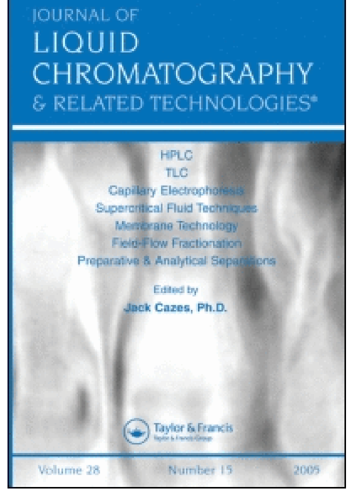
This article was downloaded by:
On: *25 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Facile Resolution OF N-*tert*-Butoxy-Carbonyl Amino Acids: The Importance of Enantiomeric Purity in Peptide Synthesis

San Chun Chang[a]; Lisa R. Wang[a]; Daniel W. Armstrong[a]
[a] Department of Chemistry, University of Missouri-Rolla, Rolla, Missouri

To cite this Article Chang, San Chun , Wang, Lisa R. and Armstrong, Daniel W.(1992) 'Facile Resolution OF N-*tert*-Butoxy-Carbonyl Amino Acids: The Importance of Enantiomeric Purity in Peptide Synthesis', Journal of Liquid Chromatography & Related Technologies, 15: 9, 1411 — 1429
To link to this Article: DOI: 10.1080/10826079208018297
URL: http://dx.doi.org/10.1080/10826079208018297

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(9), 1411-1429 (1992)

FACILE RESOLUTION OF N-*tert*-BUTOXY-CARBONYL AMINO ACIDS: THE IMPORTANCE OF ENANTIOMERIC PURITY IN PEPTIDE SYNTHESIS

SAN CHUN CHANG, LISA R. WANG,
AND DANIEL W. ARMSTRONG
Department of Chemistry
University of Missouri-Rolla
Rolla, Missouri 65401

Downloaded At: 09:22 25 January 2011

ABSTRACT

Twenty five pairs of N-*tert*-butoxycarbonyl (N-*t*-Boc) protected racemic amino acids were separated using a hydroxypropyl derivatized β-cyclodextrin bonded phase column in the reversed-phase mode. Most of the N-*t*-Boc-amino acids are base-line separated in 30 minutes or less. The retention behavior, the effect of structure on chiral recognition, and the effect of mobile phase composition are discussed. It was found there is a maximum resolution value when the mobile phase contains from 5% to 10% acetonitrile (or ~20% methanol) for many of the N-*t*-Boc-amino acids. Others were found to resolve better with acetonitrile concentrations between 90 - 97%. It was found that the higher the pH, the shorter the retention and the better the resolution. The optimum concentration of buffer solution was 1% triethylammonium acetate. N-*t*-Boc-amino acids are used extensively in peptide synthesis. Relatively small enantiomeric impurities in these starting materials can translate into peptide products containing significant, often unacceptable impurities. The relationship between the enantiomeric purity of the starting material (N-*t*-Boc-amino acids), the number of synthetic steps and the stereochemical purity of the final product is discussed.

Copyright © 1992 by Marcel Dekker, Inc.

INTRODUCTION

The *tert*-butoxycarbonyl (*t*-Boc) group is one of the most important amino-protecting groups in peptide synthesis. N-*t*-Boc-amino acids are resistant to racemization during peptide synthesis and the *t*-Boc-group can be removed easily by acid-catalyzed hydrolysis (1). These properties make the *t*-Boc protecting group particularly useful in solid phase peptide synthesis (2). First, the N-*t*-Boc-amino acids are attached to a stable, solid support (*i.e.*, a resin). These form the beginning of potentially elaborate peptide chains. The growing chain usually remains attached to the resin throughout the synthetic process and is separated from soluble reagents and solvents by simple filtration and washing. Finally, the desired peptide is detached from the resin. Unfortunately, each monomer amino acid contains small amounts of enantiomeric impurities. This can be a problem for larger peptides because, as will be shown, the stereochemical purity of the final product decreases exponentially as the number of amino acids in the peptide increases.

Probably more attention has been focused on the resolution of enantiomeric amino acids than any other class of compounds. Native underivatized amino acids can be resolved chromatographically using ligand exchange methods (3, 4), chiral crown ether stationary phases (5) or α-cyclodextrin bonded phases for aromatic amino acids (6). Blocking or derivatizing the amine functional group generally renders these methods less effective or ineffective. Fortunately, a variety of other chiral stationary phases can be used to resolve aromatic derivatized amino acids (7-10). In fact, most of these chiral stationary phases require that an aromatic functional group be present in the analyte for successful resolution. However, the *tert*-butoxycarbonyl protecting group is not aromatic. Consequently, N-*t*-Boc-amino acids generally cannot be resolved using the techniques developed for aromatic amino acid derivatives. In fact, there are relatively few reports on the chromatographic resolution of N-*t*-Boc-amino acids on chiral stationary phases. There have been at least two reports on the use

Downloaded At: 09:22 25 January 2011

of chiral mobile phase additives to resolve N-*t*-Boc-phenylalanine, an aromatic amino acid (11, 12). In this work we report the first widely applicable chiral stationary phase for resolving N-*t*-Boc-amino acids. The resolution and optimization of 25 racemates on a hydroxypropyl derivatized β-cyclodextrin column is demonstrated and discussed.

