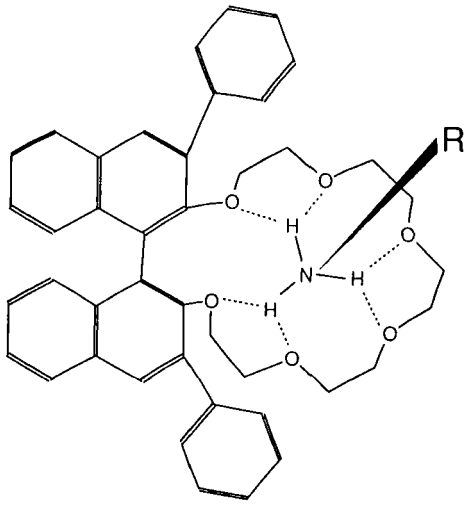



Figure 1 The chiral crown ether utilized in the column interacting with an ammonium group. (Adapted from references 9 and 13).



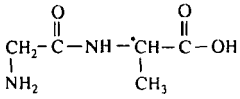
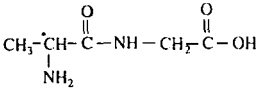
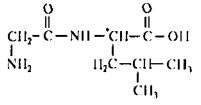
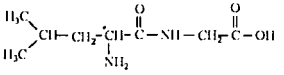
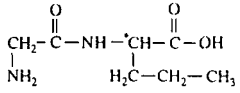
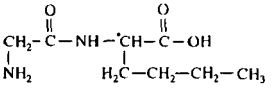
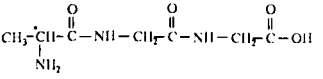
Flow rate varied from 0.1 to 1.0 ml/min. The temperature variation was range used was from 5 °C to 25 °C. The column was a Crownpak CR(+) supplied by Dr. Michael Henry of J.T. Baker Inc., (Phillipsburg, NJ).

## RESULTS AND DISCUSSION

In order to achieve enantiomeric separations with a "crown-6"-type-ether stationary phase it is necessary that the compound contain a primary amine functional group.[12-14] Neither secondary and tertiary amines nor other functional groups provide the type of association (inclusion complexation) required for enantioselective separations. The requisite interaction is both charge and size dependant. An acidic mobile phase assures protenation to form the ammonium ion which fits into the cavity of the crown ether as seen in Figure 1. In dipeptides made up of two chiral amino acids, the stereogenic centers are either $\alpha$ or $\delta$ to the primary interaction site.

**Table 1**

**Enantiomeric Peptides Containing One Stereogenic Center**

| Compound | Temp. | Flow Rate | $k'_1$ | $\alpha$ | $R_s$ |
|---|---|---|---|---|---|
| Glycyl-DL-alanine | 25 | 0.1 | 0.3 | 1 | ++ |
|  | 5 | 0.5 | 1.4 | 1.3 | 1.2 |
| DL-alanylglycine | 25 | 0.1 | 0.1 | 5.0 | 2 |
|  | 5 | 0.5 | 0.3 | 5.6 | 5.3 |
| Glycyl-DL-leucine | 25 | 0.1 | 2.0 | 1.0 | ++ |
|  | 5 | 0.5 | 10.2 | 1.1 | 0.9 |
| DL-leucylglycine | 25 | 0.1 | 0.3 | 2.0 | 1.5 |
|  | 5 | 0.5 | 0.6 | 3.8 | 8.7 |
| Glycyl-DL-norvaline | 25 | 0.1 | 2.1 | 1.1 | 0.8 |
|  | 5 | 0.1 | 6.9 | 1.4 | 0.8 |
| Glycyl-DL-norleucine | 25 | 0.1 | 5.6 | 1.1 | 0.8 |
|  | 5 | 0.1 | 12.3 | 1.2 | 0.8 |
| DL-alanylglycylglycine | 5 | 0.5 | 0.1 | 7 | 3.3 |
|  | | | | | |

