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Determination of Enantiomeric Purity of Commercial ^{14}C- and ^{3}H-Labeled L-α-Amino Acids†

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SUMMARY

The enantiomeric purity of twelve commercial ^{14}C- and ^{3}H-labeled L-α-amino acids was determined using reverse isotope dilution analysis. The technique utilized reversed-phase (RP) thin-layer chromatography (TLC) and beta-cyclodextrin (β-CD) in the mobile phase to separate D- and L-amino acids as their 5-dimethylamino-1-naphthalene sulfonyl (dansyl, DNS) derivatives. In all cases, the L-amino acid was contaminated with the D-isomer. This is the first report of the resolution of *N*-DNS-DL-tyrosine and *N*-(α)-DNS-DL-lysine using this methodology.

Keywords: ^{14}C-labeled amino acids, ^{3}H-labeled amino acids, beta-cyclodextrin, dansyl amino acids, resolution, thin-layer chromatography.

INTRODUCTION

Those who investigate biosynthetic pathways in the production of secondary metabolites using ^{14}C- and ^{3}H-labeled amino acids often need enantiomerically pure substrates. This is especially true when probing stereochemical processes (1-3). For example, a precursor composed of L-[4,5-$^{3}H_2$]/DL-[6-^{14}C]lysine with a $^{3}H/^{14}C$ ratio of 6.8 was incorporated into the alkaloid sedamine (3). The resulting product showed a doubling of the $^{3}H/^{14}C$ ratio indicating a preference for L-lysine over the D-isomer. An experiment such as this one can be interpreted correctly only if the stereochemical integrity of the lysine precursors is intact. Normally, information on the enantiomeric purity of commercial ^{14}C- and ^{3}H-labeled amino acids is not provided by the manufacturer. Indeed, for all of the amino acids analyzed here, no such information was provided. Described in this paper is a modified reverse isotope dilution technique that was used to analyze

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commercial radioactive L-amino acids for enantiomeric purity on approximately one μCi of amino acid. The process involves the following four steps:

1. Dilution of the radioactive-L-amino acid with the corresponding non-radioactive DL-racemate.
2. Derivatization of the mixture using dansyl chloride (DNS-Cl), as shown in Figure 1.
3. Resolution of the enantiomeric DNS-DL-amino acids by RP-TLC using the chiral selector β-CD in an aqueous mobile phase containing either methanol (MeOH) or acetonitrile (CH_3CN).
4. Analysis of the pure DNS-D- and L-amino acids for radioactive content by liquid scintillation (LS) counting.

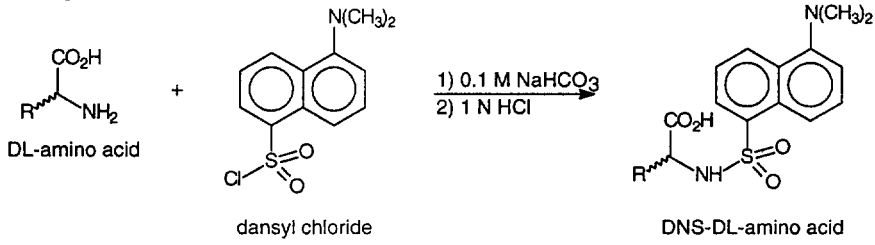

Figure 1. Formation of dansyl amino acids

The dansylation reaction was first introduced for *N*-terminal analysis of proteins and peptides (4). It produces highly fluorescent DNS-amino acids after acid hydrolysis. In normal-phase TLC, detection limits are 10^{-10} moles (5). In HPLC using fluorescence detection, sensitivities of less than 100 femtomoles have been attained (6). More recently, the dansylation reaction has been used to prepare derivatives of free amino acids whose D- and L-forms have been separated by HPLC (7-9), micellar electrokinetic chromatography (10), and RP-TLC (11-13).

