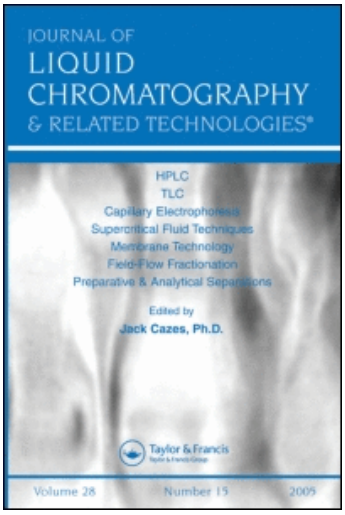

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Applications of tert-Butyloxycarbonyl-L-amino Acid-N-Hydroxysuccinimide Esters in the Chromatographic Separation and Determination of D,L-Amino Acids and Diastereomeric Dipeptides

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APPLICATIONS OF tert-BUTYLOXYCARBONYL-L-AMINO ACID-N-HYDROXYSUCCINIMIDE ESTERS IN THE CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF D,L-AMINO ACIDS AND DIASTEREOMERIC DIPEPTIDES

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ABSTRACT

DL-Amino acids are converted into the LD, and LL diastereomeric dipeptides after reaction with a Boc-L-AA-OSu derivative. The dipeptides are subsequently separated by liquid chromatography using a reversed, bonded stationary phase. If the sample is a DL,DL-dipeptide, the products of the reaction are the LLL, LDD, LLD, and LDL tripeptide. Reaction conditions, structure of the BOC reagent, eluting conditions, and calibration procedures are the major parameters studied. In general, per cent conversion is greater than 95%; the determination of minor concentrations of one enantiomer in the presence of the other is possible.

INTRODUCTION

The unique qualities of high performance liquid chromatography (HPLC) are particularly suitable to the many difficult separation problems routinely encountered in peptide chemistry. Several recent investigations have focused on evaluating those parameters which influence the interactions between stationary phases and amino acids, peptides, and their derivatives while others were concerned primarily with established optimum eluting conditions for their separation ([1-8]).

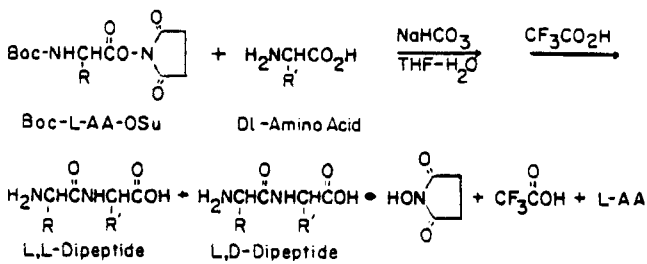
is the column void volume. The column void volume was determined by measurement of the retention volume for a sample or solvent that is not retained by the column.

Quantitation was achieved by peak area and peak height measurements using a direct calibration curve and an internal standard calibrative curve. This was done both manually and with a Spectra Physics SP-4100 Computing Integrator.

RESULTS AND DISCUSSION

Boc Reagents

The Boc-L-AA-N-hydroxysuccinimide ester will undergo a reaction with a DL-amino acid in the presence of $NaHCO_3$ to give the Boc protected L,L-dipeptide and L,D-dipeptide. Removal of the Boc group is accomplished by treatment with CF_3CO_2H. The final reaction mixture after work-up is then composed of the L,L and L,D-dipeptides, N-hydroxysuccinimide, CF_3CO_2H, the L-amino acid from the excess Boc reagent, and their sodium salts. These reactions are summarized below.



