Journal of Chromatography, 386 (1987) 251–272
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 975

DETERMINATION OF NON-PROTEIN α-ALKYL-α-AMINO ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN COMPARISON WITH ION-EXCHANGE AND CAPILLARY GAS CHROMATOGRAPHY

HANS BRÜCKNER* and INGRID BOSCH
Institute of Food Technology, University of Hohenheim, D-7000 Stuttgart 70 (F.R.G.)
and
THEODOR GRASER and PETER FÜRST
Institute of Biological Chemistry and Nutrition, University of Hohenheim, D-7000 Stuttgart 70 (F.R.G.)

SUMMARY

The separation of 18 non-protein α-alkyl-α-amino acids of structure $H_2NC(R_1R_2)COOH$ (R_1 = alkyl, R_2 = alkyl, alkaryl) was performed comparatively using automated ion-exchange chromatography and detection with ninhydrin, reversed-phase high-performance liquid chromatography and fluorescence detection of their *o*-phthaldialdehyde–2-mercaptoethanol (OPA–2-ME) derivatives, and capillary gas–liquid chromatography (GLC) of N(O)-pentafluoropropionyl and N(O)-heptafluorobutyryl *n*-propyl esters. Using analytical conditions common for α-amino acids, most α-alkyl-α-amino acids exhibit drastically decreased reactivity, manifested in very low colour yields with ninhydrin, low relative fluorescence with OPA–2-ME, strongly dependent on temperature and reaction time, and incomplete derivatization in GLC. These shortcomings could be partly overcome by applying more severe conditions, *i.e.*, prolonged reaction time, increased temperature and excess of reagents. This behaviour, attributable to steric effects of geminal C_α-alkyl (alkaryl) groups, is discussed in detail with regard to the structure and reactivity of α-alkyl-α-amino acids.

INTRODUCTION

Replacement of the α-hydrogen atoms of α-amino acids by alkyl groups yields α-alkyl-α-amino acids of the general formula $H_2NC(R_1R_2)COOH$ (R_1 = alkyl, R_2 = alkyl, alkaryl), also designated in the literature as 2-alkylamino acids, α,α-dialkyl-α-amino acids or $C_{\alpha,\alpha}$-dialkylglycines.

Interest in these compounds dates back to the beginning of this century[1–4], but still continues owing to the severe resistance to cleavage of the peptide bond by most *exo-* and *endo*-peptidases when α-alkyl-α-amino acids are present in naturally occurring[5] or synthetic[6,7] polypeptides.

In addition to the presence of α-aminoisobutyric acid (Aib) and DL-isovaline (Iva) in extra-terrestrial material[8] and their formation in electric discharge experi-

ments simulating primitive atmospheric conditions[9], the presence of α-methylserine in the antibiotic amicetin[10] and, in particular, of large amounts of Aib in a unique group of fungal polypeptide metabolites referred to as "peptaibol" mycotoxins (or antibiotics)*, has attracted considerable attention from the biophysical[11], sequential [12,13], synthetic[14], conformational[15–21] and chromatographic[22,23] points of view.

Incorporation of hydrophobic α-alkyl-α-amino acids into peptides may force them into very specific and stable conformations (*e.g.*, α- or 3_{10}-helical secondary structures). This is partly due to severe impediments imposed on their conformationally restricted polypeptide torsion angles φ (rotation angles of the C_α–C bonds) and ψ (rotation angles of the C_α–N bonds). These amino acids must ultimately be responsible for the antibiotic and membrane-modifying activities of peptaibol mycotoxins and may also be promising tools for both peptide modelling and drug design[24]. α-Alkyl-α-amino acids are also routinely used as non-metabolizable amino acids in studies of food digestion and amino acid metabolism[25,26], and the L-isomer of α-methyl-dopa has been shown to exhibit hypotensive activity[27]. However, despite their steadily increasing importance, as indicated in the above selection of topics, very little systematic work has been carried out on the analytical methodology of α-alkyl-α-amino acids. This work has focused mainly on the separation of enantiomers[28–30].

In addition to the challenge of including them in the rapidly growing field of high-performance liquid chromatographic (HPLC) applications, we believe that comparative determinations of α-amino acids and their alkylated counterparts will also lead to a better understanding of the fundamental analytical separation and reaction mechanisms as exemplified below with the ninhydrin and *o*-phthaldialdehyde reaction. Finally, from the occurrence of Aib, Iva and α-Me-Ser (α-methylserine) as fungal metabolites, it is evident that biochemical pathways for their syntheses exist, and probably many more naturally occurring α-alkyl-α-amino acids can be expected.

In this paper, the separation and quantification of 18 α-alkyl-α-amino acids from 15 amino acids and two amino alcohols by reversed-phase HPLC after *o*-phthaldialdehyde–2-mercaptoethanol (OPA–2-ME) derivatization is compared with ion-exchange chromatography involving automated amino acid analysis with ninhydrin reagent. In conclusion, the determination by capillary gas–liquid chromatography of N(O)-pentafluoropropionyl and N(O)-heptafluorobutyryl α-alkyl-α-amino acid *n*-propyl esters is discussed.

EXPERIMENTAL

Sources and abbreviations of α-alkyl-α-amino acids and α-amino alcohols

α-Aminoisobutyric acid (α-methylalanine, 2-methylalanine; Aib) was purchased from Fluka (Buchs, Switzerland); DL-isovaline (DL-α-ethylalanine, DL-α-methyl-α-amino-*n*-butyric acid; Iva) was prepared according to the literature[20] and also purchased from Serva (Heidelberg, F.R.G.; currently out of stock); DL-α-methylleucine was obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). DL-α-methylas-

* The name peptaibol is derived from *pept*ides containing *Aib* and an amino alco*hol*, as exemplified by the sequences of the polypeptide mycotoxin paracelsin A (B): Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val(Leu)–Aib–Gly–Aib–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol[13].

partic acid (α-Me-Asp), α-methyl-dopa [DL-2-methyl-3-(3,4-dihydroxyphenyl)alanine, (α-Me-Dopa)], DL-α-methylglutamic acid · $0.5H_2O$ (α-Me-Glu), DL-α-methylhistidine · 2HCl (α-Me-His), DL-α-methylmethionine (α-Me-Met), DL-α-methylornithine · 2HCl · H_2O (α-Me-Orn), DL-α-methyl-*m*-methoxyphenylalanine (α-Me-PheOCH_3 or α-Me-PheOMe), DL-α-methylphenylalanine (α-Me-Phe), DL-α-methylserine (α-Me-Ser), DL-α-methyltryptophan (α-Me-Trp) and DL-α-methyltyrosine (α-Me-Tyr) were obtained from Sigma (St. Louis, MO, U.S.A.). DL-α-Methylvaline (α-Me-Val), DL-α-methylnorvaline (α-Me-Nva) and DL-α-methylnorleucine (α-Me-Nle)[29] were gifts from E. Gil-Av (Weizman Institute of Science, Rehovot, Israel) and dipropylglycine[31,32] (Dpg or Prg) was obtained from P. M. Hardy (University of Exeter, U.K.). L-Phenylalaninol (Pheol) and L-valinol (Valol) were purchased from Aldrich (Steinheim, F.R.G.).

Automatic amino acid analysis by ion-exchange chromatography

Amino acid analysis was performed using a Model LC 6001 automatic amino acid analyser, equipped with a Model 7040 sample injector and a 50-μl sample loop (Biotronik, Maintal, F.R.G.) and a C-R3A integrator (Shimadzu, Kyoto, Japan). The cation exchanger used (BTC 2710) (Biotronik) was packed in a 300 mm × 4 mm I.D. column with a water-jacket, connected to a 25 mm × 10 mm I.D. pre-column, packed with BTC F (Biotronik) resin. A standard programme for physiological fluids was used with a temperature programme and step gradients of lithium citrate buffers: (A) pH 2.82, 0.1 *N*; (B) pH 3.05, 0.12 *N*; (C) pH 3.59, 0.18 *N*; (D) pH 4.02, 0.40 *N* and (E) pH 3.50, 1.40 *N* (for details, see Fig. 1). The flow-rate of the buffer pump was 21 ml/h and that of the ninhydrin pump was 20 ml/h. The reaction coil was 27.5 m × 0.3 mm I.D. The ninhydrin concentration used was 20 g/l. All chemicals for amino acid analysis were purchased from Merck (Darmstadt, F.R.G.) and Sigma (Deisenhofen, F.R.G.). Full details of the instrumental design and buffer preparations are published in the Biotronik LC 6001 Manual.

