Journal of Chromatography A, 653 (1993) 293–302
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 452

Reversed-phase thin-layer chromatographic separations of enantiomers of dansyl-amino acids using β-cyclodextrin as a mobile phase additive[☆]

Joseph W. LeFevre[☆☆]

Chemistry Department, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061 (USA)

(First received June 8th, 1993; revised manuscript received July 25th, 1993)

ABSTRACT

The DL-racemates of nine proteinogenic amino acids were resolved by reversed-phase thin-layer chromatography after conversion to their 5-dimethylamino-1-naphthalene sulfonyl (dansyl) derivatives. The chiral selector β-cyclodextrin was used in the mobile phase along with either aqueous acetonitrile or aqueous methanol as an organic modifier. DL-Histidine was separated as both its mono- and didansyl derivatives. It was determined that it was the cyclic nature of two imino acids DL-proline and DL-pipecolic acid that prevented their enantiomeric separations under the conditions employed. The DL-racemates of four non-proteinogenic amino acids, two of which are involved in the urea cycle, were also separated.

INTRODUCTION

The resolution of racemates using optically active cyclodextrins (CDs) as chiral selectors continues to be a topic of much interest. The cyclodextrins, which are designated α, β or γ, are cyclic molecules consisting of either six, seven or eight α-D-glucose units, respectively, bonded through 1,4-linkages. These molecules have been used to separate a wide variety of racemates by high-performance liquid chromatography (HPLC) [1], gas chromatography (GC) [2], high-performance capillary electrophoresis (HPCE) [3], and thin-layer chromatography (TLC) [4]. The cyclodextrins form diastereomeric inclusion complexes which allow hydrogen bonding interactions to occur with the unidirectional 2- and 3-hydroxyl groups located at the mouth of the cyclodextrin cavity [5].

The separation of 5-dimethylamino-1-naphthalene sulfonyl (dansyl, Dns)-DL-racemates of several amino acids by reversed-phase TLC using β-cyclodextrin (β-CD) in the mobile phase was first reported by Armstrong *et al.* [4]. This work was extended by Lepri *et al.* [6] who compared two different types of reversed-phase TLC. Both groups were successful in resolving DL-racemates of the same eight proteinogenic and the same three non-proteinogenic amino acids. Armstrong achieved only partial resolution of Dns-DL-tryptophan and Lepri failed to resolve it at all. This constitutes the first report on the application of this methodology to the resolution of the DL-racemates of the remaining ten common amino acids.

Complete resolution of nine of the remaining ten proteinogenic amino acid DL-racemates is reported in this paper. DL-Proline was only

[☆] In memory of my father, the late David A. LeFevre, and his contributions to electrochemistry.

[☆☆] Permanent address: Chemistry Department, State University of New York at Oswego, Oswego, NY 13126, USA.

0021-9673/93/$06.00

TABLE I

REVERSED-PHASE TLC DATA FOR THE SEPARATION OF Dns-AMINO ACIDS

Solvents: A = MeOH–0.20 *M* β-CD (35:65, v/v); B = CH_3CN–0.20 *M* β-CD (32:68, v/v); C = CH_3CN–0.2 *M* β-CD (20:80, v/v); D = MeOH–0.2 *M* β-CD (55:45, v/v); E = MeOH–saturated β-CD (60:40, v/v); F = MeOH–0.2 *M* β-CD (50:50, v/v).

Dns-Amino acid	Abbreviation	$R_{F(D)}$	$R_{F(L)}$	α[a]	R_s[b]	Solvent[c]
DL-Alanine	Dns-DL-Ala	0.47	0.40	1.43	1.64	A
DL-*allo*-Isoleucine	Dns-DL-*allo*-Ile	0.38	0.30	1.43	3.25	B
DL-Asparagine	Dns-DL-Asn[d]	0.69	0.60	1.39	1.53	C
DL-Arginine	Dns-DL-Arg	0.65	0.55	1.52	1.69	C
DL-Citrulline	Dns-DL-Cit	0.63	0.54	1.45	1.52	C
DL-Cystine	N,N'-Di-Dns-DL-Cys-Cys	0.42	0.37	1.23	1.52	D
DL-Glutamine	Dns-DL-Gln	0.66	0.57	1.46	1.86	C
DL-Histidine	N-(α)-Mono-Dns-DL-His	0.64	0.58	1.28	1.13	C
	N-(α),N-(im)-Di-Dns-DL-His[e]	0.22	0.19	1.20	0.94	E
DL-Isoleucine	Dns-DL-Ile	0.40	0.33	1.35	1.71	B
DL-Lysine	N,N'-Di-Dns-DL-Lys	0.39	0.35	1.19	1.02	E
N-Methyl-DL-valine	Dns-DL-N-Me-Val[e]	0.28	0.24	1.18	0.94	F
DL-Ornithine	N,N'-Di-Dns-DL-Orn	0.40	0.35	1.24	1.20	E
DL-Pipecolic acid	Dns-DL-Pip	0.25	0.25	1.00	0	F
DL-Proline	Dns-DL-Pro	0.41[f]	0.39[f]	1.10[f]	0.63[f]	F
DL-Tyrosine	N,O-Di-Dns-DL-Tyr	0.26	0.23	1.15	0.94	E

