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High-performance liquid chromatographic separation of DL-amino acids derivatized with chiral variants of Sanger's reagent[a]

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ABSTRACT

Substitution of one fluorine atom in 1,5-difluoro-2,4-dinitrobenzene by L-alanine amide yields N^2-(5-fluoro-2,4-dinitrophenyl)-L-alanine amide (FDNP-Ala-NH_2, "Marfey's reagent"). This reagent (or analogues, termed FDNP reagents, in which Ala is replaced with other chiral α-amino acids (AAs), oligopeptides or amino components) might be considered as a chiral variant of "Sanger's reagent" (1-fluoro-2,4-dinitrobenzene). The remaining fluorine atom in FDNP reagents can be substituted by chiral AAs to furnish diastereomeric derivatives which are separable by high-performance liquid chromatography (HPLC). A number of chiral variants of Sanger's reagent have been synthesized with the general structures (a) FDNP-Val-NHR (R = H, *tert.*-butyl, chiral aralkyl, phenyl, *p*-nitrophenyl), (b) FDNP-Val-OR (R = H, CH_3, *tert.*-butyl), (c) FDNP-$(Ala)_n$-NH_2, $n = 1,2$, and (d) FDNP-NHR (R = aralkyl and hydroxyalkyl), and their ability to resolve certain DL-AAs as diastereomers in reversed-phase HPLC was investigated. Reagents with the structures (a), (b) and (c), but not (d), made possible the separation of DL-AAs as diastereomers. From the results obtained and from observations of Corey–Pauling–Koltun (CPK) space-filling molecular models of the diastereomers, it is concluded that the AA side-chains and the carboxy and carboxamide substituents in the reagents have an influence on the retention times of diastereomers and that the differences in free energy as a result of the formation of an intramolecular hydrogen bridge in the L–L and non-formation in the D–L diastereomers (first letter refers to the configuration of the amino acid to be analysed) are in particular responsible for large differences in the retention times of certain diastereomers in HPLC.

INTRODUCTION

In 1945, Sanger [1] reported that 1-fluoro-2,4-dinitrobenzene (FDNB, later called "Sanger's reagent") was a highly suitable compound for the masking of free amino groups in insulin and that the respective dinitrophenyl amino acids (DNP-AAs) released under the conditions of acidic total hydrolysis were separable chromatographically and could be identified by comparison with authentic samples of the

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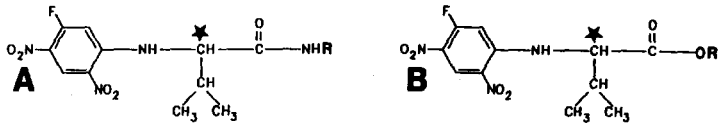
DNP-AAs. Continuation of this work finally led to the sequence determination of insulin [2], the first sequence of a protein to be elucidated. Encouraged by this work, many attempts were undertaken in the following years to increase the sensitivity of the reagent and to alter the polarity by synthesizing substituted fluorodinitrobenzenes [3–5].

Zahn [6] and Zahn and Meienhofer [7] were the first to use a bifunctional variation of Sanger's reagent, namely 1,5-difluoro-2,4-dinitrobenzene (DFDNB), for the cross-linking of proteins such as wool, silk and insulin. DFDNB was later also used, *e.g.*, for the intramolecular cross-linking of bovine pancreatic ribonuclease A [8] and basic myelin protein [9]. It is noteworthy that Zahn and Meienhofer [7] prepared by reaction of DFDNB with AA esters monofunctional, bifunctional and mixed bifunctional derivatives which were investigated by paper chromatography. They also reported on attempts to separate certain diastereomeric derivatives by fractionated crystallization [7].

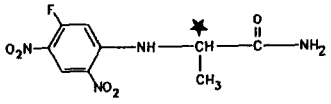
Marfey [10] showed that monosubstitution of DFDNB by L-alanine amide yielded a chiral variant of Sanger's reagent, namely 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide {N^2-(5-fluoro-2,4-dinitrophenyl)-L-alanine amide [11], FDNP-Ala-NH_2; *cf.*, Fig. 1i}. The remaining reactive fluorine atom in this reagent was susceptible to nucleophilic substitution by DL-AAs to furnish the respective diastereomeric derivatives which could be separated by high-performance liquid chromatography (HPLC). This reagent, referred to as "Marfey's reagent" in this paper, has been used for the separation of (3*R*)- and (3*S*)-β-leucine [11], in racemization studies of AAs and peptides [12,13], for the determination of the enantiomeric error frequency of aspartase aminotransferase via quantification of the racemization of L- and D-glutamate [14] and for the HPLC resolution of a mixture of twenty L-AAs [15].

We have used this reagent for the HPLC separation of diastereomers of non-proteinogenic α-hydroxymethyl-α-amino acids and α-alkyl-α-amino acids (AAAs) [16,17], which were also separable as diastereomeric esters by gas–liquid chromatography [18]. It was found that the diastereomers of AAAs formed with Marfey's reagent, like those of proteinogenic AAs, showed exceptionally large differences in retention times (Δt_R) in HPLC in comparison with other methods such as the formation of diastereomers by derivatization with *o*-phthaldialdehyde and chiral thiols [19,20].

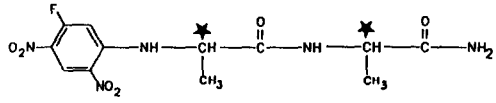
In continuation of the above work, we have recently synthesized a series of new, systematically modified chiral variants of Sanger's reagent by substitution of one fluorine atom of 1,5-difluoro-2,4-dinitrobenzene with selected L-AA amides (*viz.*, H-Val-NH_2, H-Phe-NH_2 and H-Pro-NH_2) and showed that FDNP-Val-NH_2 in particular gave large Δt_R values for diastereomers of many DL-AAs [21]. With the aims of determining the structural parameters responsible for the large Δt_R values of AA diastereomers and to design new reagents for the optimum separation of certain AAs, we now report the syntheses of a number of substituted FDNP-Val amides, FDNP-Val esters, FDNP-amines and an FDNP-dipeptide amide (*cf.*, Fig. 1) and the HPLC investigation of the diastereomers formed by reaction of these novel FDNP reagents with selected DL-AAs.



