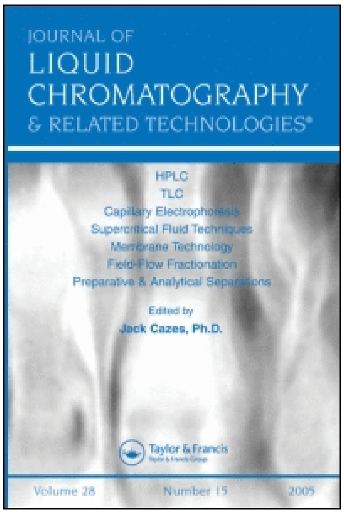

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HPLC Determination of Enantiomeric Purity of Protected Amino Acid Derivatives Used in Peptide Synthesis

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HPLC DETERMINATION OF ENANTIOMERIC PURITY OF PROTECTED AMINO ACID DERIVATIVES USED IN PEPTIDE SYNTHESIS

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ABSTRACT

An improved RP-HPLC method on ODS-Hypersil column with precolumn derivatization with Marfey's reagent were used to monitor racemization in N-, C- and/or side-chain protected amino acid derivatives by separation of a series of new diastereoisomeric Marfey's compounds. Chromatographic samples were obtained by partial deprotection of different starting materials. In a simple two-step procedure (deprotection and derivatization) the compounds of amino acids formed stable diastereomeric derivatives having facile resolutions.

INTRODUCTION

Synthetic, biologically active peptides should be very pure stereochemically. The therapeutic and biological applications require very high chiral (optical) purity from peptides to be investigated. Their synthesis also requires optically pure amino acid derivatives as starting material. Unfortunately, the racemization is one of the more common side reaction that occur during a peptide synthetic work. There is an increased demand for a method to detect and quantitate minor amounts of enantiomeric impurity in the N or C protected amino acids used to prepare these peptides. The up-to-date solid phase synthesis methods apply BOC or FMOC-chemistry (29, 30), so it became important to check the enantiomeric purity in intermediate amino acid derivatives as BOC and FMOC protected starting compounds. HPLC is the method most frequently used for this purpose. For enantiomer resolution of chiral amino acids or their derivatives, direct techniques can be applied, in which either chiral stationary phase (19-21) or chiral additives to the mobile phase are used (22-24). Derivatization procedures with chiral reagents have also been developed to produce diastereomeric derivatives, which can be separated by ordinary stationary phases and eluents. Using enantiomeric derivatization more favourable resolutions could be achieved (usually with higher α and R_S values), than applying very specific and not always available chiral columns.

After hydrolysis the chiral amino acid analysis also gives the possibility to check the mentioned intermediates. The chiral reagents used in amino acid analysis (for derivatization via amino or carboxyl group) include

2,3,4,6-tetraacetyl-D-glycopyranosyl isothyocyanate (6)
(-)-α-methoxy-α-methyl-1-naphthalene-acetic acid (7a)
(+)-1-aminoethyl-4-dimethylaminonaphthalene (7b)
(+)-1-(9-fluorenyl)-ethyl-chloroformate (8)

and some amino acid derivatives, such as the N-protected cysteine and o-phthalaldehyde (9, 13) or napthalenedicarbaldehyde (11) adduct, tert.-butoxycarbonyl-L-amino acid N-hydroxysuccinimide esters, N-carboxy-L-leucine anhydride (10, 18), D or L-O-(4-nitrobenzyl)tyrosine methylester (12) and Marfey's reagent (1) and its analogs: structurally related chiral variants of Sanger's reagent (14-15) and monohalo-s-triazine-L-alanine amide (16).

In the present paper we describe some applications of the separation method for new Marfey's derivatives in improved analyses of optical purity and racemization of amino acid derivatives.

MATERIALS

The amino acid derivatives studied (Table 1) were synthesized by the Research Group for Peptide Chemistry of the Hungarian Academy of Sciences, the Department of Organic Chemistry, Eötvös University, Budapest, the Chemical Works, G. Richter, Budapest, Fine Chemicals Co. Reanal, Budapest and the Institute of Medical Chemistry, Szent-Györgyi Albert Medical University of Szeged. The abbreviations used follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (4).