Several other reactions are known which can be used to convert amino acids into dipeptides ([18]). The advantages of the Boc-L-AA-OSu over the other reagents for the type of applications

One type of problem of considerable interest is the separation of optically active amino acids. Two general types of elution modifications have been used in gas and liquid chroma-

tography to solve this problem. a) The enantiomers are separated on an optically active stationary phase. b) The enantiomers are chemically converted into diastereomers by a reaction with a suitable asymmetric reagent followed by separation on an optically inactive stationary phase. These techniques have recently been reviewed ([9-13]). A third and recent approach, which may prove to be a general one, is to use chiral eluants. Examples of this are the separation of D,L-amino acids ([14,15]) and dansyl-D,L-amino acid derivatives ([16]) using an eluant containing a metal chelate where the chelating agent is optically active.

In the present paper a procedure is described for the separation of D-and L-amino acids, AA, after their conversion; for example, to the [L-Phe[1]] dipeptides by a reaction with an N-hydroxysuccinimide ester of a tert-butyloxycarbonyl (Boc) blocked derivative of L-Phe (Boc-L-Phe-OSu). Several other Boc reagents (Boc-L-AA-OSu) can also be used. The resulting diastereomeric dipeptides are then separated on a reversed bonded phase type column. The procedures described here are also potentially useful in racemization studies, peptide synthesis, peptide purification, and related applications since this reaction is widely used in these areas.

MATERIALS

Reagents

Amino acids and dipeptides were obtained from Sigma Chemical Co. and Vega Biochemical Co. The L-Phe and L-Ala derivatives of the Boc-L-AA-OSu reagents were obtained from Sigma while the L-Val and L-Leu derivatives were from Chemical Dynamics Corp. and Tridom/Fluka Chemical Inc. Trifluoroacetic acid was purchased from Sigma. Inorganic acids, bases, and salts were analytical reagent grade. These and organic solvents were obtained from Curtin Matheson Scientific Co. Distilled water was passed through a mixed bed ion exchange column, an activated charcoal column, and finally through a 0.2 μm stainless steel filter before being used.

Instrumentation

Two liquid chromatographs were used. One was a Waters Model 202 liquid chromatograph equipped with a Model 6000 pump, a U6K injector, and a fixed wavelength UV detector. The second one used was an Altex Model 332 gradient liquid chromatograph equipped with a Model 420 Microprocessor System, two Model 110 A pumps, a Model 153 fixed wavelength detector, and a Series 210 injector. The columns were an Altex LiChrosorb RP8, 10 μm, 25 x 0.32 cm, Merck Hibar-II LiChrosorb RP-8, 10 μm, 25 x 0.46 cm, and a Waters μBondapak C18, 10 μm, 30 x 0.39 cm. Chromatographic peak areas were computed with a Spectra Physics SP-4100 Computing Integrator.

Procedures

A detailed procedure for the reaction of the Boc-L-AA-OSu reagent with an amino acid are provided elsewhere ([17]). Reaction variables (reaction time, temperature, solvent, concentration ratio, etc.) in the procedure were studied and those already suggested were found to be optimum.

Standard amino acid and peptide solutions were prepared by dissolving mg quantities in water, 0.1M HCl, or organic solvent - water solutions and stored in 6-ml Hypovials fitted with Hycar septa and sealed with aluminum caps (Pierce Chemical). The solutions were refrigerated when not in use. Pressure Lok Series B110 syringes (Precision Sampling Corporation) of 10-and 25-μL size were used to inject the samples.

All mixed solvents used in the eluting mixtures are percent by volume. Phosphate salts were used for the buffers at 0.020M; NaCl was added in appropriate amounts to maintain the ionic strength at 0.10M.