EXPERIMENTAL

Chemicals. The structures of all solutes used in this study are presented in the Table I. N-*t*-Boc-2-fluoro-L-phenylalanine, N-*t*-Boc-4-fluoro-L-phenylalanine, N-*t*-Boc-4-bromo-L-phenylalanine, N-*t*-Boc-4-methyl-L-phenylalanine, N-*t*-Boc-3-chloro-L-phenylalanine, N-*t*-Boc-5-fluoro-D-tryptophan, N-*t*-Boc-5-methyl-L-tryptophan, 4-methyl-DL-phenylalanine, and 3-chloro-DL-phenylalanine were obtain from Dr. Jean Rivier, of the Clayton Foundation Laboratories for Peptide Biology (La Jolla, CA). All other reported amino acids and N-*t*-Boc-amino acids (if commercially available) were purchased from Sigma (St. Louis, MO). Some N-t-Boc-amino acids were made from the corresponding amino acids following a previously reported procedure (13). All solvents including acetonitrile, methanol, triethylamine, and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA).

Methods. The HPLC was performed at room temperature with a Shimadzu model LC-6A solvent delivery module, SPD-6A UV detector, and C-R2AX Chromatopac recorder. The UV detector wavelengths varied from 210 nm to 230 nm (and up to 254 nm, if the solute is an aromatic amino acid). The RAC-(2)-hydroxypropyl β-CD bonded phase column was a Cyclobond I RSP column (250 x 4.6 mm i.d., 5 mm particle diameter) which was obtained from Advanced Separation Technologies, Whippany, NJ (14). The mobile phases were mixtures of buffers and acetonitrile (or methanol) by volume ratios. The aqueous portion of the mobile phase (*i.e.*, the buffer) was made by dissolving the desired amount of pure triethylamine in HPLC-grade water and then adding glacial acetic acid to achieve the desired pH.

Downloaded At: 09:22 25 January 2011

RESULTS AND DISCUSSION

Chromatographic Results

The chromatographic data for 25 pairs of N-*t*-Boc-amino acids resolved on the hydroxypropyl derivatized β-CD bonded phase are summarized in Table I. All the enantiomers of these amino acids could be separated in < 30 minutes with resolution factors (Rs) up to 5.1 in some cases. In all cases, it was found that the L-enantiomer eluted first.

Interestingly, nonaromatic amino acids (*i.e.*, those with aliphatic R-groups) tended to give the best separations on this stationary phase (see isoleucine, Table I). N-*t*-Boc-amino acids with aromatic R-groups, although easily resolved, tended to have longer retention times and smaller α-values when separated in the reversed phase mode (Table I). N-*t*-Boc-amino acids having polar R-groups had the shortest retention in the reversed phase mode. But many of the polar Boc-amino acids were best resolved with a mobile phase containing between 92 and 97% acetonitrile.

Mobile Phase Effects

In this investigation, N-*t*-Boc-leucine (N-*t*-Boc-Leu), N-*t*-Boc-phenylalanine (N-*t*-Boc-Phe) and N-*t*-Boc-glutamine (N-*t*-Boc-Gln) represent typical analytes containing alkyl, aromatic, and polar functional R-groups, respectively. Effects of mobile phase on the resolution process was studied by varying the mobile phase composition (type and percentage of organic modifiers, pH and ionic strength of buffer). In all cases, the operative conditions were: room temperature (~22 ^{o}C), a flow rate of 1 ml/min, and a UV detector wavelength of 225 nm.

Downloaded At: 09:22 25 January 2011

TABLE I. CHROMATOGRAPHIC DATA FOR RACEMATES OF N-*t*-BUTOXYCARBONYL AMINO ACIDS RESOLVED IN THE REVERSED-PHASE MODE ON A RACEMIC (2)-HYDROXYPROPYL β-CD STATIONARY PHASE

$$-R = -\underset{O}{\overset{\|}{C}}-O-C(CH_3)_3$$

COMPOUND	STRUCTURE	k'[a]	α[b]	Rs[c]	MOBILEPHASE[d] acetonitrile-buffer
1. N-*t*-Boc-isoleucine	$CH_3-CH_2-(CH_3)CH-CH(NHR)-CO_2H$	2.16	1.69	5.10	5:95
2. N-*t*-Boc-leucine	$(CH_3)_2CH-CH_2-CH(NHR)-CO_2H$	1.48	1.61	4.62	7:93
3. N-*t*-Boc-valine	$(CH_3)_2CH-CH(NHR)-CO_2H$	1.77	1.58	4.20	5:95
4. N-*t*-Boc-norleucine	$CH_3-(CH_2)_3-CH(NHR)-CO_2H$	1.88	1.37	2.80	7:93
5. N-*t*-Boc-alanine	$CH_3-CH(NHR)-CO_2H$	1.30	1.20	1.75	5:95
6. N-*t*-Boc-proline	pyrrolidine ring, N–R, C(H)COOH	0.98	1.21	1.52	5:95
7. N-*t*-Boc-histidine	imidazolyl(N–H)$-CH_2-CH(NHR)-CO_2H$	2.01	1.15	2.19	1:99
8. N-*t*-Boc-glutamine	$H_2N-C(=O)-(CH_2)_2-CH(NHR)-CO_2H$	1.14	1.20	1.70	1:99