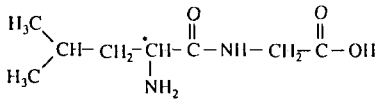
++ No resolution

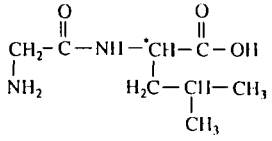
To investigate the importance of the proximity of the amine to the stereogenic center, peptides containing achiral glycine as one of the constituent amino acids were resolved. Table 1 shows the enantiomeric separations of the dipeptides that contain a single stereogenic center. In every case the selectivity was highest when the stereogenic center was $\alpha$ to the primary amine. For example, D,L-alanylglycine was much better resolved than glycyl-D,L-alanine (where the primary amine was several atoms away from the stereogenic center). In fact, in order to separate glycyl-DL-alanine the temperature had to be reduced to 5°C. DL-alanylglycine also gave a better separation at the lower temperature, but could be separated easily at 25°C as well. The difference in the ability of the crown ether to separate the DL-alanylglycine over the glycyl-DL-alanine is best explained by the difference in the proximity of the ammonium group and the stereogenic center. Closely related dimers, such as these, are ideal for studying this phenomenon. Figure 2 shows a representative chromatogram that illustrates the proximity effect. It is seen that DL-leucylglycine gives a much better separation than glycyl-DL-leucine under identical conditions.



Steric bulk is another factor that affects separation on the chiral crown ether column. It can either enhance or decrease enantioselectivity. Presumably this depends on whether or not a bulky group prevents the primary ammonium group from forming a strong inclusion complex. Thus, in the case of D,L-leucylglycine, we see a small decrease in selectivity over D,L-alanylglycine. This may be due to the bulkiness of the leucine R-group located $\alpha$ to the stereogenic center. However D,L-alanylglycylglycine shows the highest enantioselectivity ($\alpha$=7.0), possibly because of the larger glycylglycine moiety. Apparently the location of sterically bulky groups in relation to the stereogenic center affects the enantioselectivity as well.

Decreasing the temperature in these separations increased retention, usually increased $\alpha$ and often improved the resolution of compounds with one stereogenic center. However, it did not necessarily improve a separation of peptides containing two or more stereogenic centers. Glycyl-DL-alanine could

1)  DL-leucylglycine

2)  glycyl-DL-leucine

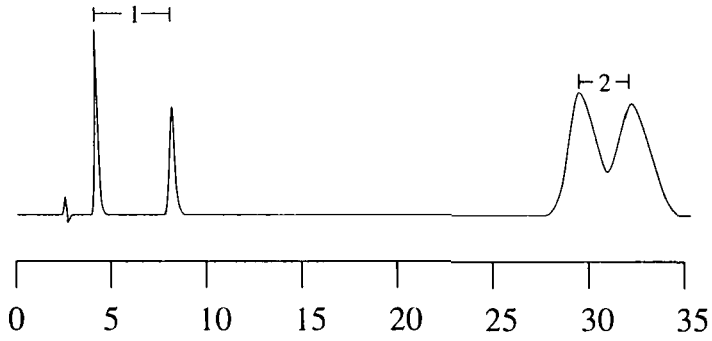


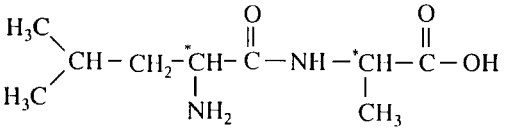

Figure 2 Under identical conditions, 5 °C, the DL-leucylglycine had better selectivity and resolution than glycyl-DL-leucine which had a longer interaction time in the column.



not be resolved at 25°C but was at 5°C. When separating solutes that contain two or more stereogenic centers (*i.e.* four or more peaks), other factors in addition to enantioselectivity become increasingly important. Figure 3 shows the separation of DL-leucyl-DL-alanine at the two temperatures of 5°C and 25°C. In particular, efficiency and diastereomeric selectivity must be high so as to minimize the possibility of overlapping peaks.

Table 2 shows the enantiomeric separations of the dipeptides that contain two stereogenic centers. Each separation was run three different times varying