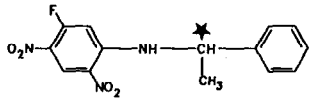
Reagent	R
(a) FDNP-Val-NH_2	-H
(b) FDNP-Val-NHtBu	$-C(CH_3)_3$
(c) FDNP-Val-PEA	$-CH(CH_3)-C_6H_5$
(d) FDNP-Val-AN	$-C_6H_5$
(e) FDNP-Val-pNA	$-C_6H_4-NO_2$
(f) FDNP-Val-OH	-H
(g) FDNP-Val-OMe	$-CH_3$
(h) FDNP-Val-OtBu	$-C(CH_3)_3$



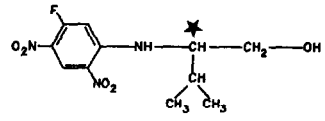
(i) FDNP-Ala-NH_2



(j) FDNP-Ala-Ala-NH_2



(k) FDNP-PEA



(l) FDNP-valol

Fig. 1. Formulae of FDNP reagents of the structure (A) FDNP-Val-NHR and (B) FDNP-Val-OR, residues R in (a)–(e) FDNP-Val-NHR and (f)–(h) FDNP-Val-OR, and structures of (i) FDNP-Ala-NH_2, (j) FDNP-Ala-Ala-NH_2, (k) FDNP-PEA and (l) FDNP-valol. Asterisks indicate chiral centres in reagents; letters (a)–(l) in parentheses correspond to those used in Fig. 2 and Table I.

EXPERIMENTAL

Instruments

A Jasco (Kyoto, Japan) HPLC system was used, consisting of a Model 880 PU single-head pump with active damper, a Model 880-02 low-pressure gradient unit, a Model 801-SC system controller, a Model 875-UV variable-wavelength spectroscopic detector and a Model D-2000 integrator from Merck–Hitachi (Darmstadt, Germany).

Sample injection was carried out with a Rheodyne (Cotati, CA, USA) Model 7125 injection valve equipped with a 20-μl sample loop.

Amino acids and chemicals

DL- and L-amino acids were purchased from either Sigma (St. Louis, MO, USA) or Fluka (Buchs, Switzerland); *tert.*-butyloxycarbonyl-L-valine hydroxysuccinimide ester (Boc-Val-OSu), L-alanyl-L-alanine amide hydrochloride (H-Ala-Ala-NH_2 · HCl), L-valine *p*-nitroanilide (H-Val-pNA), L-valine *tert.*-butyl ester hydrochloride (H-Val-OtBu · HCl) and L-valine *tert.*-butylamide hydrochloride (H-Val-NHtBu · HCl) were from Bachem (Bubendorf, Switzerland); N-methyl-DL-valine (NMe-Val-OH) was from Sigma; 1,5-difluoro-2,4 dinitrobenzene (DFDNB or FFDNB) was from Fluka, N^2-(5-fluoro-2,4-dinitrophenyl)-L-alanine amide (Marfey's reagent) was synthesized in our laboratory [21] and is also obtainable from Pierce (Rockford, IL, USA), Serva (Heidelberg, Germany) or Sigma; dimethyl sulphoxide (DMSO), trifluoroacetic acid (TFA), acetonitrile (ACN), methanol (CH_3OH), dichloromethane (DCM), light petroleum (b.p. 40–60°C), diethyl ether, N-methylmorpholine (NMM), N,N-dimethylformamide (DMF) and orthophosphoric acid (85%) of analytical-reagent grade were from Merck; triethylamine (puriss.) and L-valinol (valol) were from Fluka, (S)(−)-phenylethylamine (PEA) was from Aldrich (Steinheim, Germany) and aniline (AN) was from Sigma; DL-valine methyl or isopropyl ester [H-Val-OMe (iPrp)] were synthesized by reaction of 3 mg of DL-Val with 500 μl of 2 *M* hydrochloric acid in methanol (or isopropanol) for 1 h at 100°C.

Stationary phase and preparation of eluents for HPLC

The column (250 × 4 mm I.D.) was packed with Spherisorb ODS 2, 5 μm (Shandon, Queensferry, UK) and the precolumn (4 × 4 mm I.D.) with Microspher 100 RP-18, 5 μm (Merck).

For gradient elution, a linear gradient from 0 to 100% B in 45 min was used for separations; flow-rate, 1 ml/min at ambient temperature; absorbance (*A*), 340 nm; the mobile phase hold-up time t_0 (CH_3OH) was 1.75 min under these conditions. To prepare triethylammonium phosphate buffer (TEAP), 6.93 ml (50 m*M*) of triethylamine were dissolved in 950 ml doubly distilled water, the pH was adjusted to 3 by addition of orthophosphoric acid (85%, *ca.* 4 ml) and the volume was made up to 1 l by addition of doubly distilled water. For eluent A, 900 ml of TEAP were mixed with 100 ml of ACN; for eluent B, 200 ml of TEAP were mixed with 800 ml of ACN. In one instance (*cf.*, Fig. 2f, upper trace), the TEAP buffer was adjusted to pH 2; in another instance (*cf.*, Fig. 2c, upper trace), gradient elution was performed under the following conditions: Eluent A, 13 m*M* aqueous TFA (900 ml) and ACN (100 ml); eluent B, 13 m*M* aqueous TFA (200 ml) and ACN (800 ml); linear gradient from 0 to

100% B in 45 min; flow-rate 1 ml/min; t_0 (CH_3OH), 1.85 min. Buffers were filtered prior to use (Millipore HVLPO 4700 filter, pore size 0.45 μm) and degassed by sonification for *ca.* 15 min (Eurosonic Cobra 44 sonicator).

Corey–Pauling–Koltun (CPK) molecular models were obtained from Ealing Beck (Watford, UK).

Characterization of chiral variants of Sanger's reagent

All FDNP reagents (for syntheses see below) were found to be pure by HPLC and by thin-layer chromatography (TLC) using precoated plates (Kieselgel 60 F_{254}; Merck) and were characterized by their electron impact (EI) mass spectra (MS), usually at 70 eV, using a Varian 311 A mass spectrometer. Melting points were determined on a Büchi (Flawil, Switzerland) Model B 520 melting point apparatus. R_F values were determined at 24°C on TLC plates in glass chambers (Desaga, Heidelberg, Germany) coated with filter-paper; the distance from the start to the front on the TLC plates was 10 cm. The solvent systems were as follows (v/v): (I) ethyl acetate–light petroleum–methanol–acetic acid (30:70:14:9); (II), acetone–ethyl acetate–*n*-hexane (10:10:20); (III), chloroform–*n*-hexane–acetic acid (80:20:20). UV spectra were determined by photodiode-array detection and HPLC using instruments described elsewere [19].