(continued)

Downloaded At: 09:22 25 January 2011

TABLE I (continued)

9. N-*t*-Boc-asparagine $H_2N\text{-}C(=O)\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	0.92	1.14	1.51	1:99
10. N-*t*-Boc-methionine $CH_3\text{-}S\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	2.10	1.32	3.17	5:95
11. N-*t*-Boc-serine $HO\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	1.12	1.16	1.41	1:99
12. N-*t*-Boc-phenylglycine $C_6H_5\text{-}CH(NHR)\text{-}CO_2H$	2.89	1.28	3.33	9:91
13. N-*t*-Boc-phenylalanine $C_6H_5\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	2.97	1.21	2.68	7:93
14. N-*t*-Boc-homophenyl-alanine $C_6H_5\text{-}CH_2\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	6.12	1.13	1.40	5:95
15. N-*t*-Boc-p-methyl phenyl-alanine $H_3C\text{-}C_6H_4\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	4.67	1.23	2.09	5:95
16. N-*t*-Boc-o-benzyl serine $C_6H_5\text{-}CH_2\text{-}O\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	3.95	1.13	1.64	5:95
17. N-*t*-Boc-tyrosine $HO\text{-}C_6H_4\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	2.10	1.26	2.98	7:93
18. N-*t*-Boc-p-flouro-phenylalanine $F\text{-}C_6H_4\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	2.47	1.24	2.34	7:93
19. N-*t*-Boc-o-flouro-phenylalanine $(o\text{-}F)C_6H_4\text{-}CH_2\text{-}CH(NHR)\text{-}CO_2H$	3.46	1.23	2.72	7:93

Downloaded At: 09:22 25 January 2011

TABLE I (continued)

20. N-*t*-Boc-p-bromo-phenylalanine Br-C6H4-CH_2-CH(NHR)-CO_2H	7.07	1.14	2.07	7:93
21. N-*t*-Boc-3-chloro-phenylalanine Cl-C6H4-CH_2-CH(NHR)-CO_2H	6.10	1.15	1.74	5:95
22. N-*t*-Boc-tryptophan indolyl-CH_2-CH(NHR)-CO_2H	4.63	1.14	1.89	7:93
23. N-*t*-Boc-5-flouro-tryptophan F-indolyl-CH_2-CH(NHR)-CO_2H	4.83	1.13	1.60	7:93
24. N-*t*-Boc-5-methyl-tryptophan H_3C-indolyl-CH_2-CH(NHR)-CO_2H	4.20	1.13	1.50	7:93
25. N-*t*-Boc-3-(2-naphthyl) alanine naphthyl-CH_2-CH(NHR)-CO_2H	6.90	1.16	2.00	15:85

[a]. k' = capacity factor of the first eluted enantiomer. In all case, the first eluted solute was the L-enantiomer.

[b]. The selectivity factor, α, is equal to k_1'/k_2'.

[c].The resolution (Rs) is equal to $2(tr_2-tr_1)/(w_1+w_2)$.

[d]. Mobile phase conditions are given as volume percentages of acetonitrile:buffer. The buffer was 1% triethylammonium acetate at pH 7.1.

Downloaded At: 09:22 25 January 2011

I. Effect of organic modifier content

Mobile phase effects on the retention and resolution of N-*t*-Boc-amino acids were investigated by changing the ratio of organic modifier and buffer in the mobile phase. The buffer was 1% triethylammonium acetate at a pH of 7.1. The organic modifiers studied were acetonitrile and methanol.

