Separation of the Enantiomers of *N*-Benzyloxycarbonyl, 9-Fluorenylmethoxycarbonyl and Phthaloyl Protected α-Amino Acids and Their Ester or Amide Derivatives

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The need to determine the configuration and/or enantiomeric purity of amino acids frequently arises for those in the fields of pharmaceutical chemistry and biochemistry and many methods for these determinations have been developed.[1,2] Many of these are chromatographic in nature and require derivatization of the amino group of amino acids prior to chromatography. Owing to the growing interest in peptide synthesis, questions of the enantiomeric purities of the *N*-protected α-amino acids are increasingly asked. Derivatization of the carboxyl group of amino acids affords derivatives the enantiomers of which can be separated on chiral HPLC columns. However, many workers wish to avoid such prederivatization. We now report that a chiral stationary phase, CSP **1** (Figure 1),[3-14] enables one to separate the enantiomers of N-CBZ, FMOC and PHT protected α-amino acids without further derivatization (Figure 2).[15,16] The CBZ group is one of the most commonly used protecting groups for amino acids whereas the FMOC group provides the advantages of high sensitivity in fluorescence detection.[17,18] The PHT protecting group is seldom used but is attractive in certain instances for economic reasons. The chromatographic separations of the enantiomers of *N*-protected α-amino acids having CBZ, FMOC or PHT protecting groups have been reported using CSPs derived from proteins,[19-22] cellulose,[23-26] cyclodextrin derivatives,[27] macrocyclic antibiotics,[28-29] and, in a few cases, brush-type CSPs have been employed.[30-32] Only a few examples of enantioseparation of *N*-protected α-amino acids on CSPs using nonaqueous mobile phases have been reported.[23,25-27,32] We earlier found that the enantiomers of anilide derivatives of *N*-protected α-amino acids are readily separated on π-acidic CSPs.[33] In this study, we describe the resolution of N-CBZ, FMOC and PHT protected amino acids as well as their alkyl ester or alkyl amide derivatives on brush-type CSP **1**.[3-14,34]

Chromatographic data for the normal-phase separation of the enantiomers of several *N*-protected α-amino acids are presented in Table 1. All of the CBZ and FMOC protected α-amino acids tested afford base-line separations on CSP **1** using 5% 2-propanol in hexane containing 0.1% trifluoroacetic acid as the mobile phase. Trifluoroacetic acid is more effective than the weaker formic acid, affording higher enantioselectivities and resolution factors.[10,23] The separation factors, α, of the enantiomers of the CBZ protected α-amino acids obtained under these conditions are greater than any observed on any other brush-type CSP.[30-32] The separation factors of FMOC protected α-amino acids are comparable to those of CBZ protected α-amino acids. However, the enantioselectivities shown by the PHT protected α-amino acids are considerably lower than those of the corresponding CBZ or FMOC protected analytes except for phenylalanine (α=1.27, entry 18). Typical chromatograms of these *N*-protected α-amino acids are presented in Figure 3.

Except for phenylglycine or phenylalanine (entries 5, 13 and 18 in Table 1), the (*R*)-analytes with aliphatic side chains are selectively retained on (3*S*,4*R*) CSP **1**. A consistent elution order for the enantiomers of these analytes would suggest that they all are resolved by the same chiral recognition mechanism. Presumably, the aromatic portions of the CBZ, FMOC and PHT protecting groups serve as π-donor groups for π-π interaction with the dinitrobenzoyl (DNB) moiety of the CSP for *N*-protected α-amino acids having aliphatic side chains. It is considered that the chiral recognition of *N*-protected phenylglycine or phenylalanine is different from that of *N*-protected α-amino acids having aliphatic side chains. Therefore, the "inverted" elution orders for the enantiomers of the CBZ and FMOC derivatives of phenylglycine and the PHT derivative of phenylalanine (entries 5, 13 and 18) presumably arise from the π-π interaction between the π-basic substituent on the stereogenic center of these analytes, not the aromatic moiety of *N*-protecting groups, and the DNB group of the CSP. Since the degree of enantioselectivity does not parallel the bulkiness of the substituents on the stereogenic centers of the analytes, one infers that the analytes must undergo conformational change on binding. The greater the initial conformational

