Journal of Chromatography, 543 (1991) 299–305
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 124

Chiral high-performance liquid chromatography of aromatic cyclic dipeptides using cyclodextrin stationary phases

J. FLORANCE* and Z. KONTEATIS
BOC Group Technical Center, 100 Mountain Avenue, Murray Hill, N.J. 07974 (U.S.A.)
(First received October 18th, 1990; revised manuscript received January 16th, 1991)

ABSTRACT

A series of enantiomers of cyclic and linear dipeptides containing aromatic amino acids was prepared and chromatographed on β- and γ-cyclodextrin (CD) columns. The retention times, separation factor α and resolution values were calculated. The relevance of the distance of the chiral center from the phenyl ring for chiral resolution was studied. A model was developed using X-ray crystallographic data for an inclusion complex of β-CD and the enantiomers of cyclic(Phe-Gly).

INTRODUCTION

Separation of chiral peptides has become increasingly important due to differences in physiological activity of the resolved stereoisomers as well as regulatory requirements [1]. A variety of techniques have been used to facilitate enantiomeric separations, including chiral stationary phases, chiral eluents and chiral derivatizations [2–4]. The increasing importance of peptides as drugs has encouraged new systems for analysis of small peptide stereoisomers that are not well resolved by traditional reversed-phase columns.

Cyclic dipeptides (diketopiperazines) are an interesting class of peptides that have a variety of biological activities [5]. Previous work has been done on resolution of amino acid and dipeptide (linear and cyclic) enantiomers [6–8]. The mode of separation of enantiomers using cyclodextrin (CD) immobilized on silica high-performance liquid chromatography (HPLC) columns requires the formation of an inclusion complex that will selectively form a tighter binding complex with one isomer as opposed to the other [9–11].

Structural requirements in some systems have been defined that allow for separation of enantiomers [12–14]. In the present study a series of dipeptide compounds was synthesized. The dipeptides contained phenylalanine and phenylalanine derivatives where the phenyl ring was directly attached to the chiral center, or at one or two carbons away from the chiral center, respectively. Both linear and the cyclized dipeptides were prepared and chromatographed.

To understand better the importance of the structural requirements for separation of these dipeptides on CD, several features of these model compounds were

studied including the requirement for a phenyl ring, its proximity to the chiral center to be resolved and also the possible effects on the hydrogen bonding in CD inclusion complexes.

Molecular graphics studies of the CD inclusion complexes were also done to understand better the chromatographic results.

EXPERIMENTAL

Chromatography

Separations were performed using a Waters Assoc. (Milford, M.A., U.S.A.) HPLC system which consisted of two 510 HPLC pumps, a 481 UV detector, A WISP autoinjector and a Model 840 data acquisition and control system. The columns were β-CD and γ-CD (25 cm × 4.6 mm I.D.) from ASTEC (Whippany, N.J., U.S.A.) Mobile phases consisted of various isocratic combinations of HPLC-grade methanol and water, typically 10:90 (v/v). Additives to the aqueous phase were also tried, such as triethylamine acetate, pH 4.0. Flow-rates were 1.0 ml/min and UV detection was at 214 nm.

Materials

Solvents were HPLC grade from J. T. Baker (Phillipsburg, N.J., U.S.A.) and water was obtained from a Milli-Q system (Bedford, MA, U.S.A.). The 0.1 *M* TEAE buffer was made by titration of triethylamine with acetic acid to pH 4.0.

α-Phenyl-α-ethylglycine (racemic) was obtained from Chemical Dynamics (South Plainfield, N.J., U.S.A.). Cyclo(L-Phe-Gly) and D- and L-homophenylalanine) were purchased from Bachem (Bubendorf, Switzerland); D- and L-phenylglycine were purchased from Sigma (St. Louis, MO, U.S.A.). Boc-glycine was purchased from Peninsula Labs (Belmont, CA, U.S.A.). All other chemicals were reagent grade.

Synthesis

The linear peptides were prepared by the same general procedure as using conventional *t*-Boc peptide chemistry. Cyclic dipeptides were prepared from the corresponding linear dipeptides by cyclodehydration. This was done using phenol under non-racemizing conditions, with slight modifications from the original described by Kopple and Ghazarian [15].