Capacity factors were calculated according to

$$k' = (V_R - V_O)/V_R$$

where V_R is the elution volume for the chromatographic peak and V_O described here are several. 1. The reaction is quantitative (>95%

reaction) and proceeds without racemization. 2. The reaction conditions are relatively simple and require a ratio of 1:2:2 in amino acid:Boc-L-AA-OSu:$NaHCO_3$. A larger excess of the Boc-L-AA-OSu is necessary to ensure a quantitative conversion of the acidic and basic amino acids into the derivatives. For example, reaction with lysine requires approximately a 1:4:2 ratio. These observations were experimentally established previously ([17]) and were verified in the present study. 3. It is possible to introduce a chromophore into the dipeptide by choosing a particular Boc-L-AA-OSu reagent and thus facilitate its UV detection. For example, using Boc-L-Phe-OSu would introduce an aromatic side chain group and would easily allow detection at 254 nm. 4. The Boc-L-AA-OSu reagents are commercially available in reasonable purity and are available as different amino acid derivatives. 5. The final reaction mixture is a favorable one in that there is a considerable difference in the chromatographic behavior between the dipeptides and the by-products, thus, removal of the by-products is readily achieved. 6. The reaction is not only limited to amino acids but can also be applied to dipeptides and longer chain peptides.

Separation of Diastereomeric Peptides

In previous investigations ([7], [17]) diastereomeric dipeptides obtained via reaction 1, where Boc-L-Leu-OSu was used, were separated by using a sulfonated polystyrene-divinylbenzene type column with aqueous acidic-buffered eluting mixtures. Although resolution of L,D-dipeptide mixtures was achieved elution times are not favorable from an analytical or prep-scale viewpoint. For example, elution times of 1½ to up to 4 hours are required to elute dipeptides from the ion exchange column.

Previous work in this laboratory had demonstrated that buffered aqueous-organic solvent mixtures could be successfully used to separate peptide diastereomers on microparticle C_8 and C_{18} stationary phases ([2]) and on Amberlite XAD copolymeric adsorbents ([1]) in a matter of minutes. Since microparticle XAD columns can also be prepared which have efficiencies similar to the

alkyl-modified silica columns ([19]), both types of columns offer a significant advantage over the ion exchange column in analytical and prep-scale HPLC.

Several elution variables can be exploited in order to obtain a favorable separation of a diastereomeric peptide mixture on the bonded phase and XAD columns ([1,2]). These include adjustment of pH, organic solvent-water ratio, type of organic solvent used, addition of electrolytes and changes in the stationary phase.

Since selectivity is also strongly influenced by dipeptide structure the choice of the Boc-L-AA-OSu will influence the elution since different amino acids can be introduced into position 1 of the dipeptide with the Boc reagent. For example, a large hydrophobic side chain group in the amino acid subunit will increase the retention over that found for a less hydrophobic side chain group ([1,2]). Furthermore, the location of the hydrophobic side chain relative to the charged site in the dipeptide will influence the retention; this retention will change with pH since in acidic solution the amine terminal group exists as a cation while in basic solution the carboxyl terminal group exists as an anion.

Tables I and II illustrate many of these points. In Table I the samples were LD-amino acid mixtures and thus two dipeptide products (L,L and L,D) were obtained while in Table II the samples were individual L and D amino acids. A 254 nm wavelength was used for detecting the Phe dipeptides while 208 nm was used for the others. In most cases the peak positions were confirmed by comparison to standards. The different Boc reagents were used without modification of the procedure. All reactions appeared to provide a quantiative conversion; this was verified, when possible, by carefully examining the chromatogram at retention volumes, where unreacted AA would appear, with a high sensitivity. As expected retention increases as the CH_3CN concentration decreases and increases in acidic and basic solution where the dipeptides are not zwitterions.

The retention after reaction with a given AA is the largest when introducing Phe at position 1, this is followed by Leu and