[a] $\alpha = [(1 - R_{F(L)})/R_{F(L)}]/[(1 - R_{F(D)})/R_{F(D)}]$.
[b] $R_s = 2$ (distance between the two spots)/(sum of the widths of the two spots).
[c] Solutions contained urea and sodium chloride (see Experimental section).
[d] Part of the D-spot began to elute near the solvent front. The main body of this spot was used for these calculations.
[e] The identity of the D- *vs.* L-spot was not confirmed for this derivative.
[f] These are only approximate values because of overlap of the spots.

partially resolved by this technique. In addition, four non-proteinogenic amino acid DL-racemates were resolved. Two of these, DL-citrulline and DL-ornithine, are important compounds in the urea cycle. Table I lists the Dns-amino acids along with their abbreviations and separation data.

EXPERIMENTAL

Materials

The reversed-phase TLC was performed with glass-backed, chemically bonded octadecylsilane (C_{18}) plates. Whatman $LKC_{18}F$ plates (200 μm thickness, 20 × 5 cm) were used to separate the dansylated DL-pairs and to purify N-(α),N-imidazole (im)-Di-Dns-DL-His. A Whatman $PLKC_{18}F$ plate (1000 μm thickness, 20 × 20 cm) was used to purify N-(α)-mono-Dns-DL-His for proton nuclear magnetic resonance (^1H NMR) spectroscopy. Both types of plates contained a preabsorbent layer. Whatman $MKC_{18}F$ plates (200 μm thickness, 1 × 3 in.; 1 in. = 2.54 cm) were used to analyze the mono-and di-Dns-His samples subjected to acid hydrolysis and/or UV light. All three types of reversed-phase plates were obtained from Whatman (Clifton, NJ, USA). The normal-phase TLC plates consisted of aluminum-backed Merck Kieselgel 60 F_{254} (0.2 mm thickness, 20 × 20 cm) and glass-backed Merck Kieselgel 60 F_{254} (0.25 mm thickness, 20 × 5 cm), both available from EM Science (Gibbstown, NJ, USA). β-cyclodextrin hydrate, sodium chloride, urea and dansyl chloride (Dns-Cl), were obtained from Aldrich (Milwaukee, WI, USA). Methanol (MeOH) and acetone (both HPLC grade), and sodium hydrogencarbonate ($NaHCO_3$) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile (CH_3CN, Nanograde) was purchased from Mal-

linckrodt (Paris, KY, USA). All amino acids were purchased from Sigma (St. Louis, MO, USA) as were the following standard Dns-amino acids: Dns-L-Ala, Dns-L-Asn, Dns-L-Arg, Dns-L-Cit, N,N′-Di-Dns-L-Cys-Cys, Dns-L-Gln, N,N′-di-Dns-L-Lys, Dns-L-Pro and N,O-di-Dns-L-Tyr. A Mineralight lamp (Model UVGL-25; UVB, Inc., San Gabriel, CA, USA) was used to visualize the TLC plates at 366 nm.

Synthesis of Dns-DL-racemates

A modified version of the procedures reported by Gray [7] and Chimiak and Polonski [8] was used to prepare the following Dns derivatives: DL-Ala, D-*allo*-Ile, DL-Asn, DL-, D- and L-Ile, DL-Lys, N-Me-DL-Val, DL- and L-Orn, DL- and L-Pip and DL-Pro. To 1000 μl of a 1 m*M* solution of the amino acid in 0.1 *M* $NaHCO_3$ were added 1000 μl of 15 m*M* Dns-Cl in acetone in a 5-ml conical vial. The vial was covered and the homogeneous yellow solution was stirred for 2 h at room temperature. The excess Dns-Cl was removed by extracting one or two times with diethyl ether. The pH of the aqueous layer was adjusted to 4 using 0.2 *M* hydrochloric acid and the neutralized Dns-derivative was extracted one or two times with ethyl acetate (EtOAc). The combined EtOAc extracts were dried with anhydrous magnesium sulfate. After evaporation to dryness the crude products were dissolved in EtOAc–MeOH (95:5; 200–600 μl, depending upon the yield) for reversed-phase TLC. The same procedure was used to prepare N,O-di-Dns-DL-Tyr with the following exception. Since DL-Tyr is not soluble in 0.1 *M* $NaHCO_3$, 0.2 *M* sodium hydroxide was added dropwise to dissolve the DL-Tyr, and the pH was adjusted to 10.

A slightly different work-up procedure was used to prepare the mono-Dns derivatives of the basic amino acids DL-Arg and DL-Cit, and the N-(α)-mono-Dns derivative of DL- and L-His. After removal of the excess Dns-Cl and acidifying to pH 1, excess CH_3CN was added to the aqueous solution to form a lower-boiling azeotrope. The solution was evaporated to dryness on a rotary evaporator. The crude samples were purified (along with Dns-DL-Asn and Dns-DL-Gln) by normal-phase TLC prior to analysis by reversed-phase TLC.

