Short Communication

Novel polymeric reagent for synthesizing 9-fluorenylmethoxycarbonyl L-prolinyl derivatives for chiral high-performance liquid chromatography of amino acids

Z. Zhang, G. Malikin and S. Lam

Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461 (USA)

ABSTRACT

The synthesis of a novel polymer-supported 9-fluorenylmethoxycarbonyl-L-proline reagent for derivatizing nucleophiles is described. The new polymer, containing a 1-hydroxybenzotriazole activated ester, is highly reactive. Significant improvement in the ease of use and the rate of reaction with strong and weak nucleophiles is achieved. In this study, the utility of the solid-phase reagent for derivatizing amino acids for chiral high-performance liquid chromatography is described. Derivatization is accomplished simply by mixing a sample of the amino acid in acetonitrile with the polymer at room temperature for 10 min. Racemization of amino acids under the mild reaction conditions is not observed. Despite 9-fluorenylmethoxycarbonyl-L-prolinyl-D,L-amino acids are diastereoisomeric, the isomers are not separated by simple reversed-phase chromatography. Since these derivatives possess the necessary functional groups for metal chelation, mobile phases containing chiral Cu(II) complexes were used to resolve the optical isomers. Excellent resolution of all the D and L enantiomers of natural amino acids was achieved by using Cu(II)-L-histidine methyl ester and Cu(II)-L-proline eluents on reversed phases columns with various concentrations of acetonitrile. The separated derivatives were detected in the low-nanogram range by fluorescence at 315 nm with excitation at 275 nm.

INTRODUCTION

Chromatographic resolution of enantiomers is generally accomplished by transforming an enantiomer pair to a diastereomer pair. In the direct resolution mode, diastereomeric transformation takes place when the enantiomers form association complexes with a chiral system ligand immobilized on the stationary phase or added to the mobile phase. The chiral ligand can be a micro-molecule or a macro-molecule like protein. The forces of association, depending on the chromatographic system, may include π–π interaction, hydrogen bonding, dipole stacking, mixed ligand metal complexation, inclusion complex formation, ion pair complexation or a combination of several of these forces as in protein binding sites. In the indirect resolution mode, optical isomers are resolved by chemical derivatization with a chiral reagent to form covalently bonded diastereomers. The differences in internal energy of the diastereomers favor the partition of one isomer over the other in the chromatographic system, and as a result, chiral separation.

As the natural L-amino acids are distinct from the D-isomers with different biological implications, there is a great deal of emphases in resolving these optical isomers and identifying their stereo-config-

Correspondence to: Dr. S. Lam, Albert Einstein College of Medicine, 1303 Morris Park Avenue, Bronx, NY 10461, USA.

uration. Amino acids, with a few exceptions, are known to possess poor absorption characteristics and electrochemical properties for high-performance liquid chromatography (HPLC) detection. Chemical derivatization by pre-column or post-column techniques is a standard approach for enhancing the sensitivity of detection. Recently, chemical derivatization is also recognized as an approach for enhancing both direct and indirect chiral resolution. In the indirect resolution mode, however, the derivative must introduce an additional optical center to give a diastereomer.

Among the chiral derivatization reagent for amino acids are N^2-(5-fluoro-2,4-dinitrophenyl)-L-alanine amide and modifications substituting alanine with different amino acid [1], o-phthaldialdehyde with various chiral thiols [2,3], and a collection of other chiral reagent [4]. Recently, Patchornik and co-workers introduced polymeric active esters such as polymeric 1-hydroxybenzotriazle (HOBT) [5], polymeric 4-hydroxy-3-nitribenzophenone (P-BP) [6], and polymeric 4-(dimethylamino)pyridine (P-DMAP) [7] as acylating reagents in peptide synthesis. Following the similar idea, Krull and co-workers synthesized several of these polymeric reagents containing 9-fluorenylmethoxycarbonyl (FMOC), FMOC-L-phenylalanine, FMOC-L-proline and 3,5-dinitrophenyl (DNB) moieties for derivatization of various nucleophiles in HPLC. They used polymeric reagents P-HOBT-FMOC [8,9], P-BP-FMOC [10], P-HOBT-DNB and P-BP-DNB [11] for labelling amines and polyamines, and P-DMAP-FMOC [12], P-BP-DNB [13] and P-HOBT-DNB [13] for labelling alcohols. Polymeric reagents containing chiral labels such a P-BP-FMOC-L-proline and P-BP-FMOC-L-phenylalanine were also used for transforming enantiomeric amines to diastereomeric amines for detection and chiral separation [14,15]. No work on labelling free amino acids with chiral polymeric reagent has been reported.