TABLE I

Retention Data for Several Dipeptide Diastereomers Using Different BOC Reagents

Boc Reagent	AA	k' for Dipeptide[a] L,L	k' for Dipeptide[a] L,D	Column Conditions pH	Column Conditions %CH_3CN
L-Phe	D,L-Phe	4.76	13.0	6.4	15[b]
L-Phe	D,L-Leu	2.29	7.18	6.4	15[b]
L-Phe	D,L-Ala	0.51	1.36	6.4	10[b]
L-Leu	D,L-Phe	2.85	8.71	6.4	15[b]
L-Leu	D,L-Leu	0.98	4.32	6.4	15[b]
L-Leu	D,L-Ala	1.67	11.4	5.9	0[b]
L-Ala	D,L-Phe	0.64	1.28	6.4	10[b]
L-Ala	D,L-Leu	1.87	8.95	5.9	0[b]
L-Ala	D,L-Ala	BSP	BSP	2.3,5.9,7.5	0[b]
———	Ala-Ala	0.18	0.86	2.6	0[c]
L-Leu	D,L-Ser	BSP	0.67	5.9	2[c]
L-Phe	D,L-Ser	0.76	1.93	5.9	2[c]
L-Leu	D,L-Ser	0.70	3.17	2.6	0[c]
L-Leu	D,L-Ser	1.55	2.62	2.6	0[d]
L-Phe	D,L-Ser	2.21	5.22	5.9	0[d]

[a]BSP=the dipeptide peak appears within the by-product-solvent peaks at the void volume.

[b]Data were collected on a Merck Hibar-II LiChrosorb RP-8 Column.

[c]Data were collected on an Altex LiChrosorb RP-8 column.

[d]Data were collected on a Waters μBondapak C-18 column.

TABLE II

Retention Data for Several Dipeptide Diastereomers

Boc Reagent	AA	k'[a] 10% CH_3CN-90% H_2O;pH=6.1 L,L	L,D	5% CH_3CN-95% H_2O;pH=6.0 L,L	L,D	100% H_2O; pH=6.05 L,L	L,D
L-Leu	L-Ala;D-Ala			BSP	1.64	1.45	9.95
L-Leu	L-Val;D-Val	BSP	3.18	1.09	11.8		
L-Leu	L-Ile;D-Ile	0.92	8.57	3.27	36.0		
L-Leu	L-Leu;D-Leu	1.61	9.41	6.00	38.2		
L-Leu	L-Phe;D-Phe	5.12	19.0				
L-Leu	L-Asp;D-Asp						
L-Leu	L-Ser;D-Ser					0.72	2.21
L-Leu	L-Tyr;D-Tyr	0.59	1.26	1.69	4.18		
L-Leu	L-Lys;D-Lys	1.21	6.22	6.41	>32		
L-Leu	L-Asn;D-Asn					0.76	1.58
L-Leu	L-Arg;D-Arg			BSP	0.78	1.81	7.81
L-Leu	L-Trp;D-Trp	5.68	14.73				

[a] BSP=the dipeptide peak appears within the by-product-solvent peaks at the void volume. Data were collected on a Merck Hibar-II LiChrosorb RP-8 column.

finally by Ala. For a given Boc reagent dipeptide retention decreases as the hydrophobic nature of the AA at position-2 decreases. Thus, as shown in Table I, even when using Boc-L-Phe-OSu or Boc-L-Leu-OSu, the dipeptides obtained after reaction with a polar amino acid such as Ser have low retention and their peaks are close to those resulting from the solvent and reaction by-products. The Boc-L-Ala-OSu reagent appears to be the least useful since dipeptides derived from this reagent have the lowest retention. For example, Ala-Ala (Table I) cannot be readily separated from the

solvent-by-product peaks; the k' data listed were obtained with Ala-Ala standards.

In Table II a Boc-L-Leu-OSu reagent was used to prepare dipeptides from nonpolar, polar, and polar-acidic and basic AA. In all cases the L,D dipeptide has the greater retention and is readily baseline separated from the L,L dipeptide by carefully choosing an appropriate CH_3CN-H_2O ratio at pH=6. Also, since the structure of the dipeptides changes only at position 2 the influence of this change on retention is apparent.

If a C-18 column is used retention increases over that on the C-8 column. Only data for retention of Ser dipeptides on the C-18 column are shown in Table I. For those dipeptides whose k' values are small on the C-8 column, for example the polar dipeptides, and appear close to or are poorly resolved from the solvent-by-product peaks, the C-18 column would be preferred.

