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Journal of Chromatography A, 767 (1997) 69–75

JOURNAL OF CHROMATOGRAPHY A

Adsorption liquid chromatography on silica for the chiral separation of amino acids and asymmetric amines derivatized with optically active N-α-9-fluorenylmethyloxycarbonyl-amino acid-N-carboxyanhydrides

Martine Pugniere[a], Hélène Mattras[a], Bertrand Castro[b], Aldo Previero[a,*]

[a]*Laboratoire de Chimie Chirale et Bioorganique, ENSCM, Centre de Recherche INSERM, 70 Rue de Navacelles, 34090 Montpellier, France*

[b]*Sanofi-Chimie, 82 Avenue Raspail, 94255 Gentilly Cedex, France*

Received 8 October 1996; revised 10 December 1996; accepted 16 December 1996

Abstract

Optically pure N-α-Fmoc-amino acid-N-carboxyanhydrides (Fmoc-AA-NCAs, Fmoc=9-fluorenylmethyloxycarbonyl) are proposed as precolumn reagents for the chiral analysis of asymmetric amines including α-amino acid alkyl esters. Separation of diastereomers arising from racemic amines is better achieved by liquid–solid adsorption chromatography on silica than by reversed-phase techniques. The sample preparation is easily performed while the properties of the Fmoc group allows high sensitivity by fluorescent detection. In this mode, picomolar limits of enantiomeric excess are detected.

Keywords: Enantiomer separation; Amino acids; Amines; N-α-9-Fluorenylmethyloxycarbonyl-amino acid-N-carboxy anhydrides

1. Introduction

Analysis of enantiomer mixtures following conversion to diastereomers is a convenient approach for asymmetric compounds with functional groups that are easily derivatized. Basically, the technique involves the transformation of an enantiomeric mixture of unknown composition into an easily analyzable mixture of diastereomers.

A number of chiral reagents have been proposed for the resolution of amino acids, primary and secondary amines, while high-performance liquid chromatography (HPLC) and gas chromatography are widely used for enantioseparations [1–6].

When chiral reagents also introduce a chromophore or electroactive group into the derivatives, considerable enhancement of the sensitivity of detection may be achieved. (+)-1-(9-Fluorenyl)ethyl chloroformate, which reacts with amino acids and amines, yielding fluorescent diastereomeric derivatives, is a significant example in this field [6].

We report in this paper a family of diastereomeric derivatives of amino acids and chiral amines that are labelled with a fluorenyl group and are easily obtained using N-α-Fmoc-amino acid-N-carboxyanhydrides (Fmoc-AA-NCAs) as chiral reagents, with good analytical resolutions achieved by ad-

*Corresponding author.

PII S0021-9673(97)00021-6

sorption HPLC on silica. The use of urethane-protected amino acid N-carboxyanhydrides (UNCAs) has been proposed recently for the optical resolution of free amino acids [7]; however, our sample preparation approach, to avoid contaminating products, and our analytical procedure for improving chromatographic resolution are completely different.

2. Experimental

2.1. Chromatography apparatus

HPLC was performed using a Beckman 110 A apparatus, equipped with a Shimadzu Chromatopac C.R.6A integrator. A stainless-steel column (25× 0.46 cm) packed with 5 μm Kromasil silica and a guard column (1.5×0.32 cm) packed with the same material were from Touzart and Matignon (France). The ultrasphere ODS (C_{18}) column (25×0.46 cm) was from Beckman. Fluorescent detection was performed using a Waters 470 apparatus. The excitation and emission wavelengths were set at 263 and 313 nm, respectively.

2.2. Chemicals

All solvents were of chromatographic grade from Carlo-Erba (Milan, Italy). Enantiomers and racemic α-amino acids were pure commercial products or were taken from our collection. Methyl esters were prepared from free α-amino acids by the thionyl chloride method [8]. Fmoc-AA-NCAs were from Propeptide (Vert-Le Petit, France). Other chemicals were of pure commercial grade.