All N-*t*-Boc-amino acids showed analogous retention behavior when acetonitrile was used as the organic modifier. A retention minimum was reached between 40% and 60% (by volume) acetonitrile. Further elevations of the acetonitrile concentration caused increased retention as shown in Figure 1. Similar retention behavior was observed previously on the native cyclodextrin bonded phases (15, 16). The presence of minima indicates that the retention mechanism(s) at high buffer concentrations may differ from that at high acetonitrile concentrations. The fact that chiral recognition also increases at high acetonitrile concentration (see Figure 1) indicates that the retention increase stems largely from interaction with the cyclodextrin and not with the achiral support (i.e., residual silanol groups or the linkage chain). It was shown previously that the hydroxypropyl groups that are attached to the cyclodextrin can provide not only additional sites for hydrogen bonding but also increase the depth of cyclodextrins (14). In many cases, these properties tend to enhance chiral recognition. It is interesting to compare the retention and resolution of N-*t*-Boc-Phe and N-*t*-Boc-Gln (Figure 1). N-*t*-Boc-Phe had longer retention and better resolution than N-*t*-Boc-Gln when the mobile phases contained a low percentage of acetonitrile. On the other hand, N-*t*-Boc-Gln had longer retention and better resolution when the acetonitrile content of the mobile phase exceeded 65% (see Figures 1 and 2). This change in retention behavior may indicate a change in the relative solubilities of these analytes or a change from reversed phase to normal phase retention behavior. If the retention reversal is due to the latter of these, then the dominant retention mechanism may change from an inclusion to a noninclusion process. However, this situation was not

Downloaded At: 09:22 25 January 2011

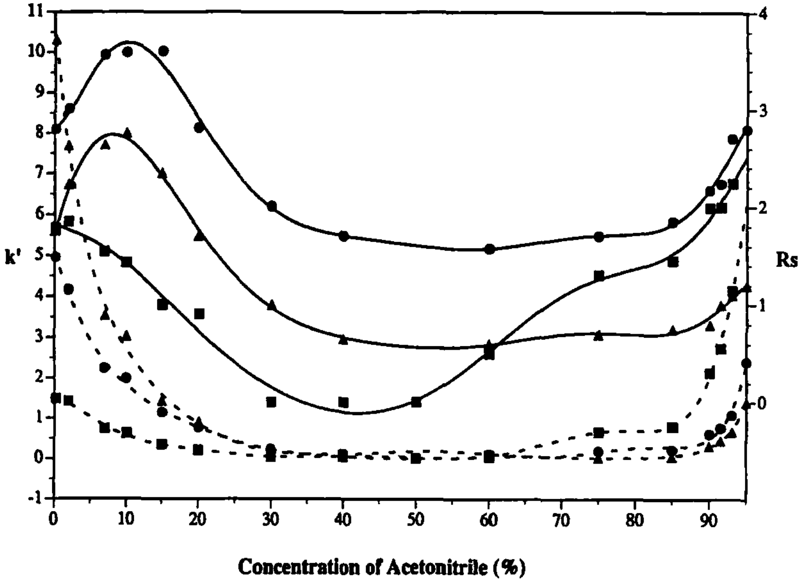


Figure 1. Effect of increasing percentage of acetonitrile in the mobile phase on the enantiomeric resolution (Rs) and retention (k') of enantiomers of N-*t*-Boc-Gln (■), N-*t*-Boc-Leu (●) and N-*t*-Boc-Phe (▲), respectively. The solid curves (——) represent the resolution (Rs) and the dashed lines (-----) represent the retention (k') of the second eluted enantiomer. One 25-cm Cyclobond I RSP column was used. The mobile phase consisted of the indicated volume percentage of acetonitrile and buffer. The buffer was 1% triethylammonium acetate (TEAA) pH = 7.1. The flow rate was 1.0 ml/min and the UV detector wavelength was 225 nm.

observed for methanol/buffer mobile phases as shown in Figure 3. The retention decreased with increasing methanol concentration. Eventually the solute eluted at or near the void volume of the column, and further increases in methanol concentration did not affect retention. This retention behavior may result from the fact that methanol, unlike acetonitrile, is a good hydrogen bond donor and

Downloaded At: 09:22 25 January 2011

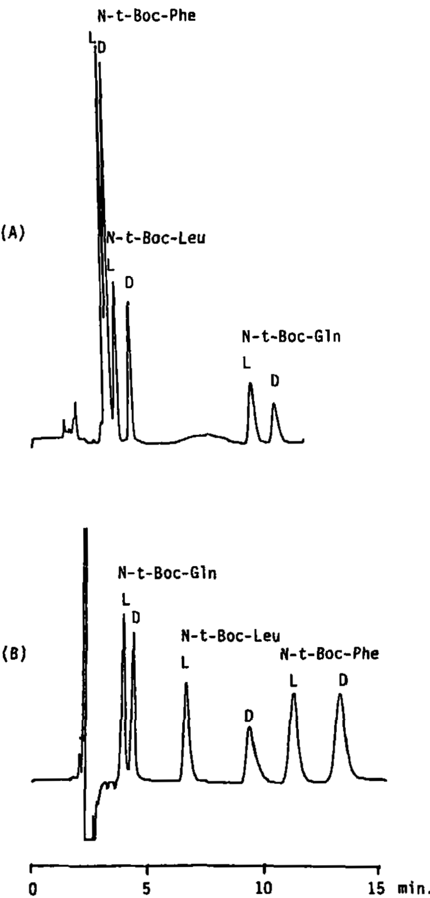


Figure 2. Chromatograms comparing the resolution and relative retention of three racemic N-*t*-Boc-amino acids in mobile phases containing high and low acetonitrile concentrations. One 25-cm Cyclobond I RSP column was used. The flow rate was 1.0 ml/min and the UV detector wavelength was 225 nm. For chromatogram A, the mobile phase was 91.5% acetonitrile/8.5% buffer (1% TEAA, pH 7.1). For chromatogram B, the mobile phase was 7% acetonitrile/93% buffer (1% TEAA, pH 7.1).