TABLE III

Retention Data for $(Ala)_3$ after Reaction of DL-Ala-DL-Ala with Boc-L-Ala-OSu

		Capacity Factor, k'				Eluting Condition[a]	
Boc Reagent	Dipeptide	LLD	LDD	LLL	LDL	pH	%CH_3CN
L-Ala	DL-Ala-DL-Ala	BSP[b]	BSP	BSP	BSP	2.6	0
———	$(Ala)_3$ Standards	0.98	0.49	0.49	1.76	2.6	0
L-Phe	DL-Ala-DL-Ala	1.12	1.50	1.50	2.87	2.6	10
L-Phe	DL-Ala-DL-Ala	3.12	3.98	4.45	9.18	2.6	5
L-Phe	DL-Ala-DL-Ala	6.18	7.58	9.14	18.3	2.6	3
L-Leu	DL-Ala-DL-Ala	1.46	1.9	1.9	4.14	2.6	10
L-Leu	DL-Ala-DL-Ala	2.22	2.70	3.06	7.00	2.6	5
L-Leu	DL-Ala-DL-Ala	5.74	7.29	8.64	22.5	2.6	3

[a] Data were collected on a Waters μBondapak C-18 column.

[b] BSP=the tripeptide peak appears within the by-product-solvent peaks at the void volume.

Reaction 1 can be applied to peptides. This is illustrated in Table III where chromatographic data are shown. For a series of tripeptides obtained after reaction of LD-Ala-LD-Ala with several different Boc reagents. The k' values for each of the tripeptides were confirmed by carrying out the reactions with L-Ala-L-Ala, D-Ala-D-Ala, L-Ala-D-Ala, and D-Ala-L-Ala. As with the dipeptides retention in Table III increases with a decrease in CH_3CN concentration, is larger in acidic and basic solution where the peptide is not a zwitterion, and is larger on the C-18 column.

The elution order in Table III for the Ala tripeptides is consistent with that observed before, that is, LDL > LLL > LDD ([2]). When the AA unit in position-1 is altered as occurs when using either the Boc-L-Phe-OSu or Boc-L-Leu-OSu reagent the elution order changes to LDL > LLL > LDD > LLD. This indicates that the hydrophobic nature of the side chain group in the AA unit at position-1 is significant and has a greater effect than that due to directional differences that are present at each chiral center ([2]) even

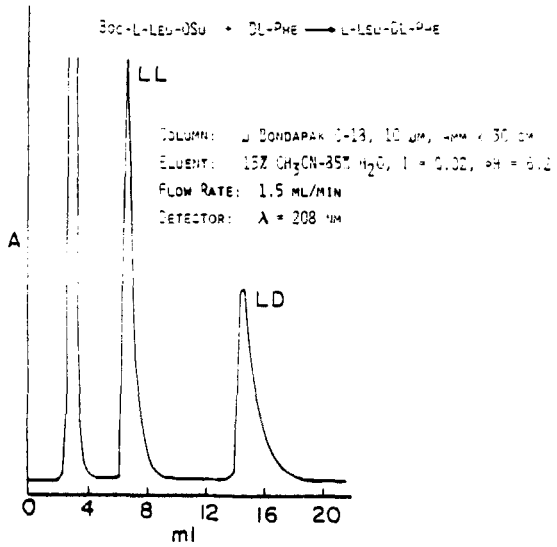


FIGURE 1
Separation of L-Leu-DL-Phe Diastereomers

though the side chain group is close to a charged site. In Table III the retention data were obtained in acidic solution where the terminal amino group at position-1 in the tripeptide is in the cationic form. The combined influence of charge, structure of the AA side chain, and their position relative to each other on retention has been discussed elsewhere ([1,2]).

Applications

Several different experiments were completed to illustrate the qualitative and quantitative scope of reaction 1.