2.3. Sample preparation

α-Amino acid methyl ester hydrochlorides ($2\cdot10^{-6}$ to $1\cdot10^{-5}$ mol), dissolved in methanol (10–50 μl), were transferred into a 1-ml vial and carefully evaporated to dryness. The residue was taken up with 35 μl of dimethylformamide (DMF) containing 28% diisopropylethylamine (DIEA) and shaken for 10 min at room temperature. The Fmoc-AA-NCA ($5\cdot10^{-6}$ to $2.5\cdot10^{-5}$ mol), dissolved in DMF (25 μl), was added and allowed to react for 10 min at room temperature. Sodium glycinate (0.25 *M*), pH 8 (200 μl), was added and after 5 min, the reaction mixture was extracted with chloroform (300 μl). Aliquots of the chloroform layer were diluted 1:10 000 to 1:50 000 with *n*-hexane; a 20-μl volume of the obtained solution was injected into the column.

Free amino acids ($0.2\cdot10^{-6}$ to $1\cdot10^{-5}$ mol) could be used as starting material provided that there was a 2-h pretreatment with 50–100 μl of a methanol–thionyl chloride (95:5, v/v) mixture at 60°C in a stoppered vial followed by careful evaporation.

Chiral amines ($2\cdot10^{-6}$ to $1\cdot10^{-5}$ mol) in free form were directly dissolved in 35 μl of DMF containing 28% DIEA and were treated with Fmoc-α-AA-NCA as described for α-amino acid methyl esters.

2.4. Chromatography procedure

Diastereomeric derivatives of amino acids and amines were separated by adsorption HPLC on a silica column, with an eluent consisting of *n*-hexane and isopropanol (of different compositions, as indicated below) under isocratic conditions. Solvent A (1% isopropanol), solvent B (2% isopropanol), solvent C (2.5% isopropanol), solvent D (3% isopropanol), solvent E (3.5% isopropanol), solvent F (5% isopropanol) and solvent G (10% isopropanol). The derivatized compounds were eluted at a constant flow-rate of 0.8 ml/min.

3. Results and discussion

Urethane-protected α-amino acid N-carboxyanhydrides (UNCAs) were first synthesized by Fuller et al. [9] and were proposed as reactive intermediates for peptide synthesis. These compounds strongly react with primary and secondary amines, yielding amides and carbon dioxide as by-products [9]. The reaction takes place without racemization so that optically active UNCA can be viewed as a chiral reagent for the enantiomeric resolution of asymmetric amines [7].

We focused on the use of optically active Fmoc-AA-NCAs since the fluorene group also makes these compounds reagents for fluorescent labelling, allowing subsequent analysis with highly sensitive detection. In particular, reaction conditions for sample preparation and chromatographic systems that would

yield a good separation of the resulting diastereomers were studied separately to optimize the overall procedure.

3.1.1. Reaction conditions

Fmoc-AA-NCAs are hydrophobic compounds, practically insoluble in water, designed for use in organic media during peptide synthesis.

The reaction with free amino acids, which are generally insoluble in non-aqueous solvents, should be excluded to avoid cumbersome empirical research on the composition of the reaction medium for each amino acid or amino acid group.

Water should also be excluded to avoid competitive hydrolysis of Fmoc-α-AA-NCAs into Fmoc-α-amino acids, which become contaminating compounds during sample preparation. This negative aspect is strongly enhanced when excess chiral and fluorescent labelling reagent is used for complete reaction of the two enantiomers of a racemic mixture. For these reasons, in the case of amino acids, we preferred to use esters, which are soluble in organic solvent and can be obtained easily in microscale quantities for analytical purposes.

The method of sample preparation is summarized in the Fig. 1.