TLC investigations of the reaction of Aib and Leu with ninhydrin

A 10.3-mg (0.1-mmol) amount of Aib [or 13.1 mg (0.1 mmol) of Leu] was dissolved in 500 μl of lithium citrate buffer (pH 2.2); this solution was mixed with 53.4 mg (0.3 mmol, 3 equiv.) of ninhydrin in 500 μl of 95% ethanol in a test-tube equipped with a screw-cap, immediately sealed and incubated in a water-bath at 70°C. After an appropriate time, 3-μl samples were spotted onto pre-coated TLC plates (Merck, Darmstadt, F.R.G.), developed with chloroform–methanol–acetic acid–water (50:35:10:5) and detected by spraying with ninhydrin reagent. With Aib the experiment was repeated with 320 mg (1.8 mmol, 18 equiv.) of ninhydrin in 500 μl of 95% ethanol.

High-performance liquid chromatography

Apparatus. The HPLC system (LKB, Bromma, Sweden) consisted of two Model 2150 pumps, a Model 2152 controller for gradient programming and a modified Model 2153 autoinjector with a 20-μl filling loop for automated on-line derivatization. A Rheodyne (Cotati, CA, U.S.A.) No. 7125 injection valve with a 20-μl filling loop was used for manual injections. Fluorescence was routinely monitored with a Model RF-530 Fluorescence Spectromonitor (Shimadzu, Kyoto, Japan) at an

excitation wavelength of 330 nm and an emission wavelength of 450 nm with a 12μl flow cell. Continuous on-line quantitation of the HPLC results was obtained with a Chromatopac C-R2AX data processor (Shimadzu).

Column. The Hyperchrome HPLC column (125 × 4.6 mm I.D.))Bischoff-Analysentechnik, Leonberg, F.R.G.) and guard column (10 × 4.6 mm I.D.) were packed with Spherisorb ODS II, 3 μ (Phase Separations, Queensferry, U.K.). The column was kept in a Model 4000 Thermobox (Bischoff) and maintained at 25°C with a Model 2761 Thermostat (Eppendorf Gerätebau Netheler & Hinz, Hamburg, F.R.G.).

Reagents. Ultra-pure water, which was exclusively used in the preparation of buffers, was generated with an Elgastat Spectrum water purification system that includes reverse osmosis, activated carbon and a nuclear-grade ionization cartridge (Elga, Lane End, U.K.). All chemicals used were of analytical-reagent grade and the solvents were of HPLC grade. Na_2HPO_4, NaH_2PO_4, methanol, tetrahydrofuran and acetonitrile were purchased from Merck.

A ready-to-use *o*-phthaldialdehyde–2-mercaptoethanol (OPA–2-ME) reagent solution (Pierce 26025) and a 1.0 *M* potassium borate buffer (pH 10.4) (Pierce 27035) were obtained from Pierce (Rockford, IL, U.S.A.).

Eluent system. Gradients were formed between two helium-conditioned solvents; 12.5 m*M* sodium phosphate at pH 7.2 (1 l of 12.5 m*M* Na_2HPO_4 was adjusted with *ca.* 0.7 l of 12.5 m*M* NaH_2PO_4 to give pH 7.2) was used to buffer the organic solvent mixtures (acetonitrile and tetrahydrofuran). Degassing was carried out by using a Model 2156 solvent conditioner (LKB). Further details of the analytical conditions are given in the figure legends.

Preparation of standard solutions. A 10-μl volume of a 2.5 m*M* solution of each α-alkyl-α-amino acid was diluted with 10 ml of water and 2 ml of potassium borate (1.0 *M*, pH 10.4). Each standard vial was filled with 1 ml of this solution and placed in the top of the thermostated autoinjector.

Automated sample injector. An LKB 2153 autoinjector was modified to permit automated pre-column derivatization and subsequent injection. Thus, the normal digitally pre-set functions of the autoinjector for flush cycles and the injection/fill cycles were changed in digitally pre-set functions for pump time (0–90 s) and derivatization time (0–990 s), respectively.

The on-line derivatization was facilitated by attaching a peristaltic pump to the autoloader. This allowed mixing of the sample with the OPA–2-ME reagent in an external mixing T-piece (reaction vessel) in the pumping mode. The prepared samples and the reagent bottle, placed in a Thermobox on the top of the autoinjector, can be held at ambient temperature or cooled to 4°C. Owing to the warming up of the reaction vessel (which is not thermostated), the temperature of the reactants is *ca.* 15°C after a 10 min reaction time or *ca.* 7°C after a 1.5 min reaction time.

In the complete HPLC system, the autoinjector is connected to the controller by an interface cable. Full control of the run time and gradient profile for each individual injection and of the number of repetitive injections from each individual vial is then achieved.

Gas–liquid chromatography

Instrumental. GLC was carried out on a Model 4100 gas chromatograph (Frac-

tovap series, Carlo Erba, Milan, Italy), equipped with a Model 410 LT programmer, EL 440 electrometer and Chromatopac C-R3A integrator/printer/plotter (Shimadzu).

Column. The capillary column employed was a 25 m × 0.22 mm I.D. wall-coated open-tubular (WCOT) fused-silica column, coated with chemically bonded CP-Sil-19 CB [methyl(85%)phenyl(7%)cyano(7%)vinyl(1%)polysiloxane] having the following specifications, as certified by the manufacturer (Chrompack, Middelburg, The Netherlands): film thickness, 0.21 μm; phase ratio, 260; capacity factor, k' (methyl decanoate, carrier gas hydrogen, 115°C), 6.4; number of theoretical plates, 112000; height equivalent to a theoretical plate (HETP), 0.233 mm; and coating efficiency, 85%.

Derivatization procedure. Each 100-μl sample of standard solutions of α-alkyl-α-amino acids (obtained by dissolving 25 μmol of the respective amino acid in 1 ml of 0.01 *N* hydrochloric acid) was transferred into a 10-ml flask, evaporated to dryness *in vacuo* at 50°C, dissolved in 600 μl of methanol, transferred quantitatively into a 1-ml heavy-walled Reactivial (Wheaton, Millville, NJ, U.S.A.) and evaporated to dryness in a stream of dry nitrogen. Then, 600 μl of 2.5 *N* hydrochloric acid in *n*-propanol were added, the vial was sealed tightly with a Teflon-lined screw-cap, heated for 1 h at 100°C in an aluminium heating block and evaporated to dryness at 40°C in a stream of dry nitrogen. The esterification procedure was repeated once, the residue was dissolved in 400 μl of dichloromethane and 150 μl of pentafluoropropionic anhydride (Pierce) or heptafluorobutyric anhydride (Fluka) were added. After 1 h at 100°C, the reagents were removed in a stream of dry nitrogen at ambient temperature and the residue was dissolved in 200 μl of dichloromethane, yielding a standard solution of α-alkyl-α-amino acids, each containing 2.5 μmol per 200 μl. Samples of 0.6–0.8 μl were applied to the injector of the gas chromatograph, using a 10-μl syringe (Hamilton, Bonaduz, Switzerland) and operating in the split mode (split ratio *ca.* 1:30).

TLC monitoring of derivatization procedures. For monitoring the course of the esterification and acylation procedure, samples of 1–2 mg of each α-alkyl-α-amino acid were dissolved in 200 μl of 2.5 *N* hydrochloric acid in *n*-propanol and treated as described above. The degree of esterification was checked after dissolving the residue in 100 μl of methanol and subjecting a 3-μl sample to TLC on pre-coated plates of silica gel 60 F_{254} (Merck). The plates were developed with *n*-butanol–acetic acid–water (3:1:1) in equilibrated glass chambers to a distance from the start to the solvent front of *ca.* 6 cm. Detection was achieved with ninhydrin reagent. Subsequently, the completeness of acylation was checked in solutions of dichloromethane by TLC as described above but with detection with chlorine–N,N,N′,N′-tetramethyl-4,4′-diaminodiphenylmethane (TDM) reagent[33] (Fluka 87800).