Consider a Phe sample where it is important to establish its optical purity. If the sample is reacted with Boc-L-Leu-OSu according to reaction 1, two dipeptides, L-Leu-L-Phe and L-Leu-D-Phe will form if the sample contained both D-and L-Phe. A typical chromatogram illustrating their separation is shown in Figure 1. If the Phe sample is optically pure only the one dipeptide that is formed will appear in the chromatogram. Thus, the appearance of either or both peaks is a qualitative estimation of optical purity while their areas correspond to a quantitative estimation of each.

Figure 2 illustrates the separation of the mixture obtained after reaction of Boc-L-Phe-OSu with LD-Ala-LD-Ala. Thus, the optical purity of an Ala-Ala sample would be established by a careful examination at appropriate sensitivity at the four retention volumes corresponding to the LLD, LDD, LLL, and LDL tripeptide.

Three independent systems were studied in order to establish the quantitative application of reaction 1. In the first two calibration curves of peak height and area versus peptide concentration were prepared using the commercially available dipeptides, L-Phe-L-Ser and L-Leu-L-Phe, as standards. Their optical purity relative to the presence of L,D and D,L dipeptides and purity relative to the presence of Phe and Leu was established by examining the separation with a high detector sensitivity at retention volumes where these impurities would appear. The absence of a Ser impurity could not be confirmed because Ser has

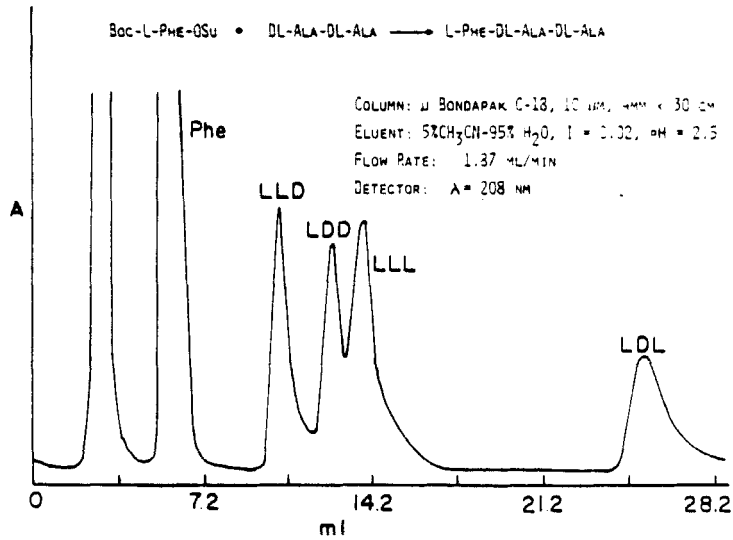


FIGURE 2
Separation of L-Phe-LD-Ala-LD-Ala Diastereomers

very little retention and its peak is not readily separated from the solvent peaks. In separate experiments known amounts of L-Ser and L-Phe were taken and converted to L-Phe-L-Ser and L-Leu-L-Phe, respectively, according to reaction 1 by using the appropriate Boc reagent. (The optical purity of the Boc reagents was verified by examining the separation with a high detector sensitivity at retention volumes where the D,L dipeptides would appear.) The concentrations of the synthesized dipeptides were obtained from the calibration curves. In general, per cent recovery of L-Ser and L-Phe, whether using peak area or peak height, was >95%.

In the third system a calibration curve was prepared using different amounts of L-Ala as the sample and L-Ser as an internal standard. The mixture were converted to the dipeptides, L-Phe-L-Ala and L-Phe-L-Ser, respectively, according to reaction 1 using Boc-L-Phe-OSu. The peak heights and areas were determined from the chromatogram and plotted as the ratio versus L-Ala concentration. Figure 3 illustrates the separation while Figure 4 illus-

trates the calibration curve using the peak height ratio. A least squares fit of the 24 data points provides a slope of 0.276 (intercept 0.0485) with a standard deviation of 0.0163. If the four data points out at the top of the curve are omitted the standard deviation is reduced to 0.00984. A similar reproducibility was found for the peak area calibration. The 24 data points represent two independent sets of Ala-Ser mixtures each of which was chromatographed at least two times after carrying out reaction 1.