Computer graphics

The molecular graphics were done on a PS390 Evans and Sutherland using SYBYL molecular modeling software [16]. The three-dimensional coordinates of β-CD were obtained from the Cambridge Crystallographic Data Base [17]. The coordinates for c(Phe-Gly) were obtained from the X-ray crystal structure of c(Tyr-Gly) which was modified by removing the hydroxyl group from tyrosine.

RESULTS AND DISCUSSION

Previous work has shown that the proximity of the chiral center to the 2′- and 3′-hydroxyls at the top of the CD cavity are important for resolution of enantiomers [12]. Modeling studies with propranolol have shown that increased hydrogen bonding allows for a more stable inclusion complex for *d*-propranolol as compared to the

l-isomer. The elution order from a β-CD column reflects this with *l*-propranolol eluting before the *d*-propranolol.

The presence of an aromatic group is beneficial but not essential for the formation of an inclusion complex with CDs. Models show that this is the portion of the molecule that inserts into the CD cavity. In the present study, an aromatic group (*i.e.*, a phenyl group), is contained in all of the cyclic dipeptides. There is also a pseudo-aromatic ring in the form of the diketopiperazine ring in these compounds. The model suggests that as with other aromatic compounds, the aromatic phenyl ring is inserted into the CD pocket.

It has been previously suggested that the distance of the chiral center to the phenyl group seems to be important for the stability of the inclusion complex. Compounds such as racemic barbiturates, in which the chiral center is contained in rings, resulted in increased resolution of enantiomers on CD. Chiral centers farther away (*i.e.*, more than one carbon) from the phenyl ring are often not well resolved on CD, although there are exceptions. Decreased bond rotation due to a shorter side chain was one suggestion for this effect [12]. Our results also agree with this observation as cyclic dipeptides are better resolved than linear dipeptides.

Studies have been conducted on a series of hydantoin enantiomers in which the length and location (5′ and 3′) of the alkyl subtituents were varied and corresponding effects on resolution of enantiomers on CD were evaluated. The best resolution was

TABLE I

RETENTION TIMES OF LINEAR AND CYCLIC DIPEPTIDES CONTAINING AROMATIC GROUPS ON γ- AND β-CD HPLC COLUMNS

Mobile phase: water–methanol, 90:10 (v/v) at 1 ml/min, 214 nm.

Compound	Retention time	
	γ-CD	β-CD
Cyclo (L-PhenylGly-Gly)	4.1	4.8
Cyclo (D-PhenylGly-Gly)	4.1	4.8
Cyclo (L-Phe-Gly)	4.9	12.2
Cyclo (D-Phe-Gly)	4.9	8.1
Cyclo (L-homoPhe-Gly)	4.6	14.6
Cyclo (D-homoPhe-Gly)	4.6	14.6
Cyclo (L-α-Phenyl-α-ethylGly-Gly)	4.9	13.6
Cyclo (D-α-Phenyl-α-ethylGly-Gly)	4.9	12.3
L-PhenylGly-Gly	4.1	4.9
D-PhenylGly-Gly	4.1	4.9
L-Phe-Gly	4.9	8.2
D-Phe-Gly	4.9	7.8
L-HomoPhe-Gly	4.7	13.3
D-HomoPhe-Gly	4.7	13.3
L-Phenylglycine	3.9	4.1
D-Phenylglycine	3.9	4.1
L-HomoPhe	4.5	8.7
D-HomoPhe	4.5	8.7
L-Phe	4.2	4.5
D-Phe	4.2	4.5

TABLE II

CALCULATED α AND RESOLUTION VALUES ON β-CD FROM DATA IN TABLE I

Compound	β-CD	
	Resolution	α
Cyclo(L-PhenylGly-Gly) Cyclo(D-PhenylGly-Gly)	0	1.0
Cyclo(L-Phe-Gly) Cyclo(D-Phe-Gly)	1.5	1.67
Cyclo(L-homoPhe-Gly) Cyclo(D-homoPhe-Gly)	0	1.0
Cyclo(L-α-phenyl-α-ethylGly-Gly) Cyclo(D-α-phenyl-α-ethylGly-Gly)	0.5	1.12

obtained with the methyl substituent; it decreased with larger substituents. The evidence points to disruption of hydrogen bonding due to longer chain substituents on 3′ or 5′ of the phenyl ring [13].