Amino Acid Derivatives Studied

BOC-Asn-OH
H-Asn-OH
BOC-Ser(Bzl-)OH
H-Ser(Bzl)-OH
Z-Asn-OMe
BOC-His(DNP)NH_2
BOC-Cys(Bzl)NH_2
BOC-Leu-NH_2

TABLE I

Chromatographic and Racemization Data of Marfey's Compounds of Amino Acid Derivatives

Amino acid (AA)	Derivatives	α-NH_2 compounds	k' L	k' D	α	D-amino acid %	RP-C18-HPLC Eluent (v/v)
Asn	BOC-Asn	Asn	1.5	5.1	3.4	0.38	MeOH-CH_3CN-0.02 M
		Asp$(OH)_2$	1.4	3.7	2.6	0.37	NaOAc (pH 4) 20:5:75
Glp	BOC-Glp-OH	Glu$(OH)_2$	2.2	5.9	2.7	19.95	20:5:75
	BOC-Glp-OPFP	Glu$(OH)_2$	2.2	5.9	2.7	0.39	20:5:75
Ser	BOC-Ser(Bzl)OH	Ser(Bzl)OH	3.0	7.4	2.47*	0.42	MeOH-0.02M NaOAc (pH 4)
		Ser(Bzl)OH	3.0	7.4	2.47	0.08	50:50
Thr	Thr(Bzl)OH	Thr(Bzl)OH	3.25	6.6	2.3	0.07	50:50
Arg	BOC-Arg(Tos)OH	Arg(Tos)OH	3.5	14.5	4.1	0.37	49.5:50.5
	FMOC-Arg(NO_2)OH	Arg(NO_2)OH	3.17	7.17	2.2	49.50	20:5:75
His	BOC-His(DNP)NH_2	His(DNP)NH_2	2.0	7.5	3.75	0.75	50:50
		His(DNP)	1.8	6.2	3.4	0.73	50:50
Cys	BOC-Cys(Bzl)NH_2	Cys(Bzl)NH_2	7.0	16.8	2.4	1.51	50:50
		Cys(Bzl)	5.8	14.1	2.4	1.54	50:50
	Cys(Acm)	Cys(Acm)	2.71	5.80	2.07	0.83	20:5:75
		Tin-OH	5.57	10.14	1.8	0.80	20:5:75
Leu	BOC-Leu-NH_2	LeuNH_2	7.1	22.5	3.2	44.0	25:10:60
		LeuOH	21.1	45.4	2.1	43.94	20:10:70
Asn	Z-Asn-OMe	Asp$(OH)_2$	1.4	3.7	2.6	0.74	20:5:75
Thr	BOC-Thr(Bzl)OH	Thr(Bzl)OH	3.25	6.6	2.3	0.18	50:50
HomoArg	BOC-HomoArg(OH)	HomoArg(OH)	11.3	4.10	3.6	41.91	20:7:73
Tin	BOC-Tin-OH	Tin-OH	5.57	10.14	1.8	0.40	20:5:75
Kpc	Kpc-OBu^t	α-AA-OH	3.46	9.76	2.5	0.71	20:5:75
Gla	Z-Gla$(OBu^t)_2$OH	Glu$(OH)_2$	2.2	5.9	2.7	0.85	20:5:75
		Gla$(OBu^t)_2$	5.75	8.75	1.52	0.9	60:40

*Using a hydroxypropyl derivatized β-cyclodextrin bonded phase column, α = 1.13 (20).

BOC-Thr(Bzl)-OH

H-Thr-(Bzl)-OH

BOC-Arg(Tos)-OH

FMOC-Arg(NO_2)-OH

KpC-OH (6-keto)-pipecoline-2-carboxylic acid)

KpC-NH_2

H-Cys(AcM)-OH

KpC-OBut,

4-thiazolidine-carboxylic acid

BOC-Tin-OH

KpCOPfp

Z-Gla(OBu^t)$_2$-OH

BOC-Homo-Arg-OH

Boc-Cys(Acm)-OH

Ac-Glp-OH.DCHA

Glp-OPcp

BOC-Glp-OH.DCHA

Glp-OPfp

Z-Glp-OH.DCHA

BOC-Glp-OPfp

Marfey's reagent was prepared by J. Horváth (Dept. Org. Chem., Eötvös University).