Another modified work-up procedure was used to prepare N-(α),N-(im)-di-Dns-DL-His. Since the imidazole dansyl group is sensitive to acid [7], the crude reaction mixture was not acidified. Instead, after removal of excess Dns-Cl by extraction with EtOAc, the solution was simply evaporated to dryness with excess CH_3CN. The crude sample was dissolved in MeOH and purified in the dark by reversed-phase TLC (see *Reversed-phase TLC methods*).

Normal-phase TLC methods

The following five mono-Dns-amino acids were purified by normal-phase TLC: DL-Asn, DL-Arg, DL-Cit, DL-Gln and DL- and L-His. Each was dissolved in approximately 500 μl of MeOH and was streaked on a glass-backed, normal-phase TLC plate. The plates were developed in a solvent system composed of either EtOAc–$CHCl_3$–MeOH–acetic acid (AcOH) 50:30:20:1, v/v) [9] for Dns-DL-Asn ($R_F = 0.10$), Dns-DL-Cit ($R_F = 0.10$) and Dns-DL-Gln ($R_F = 0.13$) or EtOAc–$CHCl_3$–MeOH–AcOH (40:30:50:2, v/v) for Dns-DL-Arg ($R_F = 0.17$) and N-(α)-mono-Dns-DL- and L-His ($R_F = 0.14$). The appropriate band was visualized using long-wavelength UV light, scraped off the plate, and each derivative was eluted with 1–2 ml of MeOH. The purified samples were evaporated to dryness and redissolved in MeOH (Dns-DL-Cit and N-(α)-mono-Dns-DL- and L-His 100 μl each, Dns-DL-Gln 200 μl, and Dns-DL-Asn and Dns-DL-Arg 300 μl each) in preparation for reversed-phase TLC analysis.

Reversed-phase TLC methods

In order to increase the solubility of β-CD in water, a saturated solution of urea in water was prepared first [4]. This solution was then made 0.6 *M* in NaCl. The appropriate amount of β-CD was then added. Less than 1 μl of each solution of Dns-amino acid was spotted on a 20 × 5 cm TLC plate and developed from 3 to 6 h at room temperature in a 225 × 54 mm I.D. chamber.

Purification of N-(α)-mono-Dns-DL-His for ^1H NMR spectroscopy was accomplished by preparative reversed-phase chromatography using MeOH–2% aqueous AcOH (65:35, v/v) as the solvent [10]. A pure sample of N-(α),N-(im)-di-

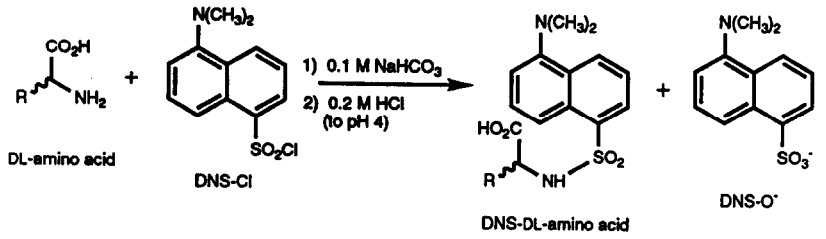

Fig. 1. General reaction for the formation of mono-Dns-DL-amino acids.

Dns-DL-His was obtained by reversed-phase chromatography in the dark using MeOH–0.01 *M* Na_2HPO_4 (75:25, v/v) [10] as the solvent. The pure samples were eluted from the silica gel with MeOH. The di-Dns derivative of DL-His was eluted in the dark. The ^1H NMR spectra were run in $C^2H_3O^2H$ using residual C^2H_3OH as an internal standard (δ4.78). A Bruker WP-270 NMR spectrometer was used to record the spectra.

RESULTS AND DISCUSSION

The preparation of the Dns-DL-racemates was straightforward (see Experimental section for details). The general reaction for the preparation of the mono-Dns derivatives appears in Fig. 1. In the cases where the side chain contained a sulfhydryl group (Cys), a phenolic hydroxyl group (Tyr), an amino group (Lys and Orn), or an imidazole group (His), a di-Dns derivative was formed (Fig. 2). Under the reaction conditions employed the guanidinium group of Arg, and the amide group in Asn, Cit and Gln showed no reactivity [7].

The separation data (R_F, α, R_s) for 15 Dns-amino acid DL-racemates appear in Table I. In each case where separation occurred, the D-isomer eluted ahead of the L-isomer (Fig. 3). This is consistent with earlier observations made on