The separation technique used in this paper was first introduced by Armstrong, *et al* (11) and was extended by Lepri, *et al* (12) and LeFevre (13). It is based on the fact that diastereomeric inclusion complexes are formed involving hydrogen bonding interactions between the DNS-amino acid enantiomers and the unidirectional 2- and 3-hydroxyl groups located at the mouth of the chiral β-CD cavity (14). The most important criterion in this four-step process is that the dansylation reaction must proceed *without any racemization*. If this were to occur, the stereochemical information in the radioactive amino acid would be lost. The extent of racemization in the dansylation of L-amino acids has been studied and no detectable amount of the D-isomer was detected by HPLC, even under prolonged reaction conditions (7).

Our method is superior to other types of analysis such as optical and enzymic methods. Milli and μCi amounts of commercial radioactive amino acids contain only 10^{-9} to 10^{-6} g of enantiomeric impurities, clearly beyond the range of detection by optical methods. Enzymic methods utilize

amino acid oxidases or decarboxylases, but a considerable amount of the enzyme must usually be employed (15). In addition, these assays may require more radioactive material than is available. Our method is also superior to the normal process of reverse isotope dilution analysis (16). That technique usually involves tedious recrystallization to constant specific activity, and sometimes coprecipitation of the wrong isomer occurs (17). Our method is simple and requires no sophisticated equipment except for a LS counter. Nanogram quantities of enantiomeric impurities can easily be detected[1]. The method is effective for analyzing amino acids with neutral, basic and acidic side chains.

RESULTS AND DISCUSSION

Enantiomeric Purity. The efficacy of the method was confirmed by analyzing a DL-racemate of [1-^{14}C]alanine. After conversion to DNS-DL-[1-^{14}C]alanine, the sample was analyzed three times via RP-TLC. After development, a narrow band (less than 1 mm wide) was scraped from between the DNS-L- and D- alanines, and showed only 11±1 counts per minute (CPM) above background. This indicated clean separation of the L- and D-isomers, each of which contained over 2000 CPM. The three plates showed 48.7, 48.9, and 48.9% of DNS-L-[1-^{14}C]alanine, and 51.3, 51.1, and 51.1% of DNS-D-[1-^{14}C]alanine, respectively. This corresponds to 48.8 ± 0.1% of the L-isomer and 51.2 ± 0.1% of the D-isomer, reported as the standard deviation of the mean. All of the amino acids analyzed in this paper were completely resolved. Therefore, the presence of radioactivity in any DNS-D-isomer was due to an enantiomeric impurity rather than contamination from the corresponding L-isomer resulting from incomplete separation.

Results of the analyses of nine ^{14}C- and four ^{3}H-labeled amino acids are listed in Table 1. In all cases, except for DL-[1-^{14}C]alanine, contamination by the D-isomer was detected, ranging from 2.2% in the case of L-[methyl-^{14}C]methionine to 48.6% in the case of L-[3,4,5-$^{3}H_3$]lysine.

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1 Assuming that 1 μCi of amino acid with a specific activity of 200 mCi/mmole was used in the dansylation reaction, a molecular weight of 119, RP-TLC analysis of 5% of the sample and a 5% D-isomer impurity, there would be only 1.5 ng of the radioactive D-isomer present as shown in the following calculation:

$$0.001\ \text{mCi} \times \frac{1\ \text{mmole}}{200\ \text{mCi}} \times 119\ \frac{\text{mg}}{\text{mmol}} \times 10^3\ \frac{\mu\text{g}}{\text{mg}} \times 10^3\ \frac{\text{ng}}{\mu\text{g}} \times 0.05 \times 0.05 = 1.5\ \text{ng}.$$