RESULTS AND DISCUSSION

Separation of α-alkyl-α-amino acids by automated ion-exchange chromatography

Nearly three decades ago the pioneering work of Spackman *et al.*[34] provided scientists with an accurate and automated method for the complete separation and quantitative determination of all protein amino acids by ion-exchange chromatography on high-resolution resins, with precisely pumped buffer systems and post-

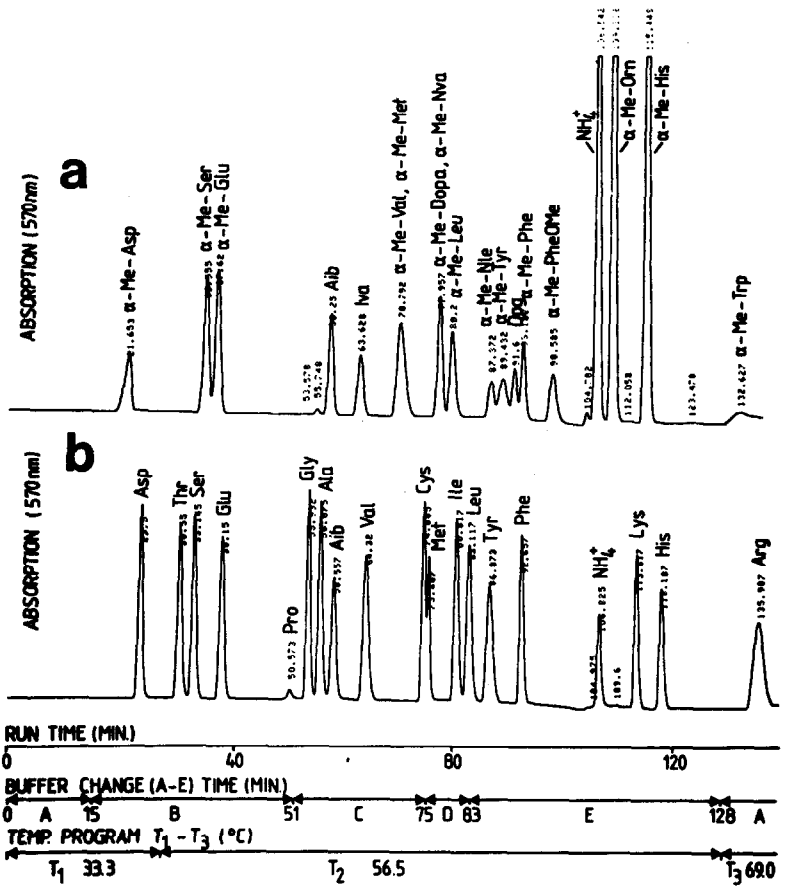

Fig. 1. Separation of (a) standards of α-alkyl-α-amino acids (125 nmol), compared with (b) standards of α-amino acids (12.5 nmol), spiked with Aib (125 nmol). Separation was performed by automated ion-exchange chromatography with lithium citrate buffers and detection with ninhydrin (for details, see Experimental). Protein amino acids are abbreviated according to *Eur. J. Biochem.*, 74 (1977) 1; for α-alkyl-α-amino acids, see Experimental.

column derivatization with ninhydrin. In retrospect, this also constitutes part of the history of HPLC. In previous papers we have demonstrated that Aib and Iva, the simplest α-alkyl-α-amino acids, could be determined by automated ion-exchange chromatography, and Iva is eluted together with Val, if both are present[35]. Iva, like Aib, exhibited an extremely low ninhydrin response. From these results it was concluded, and this is confirmed below, that a low ninhydrin response is a feature common to all α-alkyl-α-amino acids. In Fig. 1 the separation of a number of α-alkyl-α-amino acids (each representing 125 nmol) is compared with the separation of a standard of protein amino acids (12.5 nmol), spiked with a ten-fold excess of Aib (125 nmol) to yield an about equal peak area. The low ninhydrin response of the former is immediately obvious, with the exception of the basic amino acids α-Me-Orn and α-Me-His, which shows a comparatively higher colour yield. The values for

TABLE I

RETENTION TIME AND NINHYDRIN COLOUR YIELD (570 nm) RELATIVE TO LEU (= 100%) OF α-ALKYL-α-AMINO ACIDS COMPARED WITH α-AMINO ACIDS

Amino acid	*Elution time (min)*	*Colour yield (%) (Leu = 100%)*
α-Me-Asp	21.65	6.6
Asp	23.50	97.1
Thr	30.55	98.7
Ser	33.15	100.7
α-Me-Ser	35.56	11.6
α-Me-Glu	37.62	13.0
Glu	38.15	94.9
Pro	50.57	5.7
Gly	53.93	101.5
Ala	56.80	104.6
Aib	58.56	7.4
Iva	63.63	6.2
Val	64.32	101.8
α-Me-Val	70.79	2.4
α-Me-Met	70.79	12.1
Cys	74.89	90.2
Met	75.81	53.6
α-Me-Dopa	77.96	5.1
α-Me-Nva	77.96	8.2
α-Me-Leu	80.20	9.4
Ile	80.82	101.7
Leu	83.12	100.0
Tyr	86.87	92.0
α-Me-Nle	87.37	3.2
α-Me-Tyr	89.43	8.1
Dpg	91.60	4.5
Phe	92.66	88.0
α-Me-Phe	93.18	8.0
α-Me-PheOMe	98.59	7.2
α-Me-Orn	109.34	47.0
Lys	113.62	75.6
α-Me-His	115.45	39.2
His	118.19	65.7
α-Me-Trp	132.63	5.5
Arg	135.99	111.0

all the amino acids investigated and calculated with respect to Leu as a standard are compiled in Table I.

Fig. 1 shows that most α-alkyl-α-amino acids could be separated from each other in the relative order of emergence of the respective protein amino acids. However, α-Me-Val/α-Me-Met and α-Me-Dopa/α-Me-Nva were not separated. α-Me-Trp gave rise to a very broad peak with a low response, obviously requiring a higher ionic strength or pH of the elution buffer. Val and Iva were unresolved when combined, although they had slightly different elution times in the standards shown in Fig. 1. This is explainable by concentration effects. These two amino acids could not be separated, even by varying the buffer and temperature programme. This masking

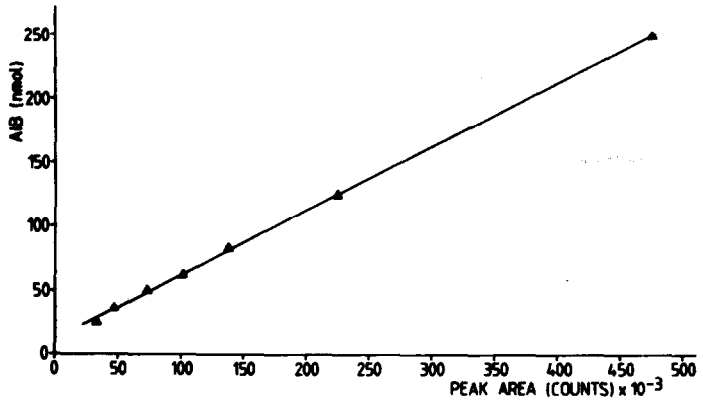

Fig. 2. Linearity of the ninhydrin response of Aib in the concentration range 25–250 nmol determined by automated ion-exchange amino acid analysis (Biotronik Model LC 6001 Analyser).

of Iva by Val until recently prevented the detection of the former in the peptaibol mycotoxins samarosporin, stilbellin and alamethicin[36,37].

Aib was very well separated from Ala and occupied a unique position, without interference from any other protein or α-alkyl-α-amino acid. This is of greatest importance when Aib is used as a very specific marker compound for the detection of polypeptide mycotoxins of the peptaibol group in culture broths or mycelia of various fungi[38]. Comparison of the elution times of α-alkyl-α-amino acids and α-amino acids (*cf.*, Table I) indicates that most of them can be analysed in a mixture by using a standard analyser programme, or that they can be separated by slight modifications of the buffer changing time, pH or temperature programme. The linearity of the colour yields of α-alkyl-α-amino acids is satisfactory, as exemplified by Aib, which shows excellent linearity in the range 25–250 nmol (Fig. 2).