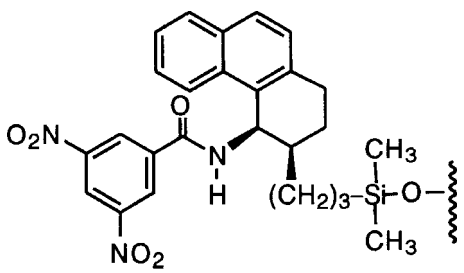

Figure 1. Structure of (3*S*,4*R*) CSP **1** used in this study.

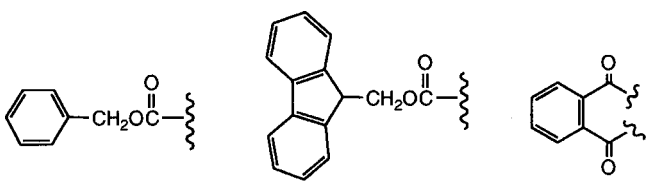

Figure 2. Structures of N-CBZ, FMOC and PHT protecting groups of α-amino acids used in this study.

Table 1. Separation of the enantiomers of several *N*-protected α-amino acids under normal-phase conditions

	Analyte	α	k'_1	Retained*
1	CBZ-Alanine	1.34	5.44	(-)(R)
2	CBZ-Valine	1.14	3.71	(-)(R)
3	CBZ-Leucine	1.26	3.38	(+)(R)
4	CBZ-Phenylalanine	1.12	6.18	(-)(R)
5	CBZ-Phenylglycine	1.71	9.79	(+)(S)
6	CBZ-Methionine	1.28	8.08	(-)
7	CBZ-Proline	1.42	8.88	(+)(R)
8	CBZ-Pipecolinic acid	1.36	6.41	(+)
9	FMOC-Alanine	1.22	7.24	(R)
10	FMOC-Valine	1.29	5.12	(R)
11	FMOC-Leucine	1.25	4.59	(+)(R)
12	FMOC-Phenylalanine	1.14	8.51	(R)
13	FMOC-Phenylglycine	1.50	14.02	(S)
14	FMOC-Serine	1.15	13.82	
15	PHT-Alanine	1.11	4.20	(+)(R)
16	PHT-Valine	1.06	2.79	(+)
17	PHT-Leucine	1.06	2.52	(+)(R)
18	PHT-Phenylalanine	1.27	4.27	(-)(S)
19	PHT-Phenylglycine	1.16	6.51	(-)

HPLC analyses were performed using a Rainin HPX solvent delivery system with a pressure monitor, a Rheodyne 7125 injector with a 20 μL sample loop, a Milton Roy LDC Monitor D fixed wavelength detector operating at 254 nm and a Kipp and Zonen BD 41 recorder. An on-line Rudolph Autopol III automatic polarimeter with a 20-cm flow cell was used to measure the signs of optical rotation.; Mobile phase: 5% 2-propanol/hexane (V/V) containing 0.1% trifluoroacetic acid; Flow rate = 2 mL/min; *indicates the absolute configuration and/or the sign of optical rotation of the more strongly retained enantiomer.