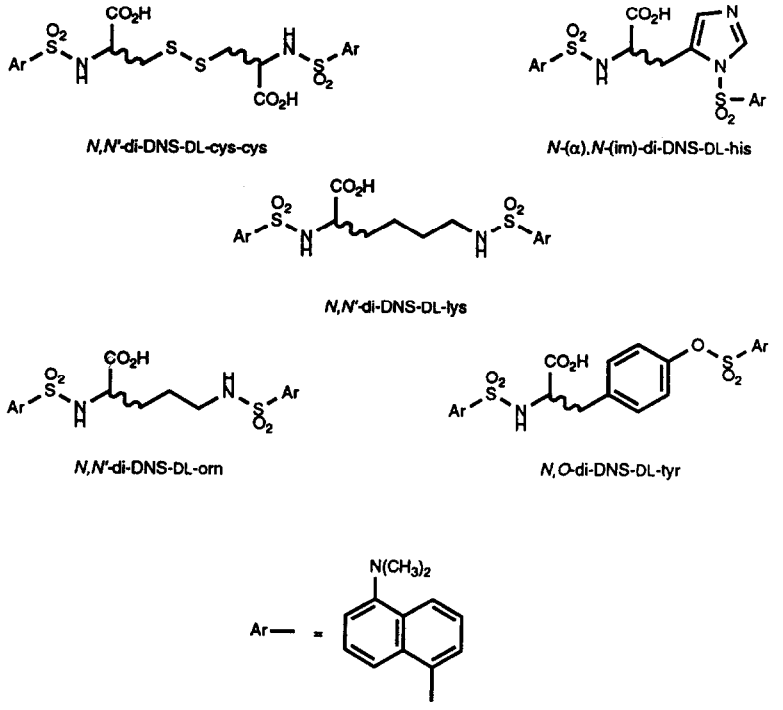

Fig. 2. Structures of the di-Dns-amino acids.

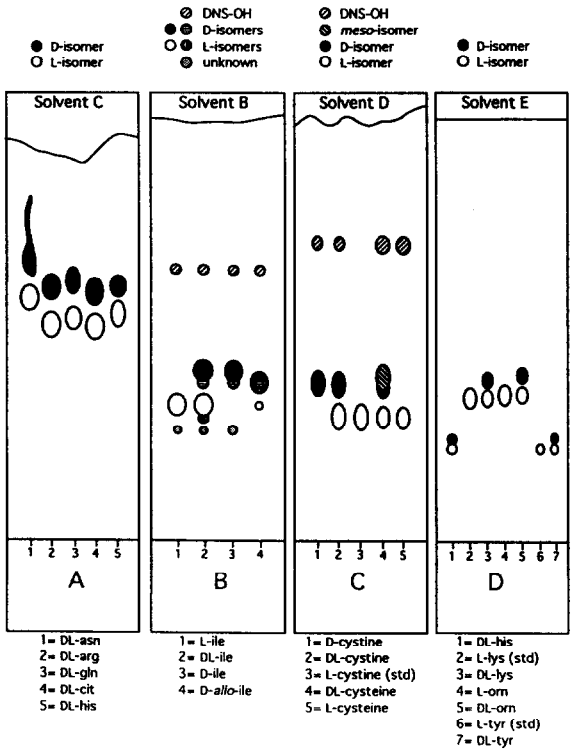

Fig. 3. Reversed-phase TLC diagrams of Dns-DL-amino acid separations. For solvents, see Table I. Plates A and B represent mono-Dns derivatives, plates C and D di-Dns derivatives.

different Dns-amino acid DL-racemates [4]. Since the separation method is believed to be based upon the preferential "fit" of one optical isomer over another into the β-CD cavity, it is obvious that the D-isomers are more tightly complexed with the β-CD in the mobile phase (higher R_F values) than the corresponding L-isomers (lower R_F values). In reversed-phase HPLC using a bonded β-CD as the stationary phase, the L-isomers elute prior to the corresponding D-isomers, again reflecting the stronger complexation of the D-isomers with the β-CD [11]. The exact mechanism of separation in either process (TLC or HPLC) is unknown. Presumably, the carboxyl group and/or the NH group of the primary sulfonamide of the D-isomer is/are capable of hydrogen bonding with the 2- or 3-hydroxyls near the mouth of the β-CD cavity to a greater extent than the L-isomer, thereby contributing to the enantioselectivity. In addition, with β-CD in the mobile phase, multiple complexation to more than one β-CD molecule is possible [12].

It was found during work-up that adjusting the pH of the crude, aqueous solution to 4 and extracting with EtOAc avoided large amounts of the fluorescent blue by-product dansyl sulfonic acid (Dns-OH) formed from the hydrolysis of Dns-Cl. This was necessary to avoid streaking upon development of the reversed-phase plates. At pH 4 the Dns-OH is essentially deprotonated (Fig. 1) and the majority remains in the aqueous layer. This extraction procedure could not be used for the mono-Dns derivatives of the basic amino acids Arg, Cit and His which were not soluble in EtOAc at pH 7 or less.

The solvent of choice for resolving the mono-Dns derivatives of DL-Asn, Arg, Cit, Gln and His was CH_3CN–0.2 *M* β-CD (20:80, v/v) (Fig. 3A). All of these compounds had similar R_F values (between 0.54 for Dns-L-Cit and 0.69 for Dns-D-Asn). The Dns-OH had an R_F value of 0.59 in this solvent system, and effectively prevented the clear measurement of α and R_s values for each of these enantiomeric pairs. It was easily removed by normal-phase TLC (see Experimental section) prior to reversed-phase TLC. It was not necessary to purify the Dns derivatives of the remaining amino acids since the Dns-OH had a much higher R_F value than the compounds of interest.