Reaction of α-alkyl-α-amino acids with ninhydrin

In the course of the sequence analysis of alamethicin, it was already realized that Aib has a very low colour constant in the ninhydrin reaction (*ca.* 12% relative to Leu, or 13.7% relative to Gly)[5], as performed by automatic amino acid analysers, based on ion-exchange chromatography according to the classical Stein and Moore procedure. Low colour constants of this order of magnitude were also found for isovaline and valinol, both being constituents of the polypeptide mycotoxin trichotoxin (see ref. 35 for an ion-exchange chromatogram of a hydrolysate). Most α-alkyl-α-amino acids investigated in this work show values of about 5–12% relative to Leu (*cf.*, Table I). Extremely low colour constants were found for α-Me-Val (2.4%), α-Me-Nle (3.2%) and Dpg (4.5%), and comparatively high constants for the basic compounds α-Me-His (39.2%) and α-Me-Orn (47%). No rational explanation for this phenomenon has been given to date.

Although details are still under investigation[39], the mechanism of the ninhydrin reaction with α-amino acids, as proposed and reviewed in great detail by McCaldin[40], is now generally accepted (Fig. 3). In a probably concerted reaction, it starts with nucleophilic attack of the amino group of the amino acids on the highly reactive geminal diol group of ninhydrin, followed by decarboxylation of the intermediate (a)

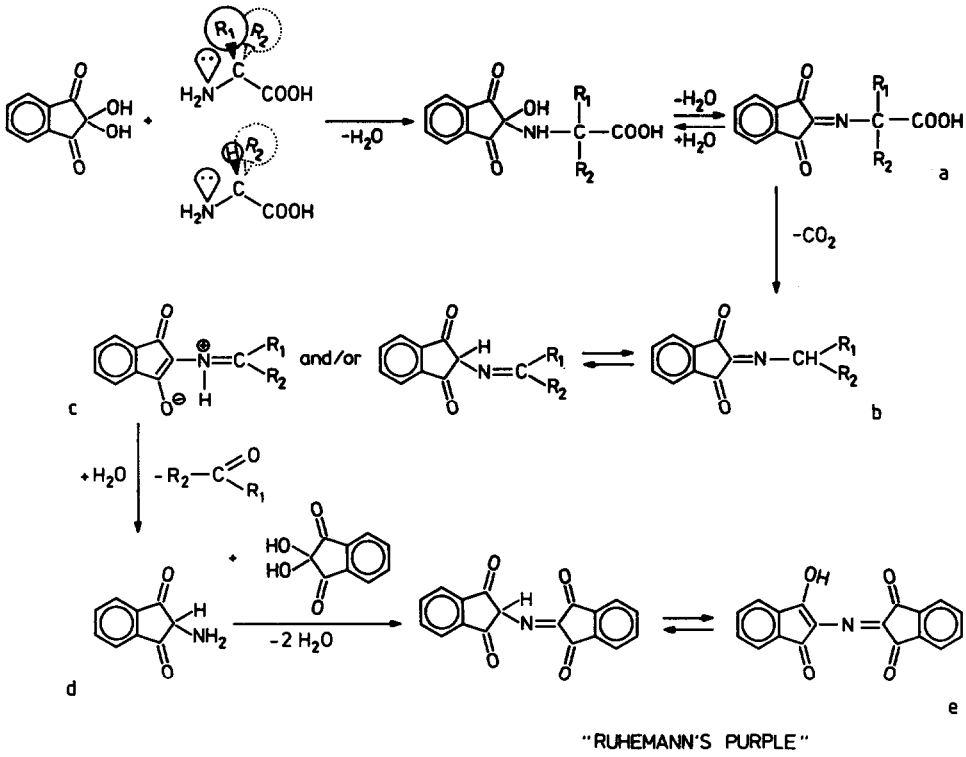

Fig. 3. Scheme of the reaction of ninhydrin with sterically hindered α-alkyl-α-amino acids (R_1, R_2 = alkyl, alkaryl), compared with α-amino acids (R_1 = H, R_2 = H, alkyl, alkaryl), yielding "Ruhemann's Purple", after decarboxylation and release of ketone (R_1COR_2) or aldehyde (R_2CHO).

formed, to yield a Schiff's base (b) or dipolar ionic enaminè (c). They are readily hydrolysed with release of an aldehyde, or ketone in the case of α-alkyl-α-amino acids (Strecker degradation), yielding an amine (d), which reacts with a second molecule of ninhydrin to form the well known blue-violet compound referred to as "Ruhemann's purple" (e). Based on this mechanism, we have postulated that the low colour yield of α-alkyl-α-amino acids results from steric hindrance of their α-amino groups, as is very well known from synthetic peptide chemistry[41]. This effect would hinder step (a) and/or lead to a stabilization of intermediates (b) and (c), thus ultimately hampering the formation of (d) and (e). Steric hindrance, as assumed in step (a), was immediately established by TLC investigation of the time-dependent reaction of Aib with ninhydrin at 70°C and comparison with Leu as a standard. In contrast to the standard, which disappeared nearly quantitatively within 2 min, most of the Aib was unchanged after 6 h and still detectable, even after reaction times of 24 and 48 h, when 3 equiv. of ninhydrin were used. However, when using 18 equiv. of ninhydrin Aib disappeared completely within 5 min.

From these results it could be concluded that the reaction of α-alkyl-α-amino acids might be pushed further towards completeness when a prolonged reaction time, higher temperature and greater excess of ninhydrin reagent are used. To test this hypothesis and to find out whether or not a routine amino acid analyser based on