In this work, we report the synthesis of a new polymeric reagent containing HOBT ester of FMOC-L-proline for derivatization and resolution of optical isomers of amino acids by HPLC.

EXPERIMENTAL

Chemical and reagents

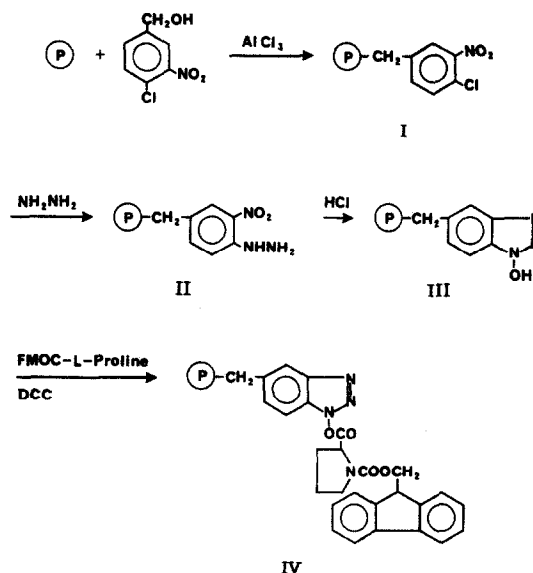Polystyrene [styrene divinylbenzene (96:4), 200–

400 mesh) was purchased from Fluka (Buchs, Switzerland) and FMOC-L-proline from Chemical Dynamics (South Plainfield, NJ, USA). The amino acids were obtained from Sigma (St. Louis, MO, USA). Dicyclohexylcarbodiimide (DCC) was bought from Aldrich (Milwaukee, WI, USA) and acetonitrile, distilled in glass, from Burdick & Jackson Labs. (Muskegon, MI, USA).

Instrumentation

The HPLC system consisted of two Altex (Berkeley, CA, USA) 110A pumps, an Altex 420 gradient microprocessor and a Rheodyne (Cotati, CA, USA) 7105 injection valve. The analytical column 15 × 0.42 cm I.D., was packed with Nucleosil-5 C_{18} (Macherey–Nagel, Düren, Germany) by the downward slurry technique. A FMOC-L-prolinyl derivatives were monitored with a Hitachi fluorescence detector, Model F-1050 (Danbury, CT, USA) at 315 nm with excitation at 275 nm. The detector signals were output to the Model 4416 data system (Nelson Analytical, Cupertino, CA, USA). Elemental microanalyses were performed by Schwarzkoff Microanalytical Laboratory (New York, NY, USA).

Synthesis of polymeric reagent

(3-Nitro-4-chloro)benzylated polystyrene I. To

IV

Ⓟ : Polystyrene

10 g of dried polystyrene copolymer [styrene–divinylbenzene (96:4), 10 g] were added 50 ml of nitrobenzene, 10 g (53.3 mmol) of 3-nitro-4-chlorobenzyl alcohol and 10 g (75 mmol) of anhydrous aluminum chloride. After stirring at 65–75°C for three days, the reaction was stopped by allowing the mixture to cool to room temperature. The polymer was filtered and washed with 1 M HCl in dioxane (3 × 50 ml), N,N-dimethylformamide (DMF) (3 × 50 ml), methanol (3 × 50 ml) and methylene chloride (3 × 50 ml), and finally dried under vacuum at 80°C. The dried polymer weighed 13.8 g. Elemental analyses of the polymer found 1.76 mmol chlorine and 1.9 mmol nitrogen per gram of dry solid, indicating that about 26% of the aromatic rings of the polymer were substituted.