Abbreviations:

BOC - tert.-butoxycarbonyl; Tin - thiazolidine-carboxylic acid; Gla - γ-carboxy-glutamic-acid; FMOC - fluorenyl methoxycarbonyl; Kpc - ketopipecolic acid, Z - benzyloxycarbonyl; HomoArg - homoarginine; Bzl - benzyl; DNP - 2,4-dinitrophenyl; OBu^t - tert-butyl; AcM - acetamidomethyl.

METHODS

High-Performance Liquid Chromatography

Separations were performed on a laboratory-assembled instrument consisting of a reciprocating piston pump (Model 1515, Orlita, Giessen, F.R.G.), a variable-wavelength UV monitor fitted to an 8 μl flow-cell (Model 212, Cecil, Cambridge, U.K.) and a sample injector; Rheodyne, Berkeley, CA, U.S.A.), or on a Knauer HPLC system consisting of two pumps Model 64 with analytical pumphead, a gradient programmer Model 50B, an injection valve with 20 µl sample loop and a spectral photometer with analytical flow cell (Knauer GmbH, Bad Homburg, FRG).

Column effluents were monitored at 340 nm (for Marfey's derivatives). The packing materials were ODS-Hypersil-ODS-6, MOS-Hypersil-6 (Shandon Southern Products, Runcorn, U.K.) and Nucleosil C-18-5 (Macherey Nagel, Düren, FRG). Peaks were recorded on a Model OH-314/1 chart recorder (Radelkis, Hungary) and the areas under them were calculated using programmed Simpson's rule. The chromatographs were operated isocratically with flow rates between 0.8 and 2.0 cm^3/min.

Deprotection of amino acid derivatives

a) Hydrolysis

Amino acid derivatives were subjected to acidic hydrolysis. The samples were treated with propionic-acid-6M HCl mixtures (1:1, v/v), or TFA-6M HCl mixtures (1:1, v/v) for 1-24 hours in sealed tubes at 105°C or some minutes (1-4) in a microwave staff. Volatile acids were removed in vacuo, samples were neutralized by $NaHCO_3$ and the acid-free hydrolysates were derivatized with Marfey's reagent.

b) BOC-derivatives (30)

1 mg of BOC-amino acid derivative was dissolved in 0.5 ml TFA. After 1 hr standing at room temperature the reaction was checked by TLC, the acid was removed in vacuo, and the acid-free hydrolysate was derivatized further.

c) FMOC-derivatives (29)

1 mg of FMOC-amino acid derivative was dissolved in 1 ml of 25% piperidine/DMF. After 1 hr standing at room temperature, the reaction was checked by TLC, the solution was evaporated and lyophilized with water.

d) Z-derivatives

1 mg of Z-amino acid derivative was deprotected by catalytic transfer hydrogenation (31-36). 10% Pd/C and 100 µl saturated NH_4 formate in MeOH were added to its solution in 200 µl MeOH. After 10 minutes the deprotection was checked by TLC (silica gel, n-BuOH-pyridine-acetic acid-water 60:20:6:24 v/v). In order to eliminate NH_4 formate, the evaporated substance was twice lyophilized from water.

Derivatization (1-5)

Derivatization was carried out according to Marfey (2) with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Pierce, Rockford, IL. U.S.A.). The hydrolysate or deprotected compound prepared from 2-5 µmol of starting material was dissolved in 100 µl of 0.5 M $NaHCO_3$ solution (if it's necessary: 50 µl MeOH, DMF or DMSO are used at first and diisopropyl-ethylamine serves as base) and 200 µl of a 1% solution of Marfey's reagent in acetone was added. The solution was incubated at 40°C for 90 min, cooled and 25 µl of 2 M HCl was added. After to 20-fold dilution in methanol or eluent, 10-20 µl aliquots were used for HPLC injection.

