Chiral Analysis of Biogenic D,L-Amino Acids Derivatized by *N*-Fluorenylmethoxycarbonyl-L-alanyl *N*-Carboxyanhydride Using High-Performance Liquid Chromatography

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Abstract

Nineteen biogenic D,L-amino acids are derivatized with highly reactive *N*-fluorenylmethoxycarbonyl-L-alanyl *N*-carboxyanhydride. Using a 0.5M borate buffer at pH 7.5 and acetone, the derivatization of amino acids is completed in 5 min at room temperature. Some of the resulting diastereomeric *N*-protected dipeptides are successfully separated on an octylsilica stationary phase using 100mM acetate buffer (pH 4.4) and acetonitrile as the eluent.

Introduction

The growth of protein and peptide pharmaceutical use and proteomics as an emerging area of research in the postgenomic era has led to the increased need for analytical methodologies that would be capable of identifying the possible racemization of their basic units (amino acids) during peptide synthesis (1). The chiral separation of amino acids has been extensively studied using chiral stationery phases in gas chromatography (GC) (2) and high-performance liquid chromatography (HPLC) (3,4) for many years. In order to enhance detectability and selectivity, a variety of derivatization reagents of amino acids have been introduced (i.e., *o*-pthalaldehyde [5–7], chiral cyanates and isocyanates [8–10], *N*-carboxyanhydrides [NCAs] [11], and *N*-fluorenylmethoxychloroformates [12]).

Urethane-protected α-amino acid *N*-carboxyanhydrides (UNCAs) have recently been introduced as highly effective reagents in peptide synthesis (13). UNCAs are very reactive, and the only side product of the derivatization reaction is carbon dioxide. Generally, the reaction of UNCAs with amino acids is achieved with very low racemization (under 0.2%), which is a great advantage of these agents (14).

The general structure of UNCAs is X–AA–NCA (X = a urethane-protecting group such as *tert*-butyloxycarbonyl, benzyloxycarbonyl, and *N*-fluorenylmethoxycarbonyl [FMOC]; AA = L- or D-amino acid [15]). Because of their UV absorption and fluorescence properties, UNCAs with FMOC urethane-protecting groups are ideal for the determination of small amounts of amino acids (e.g., to study racemization). Surprisingly, only one work has been published, which dealt partly with the analysis of FMOC–AA–NCA-derivatized amino acids (16).

The racemization of amino acids is an inherent problem in solid- and liquid-phase peptide synthesis. In almost each coupling reaction racemization can occur. FMOC-L-alanyl (Ala)-NCA was recently introduced as a highly effective reagent in peptide synthesis with a major advantage of fast reaction with amino acids and an extremely low level of racemization (< 0.2%) (13,14). The aim of this work is to develop a method that separates pairs of FMOC-L-Ala-D,L-amino acid diastereomers, which can be used for monitoring racemization after solid-phase peptide synthesis (SPPS) without any additional derivatization.

Experimental

Instrumentation

For HPLC a Gilson (Gilson Medical Electronics, Middletown, WI) chromatograph was equipped with two high-pressure pumps (Gilson 302), a mixer (Gilson 811), a manostat (Gilson 802 C), a UV detector (Gilson 116 at 260 nm), and an injector (Gilson with an injection volume of 50 µL). Separation was carried out using a Superspher 60 RP-8, 250- × 4-mm chromatographic column (Merck, Darmstadt, Germany).

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Chemicals

All chemicals were of analytical grade if not stated otherwise. FMOC-L-Ala-NCA and FMOC-L-Ala-OH were obtained from Fluka (Buchs, Switzerland). D-Histidine (His), D-asparagine (Asn), D-cysteine (Cys), D-isoleucine (Ile), D-lysine (Lys), D-threonine (Thr), and D-arginine (Arg) were obtained from Senn Chemicals International (Gentilly, France). D-Valine (Val) and D-proline (Pro) were obtained from CalBiochem (Los Angeles, CA). The other D- and L-amino acids were received from Fluka. All amino acids were certified by the suppliers to be enantiomerically pure, with the limit of detection of at least 0.1%. The stock solutions of AAs at a concentration of 1×10^{-3} mol/L were prepared daily by dissolving AA in deionized water. Phosphoric acid, boric acid, sodium phosphate tribasic, sodium tetraborate, sodium acetate, and acetic acid were obtained from Aldrich (St. Louis, MO). Acetonitrile (ACN) and acetone were obtained from Merck. Deionized water was obtained using the Milli-Q system (Millipore, Bedford, MA).