Downloaded At: 09:22 25 January 2011

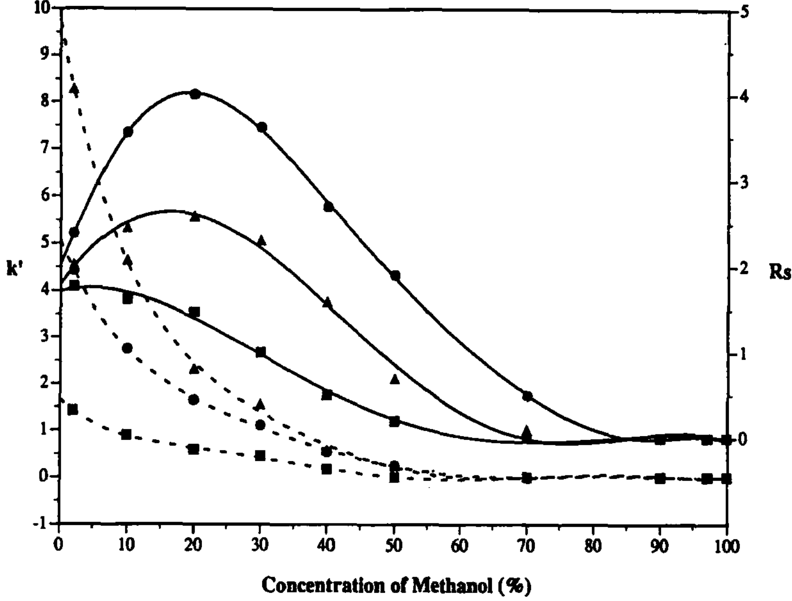


Figure 3. Effect of increasing percentage of methanol in the mobile phase on the enantiomeric resolution (Rs) and retention (k') of the enantiomers of N-*t*-Boc-Gln (■), N-*t*-Boc-Leu (●) and N-*t*-Boc-Phe (▲), respectively. The solid curves (——) represent the resolution (Rs) and the dashed lines (-----) represent the retention (k') of the second eluted enantiomer. One Cyclobond I RSP column was used. The mobile phase consisted of the indicated volume percentage of methanol and buffer (1% TEAA, pH 7.1). The flow rate was 1.0 ml/min and the UV detector wavelength was 225 nm.

acceptor molecule. Consequently, methanol can effectively compete with chiral analytes in hydrogen bonding to the cyclodextrin hydroxyl-groups or conversely compete with the cyclodextrin in hydrogen bonding to the analytes. In a previous study of aromatic carboxylic acids dissolved in methanol or included in cyclodextrins, it was shown that the Raman shift for the carbonyl stretching, ν(C=O), were similar (17). In both cases, the shifts were very different from

Downloaded At: 09:22 25 January 2011

what is found in the crystalline state of the carboxylic acid. This tends to support the supposition that high concentration of methanol in the mobile phase can in same cases negate enantioselective hydrogen bonding interactions between the cyclodextrin and chiral analytes whereas acetonitrile often does not.

II. Effect of pH

When the pH of the mobile phase was increased, the retention (k') of all N-*t*-Boc-amino acids decreased as shown in Figure 4. However, the resolution (Rs) increased with increasing pH except for N-*t*-Boc-Gln. The selectivities (α) of all three compounds increased with pH. A likely explanation for this phenomenon is that it is due to the ionization of the carboxylate group at higher pH. This ionization would result in decreased retention in the reversed phase mode. However, the negatively charged carboxylate groups could better hydrogen bond to the hydroxypropyl-cyclodextrin's free hydroxyls resulting in increased enantioselectivity.

III. Effect of buffer-salt concentration

Figure 5 shows the effect of triethylammonium acetate (TEAA) concentration (in buffer solution) on retention (k') and enantiomeric resolution (Rs). The organic modifier consisted of 15% acetonitrile. It was found that both the retention and resolution decreased somewhat at lower buffer concentration. However, at TEAA concentrations above 1%, k' and Rs were relatively constant. Although not shown in Figure 5, the selectivities (α) remained approximately constant (1.25, 1.60 and 1.21 for N-*t*-Boc-Gln, Leu, and Phe, respectively) between 0.1 - 2.0% TEAA buffer concentration. The observed increase in enantiomeric resolution with increasing ionic strength (Figure 5) has been seen many times before (8, 13, 16). It is due to the improvement in peak shape (*i.e.*, narrower band width and less tailing) that occurs when nonselective strong