RESULTS AND DISCUSSION

We have previously shown (2) that the rates of racemization in peptides can be measured by conventional HPLC using pre-column derivatization with enantiomerically very pure 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) for amino acids after hydrolysis of starting peptides (1). Marfey's reagent reacts with the optical isomers of amino acids without kinetic fractionation and racemization to form stable diastereomeric N-aryl derivatives which can be separated by RP-HPLC. The derivatives have an absorption maximum at 340 nm with an extinction coefficient of $\sim 3.10^4$, and thus can be detected with high sensitivity by UV spectroscopy, the diasteromers give identical detector response (2). Recently the HPLC separation of these derivatives has been described for all common amino acids (1-4, 37).

For rapid, accurate determination of the optical purity of the synthesized amino acid derivatives the rates of racemization were planned to be mesaured by conventional HPLC using pre-column derivatization with Marfey's reagent. In order to apply the original method elaborated for all common acids (1-4, 37), the amino acid derivatives should be hydrolyzed.

According to the acidic hydrolysis free amino acids were formed from all derivatives: X-AA-OR $\xrightarrow[H_2O]{H^{\oplus}}$ H-AA-OH

eg.: BOC-Asn-OH → Asp

BOC-Ser(OBzl)OH → Ser

Z-Gla(OBu^t)OH → Glu

BOC-Glp-OH → Glu

Kpc-OH → α-aminoadipic acid

BOC-Cys(Acm) → 4-thiazolidine-carboxylic acid

So, their determination were reduced to free amino acid measurements.

However, the hydrolysis step is also a potential source of racemization so that this step must be carefully monitored. All amino acid residues undergo some racemization during acid hydrolysis (25, 26, 3). Aspartic acid appears to be the most sensitive amino acid during acidic treatment (26, 3).

Thus, a "background" racemization must be taken into account in the exact determination of racemization rates in amino acid derivatives to be hydrolyzed.

Conventional amino acid analysis utilizes azeotropic hydrochloric acid (6 M) at 110°C for 24 hours for hydrolysis (38). We, instead, made use of microwave radiation for sample hydrolysis (27, 28). Rapid hydrolysis was achieved by using a commercial microwave oven as heating source. It was not only possible to hydrolyze amino acid derivatives quantitatively within 1 min, but also with substantially less racemization (Table II). The values are in good correlation with Chen's data (39).

Because of high "background" values we have improved the method with racemization-free, very simple deprotection steps (well-known in peptide chemistry) liberating α-amino group of the derivatives.

In the case of N-protected amino acid derivatives the N-protecting groups were quantitatively removed at first:

$$\text{X-AA-OR} \xrightarrow{-X} \text{H-AA-OR} \xrightarrow{MR} \text{diastereomeric derivatives}$$

X = FMOC, BOC, Z-

R = H, Me, Et, Bzl, (pNO_2)Bzl, Bu^t, NH_2, NH-R.

TABLE II

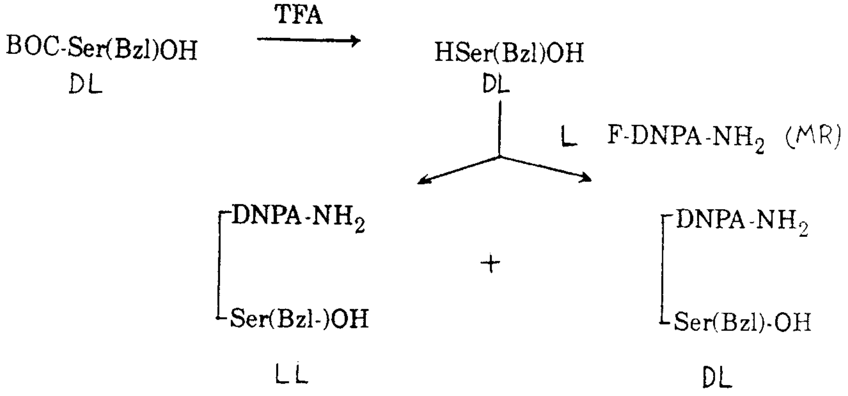
Optical Behaviour of L-Amino Acids During Acid Treatment

AA	D-enantiomer content % and time of treatment				
	105°C		microwave radiation		
	1 h	24 h	10 sec	20 sec	60 sec
Asp	1.68	11.8	0.55	0.94	1.40
Glu	0.80	2.65	0.24	0.32	0.77

The C-protected amino acid derivatives (chiefly esters) react directly via arylation with Marfey's reagent without preparation problems. Quantitative reactions were achieved after optimization (solvent pH, reaction time), using a molar reagent excess of approximately 1,4-1,6 or greater.