1-Hydroxybenzotriazole-bound polystyrene III. To 7 g of polystyrene I were added 24 ml of hydrazine hydrate–ethylene glycol monoethyl ether 4:6, v/v). The mixture was refluxed for 20 h. After cooling to room temperature, the polymer was filtered, washed thoroughly with water, and dried. The polymer, 3-nitro-4-hydrazine-benzylated polystyrene II, was then suspended in 36 ml of concentrated HCl–dioxane (1:1, v/v) and refluxed for 20 h. The polymer was filtered, washed with water (5 × 35 ml), methanol (3 × 35 ml) and diethyl ether (3 × 18 ml), and dried in vacuum at 80°C. The light brown polystyrene III weighed 6.34 g. Elemental analysis found 4.93% nitrogen, corresponding to 1.17 mmol 1-hydroxybenzotriazole functional group/g of dry solid.

Polymeric FMOC-L-proline reagent IV. To 6 g (6.2 mmol hydroxy functional group) of polystyrene III was added a solution of 4.86 g (14.4 mmol) of FMOC-L-proline in 45 ml methylene chloride. After equilibration to 0°C, the mixture was stirred for an additional 5 min before 2.97 g (14.4 mmol) of DCC in 15 ml of methylene chloride was added. The mixture was stirred at 0°C for 20 min. The polymer was filtered, washed with methylene chloride (4 × 100 ml) and dry diethyl ether (2 × 50 ml), and dried in vacuum at room temperature. The yield was 6.7 g polystyrene IV, containing 0.25 mmol FMOC-L-proline per gram of dry solid.

Derivatization of amino acid with FMOC-L-prolinyl polystyrene

Amino acids (4 µl of a 1000-ppm solution in ace-

tonitrile were derivatized by addition to a suspension of FMOC-L-prolinyl polystyrene (20 mg) in 200 µl of acetonitrile. The reaction was allowed to proceed for 10 min at room temperature. After centrifugation, 20 µl of the upper solution were injected into the HPLC system.

RESULTS AND DISCUSSION

The representative derivatization reaction of FMOC-L-prolinyl polystyrene with a nucleophile is shown below:
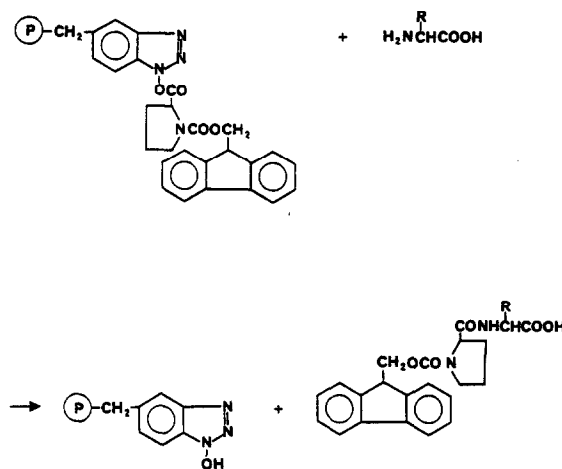
The acyl carbon in this polymer is highly reactive, due to the great electron-withdrawing inductive effect of the benzotriazole ring, that it is susceptible to attack by nucleophiles. The derivative can be used for conventional sensitivity enhancement by UV or fluorescence detection, or for chromatographic enhancement by forming diastereomers for chiral separation.

Reaction conditions

Since the benzotriazole FMOC-L-prolinyl polystyrene is highly reactive, the choice of appropriate solvent for supporting the derivatization reaction is critical. When methanol was used as the solvent for amines, rapid transesterfication of methanol was obseved as evident of the large methanol peak together with the amine peak. Methanol therefore was not an appropriate solvent for the derivatization reaction. DMF was a useful solvent for solubilizing certain amino acids, but was found to con-

tain too many impurities for a clean derivatization reaction. Acetonitrile due to its good swelling property and solvability, as reported by Gao et al. [10], was the best derivatization solvent, giving the highest yield with the smallest FMOC-L-proline reagent peak resulting from hydrolysis of the polymer. FMOC-L-proline peak was also observed when water, which also acts as a nucleophile in this case, is present in the sample or the solvent.