In these three calibration experiments a fixed 5 µl injector sample loop was used and the concentration range for the calibration curve, which were linear, was from 5 µl of 0.01 mg/ml to 5 µl of 0.5 mg/ml expressed as weight of dipeptide or Ala in the case of the internal standard calibration. Since a 0.16 AUFS detector sensitivity was used calibration at lower concentration is still possible.

Lower concentrations can still be easily detected, particularly if a 208 nm detection wavelength is used. For

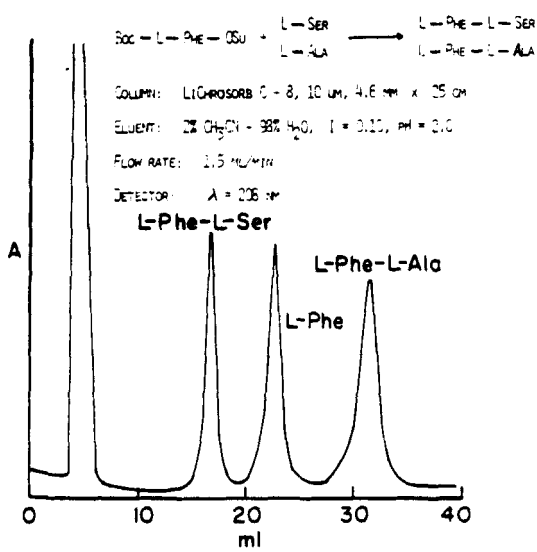


FIGURE 3
Separation of a L-Ser and L-Ala Mixture After Conversion to Dipeptides with Boc-L-Phe-OSu

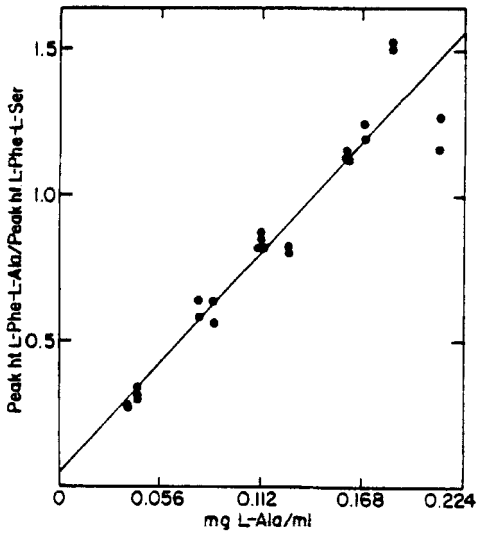


FIGURE 4
A Peak Height Calibration Curve for L-Ala Using L-Ser as Internal Standard after Conversion to the Dipeptides with Boc-L-Phe-OSu

example, solutions were prepared in which the ratio of L to D Phe was 100 to 1 and 1 to 100. After reaction with Boc L-Leu-OSu according to reaction 1 the peptide at the 1 pph concentration was readily detected. Detection at 1 ppt levels should also be possible.

Since reaction 1 provides a quantitative conversion it is widely used in peptide synthesis. The eluting conditions outlined in Tables I to III are not only useful for a rapid, efficient separation of products from the mixture but they also provide a basis for selecting eluting conditions for the purification of small chain peptides.

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REFERENCES

1. Kroeff, E. P. and Pietrzyk, D. J., Investigation of the Retention and Separation of Amino Acids, Peptides, and Derivatives on Porous Copolymers by HPLC, Anal. Chem. 50, 502 (1978).

2. Kroeff, E. P. and Pietrzyk, D. J., HPLC Study of the Retention and Separation of Short Chain Peptide Diastereomers on a C_8 Bonded Phase, Anal. Chem. 50, 1353 (1978).