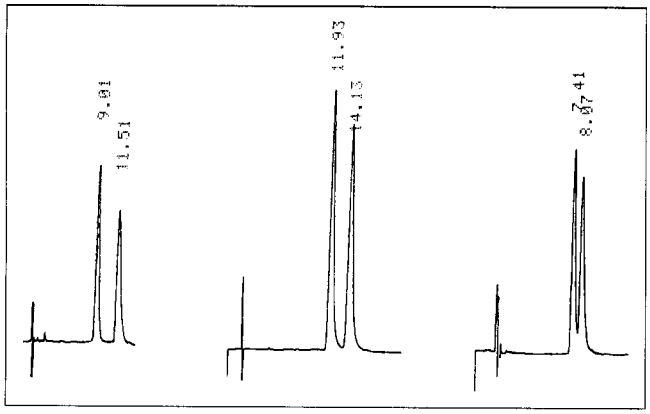

Figure 3. Direct resolution of racemic N-CBZ, FMOC and PHT alanine, respectively; See Table 1 for chromatographic conditions.

preference, the less able the analyte is to adopt the conformation imposed by the selector. The implication here is that the interaction sites in CSP **1** are not optimally placed in terms of spatial positioning, and that selectors better suited for these analytes might be designed.

Reversed-phase chromatographic data are shown in Table 2. The enantioseparations of *N*-protected α-amino acids under reversed-phase conditions are improved by adding an achiral ion-pair reagent to the aqueous methanol as the mobile phase.[35] The enantioselectivities for FMOC α-amino acids are, in general, larger than those of CBZ α-amino acids. It may be that hydrophobic interaction between the FMOC and DNB groups leads to a stronger bonding interaction than occurs for the CBZ group in the reversed mobile phase. The enantiomers of PHT α-amino acids are not separable under these conditions except for phenylalanine (α=1.10).

Table 2. Separation of the enantiomers of several *N*-protected α-amino acids under reversed-phase conditions

	Analyte	α	k'_1	Retained*
1	CBZ-Alanine	1.08	0.63	(R)
2	CBZ-Valine	1.00	0.60	
3	CBZ-Leucine	1.08	1.02	(R)
4	CBZ-Phenylalanine	1.03	1.83	
5	CBZ-Phenylglycine	1.19	1.57	(S)
6	FMOC-Alanine	1.08	2.84	(R)
7	FMOC-Valine	1.12	0.97#	(R)
8	FMOC-Leucine	1.11	1.16#	(R)
9	FMOC-Phenylalanine	1.08	1.75#	(R)
10	FMOC-Phenylglycine	1.13	2.72#	(S)
11	FMOC-Serine	1.06	2.47	

Mobile phase: methanol/water = 70/30 (V/V) containing 7 mM cetyltrimethylammonium bromide; Flow rate = 1 mL/min; *indicates the absolute configuration of the more strongly retained enantiomer.; #Methanol/0.01 M aqueous sodium phosphate buffer (pH 6.8)=70/30 (V/V) containing 6 mM octyltriethylammonium phosphate was used as the mobile phase.

Tables 3 and 4 present chromatographic data for the separation of the enantiomers of CBZ and PHT protected α-amino acids as the ethyl (or *n*-butyl) ester, *N*-*n*-butylamide and *N*,*N*-diethylamide derivatives. In general, the separation factors for the esters of *N*-protected α-amino acids are comparable to those of the corresponding acids. Typically,

Table 3. Separation of the enantiomers of N-CBZ protected α-amino acids as ethyl ester, *N*-*n*-butyl and *N*,*N*-diethylamide derivatives

	Analyte	α	k'_1	Retained*
1a	Alanine-OEt	1.38	5.07#	(R)
1b	Alanine-NH-*n*-Bu	1.15	3.17	(R)
1c	Alanine-NEt_2	1.40	5.94	(R)
2a	Valine-OEt	1.09	3.55#	(R)
2b	Valine-NH-*n*-Bu	1.10	1.56	(R)
2c	Valine-NEt_2	1.21	2.75	(R)
3a	Leucine-OEt	1.22	3.10#	(R)
3b	Leucine-NH-*n*-Bu	1.07	1.48	(R)
3c	Leucine-NEt_2	1.55	2.49	(R)
4a	Phenylalanine-OEt	1.10	5.57#	(R)
4b	Phenylalanine-NH-*n*-Bu	1.00	2.57	
4c	Phenylalanine-NEt_2	1.19	3.64	(R)
5a	Phenyglycine-OEt	1.76	6.81#	(S)
5b	Phenyglycine-NH-*n*-Bu	1.63	4.27	(S)
5c	Phenyglycine-NEt_2	1.20	8.56	(S)

Mobile phase: 10% 2-propanol/hexane (V/V); Flow rate = 2mL/min; *indicates the absolute configuration of the more strongly retained enantiomer.; # 5% 2-propanol/hexane (V/V).