In chiral derivatization, racemization of one isomer to another under drastic reaction conditions is a major concern. Since the reaction conditions were mild and HOBT ester suppresed racemization, D-isomer was not detected when L-lysine was allowed to react with the polymeric reagent for up to 30 h (Fig. 1).

Chromatography of amino acids

By attaching a FMOC-L-proline group to free D,L-amino acids, diastereomeric acids are obtained. The diastereomeric pair in theory is chromatographically separable because of differences in chemical properties. When chromatographed under reversed-phase conditions using acetonitrile and water, enantiomeric resolution was not achieved. In the study of enantiomeric amines and amino alco-

hols, chiral separation was achieved only when organic mobile phases were used [15]. Apparently, these derivatives with multiple keto and amino groups are much better candidates for hydrogen-bonding interaction in normal-phase separation than reversed-phase partition chromatography.

In our previous study, ligand-exchange chromatography has proven to be a most effective approach for resolving amino acid isomers. We therefore attempted the separation of FMOC-L-prolinyl amino acids using mobile phases that contained chiral copper complexes. We used L-histidine methyl ester copper complexes, since it gave both excellent
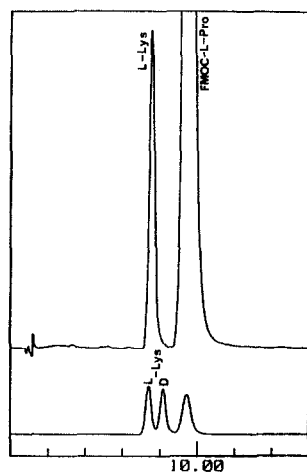

Fig. 1. Chromatogram of L-lysine after a 30-h reaction with FMOC-L-prolinyl polystyrene showing no D-isomer from racemization (upper tracing); D,L-lysine standards (lower tracing). Column: Nucleosil 5 C_{18}, 15 × 0.42 cm I.D. Mobile phase: 2.5 mM Cu(II)–L-histidine methyl ester complex and 2 g/l ammonium acetate in acetonitrile–water (25:75). pH 7.0. Flow-rate: 2.0 ml/min. Time in min.
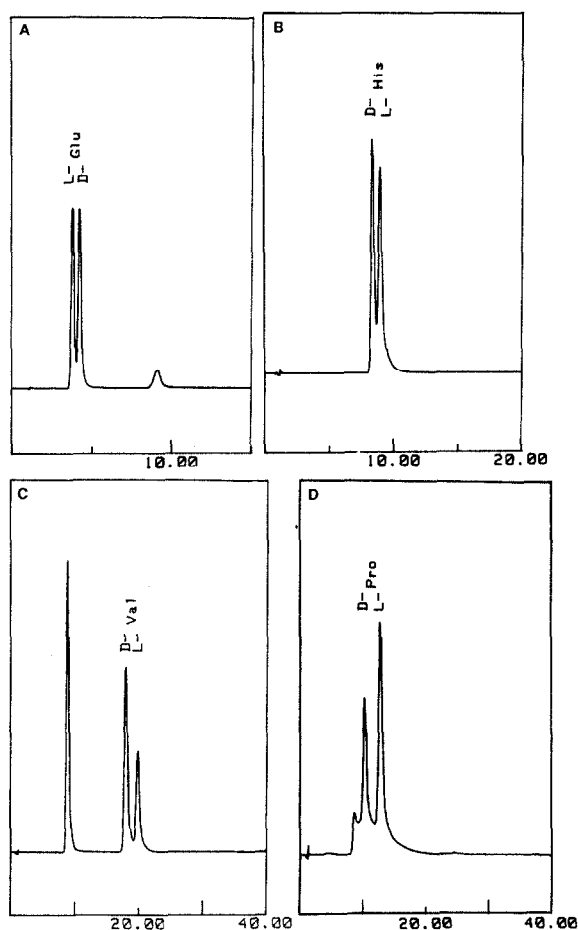

Fig. 2. Representation chromatogram of FMOC-L-prolinyl amino acids with different substituents. (A) Glutamic acid; (B) histidine; (C) valine; (D) proline. The peak at 9.5 min is FMOC-L-proline, the derivatizing agent. Conditions as in Fig. 1.

chiral and achiral selectivity in the separation of dansyl amino acids [2].