Enantiomers of amino acid esters or asymmetric amines react with an excess (two–five equivalents) of optically active Fmoc-AA-NCA, yielding two diastereomeric dipeptides that are protected at their N- and C-termini. Subsequent treatment with sodium glycinate converts the excess Fmoc-AA-NCA into a

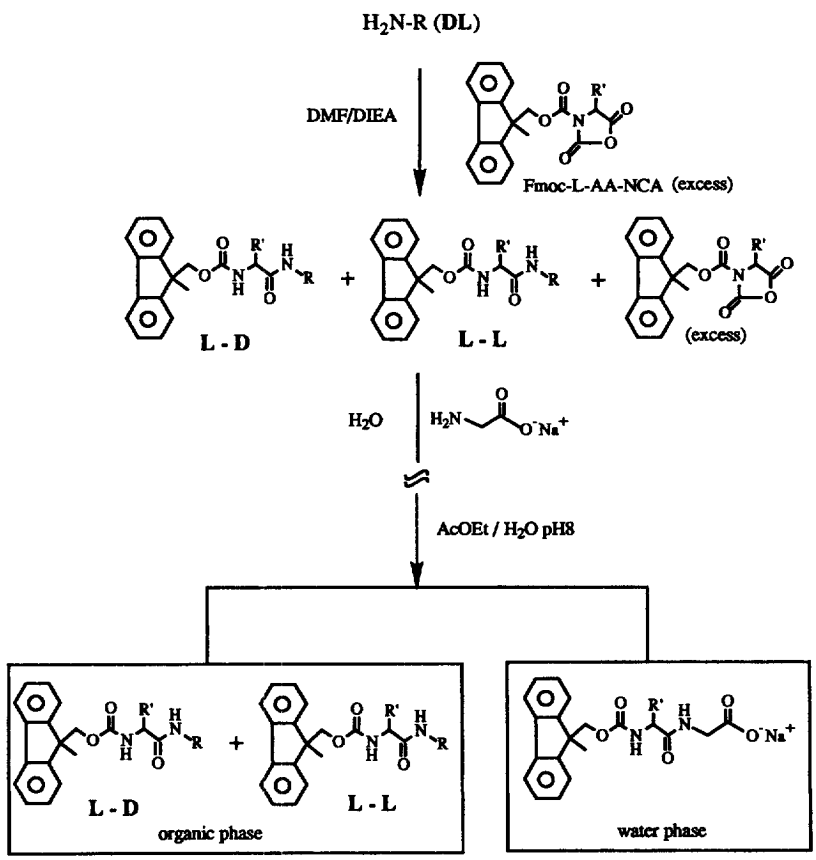

Fig. 1. Method for the preparation of diastereomers by reaction of racemic amino acid esters and amines with Fmoc-L-AA-NCA.

dipeptide free acid that is easily separated by washing with weakly alkaline water, allowing complete dissolution of the samples in the organic phase.

3.1.2. Chromatographic analysis

Separation of diastereomeric derivatives for analytical purposes is usually performed by HPLC. Gas chromatography also gives excellent results, even though sample volatility is a serious limitation for general applications. For HPLC, several separation procedures can be used, depending on the type of interaction between the stationary phase, mobile phase and sample. A literature survey showed that reversed-phase chromatography (where the stationary phase linked to the support is less polar than the mobile phase) is the most widely used system for separating diastereomeric compounds [10]. In our case, however, reversed-phase chromatographic analysis did not give good resolution. The separation factors obtained using an ultrasphere ODS (C_{18}) column were satisfactory only in some cases (Table 1), whereas for the great majority of amino acid esters and chiral amines, the results were not good enough.

By contrast, good separations were obtained using adsorption chromatography on silica, where the stationary phase can be defined as the liquid–solid interface.

Data in Table 2 give the enantiomeric separations of amino acid esters on silica (as the adsorbent) using *n*-hexane as the eluting solvent.