There was some question as to whether N-(α)-mono-Dns-DL-His or N-(α),N-(im)-di-Dns-DL-His was initially isolated and separated (Fig. 3A). Some earlier reports using similar reaction conditions indicated that the di-Dns derivative was formed [13–15], but in one study the mass spectrum showed no parent ion at molecular mass 621 [14]. Other researchers [10,16] simply stated that Dns-His was formed without indicating whether it was the mono- or di-Dns derivative. Furthermore, the reversed-phase TLC conditions used in this work for separating D- and L-Dns-His (Table I, solvent C) were very different from the conditions used to separate the D- and L-isomers of the di-Dns derivatives of Lys, Orn and Tyr (Table I, solvent E). The Dns-His sample was much more polar than these three di-Dns derivatives, showing similar polarity to the mono-Dns derivatives of the amino acids Asn, Arg, Cit and Gln.

Since no standard samples of either mono- or di-Dns-His were readily available, the identity of

the initial Dns-DL-His sample (Fig. 3A) was confirmed as N-(α)-mono-Dns-DL-His by TLC, specific chemical reactions, and ^{1}H NMR spectroscopy as described below.

First, it is known that the dansyl group on the α-nitrogen atom of both mono- and di-Dns-His is stable to acid hydrolysis, but the one on the imidazole nitrogen atom of di-Dns-His is not [7]. Therefore, hydrolysis of di-Dns-His produces N-(α)-mono-Dns-His and Dns-OH. A sample of Dns-DL-His was subjected to hydrolysis with 6 *M* HCl at 110°C for 12 h. Normal-phase TLC of the starting material using 1-butanol saturated with 0.2 *M* NaOH [17] showed a single, green fluorescent spot at $R_F = 0.15$. If di-Dns-His was present, acid hydrolysis would have resulted in the disappearance of this spot and the appearance of two new spots, one at a lower R_F value [17] (corresponding to the more polar compound N-(α)-mono-Dns-DL-His) and a second new spot at $R_F = 0.56$ (corresponding to Dns-OH). In the event, acid hydrolysis resulted in a TLC that was essentially unchanged from that of the starting material. When a sample of di-Dns-DL-His, which was prepared later, was subjected to the same acid hydrolysis conditions, the starting material at $R_F = 0.36$ (same solvent system) had disappeared after 30 min and two major spots were produced, corresponding to N-(α)-mono-Dns-DL-His at $R_F = 0.15$ and Dns-OH at $R_F = 0.56$.

The second piece of evidence for N-(α)-mono-Dns-His came from a specific, colorimetric reaction. The Pauly reaction [18] utilizes diazotized sulfanilic acid and produces a yellow to red color with compounds containing an imidazole ring whose nitrogen atom at the 1 position is not derivatized. Only N-(α)-mono-Dns-His, therefore, would be expected to react. When a sample of pure Dns-DL-His was spotted on silica gel and sprayed with the Pauly reagent [19,20], a red spot corresponding to the azo dye of N-(α)-mono-DNS-His resulted. A negative result was seen for N-(α),N-(im)-di-Dns-DL-His. These reactions are summarized in Fig. 4.

The third line of support for the mono-Dns derivative was provided by ^{1}H NMR spectroscopy. After purification of a crude sample of N-(α)-mono-Dns-DL-His by preparative reversed-phase TLC (see Experimental section), ^{1}H NMR analysis showed a six-proton singlet at δ2.86 corresponding to the N,N-dimethyl group of the disubstituted naphthalene ring, and a relatively simple aromatic region (δ7.0–8.5). A sample of di-Dns-His would be expected to show two six-proton singlets in the δ2.7–2.9 region corresponding to two sets of N,N-dimethyl groups. In fact, this was found to be the case. A pure sample of N-(α),N-(im)-di-Dns-DL-His showed two singlets at δ2.77 and 2.79 and a much more complicated aromatic region due to the presence of two disubstituted naphthalene rings rather than one. Therefore, the TLC data, the hydrolysis and Pauly reactions, and ^{1}H NMR spectroscopy clearly indicated that the Dns-His samples originally prepared and separated in this paper corresponded to N-(α)-mono-Dns-His and not the di-Dns derivative.

The N-(α),N-(im)-di-Dns-His sample is quite sensitive to UV light. Initial attempts to purify this compound by both normal- and reversed-phase TLC were unsuccessful. During development, the plates were monitored by long-wavelength UV light, which caused partial destruction of the compound. When the purification was carried out in the dark on reversed-phase TLC, a pure sample resulted. Exposure of a portion of this sample dissolved in MeOH to long-wavelength UV light for 30 min resulted in complete destruction of the starting material [$R_F = 0.49$; solvent MeOH–0.01 *M* Na_2HPO_4 (75:25, v/v), reversed-phase TLC] and the appearance of eight new spots, two of which could be identified as N-(α)-mono-Dns-DL-His ($R_F = 0.79$) and Dns-OH ($R_F = 0.89$). The behavior of the di-Dns-His on reversed-phase TLC relative to other Dns-amino acids clearly indicated that the Dns-His sample separated by Macek *et al.* [10] was di-Dns-His and not the mono-Dns derivative.