Our modification of the original procedure requires the separation of a series of new Marfey's derivatives, in which the amino acid residues are modified with other protecting groups at the C-terminal and in the side chains. These groups modify the chromatographic behaviour of the Marfey's derivatives according to their hydrophobic or hydrophilic character. So, new eluent systems should be elaborated (see Table I).

The BOC-amino acid derivatives were deprotected quantitatively and selectively at amino group by trifluoroacetic acid treatment (checked by RP-HPLC and NP-TLC). Derivatization was carried out according to Marfey:



The FMOC α-amino protecting groups were removed quantitatively by piperidinolysis. The Z groups were splitted quantitatively from α-amino groups by catalytic transfer hydrogenation. The chromatographic data of new Marfey's derivatives are summarized in Table I. It was concluded, that the all new diastereomeric compounds could be separated well on RP-columns. The chromatographic conditions were optimized to achieve large separation coefficients (α) (see Table I), and baseline separations with methanol-Na-acetate buffer (pH 4) or methanol-acetonitrile-Na-acetate buffer eluent systems, isocratically. It seems that the L-diastereomers are usually eluted before the D-isomers, and the protecting groups of ester and amide type increase the retention times because of their high hydrophobic characters (Bzl, Et, Me, OBu^t, NH_2, NH-R, etc.). So the eluent systems contain more organic modifiers (CH_3OH or CH_3CN) as usually (see e.g. $Gla(OBu^t)_2$).

The protecting groups (NO_2, Bu^t, Bzl, DNP, Tos) of side chain protected amino acid derivatives have a similar effect on the retention.

Fig.1. demonstrates the separation of D and L-Ser-OBzl-Marfey's derivatives.

Racemization of amino acid derivatives could be determined by measuring quantitatively the D-amino acid (or derivative) content of the sample, or directly the D/L compound ratio. Thus, the optical purity of amino acids used in peptide syntheses were checked very precisely: since the detection limit was about 5-8 pmol of an amino acid enantiomer, 0.02-0.05% of the racemization could be determined accurately.

Racemization rate depends on the preparation methods, of course.

It seems from Table I, that BOC-Leu-NH_2, FMOC-Arg(NO_2)OH and BOC-Homo-Arg(OH) were strongly racemized (>80%) during their synthesis because of strong basic reaction conditions. The other compounds contain only minimum quantities of D-amino acids, so their enantiomeric purity is suitable enough for peptide synthesis.

On the other hand especially important is the question of the enantiomeric purity of N-protected amino acids after activation of the C-terminal carboxyl group with peptide coupling reagents. Coupling of the carboxyl activated N-protected amino acid with an amino components can lead to epimeric products because of racemization (40).

Our improved method was applied to check the racemization level of an efficient, new amidation method of protected amino acids, when N-tert-butoxycarbonyl derivatives of L-leucine, S-benzyl-cysteine and N-(2,4-dinitrophenyl)-L-histidine were amidated with crystalline ammonium salts of 3-hydroxy-1.2.3-benztriazin-4(3H)-one and N-hydroxysuccinimide to give after activation the corresponding amino acid amides in good yields (5). Table I shows (Cys and His amides),