Table 1. Results of the Analyses of Thirteen Commercial Radioactive Amino Acids

Amino Acid	R_f		α[a]	Solvent	CPM[b]		Enantiomeric Purity (±0.1%)[c]		Vendor	Purchase Date
	L	D			L	D	L	D		
L-[1-^{14}C]alanine	0.62	0.67	1.24	A	6505±6	662±4	90.8	9.2	ICN	2/83
L-[U-^{14}C]aspartic acid	0.64	0.71	1.39	B	19295±21	2156±6	89.9	10.1	Sigma	9/88
L-[U-^{14}C]glutamic acid	0.54	0.60	1.28	C	69968±51	7420±19	89.7	10.3	Sigma	7/90
L-[3,4,5-$^{3}H_3$]lysine	0.50	0.59	1.45	C	635±2[d]	600±11[d]	51.4	48.6	CEA	unknown
L-[methyl-^{14}C]methionine	0.45	0.51	1.27	D	3361±11	74±1	97.8	2.2	RPI	8/88
L-[U-^{14}C]phenylalanine	0.39	0.45	1.29	A	9851±16	637±3	93.9	6.1	ICN	6/82
L-[^{3}H(G)]serine	0.55	0.63	1.41	E	9062±15	753±5	92.3	7.7	Sigma	10/93
L-[U-^{14}C]threonine	0.39	0.45	1.28	F	702±6	39±1	94.7	5.3	DuPont	12/94
L-[U-^{14}C]tyrosine	0.45	0.52	1.30	G	3788±4	106±3	97.3	2.7	RPI	5/90
L-[U-^{14}C]tyrosine	0.45	0.50	1.22	G	3418±11	222±2	93.9	6.1	Sigma	10/93
L-[ring-3,5-$^{3}H_2$]tyrosine	0.51	0.58	1.33	G	9258±11	275±2	97.1	2.9	RPI	11/86
L-[ring-3,5-$^{3}H_2$]tyrosine	0.52	0.58	1.27	G	2152±10	77±2	96.5	3.5	Sigma	10/93
DL-[1-^{14}C]alanine[e]	0.39	0.46	1.33	H	2291±15	2396±7	48.9	51.1	ICN	2/83

a $\alpha = [(1-R_{f\,L})/R_{f\,L}]/[(1-R_{f\,D})/R_{f\,D}]$

b Reported as standard deviation of the mean of seven countings (S_{mean} = std dev/$\sqrt{7}$). Counts are above background.

c Reported as standard deviation of the mean based upon three analyses of DL-[1-^{14}C]alanine. (See Results and Discussion section.)

d These samples were counted three times, four minutes each.

e Data based upon one of three analyses.

Solvents: A = MeOH - 0.2 M β-CD (40:60, v/v); B = MeOH - 0.13 M β-CD (30:70, v/v); C = CH_3CN - 0.175 M β-CD (20:80, v/v); D = MeOH - 0.2 M β-CD (35:65, v/v); E = MeOH - 0.2 M β-CD (30:70, v/v); F = CH_3CN - 0.15 M β-CD (25:75, v/v); G = MeOH - 0.175 M β-CD (40:60, v/v); H = CH_3CN - 0.2 M β-CD (25:75, v/v).

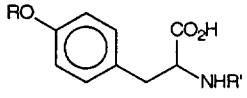
There are two reasons why commercial radioactive amino acid samples may not be enantiomerically pure. First, ^{3}H-labeled amino acids are known to racemize over time. Evans first reported that L-[ring-3,5-$^{3}H_2$]tyrosine racemizes completely after 4.5 months of storage in an aqueous solution containing 0.1% sodium formate, a known radical scavenger (18,19). Evans reported later, however, that apparently no detectable racemization occurs in aqueous solutions of tritiated amino acids when small amounts of ethanol are present (19, 20). However, our results indicate that this may not always be the case. Analyses of L-[^{3}H(G)]serine and two different samples of L-[ring-3,5-$^{3}H_2$]tyrosine indicated 7.7, 2.9, and 3.5% of radioactive D-isomer contaminant, respectively, even though these amino acids were stored in an aqueous solution containing 2% ethanol. It is possible, on the other hand, that partial racemization may have occurred during the syntheses of these compounds (19).