Downloaded At: 09:22 25 January 2011

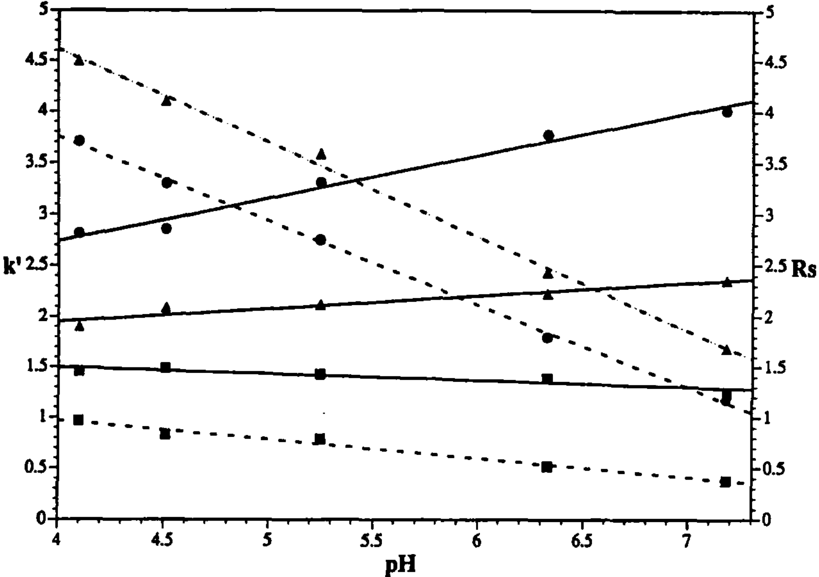


Figure 4. Effect of mobile phase pH on the enantiomeric resolution (Rs) and retention (k') of the enantiomers of N-*t*-Boc-Gln (■), N-*t*-Boc-Leu (●) and N-*t*-Boc-Phe (▲), respectively. The solid curves (——) represent the resolution (Rs) and the dashed lines (-----) represent the retention (k') of the second eluted enantiomer. One 25-cm Cyclobond I RSP column was used. The mobile phase was 15% acetonitrile and 85% buffer (1% TEAA) at the indicated pH. The flow rate was 1.0 ml/min and the UV detector wavelength was 225 nm.

adsorption effects of the stationary phase are masked by the added buffer. The observed slight increase in retention with increasing buffer capacity (Figure 5) is less common. More frequently, added buffer tends to decrease the retention of chiral analytes on cyclodextrin bonded phases in much the same manner as an organic modifier. However, in some cases, such as this one, a component of the added buffer can act as an ion interaction agent. At pH 7.1, it is thought that the triethylammonium component of the mobile phase can form a "paired-ion" with

Downloaded At: 09:22 25 January 2011

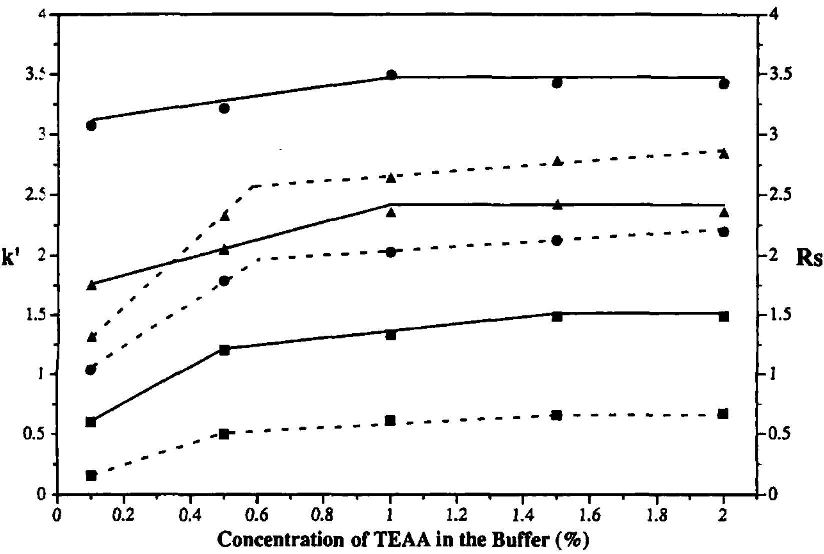


Figure 5. Effect of increasing concentration of triethylammonium acetate in the mobile phase buffer on the enantiomeric resolution (Rs) and retention (k') of the enantiomers of N-*t*-Boc-Gln (■), N-*t*-Boc-Leu (●) and N-*t*-Boc-Phe (▲), respectively. The solid curves (——) represent the resolution (Rs) and the dashed lines (-----) represent the retention (k') of the second eluted enantiomer. One 25-cm Cyclobond I RSP column was used. The mobile phase was 85% buffer (pH = 5.6) with the indicated concentration of triethylammonium acetate and 15% acetonitrile The flow rate was 1.0 ml/min and the UV detector wavelength was 225 nm.

the carboxylate portion of N-*t*-Boc-amino acids resulting in slightly increased retention.