Chromatography

Gradients were formed by the programmed gradient of a 100mM acetate buffer (pH 4.4) (eluent A) and ACN (eluent B). The gradient program was adopted from the MERCK program for the separation of amino acids derivatized by *N*-fluorenylmethoxychloroformate, and it is described in Table I.

Derivatization procedure

Procedure A

One milliliter of 1mM amino acid was added to 1 mL of a 50mM borate buffer at pH 7.5 and then mixed with 1 mL of 2mM FMOC-L-Ala-NCA (in ACN). The mixture was kept for 5 min at laboratory temperature and then analyzed.

Procedure B

One milliliter of 1mM amino acid was added to 1 mL of the borate buffer at pH 7.5 (concentration in the range of 50 to 500mM) (see the "Results and Discussion" section) and then mixed with 1 mL of 2mM FMOC-L-Ala-NCA (in acetone). The mixture was kept for 5 min at laboratory temperature and then analyzed.

Table I. The Used Gradient Program*

Time (min)	Gradient %A	Gradient %B	Flow rate (mL/min)
0.0	78	22	0.05
1.0	78	22	1.25
17.0	62	38	1.25
19.0	55	45	1.25
20.0	60	40	1.25
22.0	60	40	1.25
35.0	45	55	1.25
40.0	0	100	1.25
45.0	0	100	1.25
50.0	78	22	1.25
59.0	78	22	1.25
60.0	78	22	0.05

* Eluent A = 100mM acetate buffer (pH 4.4) and eluent B = ACN.

Results and Discussion

Derivatization

The reaction of the FMOC-L-Ala-NCA with an amino acid has to be carried out in a slightly basic medium (13). This was assured using inorganic buffers. The performance of two different derivatization procedures of the amino acids was studied using D,L-phenylalanine (Phe) (D,L-Phe-OH) as a model compound.

Derivatization procedure A, using the derivatization reagent FMOC-L-Ala-NCA dissolved in ACN results, as expected, in FMOC-L-Ala-L-Phe-OH. The unreacted L-Phe-OH was not found in the reaction mixture, thus the fractional conversion of the reaction was 1. The high stability of FMOC-L-Ala-L-Phe-OH was achieved under these conditions: relative standard deviation (RSD) = 2.68% (n = 40), and the solution was stored for 5 days at +5°C). The precision of repeated derivatizations was 4.83% for n = 5 (procedure A).