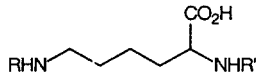
The second reason that certain radioactive amino acids may not be enantiomerically pure is that L-[U-^{14}C]amino acids are prepared by high temperature, strong acid hydrolysis of proteins biosynthesized by photosynthetic organisms or yeast (21-23). Indeed, it is known that some racemization occurs in peptides using standard hydrolysis conditions of 6 N HCl, 110 °C, for 18-24 hours (24). For example, in a study by Manning, an acid hydrolysate of natural bacitracin A contained D-leucine (6.8%) and D-*allo*-isoleucine (15.7%) which were formed by extensive racemization of L-leucine and L-isoleucine, respectively, during the course of the hydrolysis. In contrast, Manning found that free L-amino acids experience only 0.4 to 3.0% conversion to the D-isomer under standard hydrolysis conditions (25). Our results indicate that between 2.7% and 10.3% of the D-isomer contaminant was present in the seven L-[U-^{14}C]amino acids that were analyzed. Therefore, it appears that racemization occurred during hydrolysis while the amino acids were still protein bound rather than being racemized after release from the protein.

The amino acid showing the greatest amount of D-isomer contaminant was L-[3,4,5-$^{3}H_3$]lysine. The sample solvent had evaporated long before the analysis was performed. Therefore, it is not known whether the 48.6% of D-lysine contaminant was formed by racemization as a result of self-radiolysis due to the loss of solvent, or whether the original sample was actually a DL-racemate. Indeed, the latter is possible, in light of the fact that Leistner, *et al* found that a commercial sample of "L-[4,5-$^{3}H_2$]lysine" which the supplier claimed was composed of 96% of the L-isomer actually contained 49±1% L-lysine after enzymic analysis (3).

There were no trends seen regarding the age of a sample and the amount of D-contaminant. For example, the L-[U-^{14}C]phenylalanine purchased in 1982 showed 6.1% of the D-isomer, identical to that of the L-[U-^{14}C]tyrosine which was purchased in 1993 from Sigma. The results of this work suggest that blind acceptance of suppliers' claims of enantiomeric purity are not warranted, especially in experiments where a high degree of enantiomeric purity is required.

Dansyl Derivatives of Tyrosine and Lysine. The formation of the DNS-derivative of each radioactive amino acid was straightforward except for tyrosine and lysine. These amino acids contain side chains which are reactive towards DNS-Cl. Therefore, each formed three different dansyl derivatives. The structures and percentages (as determined by LS counting) of each are shown in Figure 2. The resolutions of the di-DNS-derivatives of tyrosine and lysine by RP-TLC have been reported (13). It was found that these racemates did not separate as well as those of the other mono-DNS-amino acids. In fact, under our conditions, it was not possible to separate the bands corresponding to the D- and L-isomers of these di-DNS-amino acids. However, the mono-DNS-derivatives, *N*-DNS-DL-tyrosine and *N*-(α)-DNS-DL-lysine were well resolved. Since no standard samples of these compounds were available, it was necessary to synthesize them by unambiguous routes to prove their identities. Each synthesis will be described in turn.





	Percentage		Percentage
N-DNS-tyrosine R = -H, R' = -SO_2Ar	66	*N*-(α)-DNS-lysine R = -H, R' = -SO_2Ar	43
O-DNS-tyrosine R = -SO_2Ar, R' = -H	6	*N*-(ε)-DNS-lysine R = -SO_2Ar, R' = -H	38
N,O-di-DNS-tyrosine R, R' = -SO_2Ar	28	*N*-(α)-*N*-(ε)-di-DNS-lysine R, R' = -SO_2Ar	19

Figure 2 Dansyl derivatives of tyrosine and lysine

***N*-DNS-L-Tyrosine.** The starting material, *O*-carbobenzyloxy(CBZ)-L-tyrosine, was converted to *N*-DNS-*O*-CBZ-L-tyrosine using DNS-Cl. The CBZ group was then selectively removed (26), yielding *N*-DNS-L-tyrosine as shown in Figure 3. The sample was analyzed by normal-phase TLC using solvent 1, Table 2. A major fluorescent spot at R_f = 0.19 appeared, which was identical to

that of the major product of the dansylation of unprotected DL-tyrosine. Additional evidence was obtained for the identity of *N*-DNS-L-tyrosine by converting it to *N,O*-di-DNS-L-tyrosine using standard dansylation conditions. The product of this reaction was analyzed using normal-phase TLC and a standard sample of *N,O*-di-DNS-L-tyrosine (solvent 1, Table 2). The starting material at R_f = 0.19 disappeared and a new spot at R_f = 0.26 appeared, matching that of the di-DNS-standard. This result was confirmed by a RP-TLC system using solvent 3, Table 2 (27). The starting material at R_f = 0.73 disappeared and a new spot at R_f = 0.26 appeared, again matching that of the standard.