Good separations were obtained for all proteinogenic amino acids, with the exception of proline. Non-proteinogenic amino acids, which have attracted increased interest as chiral building blocks in asymmetric synthesis, were also well resolved enantiomerically. The eluted peaks were clean and chromatograms were free from excess reagent (Fmoc-Leu-NCA) or its hydrolysis products. They both have been carefully eliminated following the proposed procedure of sample preparation. Contaminating compounds have been systematically found when Boc or Fmoc-AA-NCA were used as chiral reagents for free amino acids in aqueous media [7]. However, this procedure did not allow the analysis of water-soluble arginine and histidine. The elution order of amino acid esters was (L) before (D) in all cases using Fmoc-L-Leu-NCA as the chiral reagent, and this order was of course reversed when using Fmoc-D-Leu-NCA, as observed in separate experiments. The extent of the resolution of Fmoc derivatives was dependent on the concentration of isopropanol in the eluent, which was optimized to obtain complete enantiomeric resolution.

The use of Fmoc-L-Leu-NCA was not limiting, as shown in Table 3, which gives the enantiomeric resolution of amino acids by Fmoc-L-Ala, Fmoc-L-Val and Fmoc-L-Phe.

Asymmetric amines easily reacted with optically active Fmoc-AA-NCA, yielding covalent diastereomers that were resolved by adsorption chromatography, as shown in Table 4. In this case, the isomer elution order was not systematic as for amino acids and was not foreseeable for the homologous series. For example, the *R*-enantiomer of 2-aminobutane was the first to elute, while the opposite was true for 2-aminopentane and 2-aminoheptane.

A number of illustrative examples are reported in Fig. 2. Resolutions were generally better than those observed for analogous compounds using a reversed-phase HPLC technique.

Success in quantitative enantiomeric analysis also depends on the optical purity of the chiral reagent. The commercial Fmoc-AA-NCAs used in this study were found to be optically pure; only one peak was observed in all cases when a pure amino acid enantiomer was used under highly sensitive detection. The fluorogenic properties of the Fmoc group allows the analytical determination of picomole amounts, depending on the sensitivity of the fluorescence detector. The apparatus used in this work (see

Table 1
Capacity factors (k') and separation factors (α) of DL-amino acids derivatized with Fmoc-L-Leu-NCA

DL-Amino acid	k'(L)	k'(D)	α
Ala	1.87	1.87	1.00
Val	2.7	2.94	1.07
Leu	3.28	3.49	1.06
NVa	2.73	2.88	1.06
NLe	3.29	3.53	1.07
Abu	2.20	2.30	1.05
Thi	3.06	3.06	1.00

HPLC conditions: Ultrasphere ODS (C_{18}) column.
Eluent composition: acetonitrile–buffer, pH 2.6 (0.8 g of phosphoric acid and 0.28 g of triethylamine per litre), 25:75 (v/v).
α=separation factor (k_D'/k_L').

Table 2
Resolution by adsorption liquid chromatography on silica of DL-amino acids derivatized with Fmoc-L-Leu-NCA

Amino acid[a]	k'(L)	k'(D)	α	R_s	Mobile phase[b]
Alanine	5.37	5.97	1.11	1.58	B
Valine	2.20	2.60	1.18	2.53	B
Leucine	1.98	2.37	1.20	1.84	B
Isoleucine	2.11	2.60	1.23	1.49	B
Phenylalanine	2.55	4.91	1.92	4.20	B
Methionine	3.35	4.91	1.46	5.37	B
Lysine	1.97	3.03	1.54	4.28	F
Glutamic acid	5.04	7.8	1.54	4.95	D
Proline	not resolved				
Aspartic acid	6.24	7.35	1.18	2.36	D
Serine	3.27	3.59	1.10	1.20	F
Tyrosine	1.35	1.66	1.23	2.01	G
Tryptophan	1.42	1.72	1.21	1.65	G
Threonine	1.70	1.94	1.14	1.33	G
NVa	2.60	3.18	1.22	2.61	B
NLe	2.24	2.75	1.23	2.28	B
HomoPhe	2.58	3.35	1.30	3.26	B
p-ChloroPhe	2.98	4.71	1.58	5.66	B
3,4-DichloroPhe	3.12	4.88	1.56	7.55	B
Phg	2.88	3.42	1.19	2.18	B
Thi	2.94	4.14	1.40	4.80	B
Abu	3.61	4.34	1.20	2.64	B
Nal(1)[c]	2.50	3.51	1.41	5.21	B
Nal(2)[c]	2.87	4.42	1.51	8.62	B
Tic	3.38	4.11	1.22	1.69	B

R_s was calculated from the following equation: $R_s=(t_D-t_L)/(W_{D/2}+W_{L/2})$, where t_D and t_L are retention times, $W_{D/2}$ and $W_{L/2}$ are widths at half-height.