3. Horvath, C., Melandar, W., and Molnar, I., Solvophobic Interactions in Liquid Chromatography with Nonpolar Stationary Phases, J. Chromatogr. 125, 129 (1976).

4. Krummen, K. and Frei, R. W., The Separation of Nonapeptides by Reversed-Phase HPLC, J. Chromatogr. 132, 27 (1977).

5. Hansen, J. J., Greibrokk, T., Currie, B. L., Nils-Gunner Johnansson, K., and Folkers, K., HPLC of Peptides, J. Chromatogr. 135, 155 (1977).

6. Hearn, M. T., Grego, B., and Hancock, W. S., HPLC of Amino Acids, Peptides, and Proteins XIX. Investigation of the Effect of pH and Ion Pair Formation on the Retention of Peptides on Chemically Bonded Hydrocarbonaceous Stationary Phases, J. Chromatogr. 185, 429 (1979).

7. Kent, S. B. H., Mitchell, A. R., Barany, G., and Merrifield, R. B., Test for Racemization in Model Peptide Synthesis by Direct Chromatographic Separation of Diastereomers of the Tetrapeptide Leucylalanylglycylvaline, Anal. Chem. 50, 155 (1978).

8. Walton, H. F., Ion Exchange and Liquid Column Chromatography, Anal. Chem. 50, 36R (1978).

9. Gil-Av, E. and Nurok, D., Advances in Chromatography, Vol.10, Giddings, J. C. and Keller, R. A., Ed., M. Dekker, New York, 1974.

10. Lochmuller, C. H. and Souter, R. W., Chromatographic Resolution of Enantiomers:Selective Review, J. Chromatogr. 113, 283 (1975).

11. Krull, I. S., Advances in Chromatography, Vol. 16, Giddings, J. C. and Keller, R. A., Ed., M. Dekker, New York, 1978.

12. Lefebvre, B., Audebert, R., and Quivoron, C., Use of New Chiral Hydrophilic Gels for the Direct Resolution of α-Amino Acids by HPLC, J. Liq. Chromatogr. 1, 761 (1978).

13. Foucault, A., Caude, M., and Oliveros, L., Ligand-Exchange Chromatography of Enantiomeric Amino Acids on Copper Loaded Chiral Bonded Silica Gel and of Amino Acids on Copper(II) Modified Silica Gel, J. Chromatogr. 185, 345 (1979).

14. Hare, P. E. and Gil-Av, E., Separation of D and L Amino Acids by Liquid Chromatography:Use of Chiral Eluants, Science 204, 1226 (1979).

15. Gilon, C., Leshem, R., Tapuhi, Y., and Grushka, E., Reversed Phase Chromatographic Resolution of Amino Acid Enantiomers with Metal-Aspartame Eluants, J. Am. Chem. Soc. 101, 7612 (1979).

16. Lindner, W., LePage, J. N., Davies, G. Seitz, D. E., and Karger, B. L., Reversed Phase Separation of Optical Isomers of Dns-Amino Acids and Peptides Using Chiral Metal Chelate Additives, J. Chromatogr. 185, 323 (1979).

17. Mitchell, A. R., Kent, S. B. H., Chu, I. C., and Merrifield, R. B., Quantitative Determination of D-and L-Amino Acids by Reaction with tert-Butyloxycarbonyl-L-Leucine-N-Hydroxysuccinimide Ester and Chromatographic Separation as L,D and L,L Dipeptides, Anal. Chem. 50, 637 (1978).

18. Bodanszky, M., Klausner, Y. S., and Ondetti, M. A., Peptide Synthesis, 2nd Ed., Wiley-Interscience, New York, 1976.

19. Baum, R. G., Saetre, R., and Cantwell, F. F., Liquid Chromatography on Microparticle Amberlite XAD-2, Anal. Chem. 52, 15 (1980).