Syntheses and characterization of reagents

The general procedure was as follows. Under protection from light, DFDNB was dissolved in acetone and distilled water was added; 0.5–0.7 equiv. of the amino component were dissolved in 0.6 *M* $NaHCO_3$ (in subsequent text, $NaHCO_3$ refers to this concentration unless stated otherwise) and the solution was slowly added dropwise (*ca.* 30 min) at room temperature (RT) to the vigorously stirred solution of DFDNB and heated for 1–2 h at 40–50°C (see below.) In the course of the reaction the products precipitated either as solids or oils or remained in solution. Acetone was removed *in vacuo* and the remaining aqueous phase was diluted by addition of water. Precipitated solids were isolated by filtration, washed several times with water and dried in an desiccator over P_2O_5 and KOH pellets *in vacuo* (*ca.* 0.02 mmHg). When oily products formed in the course of the reaction or after addition of water the supernatant was discarded, the oil was dissolved in ethyl acetate (EA) and the organic phase was washed three times each with about half of the volume of aqueous $NaHCO_3$ (5%), $KHSO_4$ (5%) and water. The organic phase was dried over anhydrous Na_2SO_4, evaporated to dryness and the residue was precipitated or crystallized from organic solvents (see below.) In those instances where the product remained in solution after addition of water the reaction mixture was extracted three times with EA and the combined extracts were washed and treated as described above. Reagents and solutions were stored at 4°C and protected from light. The reagents synthesized were pure according to TLC and HPLC and were characterized as described below.

Individual syntheses of reagents are given below; for structures of reagents, see Fig. 1; for syntheses of FDNP-Ala-NH_2 and FDNP-Val-NH_2 see ref. 21. FDNP-Val-NH_2 is obtainable form Novabiochem (Läufelfingen, Switzerland) on request; letters (a)–(l) in parentheses for the synthetic compounds correspond to the designations of the FDNP reagents in Table I and Fig. 2; AAs and derivatives are abbre-

viated as usual in peptide chemistry [22], consequently FDNP is considered as an amino blocking group. Syntheses were not optimized with respect to highest yields.

(a) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-valine amide (FDNP-Val-NH_2). For syntheses, see ref. 20.

(b) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-valine tert.-butylamide (FDNP-Val-NHtBu). A 122.5 mg (0.6 mmol) amount of DFDNB was dissolved in 2 ml of acetone and 0.5 ml of water, 62.7 mg (0.3 mmol) of H-Val-L-NHtBu · HCl in 2 ml of $NaHCO_3$ were added and the mixture was stirred for 18 h at 40°C. On addition of 10 ml of water a yellow oil precipitated, which was then treated according to the general procedure above. The remaining yellow oil yielded an amorphous powder after being stirring with light petroleum. Yield, 73 mg (68.3%); m.p. 103°C; R_F (I) 0.80, R_F (II) 0.62, R_F (III) 0.84; UV (nm), 335, 215, 263; MS, *m/z* 357 (MH^+), 256, int. (M − $COOCMe_3$), 284/285 (M − $OCMe_3$).

(c) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-valine phenylethylamide (FDNP-Val-PEA). To 816.4 mg (4 mmol) of DFDNB in 30 ml of acetone, 10 ml of water and 896 mg (2.7 mmol) of H-Val-PEA · TFA (n) in 20 ml of $NaHCO_3$ were added dropwise; (c) started to precipitate during the addition. After 1 h at 40°C, 60 ml of water were added, the precipitate was filtered and washed as described under the general procedure above. Yield, 978 mg (89.6%); m.p. 189°C; R_F (I) 0.79, R_F (II) 0.58, R_F (III) 0.85; UV (nm), 213, 334, 263; MS, *m/z* 405 (MH^+), 257, int. (M − $CONHCH(Me)C_6H_5$ + H].

(d) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-valine anilide (FDNP-Val-AN). A 408.2 mg (2 mmol) amount of DFDNB was dissolved in 10 ml of acetone and 4 ml of water and 420 mg (1.4 mmol) of H-Val-AN · TFA (p) in 10 ml of $NaHCO_3$ were added. An orange oil formed immediately. After 1 h at 40°C the mixture was treated as described under the general procedure above, yielding a dark orange oil which slowly crystallized. The product was dissolved in CH_3OH and recrystallized by addition of diethyl ether and light petroleum. Yield, 504 mg (95.6%); m.p. 156°C; R_F (I) 0.79, R_F (II) 0.58, R_F (III) 0.79; UV (nm), 237, 333, 264 (shoulder); MS, *m/z* 376 (M^+), 257, int. (M − $CONHC_6H_5$ + H).

(e) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-valine p-nitroanilide (FDNP-Val-pNA). To 122.5 mg (0.6 mmol) of DFDNB in 2 ml of acetone and 0.5 ml of water a suspension of 71.2 mg (0.3 mmol) of H-Val-pNA in 2 ml of $NaHCO_3$ was added with stirring and the mixture was heated for 3 h at 40°C. A yellow oil was formed which crystallized after being stirred overnight. The fine crystal needles were collected by centrifugation. Yield, 98 mg (77.5%); m.p. 224°C; R_F (I) 0.77, R_F (II) 0.51, R_F (III) 0.56; UV (nm), 329, 226; 266; MS, *m/z* 422 (MH^+), 256 (M − $CONHC_6H_5NO_2$).

(f) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-valine (FDNP-Val-OH). A 1.0 g (2.8 mmol) amount of FDNP-Val-OtBu was dissolved in 15 ml of DCM, 15 ml of TFA were added and the solution was stirred at RT for 90 min. Solvents were removed *in vacuo*, the residue was dissolved in diethyl ether and the product was precipitated by addition of light petroleum. Yield, 666 mg (79%); m.p. 129°C; UV (nm), 338, 216, 264; R_F (I) 0.62, R_F (III) 0.59; MS, *m/z* 301 (MH^+), 256, int. (M − COOH).

(g) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-valine methyl ester (FDNP-Val-OMe). To 20 mg (0.07 mmol) of FDNP-Val-OH in 200 µl of CH_3OH, 4 ml of a solution of diazomethane in diethyl ether were added and the solvents were removed after 10 min in a stream of nitrogen; completeness of reaction was checked by TLC. R_F (I) 0.80; R_F (II) 0.65; MS, *m/z* 316 (MH^+), 255 (M − COOMe).