A portion of the N-(α),N-(im)-di-Dns-DL-His sample sample was subjected to reversed-phase TLC using β-CD in the mobile phase (Fig. 3D). Although resolution of the D- and L-isomers did occur ($\alpha = 1.20$, $R_s = 0.94$), the separation was not as efficient as the one for N-(α)-mono-Dns-DL-His ($\alpha = 1.28$, $R_s = 1.13$). Therefore, the NH group at the 1 position of the free imidazole ring of the mono-Dns derivative contributes signifi-

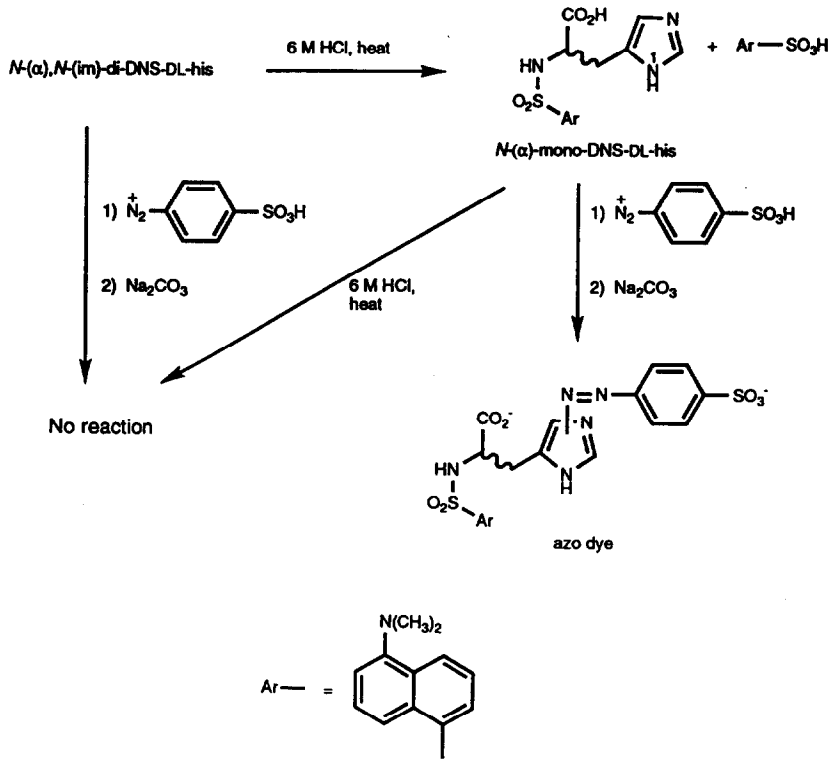

Fig. 4. Reactions of the mono- and di-Dns derivatives of DL-histidine.

cantly to the enantioselection, presumably by hydrogen bonding to the β-CD.

Because of the presence of two chiral centers Dns-Ile exists in four stereoisomeric forms (Fig. 5). All four stereoisomers were separated by this technique (Fig. 3B). The sample of DL-Ile from Sigma used in the preparation of the derivatives was labeled as containing all four stereoisomers. Reversed-phase TLC analysis showed two major spots and three minor spots, plus Dns-OH. The two major spots corresponded as expected to Dns-D- and L-Ile. The first major spot (R_F = 0.33) was identified as Dns-L-Ile by comparison with a sample synthesized from L-Ile. The second major spot (R_F = 0.40) was identified as Dns-D-Ile by comparison with a sample synthesized from the corresponding D-amino acid (which also contained traces of its diastereomer the non-proteinogenic amino acid D-*allo*-Ile). The first minor spot (R_F = 0.38), corresponding to Dns-D-*allo*-Ile, was identified by comparison with a sample synthesized from D-*allo*-Ile (which also contained traces of the diastereomer L-Ile). The

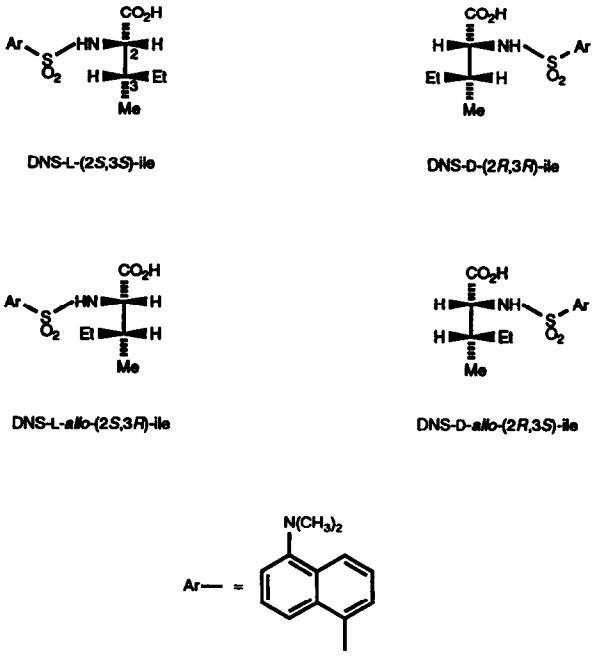

Fig. 5. Structures of the Dns-DL-isoleucines.

second minor spot at $R_F = 0.30$ corresponded to the remaining isomer L-*allo*-Ile. The third minor spot was not identified.