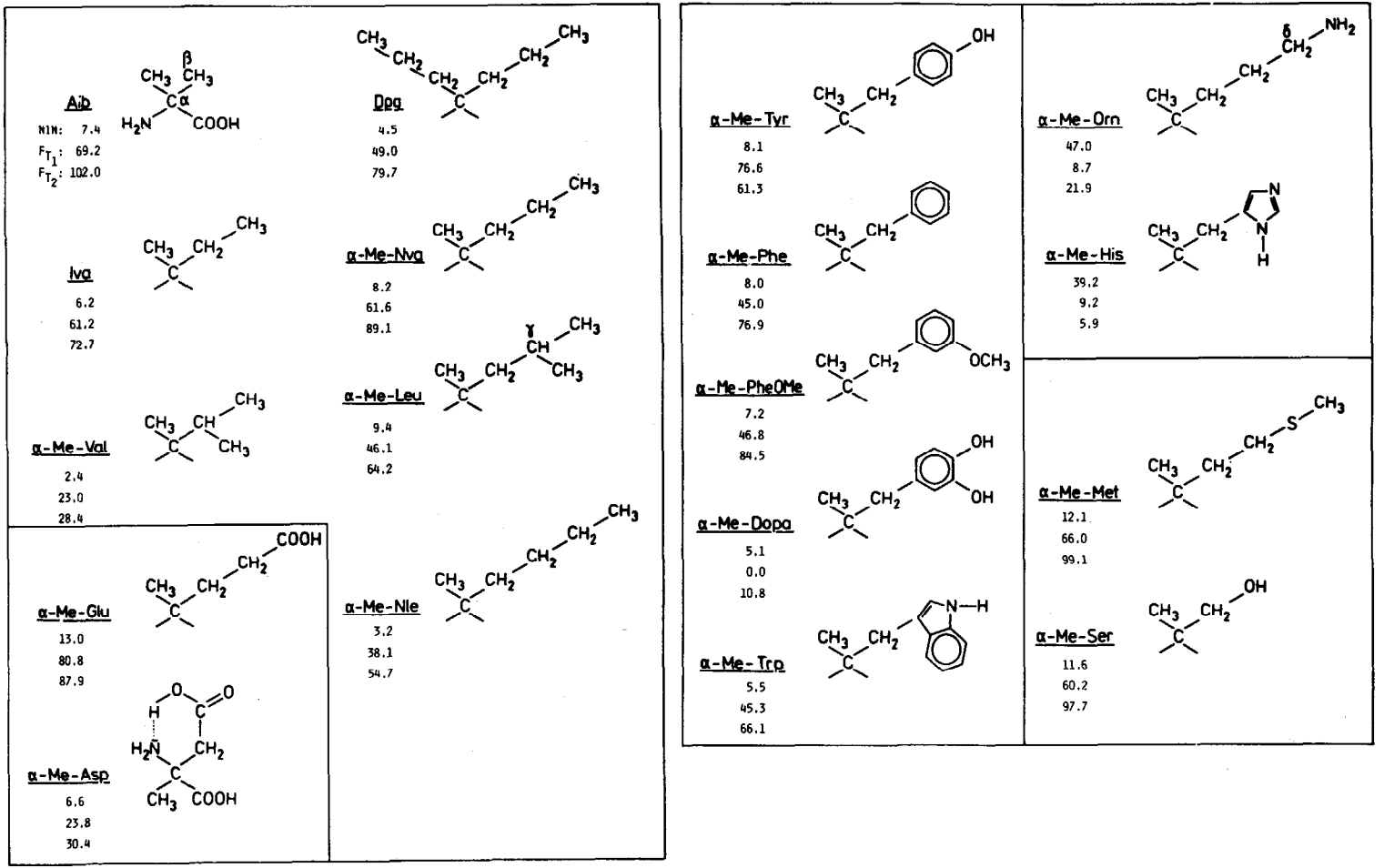

Fig. 4. Correlation of side-chain structures of α-alkyl-α-amino acids and their colour ratios with ninhydrin (NIN) and relative fluorescence F_{T1} (15°C) and F_{T2} (25°C) after derivatization with OPA–2-ME. Numbers below the amino acid symbols represent percentage colour yields and relative fluorescence values relative to Leu (= 100%). Ninhydrin colour yields were determined by using an amino acid analyser standard programme, and the relative fluorescence by HPLC. With the exception of Aib and α-Me-Asp, amino and carboxy groups linked to the C_α atom are not shown in the formulae. For explanation of the amino acid symbols, see Experimental.

ion-exchange chromatography and operated with ninhydrin can be used for more sensitive and accurate analyses of α-alkyl-α-amino acids, simple variations of the buffer and ninhydrin flow-rates were made. This should control the reaction time in the coil and the ninhydrin concentration. A test mixture of Aib (125 nmol) and Leu (12.5 nmol) (the 10:1 molar ratio yielding about equal peak areas in standard runs) was analysed, and the colour yields were calculated for equimolar (12.5 nmol) amounts. Halving both the buffer and the ninhydrin flow-rates increased the reaction time in the coil from 2.9 to 4.8 min, as calculated from the flow-rate and coil volume, and yielded a slight increase in the Aib colour constant from 8.5 to 9.3% relative to Leu. However, halving the buffer flow-rate without changing the ninhydrin flow-rate strikingly resulted in an increase of the Aib colour yield to 31.5% relative to Leu in the same run. Under these conditions Leu also showed a drastic increase in colour ratio to 136.8% in comparison with the routine analysis.

These experiments probably also explain the slight differences reported in the literature concerning the relative colour ratio (7–12%) of Aib, probably resulting from specific instrumental design. The highest possible ninhydrin concentration (limited by solubility and formation of precipitates in the reaction coil), possibly combined with a prolonged reaction time and a higher coil temperature (the latter being achieved by using an oil bath and a reaction coil designed for overpressure), should overcome the discussed analytical shortcomings. This might be also of importance in achieving an increase in the sensitivity of detection of Aib, which is used as a marker compound in the detection of the initially mentioned group of polypeptide mycotoxins[38].

Ninhydrin reaction and side-chain structures

The conspicuously lower colour yield of α-alkyl-α-amino acids compared with their non-alkylated analogues was explained above by the steric hindrance, attributed mainly to the α-methyl (alkyl) groups, However, their relative difference must be caused by the spatial arrangement around the C_α atom. As illustrated in Fig. 4, in the series Aib (7.4%), Iva (6.2%) and α-Me-Val (2.4%), the last compound shows the lowest colour yield of all the α-alkyl-α-amino acids investigated. An explanation based on the unique branching of the C_β atom is supported by a comparison with α-Me-Leu (9.4%), which has a methylene group as spacer for the isopropyl group. In comparison with α-Me-Leu (9.4%), the colour yield of α-Me-Nva (8.2%) is only slightly different, demonstrating that the alkyl-chain branching at the C_γ atom is much less important than that of the C_β atom in α-Me-Val (2.4%). At first glance unexpected, lengthening of the side-chain of α-Me-Nva (8.2%) by a methylene group, yielding α-Me-Nle (3.2%), results in a very drastic decrease in colour yield. As suggested by space-filling models, this is probably due to the ability of the *n*-butyl group of α-Me-Nle to form a bend, thus shielding the α-amino group. There is a much smaller possibility of this occurring in α-Me-Nva. Replacement of the α-methyl group of α-Me-Nva by an *n*-propyl group yields dipropylglycine, Dpg (4.5% colour yield). The comparatively low colour ratio is explainable as the shielding of the amino group by two bulky (but unbranched) *n*-propyl groups. This results in a lower color yield than that of Iva (6.2%).

From these results it is predicted that, when the most highly branched side-chains, *i.e.*, *tert.*-butyl, are attached to the C_α atom of α-amino acids, almost no

colour yield should be obtained. Among the α-methylamino acids having aromatic side-chains, all bear a methylene spacer, thus separating the aromatic ring from the C_α atom. Comparison of α-Me-Tyr (8.1%) and α-Me-Phe (8.0%) indicates that substitution in the *para* position by a hydroxy group has no effect, contrary to *meta* substitution, as demonstrated with α-Me-PheOMe (7.2%) and α-Me-Dopa (5.1%). The acidic phenol group is obviously more effective than the methoxy group. The colour yield of α-Me-Dopa (5.1%) is roughly equal to that of α-Me-Trp (5.5%), despite the voluminous indolyl group of the latter.

All α-alkyl-α-amino acids having polar side-chain groups capable of forming hydrogen bonds have colour yields of about the same order of magnitude as α-Me-Ser (11.6%), α-Me-Met (12.1%) with a more flexible side-chain and the acidic α-Me-Glu (13.0%). Remarkably, α-Me-Asp (6.6%) shows a much lower colour ratio than α-Me-Glu. This is explained by the more rigid side-chain structure permitting the formation of an energetically favoured intramolecular hydrogen bond, which includes the C_β-carboxy group and the α-amino group of α-Me-Asp (*cf.*, Fig. 4).

The trifunctional basic amino acids α-Me-His (39.2%) and α-Me-Orn (47%) give the highest colour yields among the α-alkyl-α-amino acids investigated, explainable by additional basic groups, which are not blocked by the α-methyl groups. The assumption of the nearly unhindered reaction of the α-amino group of α-Me-Orn is also supported by comparison of the colour yields of α-Me-Orn (47%) and α-Me-Nle (3.2%), both having approximately the same space-filling properties of side-chain groups.

In conclusion, the stereochemical approach explains even minor differences in the colour yield of individual α-alkyl-α-amino acids, and the quantitative ninhydrin reaction may therefore also serve as a sensitive probe in revealing slight differences in chemical reactivity. However, it should be kept in mind that in addition to the obviously dominant effect of the spatial arrangement of geminal side-chain groups, inductive and mesomeric effects must also be considered.

This stereochemical approach in general also explains the temperature- and time-dependent relative fluorescence of OPA-derivatized α-alkyl-α-amino acids, as discussed below.