(h) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-valine tert.-butyl ester (FDNP-Val-O-tBu). A 682 mg (3.27 mmol) amount of H-Val-OtBu · HCl was dissolved in 10 ml of $NaHCO_3$ and 0.36 ml (3.27 mmol) of NMM in 3 ml of $NaHCO_3$ and 1.33 g (6.5 mmol) of DFDNB in 45 ml of acetone were added. The product precipitated as an oil. The mixture was stirred for 18 h at 40°C and the oil was treated as described under the general procedure above. The dark yellow oil obtained was dissolved in 5 ml of acetone and the product was precipitated by dropwise addition of water. Yield, 1.09 g (93.3%) (yellow solid); m.p., 82°C; R_F (I) 0.85, R_F (II) 0.72; UV (nm), 330, 263, 225; MS, *m/z* 357 (M^+), 302 (M − CMe_3), 285 (M − $OCMe_3$), 255, int. [M − COOC $(Me)_3$].

(i) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-alanine amide (FDNP-Ala-NH_2). See refs. 10 and 21.

(j) N^2-(5-Fluoro-2,4-dinitrophenyl)-L-alanyl-L-alanine amide (FDNP-Ala-Ala-NH_2). A 122.5 mg (0.6 mmol) amount of DFDNB was dissolved in 2 ml of acetone and 0.5 ml of water and 58.7 mg (0.35 mmol) of H-Ala-Ala-NH_2 · HCl in 2 ml of $NaHCO_3$ were added. The mixture was heated for 1 h at 40°C, 10 ml of distilled water were added and the crystalline precipitate was treated as described under the general procedure above. Yield, 83.3 mg (69.4%); m.p. >244°C (decomp.); R_F (I) 0.53, R_F (II) 0.06, R_F (III) 0.15.; UV (nm), 336, 212, 264; MS, *m/z* 344 (MH^+), 300 (M − $CONH_2$), 228, int. (M − $CONHCH(CH_3)$-$CONH_2$.

(k) N^2-(5-Fluoro-2,4-dinitrophenyl)-(S)(−)-phenylethylamide (FDNP-PEA). A 408.2 mg (2 mmol) amount of DFDNB was dissolved in 5 ml of acetone, mixed with 15 ml of $NaHCO_3$ and 129 µl (1 mmol) of (*S*)(−)-phenylethylamine in 10 ml acetone were added to the suspension. After 20 h at RT the solution was treated as described under the general procedure above, the resultant yellow residue was dissolved in ethanol and the product was precipitated by addition of light petroleum ether. Yield, 199 mg (65.2%), amorphous powder; m.p. >290°C (decomp.); R_F (I) 0.59; UV (nm), 345, 232, 257; MS, *m/z* 305 (M^+), 228 (M − C_6H_5), 202, int. (M − $CHCH_3C_6H_5$ + 2H).

(l) N-(5-Fluoro-2,4-dinitrophenyl)-L-valinol (FDNP-valol). To 408.2 mg (2 mmol) of DFDNB in 15 ml of acetone and 15 ml of $NaHCO_3$ a solution of 103.2 mg (1 mmol) of L-valinol in 10 ml of $NaHCO_3$ were added and the mixture was heated for 1 h at 40°C. Solvents were removed *in vacuo*, the residue was dissolved in ethanol and the compound was crystallized by addition of light petroleum. Yield, 164 mg (57.1%); m.p. >200°C (decomp.); R_F (I) 0.59, R_F (III) 0.83; UV (nm), 343, 227, 258; MS, *m/z* 256, int. (M − CH_2OH).

(m) N^2-tert.-Butyloxycarbonyl-L-valine phenylethyl amide (Boc-Val-PEA). To 943 mg (3 mmol) of Boc-Val-OSu in 10 ml of ACN, 774 µl (6 mmol) of PEA in 3 ml of ACN were added. After 20 h at RT the solvents were removed *in vacuo*, EA was added and the organic phase was washed as described under the general procedure above. Yield, 884 mg (92%) (white crystals). The product was used without further characterization for the synthesis of (n).

(n) L-Valine phenylethylamide trifluoroacetate (H-Val-PEA · TFA). A 938 mg (2.93 mmol) amount of Boc-Val-PEA (m) was dissolved in 5 ml of DCM and 15 ml of TFA were added. The solvent was removed *in vacuo* and the oily residue was stirred with diethyl ether and light petroleum. The solvents were removed *in vacuo*, leaving a white foam. Yield, 941 mg (96%); the product was used for the synthesis of (c).

(o) N^2-tert.-Butyloxycarbonyl-L-valine 5-fluoro-2,4-dinitroanilide (Boc-Val-AN). To 943 (3 mmol) of Boc-Val-OSu in 20 ml of ACN, 1.70 g (18.3 mmol) of aniline in 3 ml of ACN were added and the mixture was heated for 20 h at 40°C. The solvents were removed *in vacuo*, EA was added and the organic phase was washed as described under the general procedure above. As the material obtained after removal of EA contained unreacted Boc-Val-OSu according to TLC, it was treated with a mixture of 50 ml of aqueous Na_2CO_3 (10%) and 25 ml of CH_3OH for 1 h at RT. EA was added and the organic phase was washed as described under the general procedure above. Yield, 447 mg (51%). The product was used for the synthesis of (p).

(p) L-Valine anilide trifluoroacetate (H-Val-AN · TFA). To 482 mg (1.65 mmol) of Boc-Val-AN (o) in 5 ml of DCM, 10 ml TFA were added and the solvents were removed *in vacuo* after 30 min at RT. The oily residue was dissolved in diethyl ether, light petroleum was added and the mixture was stirred overnight at RT. Yield, 463 mg (91.7%) (white crystals); the product was used for the synthesis of (d).

Preparation of stock solutions and standards

Chiral reagents. Solutions (100 m*M*) of chiral reagents in acetone were prepared with the exceptions of FDNP-Ala-NH_2, FDNP-Ala-Ala-NH_2 and FDNP-Val-PEA, for which 33.3 m*M* solutions in acetone were used.

DL-amino acids. Standards of 200 m*M* AA in 1 *M* HCl were prepared.