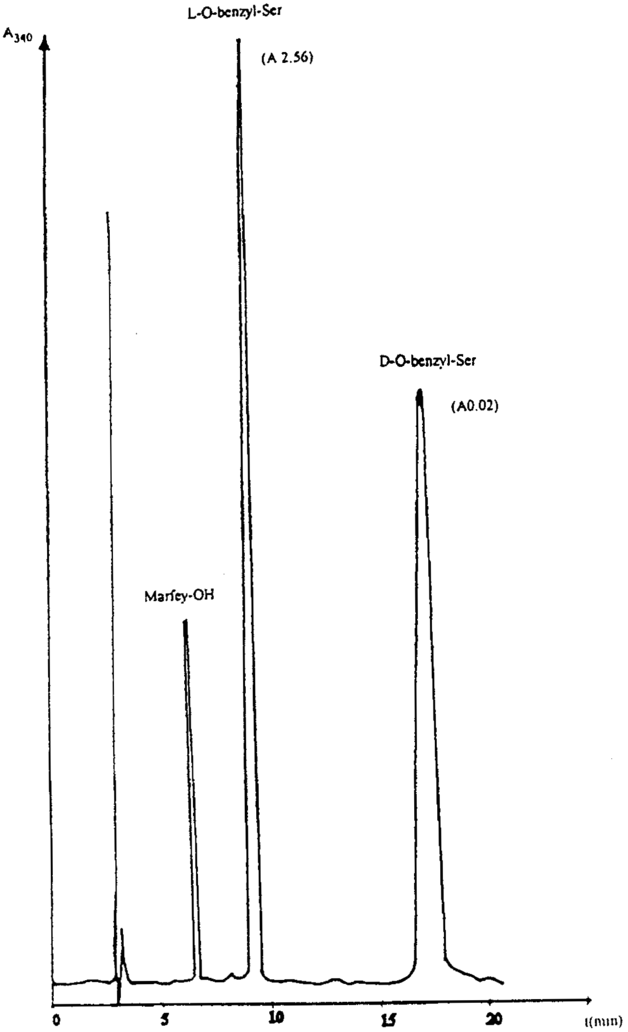


FIGURE 1. Chromatographic pattern of racemization of O-benzyl-L-serine (Fine Chemicals, REANAL, Hungary)
Column: Nucleosil C18-5 μm. Eluent: methanol - 0.02 M sodium acetate buffer (pH 4) 50:50 (v/v). Flow rate: 1.0 mL/min. Detection: 340 nm. Derivatization: 5 mmol. D-isomer content: 0.34%.

that only minimal quantities of D-amino acids could be detected using the novel amidation reagents. The endproducts have high enantiomeric purity.

The method can be used for determination of L-impurities in D-compounds, of course. Our data were supported by optical rotation measurements, too. If we took into account the enantiomer-contents as corrections, the optical rotation data of the samples became identical with the literature ones (e.g. in the case of Z-Gla(OBu^t)$_2$).

In order to validate this technique, it was found, that the values of D isomer content obtained after hydrolysis and deprotection were practically identical in the case of BOC-Asn, BOC-Leu-NH$_2$, BOC-Cys(Bzl)NH$_2$ and BOC-His(DNP)NH$_2$ (see Table I).

It seems from the recent papers, that the popularity of Marfey's reagent is increasing further in the laboratories of peptide chemists (41, 42).

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REFERENCES

1. P. Marfey, Carlsberg Res. Commun. 49: 591-596 (1984)
2. Gy. Szókán, G. Mező, F. Hudecz, J. Chromatogr. 444: 115-122 (1988)

3. Gy. Szókán, G. Mező, F. Hudecz, Zs. Majer, I. Schön, O. Nyéki, T. Szirtes, R. Dölling, J. Liquid. Chrom. 12: 2855-2875 (1989)

4. Gy. Szókán, G. Mező, F. Hudecz, in Peptides, B. Penke, A. Török, eds., Walter de Gruyter, Berlin, 1988, pp. 83-86.

5. Cs. Somlai, Gy. Szókán, L. Baláspiri, Synthesis, 285-287 (1992)

6. N. Nimura, H. Ogura, T. Kinoshita, J. Chromatogr. 202: 375 (1980)

7a. J. Goto, M. Hasegawa, S. Nakamura, K. Shimada, T. Nambara, J. Chromatogr. 152: 413-419 (1978)

7b. T. Nambara, S. Ikegawa, M. Hasegawa, J. Goto, Anal. Chim. Acta, 101: 111-118 (1978)