A mobile phase containing 2.5 mM Cu(II)–L-histidine methyl ester complex in acetonitrile–water (25:75) was used to effect the separation of FMOC-L-prolinyl amino acids (Fig. 2). As shown in Table I, all the amino acids are resolved into their enantiomeric pairs. FMOC-L-Prolinyl amino acids were similarly separated when 2.5 mM Cu(II)–L-proline was used. However, the capacity ratios were smaller than those observed in the histidine methyl ester system, reflecting the retention of a less hydrophobic mixed complex as a result of the contribution of a less hydrophobic system ligand (Table II). In agreement with the work on dansyl-amino acids, the higher the carbon content of the amino acids, the bulkier the alkyl substituent on the α-carbon, the longer the retention.

The stereoselectivity is dependent on the immediate micro-environment, affected by the substituent on the α-amino acids. Amino acids with a basic or acidic side-chain capable of participation in metal complexation showed reversed selectivity as the amino acids with aliphatic substituents. These observations suggest that the amino acids form glycine-like coordination with the system ligand, and the prolinyl substitution may contribute axial coordination in the mixed metal complex.

Proline, unlike the other amino acids, is an imino acid. The FMOC-L-prolinyl-proline does not possess a dissociable amine proton. Like dansyl-proline, it is not expected to form a glycine-like mixed complex with copper and give chiral separation. Instead proline is resolved with an exceptional large α value. The carbonyl group on the peptide linkages contributed by the FMOC-L-prolinyl moiety must be an important participant in metal coordination. The mechanism of metal complexation of the FMOC-L-prolinyl-amino acid is under investigation.

TABLE I

CAPACITY RATIO k' AND SELECTIVITY ($\alpha = k'_D/k'_L$) OF FMOC-PROLINYL AMINO ACIDS

Mobile phase: 2.5 mM Cu(II)–L-histidine methyl ester complex and 2 g/l ammonium acetate in acetonitrile–water. The acetonitrile concentration is indicated. pH 7.0. Flow-rate 2.0 ml/min.

Amino acid	25% acetonitrile			30% acetonitrile		
	k'_D	k'_L	α	k'_D	k'_L	α
Glu	3.40	2.94	0.86	1.06	0.93	0.87
Asp	4.34	4.24	0.98	1.33	1.33	1.00
Asn	6.81	6.53	0.96	2.20	2.11	0.96
Ser	7.40	8.08	1.09	2.63	2.87	1.09
Lys	7.46	6.66	0.89	2.44	2.22	0.91
Ala	7.87	8.81	1.12	2.93	3.26	1.11
Arg	8.24	7.51	0.91	2.62	2.47	0.94
Thr	9.10	9.57	1.05	3.27	3.38	1.03
Pro	9.53	12.08	1.27	2.38	2.38	1.00
His	9.75	11.09	1.14	3.56	4.05	1.14
Tyr	16.25	16.64	1.02	4.96	4.96	1.00
Val	17.64	19.55	1.11	5.74	6.27	1.09
Nval	18.22	20.56	1.13	5.98	6.65	1.11
Met	19.45	21.48	1.10	6.52	7.11	1.09
Ileu	28.45	30.81	1.08	9.93	10.73	1.08
Leu	33.58	37.34	1.11	9.84	10.91	1.11
Nleu	34.72	38.75	1.12	9.81	10.86	1.11
Phe	54.15	60.34	1.11	14.90	15.93	1.07
Trp	58.66	64.77	1.10	15.20	16.36	1.08

TABLE II

CAPACITY RATIO k' AND SELECTIVITY ($\alpha = k'_D/k'_L$) OF FMOC-PROLINYL AMINO ACIDS

Mobile phase: 2.5 mM Cu(II)–L-proline complex and 5 g/l ammonium acetate in acetonitrile–water. The acetonitrile concentration is indicated. pH 7.0. Flow-rate 2.0 ml/min.