Derivatization of DL-amino acids with chiral reagents

A volume of 25 µl (5µmol) of DL-amino acid standard solution, 25 µl of 1 *M* $NaHCO_3$, 50 µl (or 150 µl in the cases of 33.3 m*M* reagent solutions) (5 µmol) of reagent solutions and further 40 µl of 1 *M* $NaHCO_3$ were heated for 1 h at 40°C, then 20 µl of 2 *M* HCl were added and the mixture was dried for 18 h *in vacuo* over KOH–P_2O_5. The residue was dissolved in 1 ml of DMSO, filtered (Anotop 0.2-µm filter, Merck) and a 1-µl aliquot was used for HPLC (this corresponds to 2.5 nmol of the respective diastereomers calculated for a 100% derivatization yield).

RESULTS AND DISCUSSION

Derivatization of DL-amino acids with FDNP-Val-NHR and FDNP-Val-OR

From the Δt_R values of diastereomers obtained by reaction of DL-AAs with reagents of the structure FDNP-L-AA-NH_2 (AA = Ala, Val, Phe, Pro) [21], it was concluded that exchange of further AAs in FDNP-AA amides would not lead to reagents giving rise to substantially larger Δt_R values for DL-AAs.

As FDNP-Val-NH_2 gave the largest Δt_R value for many diastereomers of DL-AAs [21], substituted FDNP-Val amides and esters of the structures FDNP-Val-NHR (R = *tert.*-butyl; H_2NR = phenylethylamine, aniline, *p*-nitroaniline), FDNP-L-Val-OR (R = H, methyl, *tert.*-butyl) and FDNP-L-Ala-L-Ala-NH_2 were synthesized. Furthermore, FDNP reagents were synthesized by substitution of one fluorine atom of DFDNB with the chiral amines (*S*)(−)-phenylethylamine and L-valinol (Fig. 1.).

The reagents were used for the derivatization of selected DL-AAs, *viz.*, DL-Val (branched, neutral side-chain), Ser (neutral side-chain with hetereoatom), Glu (trifunctional, acidic AA) and Lys (trifunctional, basic AA). Net retention times, t'_R, and

Δt_R values calculated therefrom, determined under same gradient conditions by HPLC, were compared with those of FDNP-Val-NH_2 and FDNP-Ala-NH_2 as shown in Table I; sections of the chromatograms of the diastereomers of DL-Val (which are best resolved in most cases) are shown in Fig. 2a–h. The HPLC investigation of the diastereomers formed by reaction of selected analyte DL-AAs with FDNP reagents (*cf.*, Table I) is discussed below.

Resolution of DL-Val

By derivatization of DL-Val with reagents (a)–(j) (Table I), all diastereomers formed were baseline resolved with Δt_R values from 1.36 to 8.78 min. The largest Δt_R values were obtained by derivatization with FDNP-Val-OH and FDNP-Val-NH_2 and the smallest by derivatization with FDNP-Val-NHtBu and FDNP-Val-OtBu. Derivatization with FDNP-Val-OtBu and FDNP-Val-PEA gave rise to intense reagent peaks in the chromatograms, indicating that the reactions are less complete in comparison with the other reagents as a result of steric hindrance. Kinetic discrimination is also a feasible explanation that the peak areas of certain diastereomers are not equal (*cf.*, Fig. 2). In several instances, such as derivatization of DL-Val with FDNP-Val-PEA and FDNP-Val-pNA, the peak of the hydrolysed reagent and one of the diastereomers formed eluted together under the conditions used (Fig. 2c, lower trace, and Fig. 2e, respectively). Diastereomers and reagent were separated, however, using a TFA gradient (Fig. 2c, upper trace; for conditions see Experimental). Derivatization of DL-Val with FDNP-Val-OH resulted in the elution of the diastereomer formed with D-Val (but not with L-Val) as a very broad and asymmetric peak in HPLC using the TEAP pH 3 buffer (Fig. 2f, lower trace). The peak symmetry was improved considerably by adjusting the buffer to pH 2, probably as a result of the protonation of carboxy groups (Fig. 2f, upper trace). For a discussion of the resolution mechanisms, it is crucial that diastereomers of DL-Ala, DL-Val, DL-Ser and DL-Glu, obtained by derivatization with FDNP-PEA, and FDNP-valol, were not resolved under the HPLC conditions used (for HPLC of diastereomers of these reagents formed with DL-Val, see Fig. 2k–l).

Resolution of DL-Glu

Baseline separation of DL-Glu was achieved for diastereomers formed by reactions with most reagents, with the exception of FDNP-Val-OH, FDNP-Val-OMe and FDNP-Val-OtBu, which gave unsatisfactory resolution under the HPLC conditions used. With the exception of FDNP-Val-NHtBu, FDNP-Val-pNA and FDNP-Ala-Ala-NH_2, intense peaks of hydrolysed reagents appeared in the chromatograms, indicating low derivatization yields for the other reagents.

Resolution of DL-Ser

Baseline separation of DL-Ser was possible by formation of the diastereomers obtained with FDNP-Ala-NH_2 and FDNP-Val-NH_2, and almost beaseline separation occurred by derivatization with FDNP-Val-OH and FDNP-Val-pNA. Unsatisfactory resolution was obtained by derivatization with FDNP-Val-OMe and FDNP-Val-PEA, and no or only very slight separation was obtained with FDNP-Val-NHtBu, FDNP-Ala-Ala-NH_2, FDNP-Val-AN and FDNP-Val-OtBu. Relatively low derivatization yields were realized with the use of FDNP-Val-OtBu, FDNP-Val-PEA

TABLE I

NET RETENTION TIMES OF FIRST ($t'_{R(L-L)}$) AND SECOND ($t'_{R(D-L)}$) ELUTED DIASTEREOMER FORMED BY REACTION OF DL-Val, DL-Glu, DL-Ser, AND DL-Lys WITH CHIRAL FDNP REAGENTS, AND DIFFERENCES IN ELUTION TIMES (Δt_R).

For monosubstituted DL-Lys net retention times $t'_{R1,2,3}$ are given. Experiments were carried out under same gradient elution conditions in HPLC (*cf.*, Experimental). mono., di. = Mono- and disubstituted Lys, respectively.