In the present study, we observed no resolution of enantiomers with the phenyl ring attached directly to the chiral center when hydrogen is the other substituent (*i.e.*, c(D- and L-PhenylGly-Gly). Some resolution is observed when the substituent is ethyl (*i.e.*, D- and L-cyclo(α-Phe-α-ethylGly-Gly) (CPEGG) separation factor (α) = 1.12 and resolution R_s = 0.5.

The retention times of the cyclic dipeptides c(Phe-Gly), c(homoPhe-Gly) and CPEGG were in the range 8.1–14.6 min as seen in Table I. The retention times seem to be more related to the relative hydrophobicity of the compound rather than the chiral discrimination, although the enantiomers of c(Phe-Gly) seem to be an exception.

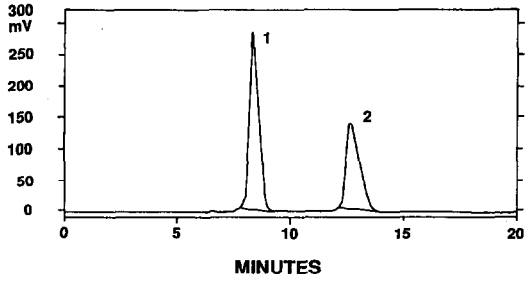
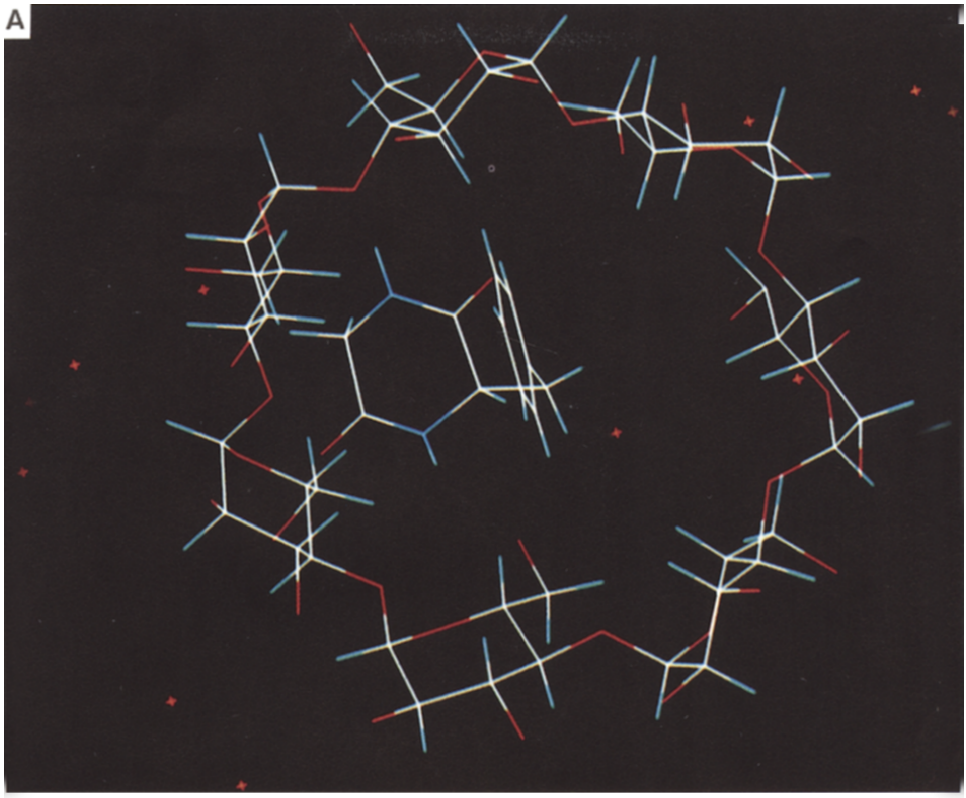
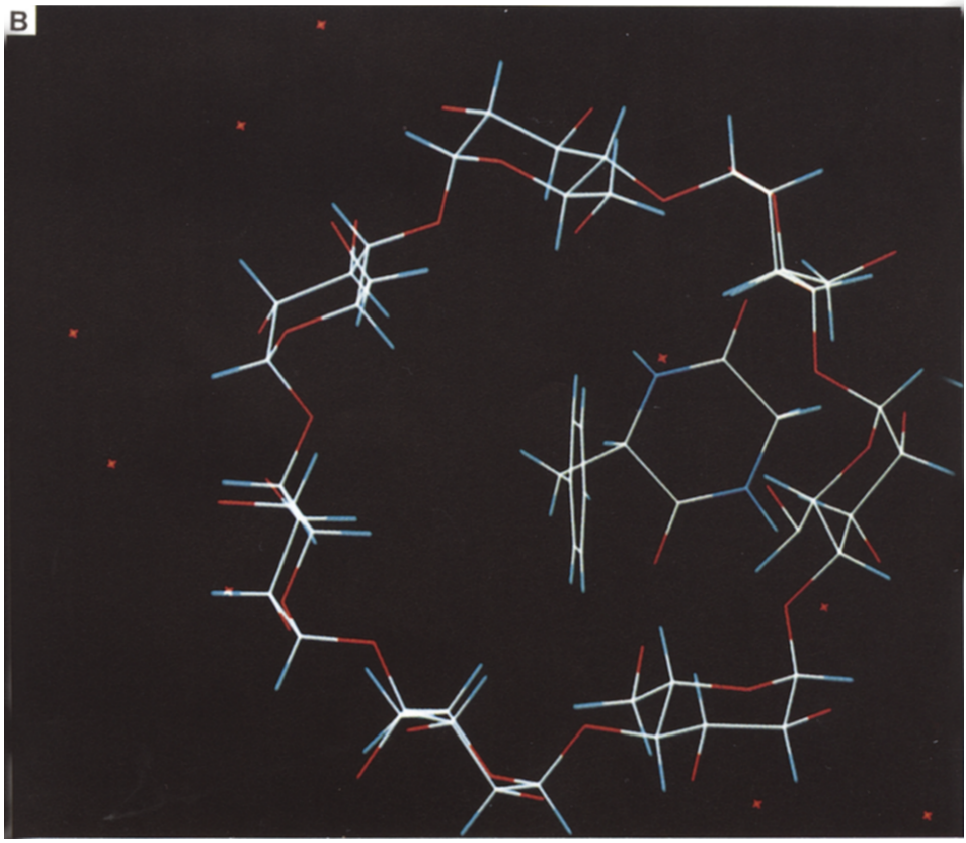