Separation of α-alkyl-α-amino acids, α-amino acids and α-amino alcohols by RP-HPLC

The large number of reports dealing with various methods of determining amino acids by HPLC[42] testifies to the leading role played by this technique. It is bolstered by its flexibility in terms of pre- and post-column derivatization, choice of stationary phases, eluents and detection systems, and the benefit of short analysis time combined with high sensitivity. Among the methods taking advantage of the facile formation of highly fluorescent derivatives of amino acids, the routine OPA derivatization method[43–47] is the most promising as an alternative to the Stein and Moore procedure. As demonstrated in great detail previously[48], we were able to separate, detect fluorimetrically and quantify 26 physiological amino acids after derivatization with *o*-phthaldialdehyde reagent by HPLC with a sodium phosphate buffer–acetonitrile gradient. As demonstrated in Fig. 5, this method achieved the almost complete separation of 18 α-alkyl-α-amino acids, 15 α-amino acids and 2 relevant α-amino alcohols, with a gradient of 2% acetonitrile–1% tetrahydrofuran

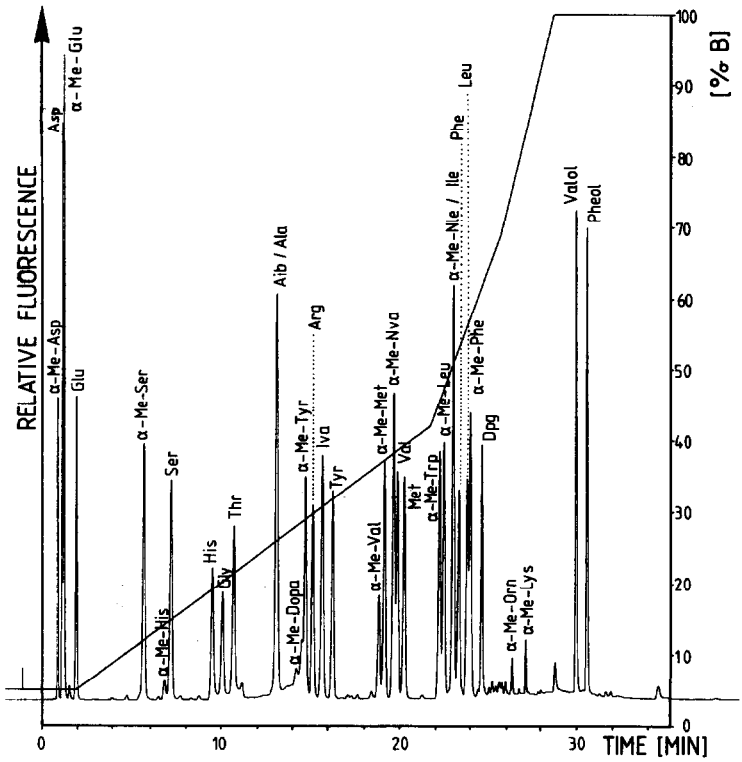

Fig. 5. Elution profile of an OPA–2-ME-derivatized standard, composed of α-alkyl-α-amino acids, α-amino acids and α-amino alcohols. Conditions: Spherisorb ODS II column (12.5 cm × 4.6 mm I.D.) with a 10 mm × 4.6 mm I.D. guard column; particle size, 3 μm; eluent A, 2% acetonitrile–1% tetrahydrofuran; eluent B, 50% acetonitrile in 12.5 mmol sodium phosphate buffer (pH 7.2); flow-rate, 1.5 ml/min; pressure, 18 MPa (180 bar); injection, 28.5 pmol of each component in 20 μl after automated pre-column derivatization for 10 min at 25°C; temperature, ambient.

to 50% acetonitrile in 12.5 mmol sodium phosphate buffer (pH 7.2). Only Aib/Ala and Ile/α-Me-Nle were not separated under the conditions used.

Most interestingly, the elution times of α-alkyl-α-amino acids were, with the exception of α-Me-Phe, lower than those of their non-alkylated analogues. This is most surprising; in view of their relatively greater hydrophobicity and higher pK values, owing to the positive inductive effect of the additional α-alkyl groups, one would expect just the reverse.

To explain this phenomenon, it could be assumed that, in accordance with restricted φ/ψ angles in the Ramachandran map of peptide-bonded α-alkyl-α-amino acids[16,17,19], there are restrictions on the free rotation of the N–C bond of their isoindolyl adduct. This would prevent to a certain extent optimal hydrophobic interaction with the stationary phase in HPLC, in contrast to the adducts of α-amino acids, which have greater rotational freedom.

The applicability of our method to the determination and quantification of amino acids in naturally occurring polypeptides is demonstrated by the total hydrolysates of paracelsin A and B (for the structures see the footnote on p. 252) and

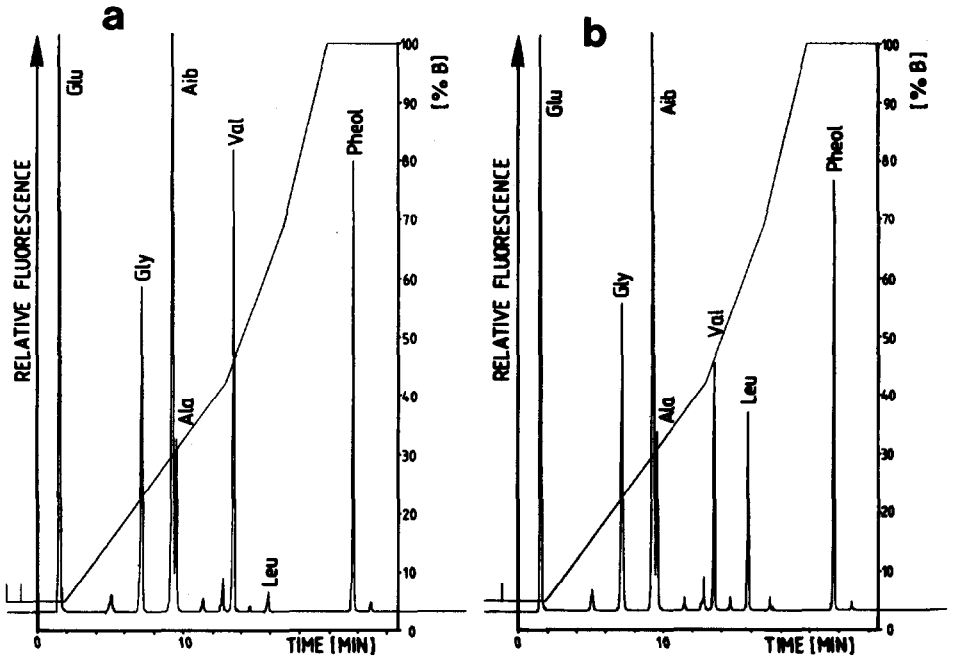

Fig. 6. HPLC separation of hydrolysates (6 *N* HCl, 110°C/24 h) of (a) polypeptide mycotoxin paracelsin A and (b) paracelsin B. Column temperature, 50°C; pressure, 10 MPa (100 bar); other conditions as in Fig. 5.

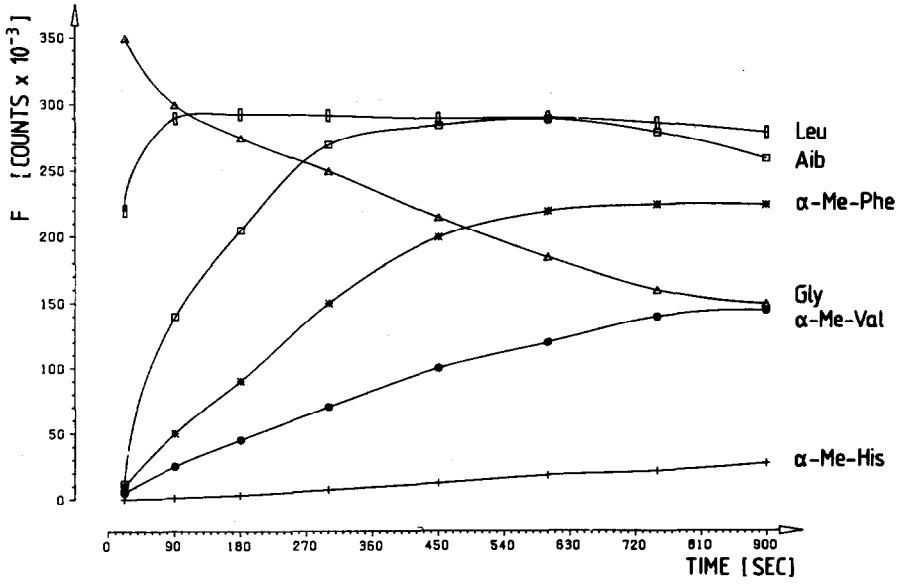

Fig. 7. Time dependence of the relative fluorescence (*F*) of selected α-alkyl-α-amino acids compared with standard Leu and Gly with OPA–2-ME at 25°C.

derivatization with OPA–2-ME (Fig. 6). Paracelsin B, having the composition Glu (3), Gly (1), Ala (3), Aib (9), Val (1), Leu (1), Pro (1) and Pheol (1), was used as the standard. The ratio of paracelsin A to B was Glu (3.08), Gly (1.0), Ala (2.94), Aib (8.82), Val (1.86), Leu (0.12, contamination), and Pheol (1.05). Proline, a secondary amino acid, could not be determined routinely by the method used (Fig. 6). The precise determination of α-alkyl-α-amino acids and the α-amino alcohols Pheol and Valol in single runs is of great importance in the sequence determination of polypeptides of the peptaibol group.