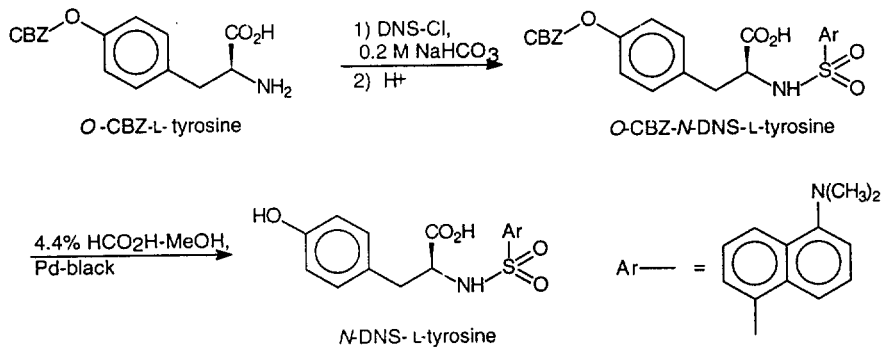


Figure 3 Synthesis of *N*-DNS-L-tyrosine

Table 2. TLC Solvents Used in the Syntheses of *N*-DNS-Tyrosine and *N*-(α)-DNS-Lysine

Solvent	Composition
1[a]	Toluene-pyridine-acetic acid (40:10:1, v/v/v)
2[a]	*n*-Butanol saturated with 0.2 M NaOH
3[b]	Methanol-2% aqueous acetic acid (75:25, v/v)

[a]Normal-phase TLC solvent
[b]Reversed-phase TLC solvent

***N*-(α)-DNS-D- and L-Lysines.** The synthesis was performed on both the D- and L-isomers of *N*-(ε)-CBZ-lysine. The synthesis of the L-isomer is described here. The starting material was converted to *N*-(α)-DNS-*N*-(ε)-CBZ-L-lysine as shown in Figure 4. Selective removal of the CBZ

group yielded *N*-(α)-DNS-L-lysine, which was analyzed by normal-phase TLC using solvent 2, Table 2 (28). A major fluorescent spot at R_f = 0.19 appeared, which was identical to that of the major product that resulted from dansylation of unprotected DL-lysine. Additional evidence for *N*-(α)-DNS-lysine was obtained by converting the D-isomer to *N*-(α)-*N*-(ε)-di-DNS-D-lysine using DNS-Cl. Under analysis via normal-phase TLC in solvent 2, Table 2, the spot corresponding to *N*-(α)-DNS-D-lysine disappeared and a new spot at R_f = 0.49 appeared, matching that of a standard sample of *N*-(α)-*N*-(ε)-di-DNS-L-lysine. Additional support was provided by RP-TLC using solvent 3, Table 2. The spot at R_f = 0.73 corresponding to *N*-(α)-DNS-D-lysine disappeared and a new spot appeared at R_f = 0.45, matching that of the di-DNS-standard sample.