8. S. Einarsson, B. Josefsson, P. Möller, D. Sanchez, Anal. Chem. 59: 1191-1198 (1987)

9. R.H. Buck, K. Krummen, J. Chromatogr. 315: 279-285 (1984)

10. A.R. Mitchell, S.B.H. Kent, I.C. Chu, R.B. Merrifield, Anal. Chem. 50: 637-640 (1978)

11. T. Takaya, Y. Kishida, S. Sakakibara, J. Chromatogr. 215: 249-256 (1981)

12. S. Görög, B. Herényi, M. Löw, J. Chromatogr. 353: 417-424 (1986)

13. H. Brückner, R. Wittner, H. Godel, Chromatographia, 32: 383-388 (1991)

14. H. Brückner, C. Keller-Hoehl, Chromatographia 30: 621-629 (1990)

15. H. Brückner, C. Gah, J. Chromatogr. 555: 81-89 (1991)

16. H. Brückner, B. Strecker, J. Chromatogr. 627: 97-105 (1992)

17. IUPAC-IUB Commission on Biochemical Nomenclature, J. Biol. Chem. 247: 977-983 (1972)

18. W.R. Cahill, Jr., E.P. Kroeff, D.J. Pietrzyk, J. Liquid Chromatogr. 3: 1319-1334 (1980)

19. W.H. Pirkle, J.E. McCune, J. Chromatogr. 479: 419-423 (1986)

20. S.C. Chang, L.R. Wang, D.W. Armstrong, J. Liquid Chromatogr. 15: 1411-1429 (1992)

21. G. Gübitz, F. Juffmann, W. Jellenz, Chromatographia, 16: 103-106 (1982)

22. S. Weinstein, Trends in Anal. Chem. 3: 16-20 (1984)

23. N. Nimura, A. Toyama, Y. Kasahara, T. Kinoshita, J. Chromatogr. 239: 671-675 (1982)

24. F. Federici, A.M. Girelli, A. Messina, I. Nicoletti, M. Sinibaldi, Anal. Letters, 23: 1565-1549 (1990)

25. A. Neuberger, Adv. Protein Chem. 4: 339-385 (1948)

26. P.J.M. van der Oetaelaar, J.R.C.M. van Beckhoven, H.J. Hoenders, J. Chromatogr. 388: 441-448 (1987)

27. L.B. Fischer, Anal. Chem. 58: 261-268 (1986)

28. S.T. Chen, S.H. Chiou, Y.H. Chu, K.T. Wang, Int. J. Peptide Protein Res. 30: 572-576 (1987)

29. E. Atherton, R.C. Sheppard, Solid Phase Peptide Synthesis, IRL Press at Oxford Univ. Press, 1989.

30. J.M. Stewart, J.D. Young, Solid Phase Peptide Synthesis, Pierce Chem. Co., Rockford, Illinois, USA, 1984.

31. S.A. Khan, K.M. Sivanandaiah, Synthesis, 750-751 (1978).

32. K.M. Sivanandaiah, S. Gurusiddappa, J. Chem. Research (S) 108-109 (1979)

33. A.M. Felix, E.P. Heimer, T.J. Lambros, C. Tzougraki, J. Meienhofer, J. Org. Chem. 43: 4194-4196 (1978)

34. M.K. Anwer, S.A. Khan, K.M. Sivanandaia, Synthesis, 751-752 (1978)

35. M.K. Anwer, A.F. Spatola, C.D. Bossinger, E. Flanigan, R.C. Lin, D.B. Olsen, D. Stephenson, J. Org. Chem. 48: 3503-3509 (1983)

36. M.K. Anwer, A.F. Spatola, Synthesis, 929-932 (1980)

37. S. Kochbar, P. Christen, Anal. Biochem. 178: 17-21 (1989)

38. D.H. Spackman, W.H. Stein, S. Moore, Anal. Chem. 30: 1190-1215 (1958)

39. S.-T. Chen, S.-H. Wu, K.-T. Wang, Int. J. Peptide Protein Res. 33: 73-75 (1989)

40. D.S. Kemp in The Peptides, E. Gross, J. Meienhofer, eds. Vol. I. Acad. Press, New York, 1979 pp. 317-383.

41. A. Scaloni, M. Simmaco, F. Bossa, Anal. Biochem. 197: 305-310 (1991)

42. J.G. Asamson, T. Hoang, A. Crivici, G.A. Lajoie, Anal. Biochem. 202: 210-214 (1992)

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