FDNP reagent[b]	Retention time (min)														
	Analyte amino acid														
	DL-Val			DL-Glu			DL-Ser			DL-Lys (mono.)[a]			DL-Lys (di.)		
	$t'_{R(L-L)}$	$t'_{R(D-L)}$	Δt_R	$t'_{R(L-L)}$	$t'_{R(D-L)}$	Δt_R	$t'_{R(L-L)}$	$t'_{R(D-L)}$	Δt_R	t'_{R_1}	t'_{R_2}	t'_{R_3}	$t'_{R(L-L)}$	$t'_{R(D-L)}$	Δt_R
(a) FDNP-Val-NH_2	19.31	24.47	5.16	15.80	16.92	1.12	14.94	15.72	0.78	12.89	13.03	14.57	27.62	29.45	1.83
(b) FDNP-Val-NHtBu	33.33	34.96	1.63	22.08	22.94	0.86	21.98	21.98	0.00	17.64	17.98	19.04	41.57	42.13	0.56
(c) FDNP-Val-PEA	33.91	36.13	2.22	24.31	25.18	0.87	23.81	24.13	0.32	19.79	20.75	–	42.35	42.94	0.59
(d) FDNP-Val-AN	33.73	35.99	2.26	23.75	25.80	2.05	24.09	24.31	0.22	19.25	20.04	20.46	41.32	42.68	1.36
(e) FDNP-Val-pNA	34.13	37.00	2.87	25.52	27.81	2.29	24.79	26.48	1.69	21.01	21.60	22.16	40.57	42.91	2.34
(f) FDNP-Val-OH	22.68	31.46	8.78	17.73	18.08	0.35	16.63	17.49	0.86	13.88	15.67	–	29.99	32.18	2.19
(g) FDNP-Val-OMe	33.02	34.95	1.93	21.87	22.05	0.18	21.42	21.85	0.43	17.49	17.68	18.69	–	–	–
(h) FDNP-Val-OtBu	40.29	41.65	1.36	29.66	29.95	0.29	30.40	30.58	0.18	22.96	23.75	–	49.94	50.93	0.99
(i) FDNP-Ala-NH_2	17.49	20.27	2.78	12.57	13.68	1.11	11.15	11.81	0.66	9.86	11.36	–	18.57	19.73	1.16
(j) FDNP-Ala-Ala-NH_2	16.76	18.49	1.73	12.50	13.67	1.17	11.37	11.31	0.00	10.00	11.60	–	20.62	21.76	1.14
(k) FDNP-PEA[c]	22.35	22.35	0.00												
(l) FDNP-valol[c]	23.07	23.07	0.00												

[a] Δt_R not calculated.

[b] Amino acids in reagents are of the L-configuration, for abbreviations and structures, *cf.*, Experimental and Fig. 1, respectively.

[c] Δt_R = 0.00 for DL-Glu, DL-Ser, DL-Lys and DL-Ala; – = t'_{R_3} not found under conditions used; t_0 (CH_3OH) 1.75 min under HPLC conditions used (*cf.*, Experimental); first letter in diastereomers L–L and D–L, respectively, refers to the configuration of the amino acid to be analyzed, second to that of the amino acid in FDNP reagent.