[a] Abbreviations used: NVa=2-aminopentanoic acid, NLe=2-aminohexanoic acid, HomoPhe=2-amino-4-phenylbutyric acid, *p*-ChloroPhe=β-(*p*-chlorophenyl)alanine, 3,4-dichloroPhe=β-(3,4-dichlorophenyl)alanine, Phg=α-aminophenylacetic acid, Thi=β-(2-thienyl)alanine, Abu=α-aminobutyric acid, Nal(1)=β-(1-naphthyl)alanine, Nal(2)=β-(2-naphthyl)alanine, Tic=1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid.

[b] For eluent composition, see Section 2.

[c] UV detection.

Table 3
Resolution by adsorption liquid chromatography on silica of DL-amino acids derivatized with different Fmoc-L-amino acid-NCAs

Derivative	k'(L)	k'(D)	α	R_s	Mobile phase[a]
Fmoc-L-Ala-DL-AlaOMe	9.24	10.4	1.12	1.67	E
Fmoc-L-Ala-DL-LeuOMe	not resolved				E
Fmoc-L-Ala-DL-ValOMe	4.16	4.54	1.09	1.29	E
Fmoc-L-Ala-DL-PheOMe	4.81	5.49	1.14	2.04	E
Fmoc-L-Val-DL-AlaOMe	5.00	5.62	1.12	1.59	C
Fmoc-L-Val-DL-LeuOMe	2.07	2.33	1.13	1.24	C
Fmoc-L-Val-DL-ValOMe	2.20	2.58	1.17	1.80	C
Fmoc-L-Val-DL-PheOMe	2.51	3.24	1.29	3.10	C
Fmoc-L-Phe-DL-AlaOMe	not resolved				C
Fmoc-L-Phe-DL-LeuOMe	2.12	2.40	1.13	1.29	C
Fmoc-L-Phe-DL-ValOMe	2.39	2.74	1.14	1.80	C
Fmoc-L-Phe-DL-PheOMe	2.72	3.73	1.37	4.06	C

[a] For eluent composition, Section 2.

Table 4
Resolution by adsorption liquid chromatography on silica of chiral amines derivatized with Fmoc-L-Leu NCA

Amines	$k'(S)$	$k'(R)$	α	R_s	Mobile phase[a]
2-Aminobutane	5.32	4.66	1.14	1.90	A
2-Aminopentane	3.41	4.14	1.21	2.40	A
2-Aminoheptane	2.51	3.12	1.25	2.80	A
2-Methylpiperidine	7.68	6.83	1.33	1.80	A
3-Methylpiperidine	6.39	5.73	1.12	1.34	A
1-Phenylethylamine	5.32	4.19	1.25	3.27	A

[a]For eluent composition, see Section 2.

Section 2) easily allowed analysis to be performed in the range of 10^{-12} to 10^{-9} moles of enantiomers. Optical impurities can thus be detected up to 0.1%. Furthermore, the possibility of inverting the elution order of enantiomers using the L- or D-Fmoc-AA-NCA may be of some interest, since the quantitative detection of a contaminant enantiomer is easier when it arises before the dominant peak.

It is also possible that one enantiomer could react with the chiral reagent more rapidly than its antipode. This seemed to be excluded using the derivatization procedure illustrated in Section 2. Quantitative integration of the two chromatographic peaks obtained from pure racemic compounds were identical in all cases when detection was performed by adsorption at 254 nm. UV adsorption was used to avoid possible differences in fluorescence emission of two diastereomers.