Time and temperature dependence of the relative fluorescence of α-alkyl-α-amino acids and α-amino acids

In previous studies[48–50], the maximal relative fluorescence of most OPA–3-MPA α-amino acid adducts was found 2.5 min after onset of the reaction at 4°C, except for Gly, which showed a very rapid quenching of fluorescence. The derivatization of α-amino acids with OPA–2-ME is routinely carried out in most laboratories for 1 min at ambient temperature, or for 1.5 min at 4°C, the lower temperature being more favourable when dealing with physiological fluids. α-Alkyl-α-amino acids show a different, in general much lower, reaction rate at 25°C than the α-amino acids, as illustrated for selected amino acids in Fig. 7 and summarized for all the compounds investigated in Table II. Contrary to the standard, Leu (*cf.*, Fig. 7), which reached the maximal relative fluorescence after 1.5 min and remained on the plateau of the reaction curve for about 15 min, and Gly, which showed its usual, rapidly decreasing fluorescence, the α-alkyl-α-amino acids reveal individual reaction curves. The gradually ascending slopes of their time-dependent relative fluorescence indicate that the formation of the OPA–2-ME adducts of α-alkyl-α-amino acids proceeds considerably

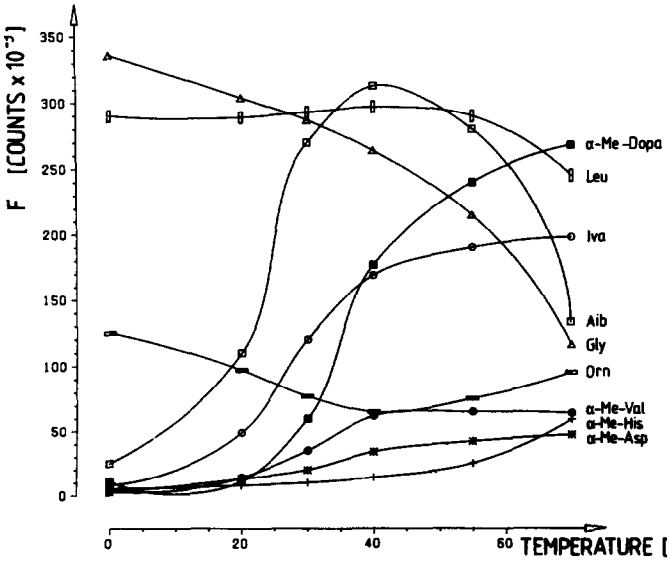

Fig. 8. Temperature dependence of the relative fluorescence (F) of selected α-alkyl-α-amino acids and α-amino acids with OPA–2-ME after a 10-min reaction time.

more slowly than that of α-amino acids, as exemplified by α-Me-Phe, α-Me-Val and α-Me-His. In comparison with Aib, α-Me-Phe and α-Me-Val show a significantly diminished reaction velocity, reaching the plateau of their relative fluorescence after about a 15-min reaction time. From these results a reaction time of $\geqslant 15$ min would be reasonable. With Aib, the plateau of the reaction curve is attained after 10 min with the relative fluorescence of Leu and it shows a slight decrease with prolonged reaction time. With regard to the most important Aib, 10 min was chosen as an acceptable compromise (*cf.*, Fig. 7).

The temperature dependence of the relative fluorescence for a 10-min reaction time in the range 0–70°C for OPA–2-ME adducts of selected α-alkyl-α-amino acids

TABLE II

RETENTION TIMES, *t* (min), OF α-ALKYL-α-AMINO ACIDS AND α-AMINO ACIDS AND TEMPERATURE DEPENDENCE OF THEIR RELATIVE FLUORESCENCE, F_{T_1} AND F_{T_2} COMPARED WITH LEU (= 100%), AFTER DERIVATIZATION WITH OPA–2-ME FOR 10 min

Amino acid	*t (min)*	F_{T_1} *(15°C)*	F_{T_2} *(25°C)*
α-Me-Asp	1.24	23.8	30.4
Asp	1.6	77.6	93.3
α-Me-Glu	1.75	80.8	87.9
Glu	2.19	92.8	103.2
α-Me-Ser	6.49	60.2	97.7
α-Me-His	7.47	9.2	5.9
Ser	8.07	95.3	94.4
His	10.09	74.1	53.6
Gly	10.97	64.6	66.2
Thr	11.46	93.8	94.5
Ala	13.93	87.4	92.2
Aib	13.98	69.2	100.2
α-Me-Dopa	15.27	0	10.8
α-Me-Tyr	15.55	76.6	61.3
Arg	15.81	80.0	64.8
Iva	16.64	61.2	72.7
Tyr	17.11	92.7	99.0
α-Me-Val	19.96	23.0	28.4
α-Me-Met	20.33	66.0	99.1
α-Me-Nva	20.82	61.6	89.1
Val	21.11	98.9	110.3
Met	21.5	99.3	108.1
α-Me-Trp	23.7	45.3	66.1
α-Me-Leu	23.87	46.1	64.2
α-Me-Nle	24.28	38.1	54.7
Ile	24.33	104.8	108.2
Phe	24.56	95.3	100.3
Leu	24.84	100	100
α-Me-Phe	24.93	45.0	76.9
α-Me-PheOCH$_3$	25.04	46.8	84.5
Dpg	25.34	49.0	79.7
α-Me-Orn	26.78	8.7	21.9
Lys	27.54	37.2	23.1
Valol	30.64	85.3	117.9
Pheol	31.36	96.2	136.5

is shown in Fig. 8 and compared with Leu, Gly, and Orn. In agreement with the high reactivity, the maximal fluorescence of Leu is reached within less than 1 min and remains nearly independent of the reaction temperature up to 50°C. Gly, as expected, shows a rapid, temperature-accelerated decrease, owing to the usual quenching effect. Orn, as an example of a basic amino acid (and, in principle, in agreement with Lys), shows a minimum of the temperature-dependent fluorescence curve at 40°C. In contrast, all α-alkyl-α-amino acids reveal a sometimes very dramatic temperature dependence of their relative fluorescence, and have individual reaction rates, as indicated by the slopes of their reaction curves. Aib, very uniquely, reveals an extremely strong temperature dependence, reaching a maximum, and even exceeding slightly that of Leu, at 40°C, followed by a rapid decrease to about half the value at 70°C.

Comparison of the relative fluorescence of α-alkyl-α-amino acids, α-amino acids and α-amino alcohols

The relative fluorescence values at two temperatures of 18 α-alkyl-α-amino acids, 15 α-amino acids and 2 relevant α-amino alcohols are listed in Table II. The influence of temperature on the relative fluorescence after OPA–2-ME derivatization was determined by HPLC after a 10-min reaction time. The temperature of the reaction vessel of an autosampler was kept at 25°C (T_2) or, in another series of experiments, at an average temperature of 15°C (T_1), which should, according to Van't Hoff's rule, lead to an approximate halving of the reaction velocity. When the individual values listed in Table II are compared, it is apparent that, in general, the relative fluorescence of α-alkyl-α-amino acids, α-amino acids and α-amino alcohols is lower at T_1; exceptions are α-Me-Tyr and the basic amino acids Lys, Arg, His and α-Me-His, the last acid having the lowest relative fluorescence (5.9% at T_2) of all the compounds investigated. α-Me-Dopa also yields a very low fluorescence of 10.8% (T_2), which is not measurable at T_1. Further, the relative fluorescence of most α-alkyl-α-amino acids is much lower than that of their non-alkylated counterparts. However, a few, namely α-Me-Ser (97.7%), Aib (100.2%) and α-Me-Met (99.1%), approach the 100% value of Leu at T_2. The α-amino alcohols Valol (117.9%) and Pheol (136.5%) even surpass the fluorescence of Leu.