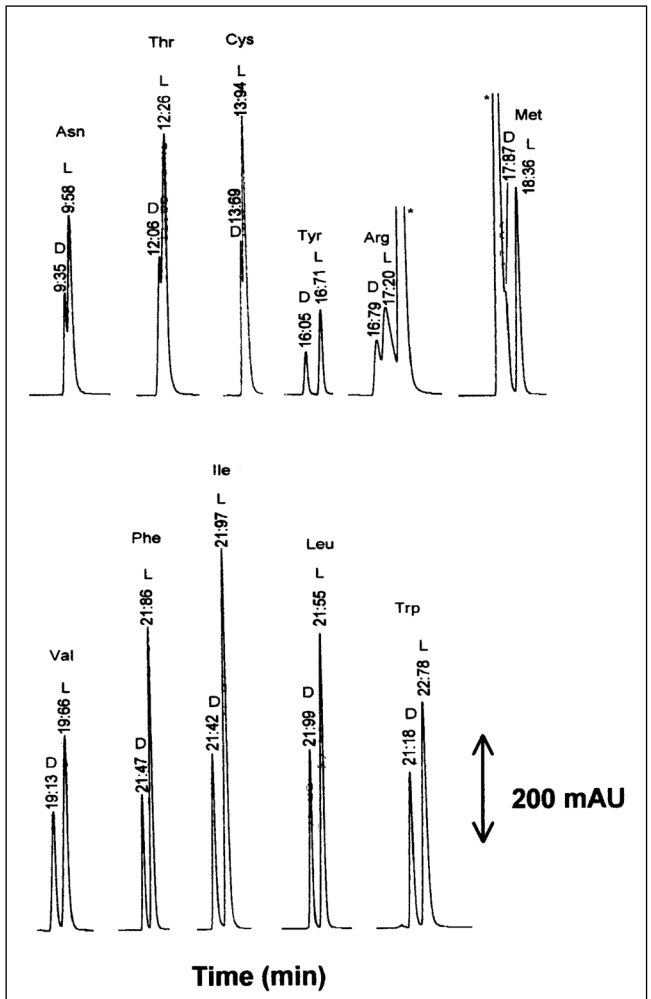

Figure 1. Section of the chromatogram of diastereomeric dipeptides formed by the reaction of free D- and L-amino acids (D/L = 1:2) with FMOC-L-Ala-NCA: (*) peak of FMOC-L-Ala-OH.

Derivatization procedure B used derivatization reagent FMOC-L-Ala-NCA dissolved in acetone. The high stability of FMOC-L-Ala-L-Phe-OH was achieved under conditions described in the "Procedure B" subsection (50mM borate, RSD = 1.07% (n = 40), solution was stored for 5 days at +5°C). The fractional conversion of the reaction was 0.6 (determined as the ratio of the area of the signal unreacted L-Phe-OH and the sum of the signals of the FMOC-L-Ala-L-Phe-OH and L-Phe-OH. The influence of the concentration of the borate buffer on the fractional conversion of the reaction in procedure B was studied. The fractional conversion of the reaction increased with the increasing concentration of the borate buffer and achieved magnitude one at the concentration of 0.5M borate. A high stability of the reaction product (RSD = 1.12% (n = 40) for FMOC-L-Ala-L-Phe-OH; solution stored at +5°C and analyzed for a period of 5 days) was achieved using optimized derivatization conditions (procedure B, 0.5M borate buffer). The precision of repeated derivatizations was 1.83% for n = 5 (procedure B; 0.5M borate buffer). Subsequent work employed procedure B with 0.5M borate as the derivatization procedure because of its higher reproducibility.

It was experimentally proved that no undesirable kinetic effects occurred when derivatizing a mixture of enantiomers with optically pure FMOC-L-Ala-NCA. For derivatized racemic D,L-Phe-OH the peak area ratio was 1:1. However, the high optical purity of the used derivatization reagent was an essential prerequisite for reliable quantitative results, especially in the determination of enantiomeric excess (studied D,L-Phe-OH (e/e) ratios of 1:2, 1:1, and 2:1). In the subsequent work the D/L ratio of amino acids 1:2 was used.

It should be noted that in using an excess of the derivatization reagent (FMOC-L-Ala-NCA), the respective *N*-protected amino acid (FMOC-L-Ala-OH) was also formed as a result of its hydrolysis. This product can be seen in chromatograms depicted in Figure 1 and is marked as "*".

Separation

Eleven of 19 amino acids were separated using HPLC, namely D,L-Phe, D,L-tyrosine (Tyr), D,L-methionine (Met), D,L-Arg, D,L-Val, D,L-leucine (Leu), D,L-Cys, D,L-Ile, D,L-Thr, D,L-tryptophan (Trp), and D,L-Asn. Chromatograms of the separation of these amino acids are depicted in Figure 1. Six amino acids were baseline separated (D,L-Phe, D,L-Tyr, D,L-Met, D,L-Leu, D,L-Ile, and D,L-Trp). It is also clear from Figure 1 that the migration order of the diastereomers was always FMOC-L-Ala-D-AA-OH and FMOC-L-Ala-L-AA-OH. It can be concluded that the interaction of the RP-C8 column with the L–L diastereomer is stronger than with the L–D diastereomer. The diastereomers are resolvable by reversed-phase (RP)-HPLC because they have different hydrophobic surface areas. The retention of L–L and L–D diastereomers depends upon the orientation of the side-chain groups on the asymmetric a-carbon atoms with regard to the dipeptide backbone. Because FMOC-L-Ala-L-AA diastereomers have a higher hydrophobic surface than FMOC-L-Ala-D-AA diastereomers, the L–D isomer is eluted more rapidly than the L–L isomer (17).