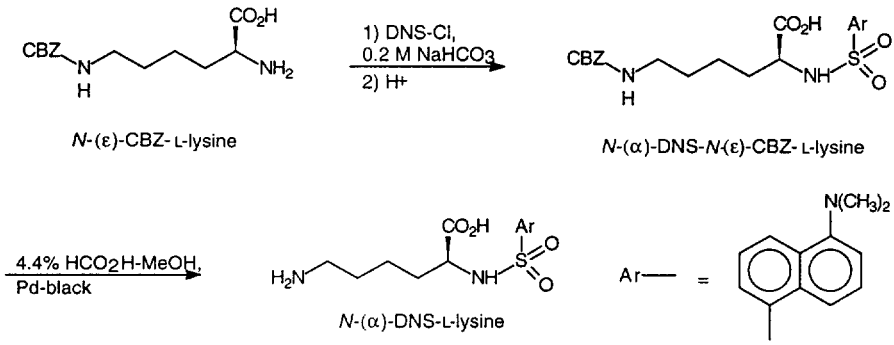


Figure 4 Synthesis of *N*-(α)-DNS-L-lysine

A preliminary RP-TLC separation using β-CD gave a clean resolution of the D- and L-isomers of *N*-(α)-DNS-DL-lysine, but overlap of the L-isomer with dansyl sulfonic acid, DNS-OH, a reaction by-product (4), occurred. Using solvent 3, Table 2, the DNS-OH (R_f = 0.81) could be separated from *N*-(α)-DNS-DL-lysine (R_f = 0.73) prior to the resolution step.

Like all of the other protein DNS-amino acids (11-13), the D-isomers eluted ahead of the L-isomers for both tyrosine and lysine. The α-values for the *N*-α-DNS-derivatives of DL-tyrosine (average of 1.28) and DL-lysine (1.45) are higher than those of the di-DNS-derivatives of these compounds, which were reported as 1.15 and 1.19, respectively (13).

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EXPERIMENTAL

Materials. L-[methyl-^{14}C]Methionine, one sample of L-[U-^{14}C]tyrosine, and one sample of L-[ring-3,5-$^{3}H_2$]tyrosine were purchased from Research Products International (Mount Prospect, IL). ULTIMA GOLD LS cocktail was obtained from Packard Instrument Co. (Meriden, CT). L-[1-^{14}C]Alanine, DL-[1-^{14}C]alanine, and L-[U-^{14}C]phenylalanine were obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). L-[U-^{14}C]Aspartic acid, L-[U-^{14}C]glutamic acid, L-[^{3}H(G)]serine, a second sample of L-[U-^{14}C]tyrosine, a second sample of L-[ring-3,5-$^{3}H_2$]tyrosine, *N*-(ε)-CBZ-D- and L-lysine, *O*-CBZ-L-tyrosine, Palladium black, and all free and DNS-amino acids were purchased from Sigma Chemical Co. (St. Louis, MO). L-[3,4,5-$^{3}H_3$]Lysine was obtained from CEA (France). L-[U-^{14}C]Threonine was purchased from DuPont (Wilmington, DE). Dansyl chloride was obtained from Aldrich Chemical Co. (Milwaukee, WI). Beta-cyclodextrin hydrate was purchased from CERESTAR, USA, Inc. (Hammond, IN). Normal-phase TLC plates with fluorescent indicator (#13181) were obtained from Eastman Kodak Co. (Rochester, NY). Whatman KC18F, 1x3", 200 μm thickness, and 5x20 cm LKC18, 200 μm thickness RP-TLC plates with no fluorescent indicator were obtained from Alltech Associates, Inc. (Deerfield, IL). A Mineralight® lamp, model UBGL-25, (San Gabriel, CA) was used to visualize all of the DNS-amino acids at 366 nm. The LS counter was an LKB model 1214 RackBeta Excel.

Preparation of DNS-Amino Acids - General Procedure. A 1 mM solution of the appropriate non-radioactive DL-amino acid in 0.1 M $NaHCO_3$ was prepared. To 1000 μL of this solution was added approximately 1 μCi of the corresponding radioactive L-amino acid. To this solution were added 500 μL of 15 mM DNS-Cl in CH_3CN. The solution was stirred for 1-2 hours at room temperature. The excess DNS-Cl was extracted with diethyl ether, and the solution was acidified with 0.1 N HCl to a pH of approximately 2. The solution was extracted with ethyl acetate (EtOAc, 3x1000 μL) and the EtOAc extracts were combined and dried with anhydrous $MgSO_4$. After filtration, the EtOAc was left to evaporate in a fume hood overnight, leaving the crude DNS-DL-amino acid.