ENANTIOMERIC PURITY IN PEPTIDE SYNTHESIS

As mentioned in the introduction, N-*t*-Boc-amino acids are the starting materials for many synthetic peptides. N-*t*-Boc-amino acids are made by

Downloaded At: 09:22 25 January 2011

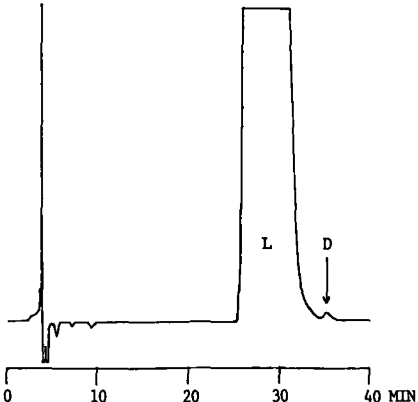


Figure 6. LC chromatogram used to evaluate the enantiomeric purity of N-*t*-Boc-L-tryptophan from Sigma. There was approximately 1.5 x 10^{-2}% N-t-Boc-D-tryptophan present. One 25-cm Cyclobond I RSP column was used. The mobile phase was 5% acetonitrile and 95% buffer (1% TEAA, pH = 7.1). The flow rate was 0.8 ml/min and the UV detector wavelength was 225 nm.

Downloaded At: 09:22 25 January 2011

derivatizing native amino acids (2). However, as has been shown previously, some finite level of the contaminating D-enantiomers is present in virtually all L-amino acids and vice versa (18). The degree of enantiomeric contamination can range from very low levels (< 0.001%) up to percent levels. Furthermore amino acids from the same source can vary considerably from batch to batch (18). For example, the N-*t*-Boc-L-tryptophan used in this study contained approximately 0.015% of the D-enantiomer. This chromatographic analysis is shown in Figure 6. The questions arise, should there be concern over enantiomeric impurities in amino acid preparations, and if so, what levels should cause concern? The answer to the first question must be yes if the amino acids end up in pharmacological products (either as derivatives or part of a peptides) or if they are to be used in any kind of accurate biological or chemical study where the possibility of stereochemical discrimination exists. The second question on

degree of purity (*i.e.*, How pure is pure?) is more difficult to assess and probably will be decided on a case by case basis.

Using simple models, one can estimate the degree to which enantiomeric impurities in individual amino acid starting materials will impact the overall purity of a peptide product. Let X = the final stereochemical purity of a peptide (e.g., 0.90 = 90%); n = the number of amino acids residues in the peptide; A, B, C,... = the enantiomeric purity of each amino acid (*i.e.*, 0.99 = 99% of the L-enantiomer and 1% of D-enantiomer); and a, b, c,... = the enantiomeric discrimination coefficients. These coefficients are correction factors that result from differences in the reactivity of two enantiomers with a chiral substrate. All coefficients are ≥ 0 and ≤ 1 (*i.e.*, $0 < a \leq 1$). It is possible for the same amino acid (or N-*t*-Boc-derivative) to have different coefficients when reacting with different chiral substrates (*i.e.*, peptide chains).

Downloaded At: 09:22 25 January 2011

Consider the simplest (ideal) case first, where all of the amino acid building blocks have approximately the same enantiomeric purity (*i.e.*, A = B = C...) and there is little or no enantiomeric discrimination during peptide synthesis (*i.e.*, a, b, c... = 1). In this case, the stereochemical purity of the final peptide (X) is a simple exponential function of number of amino acid building blocks (n):

$$X = (A)^n \tag{1}$$

Although admittedly oversimplified, this first approximation remains useful in that it shows that larger peptides are contaminated with stereochemical impurities to a much greater extent than smaller peptides (see Figure 7). For example, if the amino acid starting material is 99% enantiomerically pure, then a dimer product would have 98% stereochemical purity; a decamer, 90.4% purity and a 50-mer, 60.5% purity. However if the enantiomeric purity of the starting material is just 1% less (*i.e.*, 98%) then the stereochemical purity of the dimer, decamer and 50-mer decreases to 96, 81.7 and 36.4% respectively.

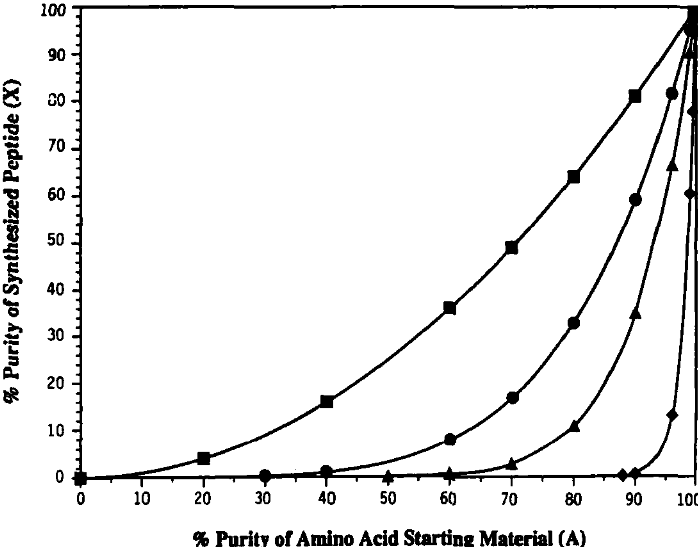


Figure 7. Illustration of the idealized relationship between the stereochemical purity of a synthesized peptide and the enantiomeric purity of the amino acid starting materials. It is assumed that all amino acids contain the same percentage of enantiomeric impurities and that the enantiomeric discrimination coefficients are equal to 1. The symbols (■), (●), (▲), and (◆) represent a peptide dimer, pentamer, decamer, and 50-mer, respectively.