The proposed analytical procedure could thus be applied in all investigations requiring rapid, precise and highly sensitive determination of enantiomeric proportions of proteinogenic and synthetic amino acids, as well as of asymmetric amines.

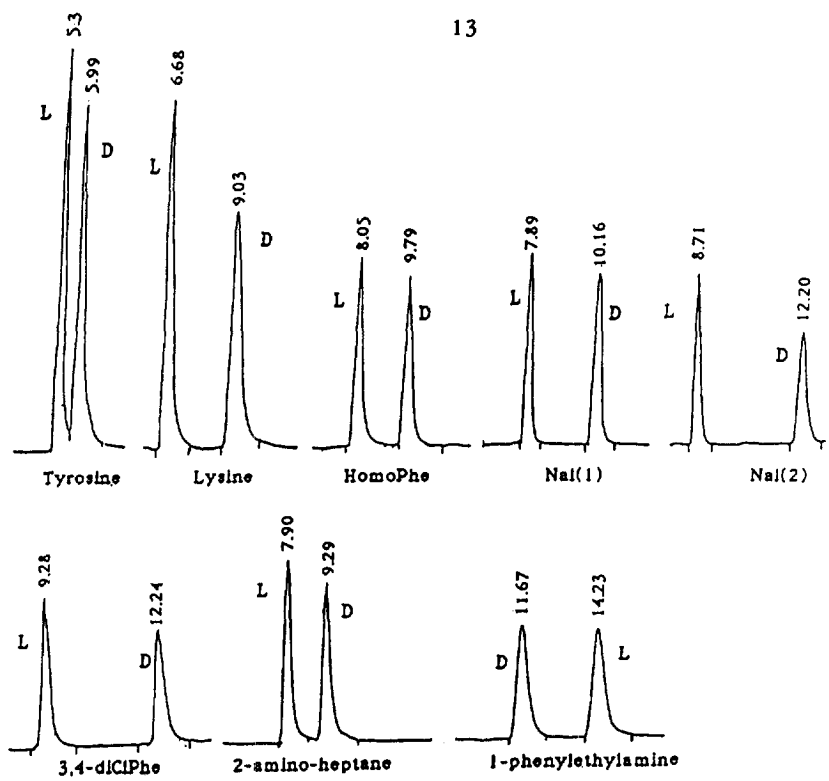

Fig. 2. Sections of chromatograms of diastereomers of selected amino acids and amines formed by reaction with Fmoc-L-Leu-NCA. Compositions of the mobile phase are listed in Tables 2 and 4 and the HPLC conditions were given in Section 2.

References

[1] J.G. Adamson, T. Hoang, A. Grivici and G.A. Lajoie, Anal. Biochem., 202 (1992) 210.
[2] N. Nimura, A. Toyama and T. Kinoshita, J. Chromatogr., 316 (1984) 547.
[3] D.S. Dunlop and A. Neidle, Anal. Biochem., 165 (1987) 38.
[4] H. Brückner and B. Strecker, J. Chromatogr., 627 (1992) 97.
[5] H. Brückner, S. Haasmann, M. Langer, T. Westhauser and R. Wittner, J. Chromatogr. A, 666 (1994) 259.
[6] S. Einarsson, B. Josefsson, P. Möller and D. Sanchez, Anal. Chem., 59 (1987) 1191.
[7] H. Brückner and M. Lüpke, Chromatographia, 40 (1995) 601.
[8] M. Brenner and W. Huber, Helv. Chem. Acta, 36 (1953) 1109.
[9] W.D. Fuller, M.P.Cohen, M. Shabankareh, R.K. Blair, T.A. Miller, M. Goodman and F.R. Naider, in E. Giralt and D. Andreu (Editors), Peptides 1990: Urethan-protected Amino Acid N-Carboxyanhydrides and their use in Peptide Synthesis, Escom, Leiden, 1990.
[10] N.L. Benoiton, Y. Lee, B. Liberek, R. Steinauer and F.M.F. Chen, Int. J. Peptide Protein Res., 31 (1988) 581.