Reversed-Phase TLC Methods. After evaporation of the EtOAc used to extract the DNS-amino acids, the crude sample was dissolved in 50 - 400 μL of MeOH. A 10 - 30 μL aliquot was delivered to a 5x20 cm RP-TLC plate. The number of CPM delivered to the plate was in the range of 10^3 - 10^4 except for L-[3,4,5-$^3H_3$]lysine and L-[U-^{14}C]threonine, where less material was available. The proportion of organic modifier (CH_3CN or MeOH) was varied according to the polarity of the DNS-amino acid. Optimum ranges for CH_3CN and MeOH were reported earlier to be 15 - 20% and 20 - 30%, respectively (11). Optimum separations were obtained using 20 - 30% CH_3CN and 30 - 40% MeOH. The aqueous solution was saturated with urea to increase the solubility of β-CD (11), and the solution was made 0.6 M in NaCl to prevent the highly aqueous solution from dissolving the binder which holds the derivatized silica gel to the glass plate. Plate development times ranged from 3-10 hours depending upon the nature of the mobile phase. The more organic modifier, the shorter the development time. Spots were visualized at 366 nm. *The 5x20 cm RP-TLC plates used for the analyses must not contain a fluorescent indicator.* Analyses of ^{14}C-amino acids done on fluorescent plates showed thousands of extraneous counts at energies lower than those of ^{14}C.

Liquid Scintillation Methods. After development, the TLC plates were allowed to dry for 1-2 hours in a fume hood. The bands corresponding to the DNS-D-and L-amino acids were sequentially scraped from the plate and transferred quantitatively to separate LS vials. Ten mL of LS cocktail were added to each vial and the samples were sonicated for 5 minutes. The silica gel was allowed to settle for at least 24 hours in the dark before the samples were counted seven times, five minutes each. Enantiomeric purity calculations were done by dividing the appropriate number of CPM for a DNS-D- or L-amino acid by the total number of CPM for both isomers.

Syntheses of DNS-L-[U-^{14}C] and DNS-L-[Ring-3,5-$^3H_2$]Tyrosines (RPI). Because DL-tyrosine is not soluble in 0.1 M $NaHCO_3$, it was necessary to add NaOH. To 5 mL of 0.5% $NaHCO_3$ was added 2 mL of 0.1 N NaOH, 18.1 mg of DL-tyrosine and 3 mL of deionized water. To 1000 μL of this 10 mM solution were added either 2 μCi of L-[U-^{14}C]tyrosine or L-[ring-3,5-$^3H_2$]tyrosine and 1000 μL of 10 mM DNS-Cl in acetone. The solution was stirred for 2 hours at room temperature and acidified with 0.1 N HCl to pH = 2. The solution was extracted with diethyl ether (3x1000 μL) and the ether extracts were combined and dried with anhydrous $MgSO_4$. After filtration, the ether was evaporated, leaving the crude mixture of mono- and di-DNS-DL-tyrosines.

The mixture was dissolved in 400 μL of MeOH and a small aliquot was analyzed by RP-TLC. The same procedure was used to synthesize L-[U-^{14}C]tyrosine and L-[ring-3,5-$^{3}H_2$]tyrosine purchased from Sigma, except that 1 mM solutions were used and the crude products were extracted with EtOAc rather than diethyl ether.

Synthesis and Purification of *N*-DNS-[3,4,5-$^{3}H_3$]Lysines. The procedure was the same as that found in the General Procedure except that the pH was carefully adjusted to the isoelectric point of lysine (9.74) with 0.1 N NaOH to ensure maximum dansylation at the *N*-(α)-position before the addition of DNS-Cl. Before RP-TLC to separate the *N*-(α)-DNS- D- and L-lysines, a portion of the crude sample in MeOH was purified on a 5x20 cm RP-TLC plate using solvent 3, Table 2 to remove DNS-OH which overlapped the D-isomer. The *N*-(α)-DNS-DL-lysine was scraped from the plate and eluted from the silica gel with 2 mL of MeOH. After evaporating the solvent, the sample was redissolved in 30 μL of MeOH and analyzed by RP-TLC.