It is more likely that the enantiomeric purity of each amino acid starting material is different. In this case:

$$X = (A)\ (B)\ (C)... \quad (2)$$

provided the enantiomeric discrimination coefficients are equal to 1. If they are not, then:

$$X = (aA)\ (bB)\ (cC)... \quad (3)$$

Downloaded At: 09:22 25 January 2011

To date, the degree to which these coefficients deviate from unity under various synthetic conditions have not been systematically evaluated to our knowledge. However, even with the use of valid coefficients, the basic conclusion is likely to be the same as above. That is, an arithmetical increase in the number of amino acid units causes a geometrical decrease in the stereochemical purity of a peptide. One way to minimize this problem is to use only high purity amino acids or derivatives. In our opinion the purity of these starting materials should be at least 99.9%, and even higher purities ($\geq$ 99.99%) are preferable.

CONCLUSION

The hydroxypropyl derivatized β-CD bonded phases exhibits high enantioselectivity for N-*t*-Boc amino acids. When using mobile phases high in buffer concentration and low in acetonitrile, the enantioselective retention appears to be due a combination of inclusion complex formation and hydrogen bonding effects. In mobile phases of predominantly acetonitrile, enantioselective retention seems to be controlled by hydrogen bonding and perhaps dipolar stacking interactions. The hydroxypropyl moity at the mouth of the cyclodextrin cavity is essential since comparable separations of N-*t*-Boc-amino acids are not obtained with underivatized, native cyclodextrins. The hydroxypropyl group is known to provide extended hydrogen bonding sites and steric interactions that do not exist on the underivatized cyclodextrin (14). Simple models can be used to estimate the stereochemical purity of peptides that are synthesized using amino acids containing known amounts of enantiomeric impurities. Unless high purity N-*t*-Boc-amino acid starting materials are used under proper conditions, significant quantities of stereochemically impure product can be produced.

Downloaded At: 09:22 25 January 2011

REFERENCES

1. T. W. Greene, " Protective groups in Organic Synthesis", John Wiley & Sons New York, (1981).

2. E. Atherton, and R. C. Sheppard, "Solid Phase Peptide Synthesis", IRL Press,Oxford, England.
3. G. Gubitz, W. Jellenz and W. Santi, . *Liquid Chromatogr.*, 4 (1981) 701.
4. G. Gubitz, F. Juffman, and W. Jellenz, . *Chromatographia*, 16 (1982) 103.
5. M. Hilton, and D. W. Armstrong, *J. Liquid Chromatogr.*, 14 (1991) 9.
6. D. W. Armstrong, X. Yang, S. M. Han, and R.A. Menges, *Anal Chem.*,59 (1987) 2594.
7. A. M. Stalcup, S. C. Chang, and D. W. Armstrong, *J. Chromatogr.*, 540 (1991) 113.
8. W. L. Hinze, T. E. Riehl, D. W. Armstrong, W. DeMond, A. Alak, and T. Ward, *Anal. Chem.*,57 (1985) 237.
9. W. H. Pirkle, and M. H. Hyun, *J. Chromatogr.*,322 (1985) 287.
10. W. H. Pirkle, T. C. Pochapsky, G. S. Mahler, R. E. Field, *J. Chromatogr.*,348 (1985) 89.
11. C. Petterson, *J. Chromatogr.*, 316 (1984) 553.
12. C. Petterson, and K. No, *J. Chromatogr.*, 282 (1983) 671.
13. Technical Information Bulletin Number AL-125, "BOC-ON", Aldrich.
14. A. M. Stalcup, S. C.Chang, and D. W. Armstrong, *J. Chromatogr.*, 513 (1990) 181.
15. S. M. Han and D. W. Armstrong, *J. Chromatogr.*,389 (1987) 256.
16. D. W. Armstrong, and W. Li, *Chromatography*, 2 (1987) 43.
17. S. C. Chang, P. M. Calahan, D. W. Armstrong, Pittsburgh Conference in Chicago, IL, March, 4, 1991. Titled 205, "*Chromatographic and Spectroscopic Investigation into Chiral Recognition Mechanism of Derivatized Cyclodextrin Chiral Stationary Phases.*"
18. D. W. Armstrong, J. D. Duncan, and S. H. Lee, *Amino Acids*, 1 (1991) 97.

Downloaded At: 09:22 25 January 2011