DL-Cysteine (Cys) was separated as the N,N'-di-Dns dimer of DL-cystine (Cys-Cys). Under the conditions employed in the dansylation reaction the –SH (thiol) group of Cys is oxidized to the corresponding –S–S– (disulfide) dimer, Cys-Cys, which subsequently is didansylated, once on each nitrogen atom [21]. Thus, dansylation of L-Cys yields N,N'-di-Dns-L-Cys-Cys and D-Cys yields N,N'-di-Dns-D-Cys-Cys. Dansylation of DL-Cys yields not only the di-Dns-L-(25%) and D-(25%) isomers of Cys-Cys, but also the *meso* compound di-Dns-D-Cys-L-Cys (50%). Therefore, reversed-phase TLC gave spots with identical R_F values for the di-Dns derivatives of both D-Cys-Cys and D-Cys (0.38) and for both L-Cys-Cys and L-Cys (0.31). When a DL-mixture of Cys was dansylated, a third major spot was visible (R_F = 0.39) corresponding to the *meso* compound (Fig. 3C).

As is evident from Table I, excellent α and R_s values were obtained for all mono-Dns-amino acids (except proline and pipecolic acid). The di-Dns-derivatives of His, Lys, Orn and Tyr did not separate as well as the others (Fig. 3D). Because of the less polar nature of these derivatives, it was necessary to use more organic modifier (MeOH) relative to aqueous β-CD to get the compounds to move up the reversed-phase plate than in the case of the mono-Dns derivatives. The lower R_s values were presumably due to the fact that derivatization of the side chain resulted in reduced hydrogen bonding potential. The Dns-OH by-product produced in the syntheses of these di-Dns derivatives eluted near the solvent front and no attempts were made to draw these complex, elongated patterns in Fig. 3D.

It was not possible to completely separate Dns-DL-Pro using this methodology. Proline is a cyclic imino acid rather than an amino acid, and thus forms a secondary sulfonamide upon dansylation rather than a primary sulfonamide. Dns-DL-Pipecolic acid (Pip), the six-membered ring analogue of proline (Fig. 6) could not be separated at all. Lam and Karmen [22] experienced similar difficulty with these two imino

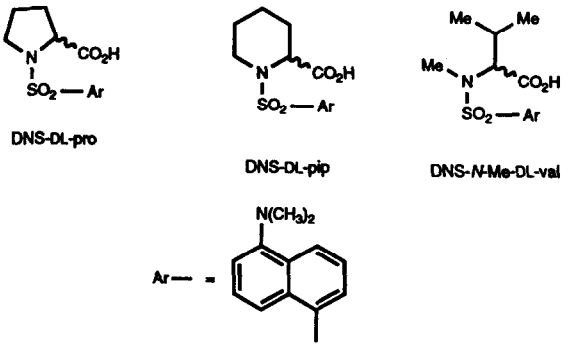

Fig. 6. Structures of the secondary sulfonamide Dns-DL-amino acids.

acids. They used a copper(II)-L-proline chiral mobile phase additive in order to form diastereomeric copper(II) complexes with enantiomeric Dns-amino acid derivatives using reversed-phase HPLC. Although the mechanism of separation using Cu(II) complexes is obviously different than with β-CD, it is interesting to note that the presence of a secondary sulfonamide was mentioned as a possible reason for the lack of formation of the proper diastereomeric copper(II) complexes, and hence, lack of separation. The Dns-D- and L-isomers of Pro and Pip were eventually separated by Lam and Karmen [22] by forming complexes with copper(II)-L-aspartame.

In order to determine whether it was the cyclic nature of these imino acids or the presence of the secondary sulfonamide that was responsible for the lack of separation, a sample of Dns-N-methyl-DL-valine (Fig. 6) was synthesized from the corresponding non-proteinogenic amino acid. This derivative is of comparable molecular mass and polarity to Dns-DL-Pro and Dns-DL-Pip and is a secondary sulfonamide, but is not cyclic. Reversed-phase TLC analysis showed nearly complete resolution of this DL-pair ($R_s = 0.94$). Presumably then, it is the cyclic nature of these imino acids that prevents enantioselection by β-CD rather than the fact that they are secondary sulfonamides. Reversed-phase TLC separation of Dns-DL-valine using the same solvent system as Dns-N-Me-DL-Val (Table I, solvent F) resulted in a much improved separation (R_s = 2.07). Dns-DL-Val is a primary sulfonamide and,

therefore, an NH group is available for hydrogen bonding to one of the 2- or 3-hydroxyl groups of β-CD thus improving the enantioselectivity. Dns-N-Me-DL-Val, being a secondary sulfonamide, lacks this NH group. The fact that this DL-pair can be resolved indicates that a primary sulfonamide group is not a necessary condition for enantioselection by β-CD.