DL-leucyl-DL-alanine



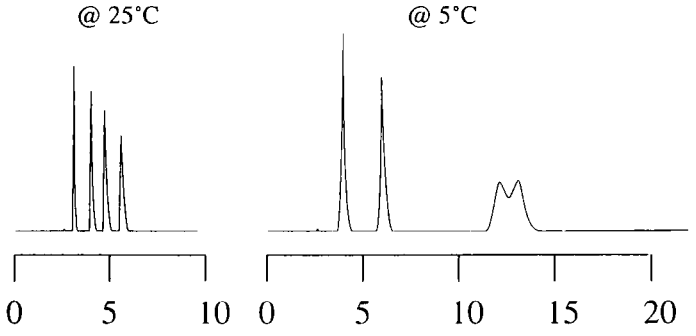


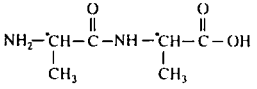
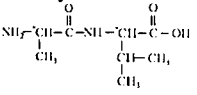
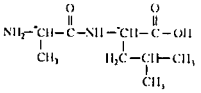
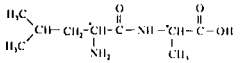
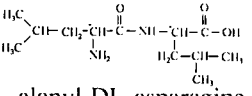
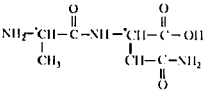

Figure 3 Inconsistent results were seen with the two temperatures of 5°C and 25°C. These results are an example but are not necessarily representative since no specific trend was followed.



the wavelength of the detector each run to further substantiate chiral separation. DL-leucyl-DL-leucine was the only dipeptide separated for which each of the pure enantiomer standards were available. The elution order for DL-leucyl-DL-leucine was DD, DL, LD, and LL respectively. Due to the lack of availability of the pure standards for the other dipeptides, no conclusions were made about the significance of their elution order. DL-alanyl-DL-asparagine has an additional primary amine functional group away from the stereogenic center. Interestingly, the capacity factors ($k_1$, $k_2$, $k_3$, and $k_4$) for this particular dipeptide were smaller than for any of the other compounds tested. Hence, there appeared to be no

**Table 2**

**Enantiomeric Dipeptides Containing Two Stereogenic Centers**

| Compound | Temp. °C | $k'_1$ | $k'_2$ | $k'_3$ | $k'_4$ |
|---|---|---|---|---|---|
| DL-alanyl-DL-alanine  | 25 | 1.1 | 1.3 | 1.9 | 2.3 |
| | 5 | 2.3 | 2.8 | 3.1 | 3.4 |
| DL-alanyl-DL-valine  | 25 | 0.7 | 0.8 | 2.3 | 5.6 |
| | 5 | 1.4 | 1.6 | 7.1 | 17.1 |
| DL-alanyl-DL-leucine  | 25 | 2.0 | 2.8 | 6.5 | 14.0 |
| | 5 | 3.0 | 4.1[a] | | |
| DL-leucyl-DL-alanine  | 25 | 0.5 | 1.5 | 2.0 | 2.5 |
| | 5 | 0.8 | 1.6 | 4.0 | 4.6 |
| DL-leucyl-DL-leucine  | 25 | 1.9 | 6.5 | 13[b] | |
| | 5 | 2.8 | 9.9 | 15 | 21 |
| DL-alanyl-DL-asparagine  | 25 | --eluted with solvent front-- | | | |
| | 5 | 0.1 | 0.2 | 0.8 | 1.3 |

a- two peaks eluted only

b- three peaks eluted only

increase in binding interaction when additional primary amines were present in the dipeptides.

## CONCLUSIONS

As in any enantiomeric separation, the position, size and nature of a chiral molecule's functional groups control its stereoselectivity on a chiral stationary phase.[15,16] In order to separate enantiomers, diastereomeric complexes must be formed between the stationary phase and the solute. In the specific interaction of a chiral crown ether and an organic analyte with an ammonium group, the closer the requisite primary amine and the stereogenic center the better is the observed enantiomeric resolution. Although selectivity is generally best when the amine functionality is adjacent to the chiral center, separation may be achieved the amine group is as far as four atoms from the stereogenic center. The steric bulk of other substituents can either enhance of decrease enantioselectivity. If both the primary amine and a bulky substituent are $\alpha$ to the stereogenic center and the bulky substituent sterically hinders the complexation of the amine by the crown ether, then enantioselectivity can be decreased. However, when the primary amine is further removed from the stereogenic center, steric bulk seems to enhance enantioselectivity. Likewise decreasing the temperature can enhance or decrease an enantiomeric separation on a chiral crown ether stationary phase. Most racemic compounds containing a single stereogenic center show increased resolution at lower temperatures. The same trend is not necessarily observed for compounds with two or or more stereogenic centers because of the problem of overlapping peaks.



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