Amino acid	25% acetonitrile			30% acetonitrile		
	k'_D	k'_L	α	k'_D	k'_L	α
Glu	2.89	2.46	0.85	0.88	0.77	0.88
Asp	2.97	2.82	0.95	0.87	0.87	1.00
Asn	6.60	6.40	0.90	2.03	2.03	1.00
Pro	6.68	9.98	1.49	2.09	2.59	1.24
Lys	6.97	6.14	0.88	2.14	1.95	0.91
Arg	7.92	7.18	0.90	2.32	2.32	1.00
Ser	8.20	8.20	1.00	2.47	2.47	1.00
Ala	8.87	10.04	1.13	3.22	3.22	1.00
His	9.05	9.81	1.08	2.99	2.99	1.00
Thr	9.84	10.33	1.05	3.04	3.04	1.00
Tyr	15.87	16.30	1.03	4.54	4.54	1.00
Val	17.53	19.84	1.13	5.23	5.82	1.11
Nval	18.63	21.25	1.14	5.40	6.08	1.13
Met	20.20	22.42	1.11	5.86	6.43	1.10
Ileu	27.60	30.37	1.10	7.65	8.34	1.09
Leu	31.57	35.15	1.11	8.33	9.27	1.11
Nleu	32.15	36.13	1.12	8.54	9.54	1.12
Phe	54.66	60.20	1.10	13.48	14.35	1.06
Trp	57.95	64.80	1.12	13.68	14.87	1.09

REFERENCES

1 H. Brückner and C. Keller-Hoehl, Chromatographia, 30 (1990) 621–629.
2 S. Lam, J. Chromatogr., 355 (1986) 157–164.
3 H. Brückner, R. Wittner and H. Godel, in G. Lubec and G. A. Rosenthal (Editors), Amino Acids: Chemistry, Biology and Medicine, ESCOM, Leiden, Netherlands, 1990, pp. 143–151.
4 M. Ahnoff and S. Einarsson, in W. J. Lough (Editor), Chiral Liquid Chromatography, Blackie, New York, 1989, p. 39.
5 R. Kalir, A. Warshawsky, M. Fridkin and A. Patchornik, Eur. J. Biochem., 59 (1975) 55–61.
6 B. J. Cohen, H. Karoly-Hafeli and A. Patchornik, J. Org. Chem., 49 (1984) 922–924.
7 Y. Shai, K. A. Jacobson and A. Patchornik, J. Am. Chem. Soc., 107 (1985) 4249–4252.

8 C.-X. Gao, T.-Y. Chóu, S. T. Colgan, I. S. Krull, C. Dorschel and B. Bidlingmeyer, J. Chromatogr. Sci., 26 (1988) 449–457.
9 T.-Y. Chou, C.-X. Gao, S. T. Colgan, I. S. Krull, C. Dorschel and B. Bidlingmeyer, J. Chromatogr., 454 (1988) 169–183.
10 C.-X. Gao, T.-Y. Chou and I. S. Krull, Anal. Chem., 61 (1989) 1538–1548.
11 A. J. Bourque and I. S. Krull, J. Chromatogr., 537 (1991) 123–152.
12 C.-X. Gao and I. S. Krull, J. Chromatogr., 515 (1990) 337–356.
13 A. J. Bourque and I. S. Krull, J. Chromatogr. Sci., 29 (1991) 489–495.
14 C.-X. Gao and I. S. Krull, J. Pharm. Biomed. Anal., 7 (1989) 1183–1189.
15 T.-Y. Chou, C.-X. Gao, N. Grinberg and I. S. Krull, Anal. Chem., 61 (1989) 1548–1558.