Table 4. Separation of the enantiomers of N-PHT protected α-amino acids as *n*-butyl ester, *N*-*n*-butyl and *N,N*-diethylamide derivatives

	Analyte	α	k'_1	Retained*
1a	Alanine-O-*n*-Bu	1.06	3.55#	(R)
1b	Alanine-NH-*n*-Bu	1.31	4.00	(S)
1c	Alanine-NEt_2	1.47	10.03	(+)(R)
2a	Valine-O-*n*-Bu	1.00	2.24#	
2b	Valine-NH-*n*-Bu	1.25	2.70	
2c	Valine-NEt_2	1.08	5.21	
3a	Leucine-O-*n*-Bu	1.04	2.03#	(R)
3b	Leucine-NH-*n*-Bu	1.52	1.92	(S)
3c	Leucine-NEt_2	1.85	4.56	(+)(R)
4a	Phenylalanine-O-*n*-Bu	1.20	3.47#	(-)(S)
4b	Phenylalanine-NH-*n*-Bu	1.08	6.82	(+)(R)
4c	Phenylalanine-NEt_2	1.48	8.15	(+)(R)
5a	Phenylglycine-O-*n*-Bu	1.00	5.49#	
5b	Phenylglycine-NH-*n*-Bu	1.14	9.48	(+)
5c	Phenylglycine-NEt_2	1.28	12.06	(-)

Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate=2 mL/min; *indicates the absolute configuration and/or the sign of optical rotation of the more strongly retained enantiomer.; #5% 2-propanol/hexane (V/V).

the *N,N*-diethylamide derivatives show enhanced retentions and the greatest enantioselectivities of the derivatives studied. This implies that the interaction between the carbonyl oxygen of the analyte and DNB NH amide of CSP is essential for chiral recognition.[10] The N-H of the secondary amides of CBZ α-amino acids would appear to contribute to chiral recognition, but in an opposite sense. For these analytes, in general, enantioselectivity is reduced relative to the *N,N*-diethylamides. In the case of the N-PHT α-amino acids derivatives examined, four analytes show reversal of elution orders (entries 1b, 3b, 4b and 4c in Table 4) whereas all others investigated afford the same elution orders as the corresponding N-PHT protected α-amino acids. It is noticeable that the enantioseparations of the esters and amides of N-FMOC α-amino acids are similar to those of N-CBZ α-amino acid derivatives. As an example, the enantiomers of FMOC-valine ethyl ester, *N*-*n*-butyl and *N,N*-diethylamide derivatives show αs of 1.21, 1.13 and 1.14, respectively, with the same elution order as the enantiomers of the corresponding acids.

In conclusion, CSP 1 is proving to be capable of separating the enantiomers of a broad array of compounds including N-CBZ, FMOC and PHT protected α-amino acids and the esters or amide derivatives thereof under both normal and reversed phase conditions. In particular, all of the N-CBZ and FMOC protected α-amino acids studied are base-line resolved using 5% 2-propanol in hexane containing 0.1% trifluoroacetic acid. Consequently, to our knowledge, these separation factors of the enantiomers of N-CBZ and FMOC protected α-amino acids on CSP 1 are greater than those on any other brush-type CSP.[30-32] In general, the *N,N*-diethylamide derivatives show the greatest enantioselectivities among the derivatives studied. CSP 1 should prove useful for the determination of the enantiomeric purity of *N*-protected α-amino acids as well as their ester or amide derivatives.

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