Zukowski *et al.* [23] recently reported the enantiomeric separations of DL-Pro, DL-Pip and other imino acids as their 9-fluorenylmethyl chloroformate (FMOC) derivatives using an (*R*)-(−)-1-(1-naphthyl)ethyl carbamoylated-β-CD column and HPLC. Baseline separations were obtained using non-aqueous polar mobile phases containing triethylamine, AcOH and CH_3CN. The chiral recognition mechanism under non-aqueous conditions is still not clear, but it has been hypothesized that rather than inclusion complex formation, external adsorption at the mouth of the cyclodextrin cavity may be occurring [24].

An enantioseparation of DL-Pro has also been performed by LeFevre [25], who made diastereomeric Mosher amides of the methyl esters of DL-Pro. A normal-phase HPLC column was used for the baseline separation using *n*-heptane and diethyl ether in the mobile phase.

Finally, it was of interest to separate the DL-enantiomers of the non-proteinogenic amino acids DL-citrulline and DL-ornithine since these are important compounds in the urea cycle. Dns-DL-Cit ($R_s = 1.52$, Fig. 3A) and di-Dns-DL-Orn ($R_s = 1.20$, Fig. 3D) were both completely resolved by this technique as was Dns-DL-Arg ($R_s = 1.69$, Fig. 3A).

CONCLUSIONS

The reversed-phase TLC method described above, which uses β-cyclodextrin as a chiral selector, provides a simple, sensitive technique for separating enantiomers of dansyl amino acids. The system is capable of separating both mono- and di-dansyl derivatives with the former showing better R_s values. This work, in conjunction with that of Armstrong *et al.* [4], makes possible the resolution of all but two (proline and tryptophan) of the nineteen common, optically active amino acids found in proteins by reversed-phase TLC. Other applications of this method are currently being investigated.

ACKNOWLEDGEMENT

Financial support from the Chemistry Department at the State University of New York at Oswego is gratefully acknowledged as is Dr. David G.I. Kingston of Virginia Polytechnic Institute and State University for supplying laboratory space and selected chemicals during my sabbatical leave.

REFERENCES

1 W.L. Hinze, T.E. Riehl, D.W. Armstrong, A. Alak and T.J. Ward, *Anal. Chem.*, 57 (1985) 237.
2 T. Koscielski, D. Sybilska and J. Jurczak, *J. Chromatogr.*, 280 (1983) 131.
3 A. Guttman, A. Paulus, A.S. Cohen, N. Grinberg and B.L. Karger, *J. Chromatogr.*, 448 (1988) 41.
4 D.W. Armstrong, F.-Y. He and S.M. Han, *J. Chromatogr.*, 448 (1988) 345.
5 D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, *Science*, 232 (1986) 1132.
6 L. Lepri, V. Coas, P.G. Desideri and L. Checchini, *J. Planar Chromatogr.*, 3 (1990) 311.
7 W.R. Gray, *Methods Enzymol.*, 25B (1972) 121.
8 A. Chimiak and T. Polonski, *Org. Prep. Proceed. Int.*, 5 (1973) 117.
9 N. Seiler and J. Wiechmann, *Experientia*, 20 (1964) 559.
10 K. Macek, Z. Deyl and M. Smrž, *J. Chromatogr.*, 193 (1980) 421.
11 D.W. Armstrong and W. DeMond, *J. Chromatogr. Sci.*, 22 (1984) 411.
12 D.W. Armstrong, F. Nome, L.A. Spino and T. Golden, *J. Am. Chem. Soc.*, 108 (1986) 1418.
13 Y. Tapuhi, D.E. Schmidt, W. Lindner and B.L. Karger, *Anal. Biochem.*, 115 (1981) 123.
14 N. Seiler, H.H. Schneider and K.D. Sonnenberg, *Anal. Biochem.*, 44 (1971) 451.
15 W. Lindner, J.N. LePage, G. Davies, D.E. Seitz and B.L. Karger, *J. Chromatogr.*, 185 (1979) 323.
16 D. Morse and B.L. Horecker, *Anal. Biochem.*, 14 (1966) 429.
17 M. Cole, J.C. Fletcher and A. Robson, *J. Chromatogr.*, 20 (1965) 616.
18 H. Pauly, *Z. Physiol. Chem.*, 42 (1904) 508.
19 B.N. Ames and H.K. Mitchell, *J. Am. Chem. Soc.*, 74 (1952) 252.
20 R.W. Cowgill, *Anal. Chem.*, 27 (1955) 1519.

21 E. Schulze and V. Neuhoff, *Hoppe-Seyler's Z. Physiol. Chem.*, 357 (1976) 225.
22 S. Lam and A. Karmen, *J. Liq. Chromatogr.*, 9 (1986) 291.
23 J. Zukowski, M. Pawloska and D.W. Armstrong, *J. Chromatogr.*, 623 (1992) 33.
24 D.W. Armstrong, S. Chen, C. Chang and S. Chang, *J. Liq. Chromatogr.*, 15 (1992) 545.
25 J.W. LeFevre, *Ph.D. Dissertation*, Virginia Polytechnic Institute and State University, Blacksburg, VA, 1984, pp. 47–51.