Fig. 1. Resolution of (1) c(D-Phe-Gly) and (2) c(L-Phe-Gly), on Cyclobond I, 90:10 (v/v) water–methanol at 1 ml/min.

Fig. 2. Model of the inclusion complex of c(L-Phe-Gly) (A) and c(D-Phe-Gly) (B) with β-CD using X-ray coordinates. The hydrogen bond distances in the c(L-Phe-Gly)–CD complex for the two amide hydrogens interacting with the oxygens of the 2′- and 3′-hydroxyls are 2.2 and 2.6 Å. In the D-isomer complex the same distances are 2.6 and 4.1 Å as measured with SYBYL software.


A


B

Enantiomers in which the phenyl ring and the chiral center are separated by two methylene units were not resolved on β-CD (*i.e.*, c(D- and L-homoPhe-Gly). This was expected in view of other studies indicating that when the chiral center is too far out of the CD pocket it will not interact with the 2'- and 3'-hydroxyls, thus not allowing for chiral recognition.

The enantiomers of c(Phe-Gly) were retained and well resolved with $\alpha = 1.67$ and $R_s = 1.5$ (Table II). The high resolution indicates a significant difference between the stability of the inclusion complex of CD with the D- versus the L-isomer. The difference is manifested in the elution times, with the c(L-Phe-Gly) eluting about 5 min after the c(D-Phe-Gly) as shown Fig. 1. These results suggest that optimal chiral resolution in this class of cyclic dipeptides is observed when the distance between the phenyl ring and the chiral center is one methylene unit.

In this study most of the chiral chromatography was performed with isocratic methanol–water combinations. In addition, cyclic dipeptides were chromatographed using an ion-pair buffer, triethylamine acetate, pH 4.0. The retention times on β-CD were increased slightly but no improvements in resolution were seen with this buffer system. No enantiomers were separated using this system that did not resolve with methanol–water.

In Fig. 2 a model of the inclusion complex of c(D-Phe-Gly) and c(L-Phe-Gly) with β-CD shows the phenyl ring inserted into the CD pocket. In this model the possible hydrogen bonds that can form with the primary and secondary hydroxyls of β-CD are the two amide protons from the cyclic dipeptide ring. The diketopiperazine ring is not linear but slightly puckered as determined from crystallographic data. It would be difficult to form optimal hydrogen bonds between the two carbonyls on the cyclic dipeptide ring and the CD hydroxyls, with the phenyl ring fully inserted. The hydrogen bond distances were measured in both D- and L-Phe isomers for the amide hydrogens. The preliminary results indicate that the L-isomer has the possibility for two optimal hydrogen bonds (about 2.5 Å) with the adjacent CD hydroxyls. The D-isomer seems to allow for only one optimal hydrogen bond with the phenyl ring fully inserted. Various rotations and translations of the cyclic dipeptide and β-CD were performed to locate possible hydrogen bonds. Molecular modeling studies are currently underway to refine this model of CD–dipeptide complexes. A full understanding of the dipeptide–β-CD complex would require crystallization of the complex and solution of the crystal structure by *X*-ray techniques.

ACKNOWLEDGEMENTS

We would like to thank Dr. Alphonse Galdes and Dr. Peter Maye for help with the Cambridge crystallographic files and with the SYBYL software.

REFERENCES

1 R. W. Souter, *Chromatographic Separations of Stereoisomers*, CRC Press, Boca Raton, FL, 1985.
2 P. Roumeliotis, K. K. Unger, A. A. Kurganov and V. A. Davankov, *J. Chromatogr.*, 255 (1983) 51.
3 N. Nimura, A. Toyama, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 239 (1982) 671.
4 D. W. Aswad, *Anal. Biochem.*, 137 (1984) 405.
5 S. Johne and D. Groger, *Pharmazie*, 32 (1977) 1.
6 J. Florance, A. Galdes, Z. Konteatis, Z. Kosarych, K. Langer and C. Martucci, *J. Chromatogr.*, 414 (1987) 313.

7 H. J. Isaaq, *J. Liq. Chromatogr.*, 9 (1986) 229.
8 H. J. Isaaq and C. D. Ridlon, *J. Liq. Chromatogr.*, 9 (1986) 3377.
9 D. W. Armstrong, *J. Liq. Chromatogr.*,7 (Supp. 2) (1984) 353.
10 D. W. Armstrong and W. DeMond, *J. Chromatogr. Sci.*, 22 (1984) 411.
11 *The ASTEC Informer*, Vol. 1. No. 2, Advanced Separation Technologies, Whippany, NJ, 1985.
12 D. W. Armstrong, T. J. Ward, R. D. Armstrong and T. S. Beesley, *Science (Washington D.C.)*, 232 (1986) 1132.
13 J. H. Maguire, *J. Chromatogr.*, 387 (1987) 453.
14 K. G. Feitsma, J. Bosman, B. F. H. Drenth and R. A. de Zeeuw, *J. Chromatogr.*, 333 (1985) 59.
15 K. D. Kopple and H. G. Ghazarian, *J. Org. Chem.*, 33 (1968) 862.
16 *SYBYL, Version 5.2, Molecular Modeling Software*, Tripos Associates, St. Louis, MO.
17 *Cambridge Structural Database System, Version 3.6*, Cambridge Crystallographic Data Center, University of Cambridge, U.K.