Synthesis of DNS-L-[U-^{14}C]Threonine. To 100 μL of a 1 mM solution of non-radioactive DL-threonine in 5% $NaHCO_3$ (pH = 9.5) were added approximately 1 μCi of L-[U-^{14}C]threonine and 100 μL of 5 mM DNS-Cl in acetone. After 1 hour, the solution was acidified with 30 μL of 1 N HCl. The solution was evaporated to dryness in a fume hood. The residue was dissolved in 200 μL of wet EtOAc (4) to avoid large amounts of DNS-OH, which has an R_f slightly higher than that of DNS-D-threonine in RP-TLC. Ten μL of the solution were analyzed by RP-TLC.

Synthesis of *N*-DNS-L-Tyrosine. To 4 mg of *O*-CBZ-L-tyrosine were added 10 mL of 0.2 M $NaHCO_3$ (pH = 9.5). Two mL of acetone and 12.7 mL of 5 mM DNS-Cl in acetone were added and a cloudy yellow solution resulted. To this were added 2 mL of CH_3CN and 2 mL of 0.2 M $NaHCO_3$, after which the yellow solution cleared. The solution was shaken for 1 minute and left to sit at room temperature for 1.5 hours. The solution was acidified with approximately 5 mL of 1 N HCl and extracted three times with EtOAc. After drying the EtOAc with anhydrous Na_2SO_4, the EtOAc was filtered and evaporated to dryness. The crude product (10.8 mg), *N*-DNS-*O*-CBZ-L-tyrosine, was dissolved in 0.5 mL of 4.4% HCO_2H in MeOH, and 10.8 mg of Pd-black were added. The solution was refluxed for 45 minutes, filtered to remove the Pd-black, and evaporated to dryness, leaving 5.0 mg of crude *N*-DNS-L-tyrosine. The reaction time was not optimized. The product was analyzed on a 1 x 3" RP-TLC plate using solvent A, Table 1. The major spot at R_f = 0.53 matched that of the L-spot of the DNS-L-[U-^{14}C]tyrosine (RPI) synthesized earlier. The *N*-DNS-D-tyrosine from the radioactive sample had an R_f of 0.60 in this solvent.

Approximately 2 mg of the crude *N*-DNS-L-tyrosine were converted to *N,O*-di-DNS-L-tyrosine by dissolving it in 3 mL of 0.2 M $NaHCO_3$ (pH = 9.5). To this solution were added 3.0 mL of 10 mM DNS-Cl in acetone. After 1 hour at room temperature, the solution was acidified with 1 N HCl and extracted with EtOAc. Analysis by TLC (solvents 1 and 3, Table 2) showed partial conversion to *N,O*-di-DNS-L-tyrosine by comparison to a standard sample.

Syntheses of *N*-DNS-D- and L-Lysines. To 4.0 mg of *N*-ε-CBZ-L-lysine were added 14.3 mL of 0.2 M $NaHCO_3$ (pH = 9.5) and 14.3 mL of DNS-Cl in acetone. The solution was shaken for 1-2 minutes and allowed to sit for 2 hours at room temperature. The solution was acidified with 5 mL of 1 N HCl and extracted three times with EtOAc. The EtOAc was washed two times with water, dried with anhydrous Na_2SO_4 and evaporated to dryness, leaving 10 mg of crude *N*-(α)-DNS-*N*-(ε)-CBZ-L-lysine. To 1.6 mg of the crude sample were added 0.5 mL of HCO_2H in MeOH and 4.9 mg of Pd-black. The sample was refluxed for 10-15 minutes, after which time the solution was filtered and evaporated to dryness, leaving crude *N*-(α)-DNS-L-lysine. The D-isomer was synthesized in the same way.

A 200 μL portion of the *N*-(α)-DNS-D-lysine in 4.4% HCO_2H in MeOH was evaporated to dryness. After adding 200 μL of 0.2 M $NaHCO_3$ and 200 μL of 10 mM DNS-Cl in acetone, the solution was allowed to sit for 0.5 hours. To 200 μL of the solution was added 1 drop of 1 N HCl. The solution was extracted once with EtOAc. Analysis by TLC (solvents 2 and 3, Table 2) showed complete conversion to *N*-(α)-*N*-(ε)-di-DNS-D-lysine by comparison to a standard sample of the corresponding L-isomer.

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