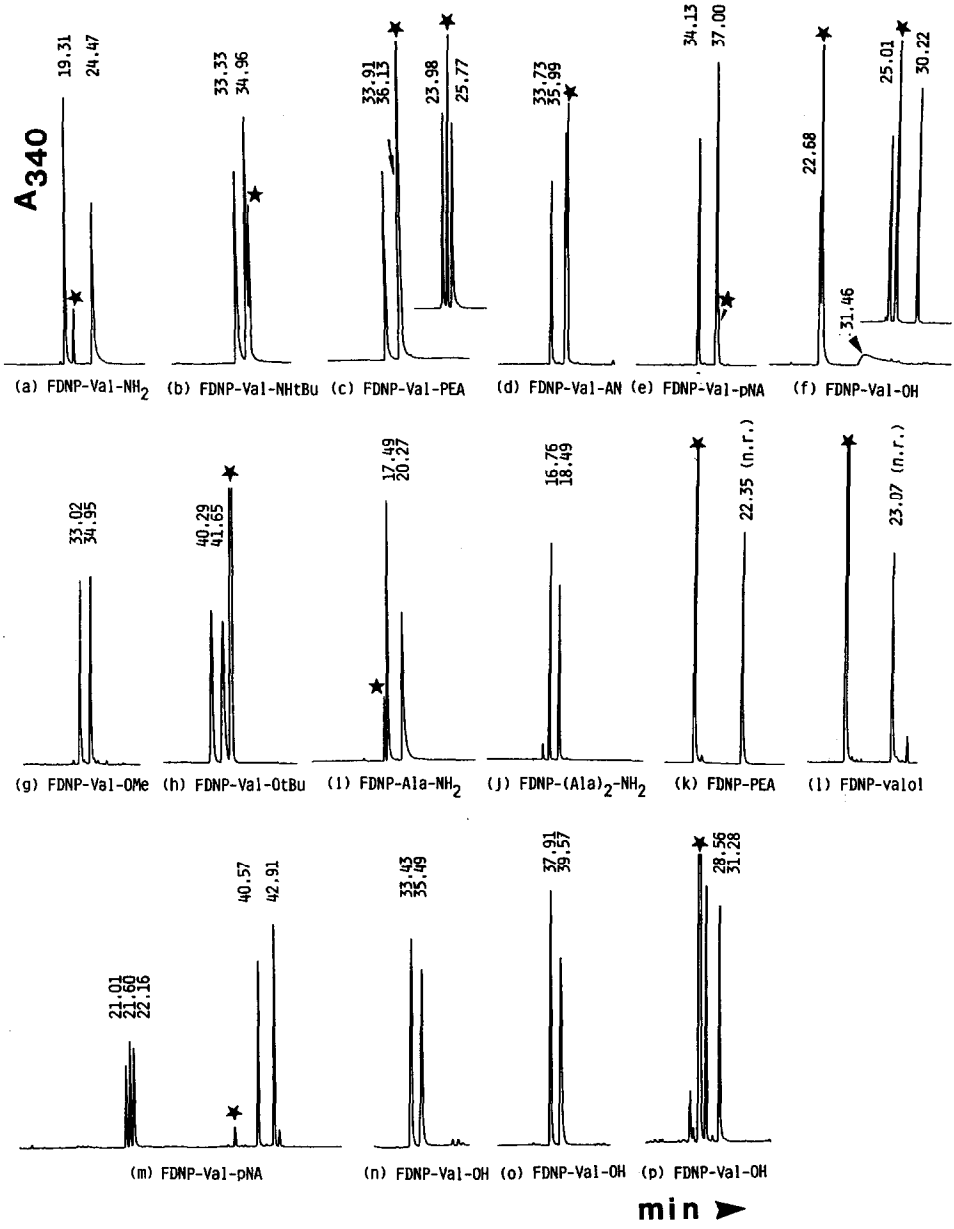


Fig. 2. Sections of chromatograms of the HPLC separation of diastereomers obtained by reaction of (a)–(l) FDNP reagents (*cf.*, Table I) with DL-Val, (m) FDNP-Val-pNA with DL-Lys and of (n)–(p) FDNP-Val-OH with (n) DL-Val-OMe, (o) DL-Val-iPrp and (p) NMe-DL-Val-OH. Net retention times, t'_R, of diastereomers are given; diastereomers of (k)–(l) are not resolved (n.r.); asterisks indicate peaks of hydrolysed reagents which are shown in chromatograms if eluted close to diastereomers. Chromatograms were taken under same standard gradient elution conditions (*cf.*, Experimental); upper traces in (c) and (f) show elution with TEAP pH 2 buffer and TFA gradient, respectively (for details, see Experimental). For structures of reagents, see Fig. 1.

and FDNP-Val-AN, as indicated by intense peaks of hydrolysed reagent in the chromatograms.

Diastereomers formed by derivatization of DL-Ser are eluted much earlier than those of DL-Val (*cf.*, Table I). This is explained by the higher polarity (hydrophilicity) of the respective derivatives; the shorter retention times might also contribute to smaller Δt_R values.

Derivatization of DL-Lys

As Lys has reactive ε- and α-amino groups, the formation of mono- and disubstituted derivatization products is, in principle, possible. The respective diastereomers are baseline separated by application of FDNP reagents (a)–(j) (Table I; FDNP-Val-OMe tested only in a less than equivalent amount yielded monosubstituted Lys).

It is of interest that derivatization of DL-Lys with certain FDNP reagents (*cf.*, Table I) gives rise to three signals at relatively low retention times in the chromatograms, assigned to monosubstitution products, and to two signals at relatively higher retention times, assumed to be disubstituted products [12] (in addition, peaks of hydrolysed reagents are seen in the chromatograms; see derivatization of DL-Lys with FDNP-Val-pNA as an example, Fig. 2m).

With monosubstitution products, the formation of four diastereomers from DL-Lys is possible, *viz.*, L–L_α, D–L_α, L–L_ε and D–L_ε (the first letter refers to the configuration of the analyte AA and the second letter to the substitution position α or ε in Lys by the reagent L-AA).

With disubstitution products, the formation of two diastereomers from DL-Lys is possible, *viz.*, L–L_α–L_ε and D–L_α–L_ε. Derivatization of either L- or D-Lys with the reagents gives two signals of monosubstitution products at relatively lower retention times. It is feasible, therefore to assume that the monosubstitution products are the unresolved diastereomeric pairs obtained by reaction of the reagents with the α- and ε-amino group, respectively, of Lys giving rise to two signals in the chromatogram. In instances where three peaks of monosubstitution products are detected in the chromatograms, one diastereomeric pair is separated and the other is not (*cf.*, Fig. 2m).

That FDNP reagents are, in principle, also suitable for the separation of DL-AA esters and N-methyl-DL-AAs is demonstrated by the resolution of diastereomers obtained by derivatization of H-DL-Val-OMe(iPrp) (Fig. 2n and o) and NMe-DL-Val-OH (Fig. 2p) with FDNP-Val-OH.

Model for resolution of diastereomers

From the results presented it is obvious that, in principle, all DL-AAs are separable as the diastereomers formed by reaction with reagents of the general structures FDNP-AA-NHR [AA = chiral AA or oligopeptides such as Ala-Ala; R = H, alkyl such as *tert.*-butyl (tBu), aralkyl such as phenylethyl, phenyl, *p*-nitrophenyl] and by derivatization with FDNP-AA-OR (R = tBu, CH_3, iPr). However, no separation of DL-AAs is achieved by derivatization with reagents of the structure FDNP-NHR (R = chiral hydroxyalkyl or chiral aralkyl).

Marfey [10] found that after derivatization of DL-AAs with FDNP-Ala-NH_2 the L-AAs (*i.e.*, L–L diastereomers) are eluted before D-AAs (*i.e.*, D–L diastereomers; the first letter refers to the configuration of the amino acid to be analysed and the

second to the L-configuration of the AA amide). He postulated that the carboxy group in the diastereomers of the respective D-AAs forms stronger intramolecular hydrogen bridges to the carboxamide group than do L-AAs. The twelve-membered ring in the respective D–L diastereomer thus formed was assumed to be more hydrophobic than the L–L diastereomer. The more hydrophobic molecule was supposed to interact more strongly with the reversed-phase, leading to higher retention times for D–L diastereomers as compared with L–L diastereomers.

From space filling Corey–Pauling–Koltun (CPK) molecular models of diastereomers (Fig. 3), however, and from experimental results, the following conclusions on the resolution mechanisms of diastereomers by RP-HPLC are drawn.

(1) It is assumed that the dinitrobenzene ring forms a stable, planar conformation. CPK models show that in L–L diastereomers the carboxy group of the analyte L-AA, and not of the D-AA, is positioned very near to the carboxamide group of the reagent L-AA amide, thus probably forming hydrogen bridges. Models also show that the formation of such an intramolecular hydrogen bridge is not possible to such an extent in the case of the D–L diastereomers (Fig. 3). This will count for an enthalpy difference of *ca.* 4 kcal/mol of diastereomers.

(2) CPK models also show that C^{β}-branching of AA side-chains, as in the case of Val, in the diastereomers further reduce the (in the case of Ala already very low) conformational freedom of the diastereomers formed. The formation (non-formation) of intramolecular hydrogen bridges of the L–L (D–L) diastereomer as pointed out above, however, is influenced to a minor extent by the structures of AA side-chains.