Basic characteristics of HPLC separation (such as retention times (t_R), retention factor (k), resolution (Rs), and number of theoretical plates (N) of diastereomeric dipeptides formed by the reaction of free D- and L-amino acids (D/L = 1:2) with FMOC-L-Ala-NCA) are summarized in Table II. It should be noted that the aim of this work was to separate pairs of L–D and L–L diastereomers, not separate diastereomers of different amino acids.

Table II. The t_R, k, Rs, and N Values of Diastereomeric Dipeptides Formed by the Reaction of Free D- and L-amino acids (D/L ~ 1:2) with FMOC-L-Ala-NCA

	t_R (min)		k			N/m (x 10^4)	
AA	D	L	D	L	Rs	D	L
Ala	14.49	14.49	4.14	4.14	0	2.29	2.29
Phe	21.47	21.86	6.51	6.64	1.05	9.85	9.64
Tyr	16.05	16.71	4.77	5.01	3.21	3.14	3.41
Serine	10.13	10.13	2.43	2.43	0	0.60	0.60
Cys	13.69	13.94	4.01	4.11	0.31	1.53	1.59
Met	17.82	18.36	5.39	5.58	n.c.*	n.c.	4.61
Val	19.13	19.66	5.74	5.92	0.97	2.51	2.65
Leu	21.99	22.51	7.06	7.25	2.12	7.47	7.83
Ile	21.42	21.97	6.26	6.45	1.24	7.09	7.45
Thr	12.06	12.26	3.12	3.18	0.26	1.59	1.65
His	15.49	15.49	4.27	4.27	0	0.26	0.26
Pro	16.38	16.38	4.79	4.79	n.c.	n.c	n.c.
Trp	22.18	22.78	6.89	7.11	1.53	4.86	5.13
Arg	16.79	17.20	4.73	4.87	0.43	0.53	0.55
Lys	14.31	14.31	3.88	3.88	0	1.03	1.03
Glutamic acid	10.58	10.58	2.74	2.74	0	0.49	0.49
Glutamine	9.79	9.79	2.51	2.51	0	0.56	0.56
Asp	9.87	9.87	2.49	2.49	0	0.18	0.18
Asn	9.35	9.58	2.32	2.40	0.55	0.86	0.91

* n.c., not calculated. Diastereomer coelutes with FMOC-L-Ala-OH.

The good separation capability of HPLC is coupled with very good linearity (measured in the concentration range of 1×10^{-5}M to 2×10^{-4}M) and sensitivity. Defined peaks, proportional to the analyte concentration, are observed for model compounds FMOC-L-Ala-L-Phe-OH and FMOC-L-Ala-D-Phe-OH. The resulting calibration plots are linear, with sensitivities of 1.48×10^9 and 1.42×10^9 mV·s·L·mol^{-1} for FMOC-L-Ala-L-Phe-OH and FMOC-L-Ala-D-Phe-OH, respectively (correlation coefficients 0.9996 and 0.9987). Intercepts were -1.9×10^3 and -8.5×10^2 mV·s, respectively. The high sensitivity and speed of the RP-HPLC did not compromise reproducibility. A series of 12 repetitive injections of a sample containing 1×10^{-5}M FMOC-L-Ala-D-Phe-OH resulted in RSDs of 2.10%.

The developed method was used for the determination of the racemization of some amino acids during their coupling to the hydroxy group of polymer resins in SPPS for commercial purposes in our laboratories. Sensitivity of the proposed method can be enhanced using fluorescence detection.

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