(3) The models also demonstrate that all reagents with the formulae FDNP-L-

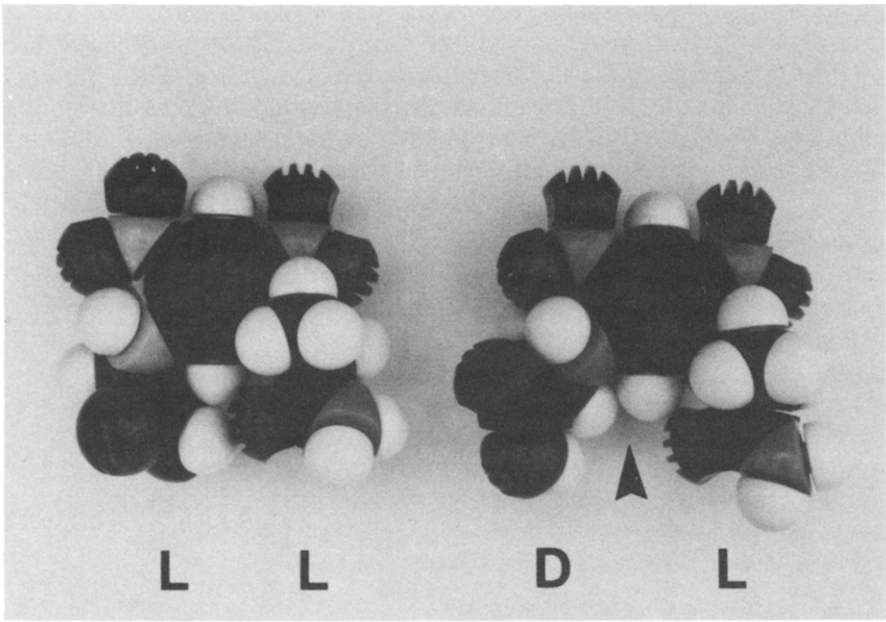


Fig. 3. Corey–Pauling–Koltun (CPK) molecular models of L–L and D–L diastereomers obtained by derivatization of L- and D-Ala with FDNP-L-Ala-NH_2. The arrow indicates the gap in the model of the D–L diastereomer.

AA-NHR, FDNP-L-AA-OR or FDNP-L-Ala-Ala-NH_2 have resolution capability as diastereomers formed by reaction with DL-AAs, as in all instances an intramolecular hydrogen bridge can be formed for the L–L diastereomer. This is not the case with reagents with the structures FDNP-NHR. The moiety R, however, alters the electronegativity of the carboxy group in CONHR and COOR residues and also the hydrophobicities of diastereomers. The model also explains that large Δt_R values are obtained by diastereomers formed by reaction of H-L-Val-OH with FDNP-L-Val-OH, since in the resulting L–L diastereomer the carboxy groups of amino acids can form hydrogen bridges to each other.

(4) The non-hydrogen-bonded, free carboxy group of the D-AA in the D–L diastereomer and the less symmetrical form of the molecule as a result of the gap in the D–L diastereomer as compared with the hydrogen-bridged, more compact L–L diastereomer (*cf.*, Fig. 3) might also explain the elution order of the respective diastereomers in HPLC. In comparison with the L–L diastereomer, the D–L diastereomer is assumed to interact more strongly with the alkyl chains of the reversed phase. It should be mentioned, however, that a CPK model is also constructable in which an intramolecular hydrogen bond is formed in the D–L diastereomer from the carboxy group of the analyte D-amino acid to the *ortho*-situated nitro group. In this instance, nevertheless, the less symmetrical shape and the gap in the D–L diastereomer remain.

In conclusion, and taking the proposed resolution mechanisms into account, under optimized separation conditions some of the new FDNP reagents might be of interest for the specific resolution of certain amino acids using the diastereomeric approach. This is also shown with the separation of DL-Val esters and NMe-DL-Val-OH by derivatization with FDNP-Val-OH (Fig. 2n–p). It is also realized that the reverse approach, *i.e.*, derivatization of DL-AA derivatives with the structures H-AA-NHR and H-AA-OR with reagents with the structures FDNP-AA-OH and subsequent HPLC separation of diastereomers is possible.

REFERENCES

1 F. Sanger, *Biochem. J.*, 39 (1945) 507.
2 F. Sanger, *Science*, 129 (1959) 1340.
3 O. Neunhoeffer and W. Ruske, *Justus Liebigs Ann. Chem.*, 610 (1957) 143.
4 E. D. Bergmann and M. Bentov, *J. Org. Chem.*, 26 (1960) 1480 and references cited therein.
5 R. L. Munier, A. M. Drapier, S. Meunier and P. Le Xuan, *Chromatographia*, 14 (1981) 289.
6 H. Zahn, *Kolloid-Z.* 121 (1951) 40.
7 H. Zahn and J. Meienhofer, *Makromol. Chem.*, 26 (1958) 126.
8 P. S. Marfey, H. Nowak, M. Uziel and D. A. Yphantis, *J. Biol. Chem.*, 240 (1965) 3264.
9 E. E. Gold and P. E. Braun, *J. Biol. Chem.*, 253 (1978) 8171.
10 P. Marfey, *Carlsberg Res. Commun.*, 49 (1984) 591.
11 D. J. Aberhart, J.-A. Cotting and H.-J. Lin, *Anal. Biochem.*, 151 (1985) 88.
12 G. Szókán, G. Mező and F. Hudecz, *J. Chromatogr.*, 444 (1988) 115.
13 G. Szókán, G. Mező, F. Hudecz, Z. Majer, I. Schön, O. Nyéki, T. Szirtes and R. Dölling, *J. Liq. Chromatogr.*, 12 (1989) 2855.
14 S. Kochhar and P. Christen, *Eur. J. Biochem.*, 175 (1988) 433.
15 S. Kochhar and P. Christen, *Anal. Biochem.*, 178 (1989) 17.
16 H. Brückner, I. Bosch, S. Kühne and S. Zivny, in G. R. Marshall (Editor), *Peptides: Chemistry and Biology*, Escom Science, Leiden, 1988, p. 195.
17 H. Brückner, S. Kühne, S. Zivny, M. Langer, Z. J. Kamiński and M. T. Leplawy, in A. Aubry, M. Marraud and B. Vitoux (Editors), *Second Forum on Peptides*, John Libbey Eurotext, London, 1988, p. 291.

18 H. Brückner and M. Langer, *J. Chromatogr.*, 542 (1991) 161.
19 H. Brückner, R. Wittner and H. Godel, *J. Chromatogr.*, 476 (1989) 73.
20 H. Brückner, C. Keller-Hoehl, H. Godel and R. Wittner, in E. Giralt and D. Andreu (Editors), *Peptides 1990*, Escom Science, Leiden, 1991, p. 334.
21 H. Brückner and C. Keller-Hoehl, *Chromatographia*, 30 (1990) 621.
22 G. C. Barrett (Editor), *Chemistry and Biochemistry of Amino Acids*, Chapman & Hall, London, 1985.