In the foregoing attempt to correlate the structure and reactivity of α-alkyl-α-amino acids, the ninhydrin colour yield (NIN) has been inserted into Fig. 4 to illustrate these connections. For comparison, the relative fluorescence (F) of OPA–2-ME adducts at 15°C (T_1) and 25°C (T_2) are also shown in Fig. 4 as F_{T_1} and F_{T_2} and are discussed below.

It is obvious that with Aib, Iva and α-Me-Val the relative fluorescence at T_1 and T_2, in agreement with the ninhydrin colour yield, shows a gradual decrease with increasing side-chain branching. By analogy with the ninhydrin reaction, α-Me-Asp exhibits a drastically lower relative fluorescence than its homologue, α-Me-Glu, and α-Me-Ser and α-Me-Met show about equal fluorescence levels. α-Alkyl-α-amino acids having an aromatic ring in the side-chain show a relative fluorescence at T_2 between 61.3% (α-Me-Tyr) and 84.5% (α-Me-PheOCH$_3$), but α-Me-Dopa shows a unique behaviour in giving no measurable fluorescence at T_1 and 10.8% at T_2. This fluorescence value is only lower for α-Me-His, which shows 5.9% at T_2 but 9.2% at T_1. The *m*-hydroxy group of α-Me-Dopa must be responsible for this particular behaviour, because α-Me-Tyr, having only a *p*-hydroxy group, exhibits a relative fluorescence of 61.3% at T_2 and 76.6% at T_1.

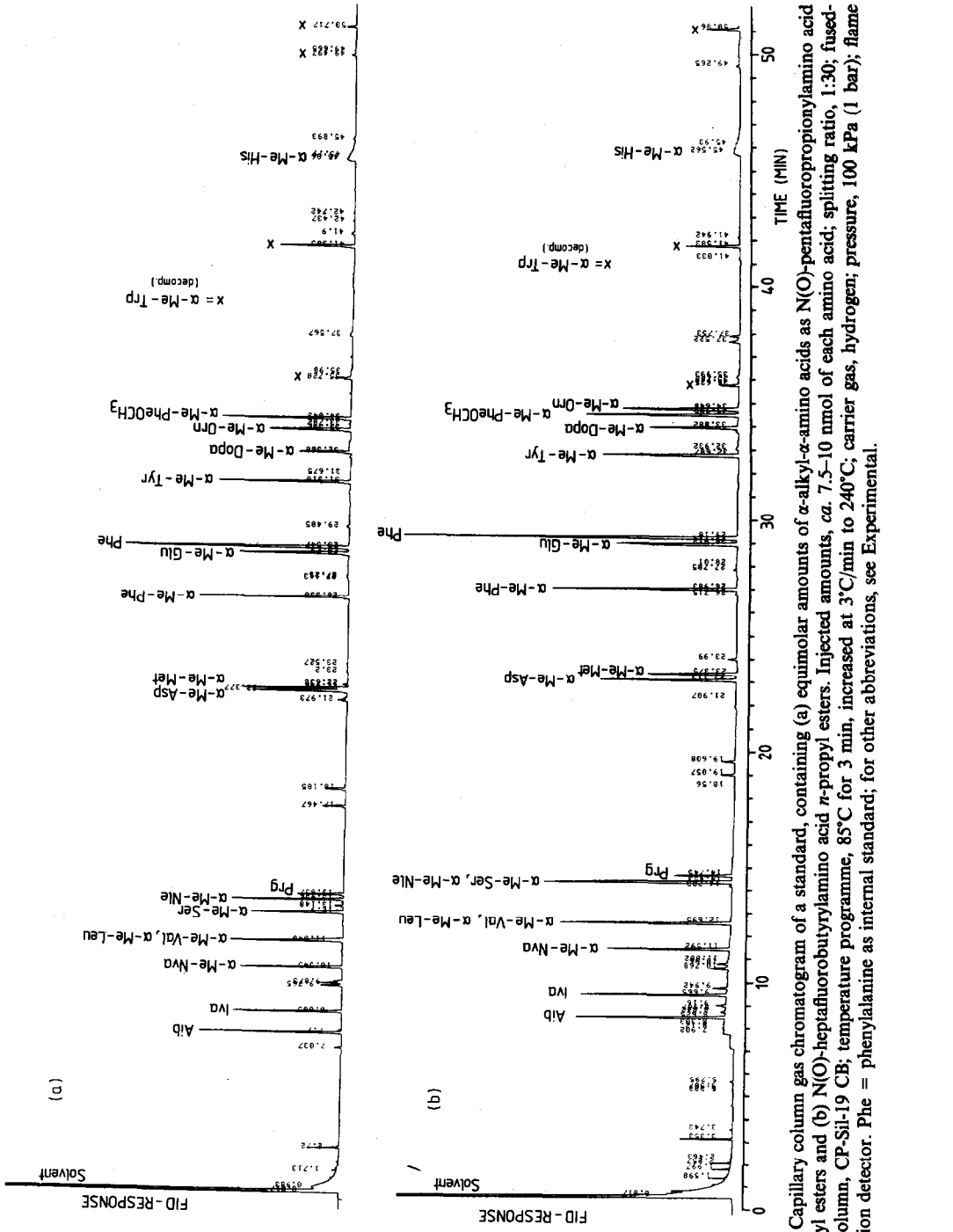

Fig. 9. Capillary column gas chromatogram of a standard, containing (a) equimolar amounts of α-alkyl-α-amino acids as N(O)-pentafluoropropionylamino acid *n*-propyl esters and (b) N(O)-heptafluorobutyrylamino acid *n*-propyl esters. Injected amounts, *ca.* 7.5–10 nmol of each amino acid; splitting ratio, 1:30; fused-silica column, CP-Sil-19 CB; temperature programme, 85°C for 3 min, increased at 3°C/min to 240°C; carrier gas, hydrogen; pressure, 100 kPa (1 bar); flame ionization detector. Phe = phenylalanine as internal standard; for other abbreviations, see Experimental.

Determination of α-alkyl-α-amino acids by capillary gas–liquid chromatography

Although hampered by the need for sufficient volatile derivatives, the determination of amino acids by GLC is still competitive with the popular Stein and Moore procedure and the great variety of existing HPLC methods. GLC will always be a most valuable complement for these methods, owing to the extremely high number of theoretical plates of capillary columns, yielding excellent separation performances of up to several hundred peaks for complex mixtures. In addition, GLC has the benefit of relatively inexpensive hydrogen as the mobile phase and the absence of toxicity or environmental problems. Using a fused-silica capillary column, coated with methyl(85%)phenyl(7%)cyano(7%)vinyl(1%)polysiloxane gum (CP-Sil-19 CB), most α-alkyl-α-amino acids could be completely separated as their N(O)-pentafluoropropionyl *n*-propyl esters (PFP esters) and N(O)-heptafluorobutyryl *n*-propyl esters (HFB esters) within 35 min (Fig. 9, Table III). This performance, complementing the HPLC methods, demonstrates the versatility of the method in separating complex mixtures of α-alkyl-α-amino acids. No separation of α-Me-Val/α-Me-Leu or α-Me-Ser/α-Me-Nle as their HFB esters was achieved under the conditions employed, but the latter could be separated as their PFP esters. Remarkably, α-Me-Orn was eluted before α-Me-PheOCH$_3$ as the PFP ester, but showed the reverse order of emergence as the HFB ester. α-Me-Trp gave rise to several peaks in the chromatogram, owing to the known decomposition of the indole ring under the acid conditions of derivatization.

TABLE III

NET RETENTION TIMES (min) OF N(O)-PENTAFLUOROPROPIONYL *n*-PROPYL ESTERS (PFP) AND N(O)-HEPTAFLUOROBUTYRYL *n*-PROPYL ESTERS (HFB) OF α-ALKYL-α-AMINO ACIDS (PHE = INTERNAL STANDARD)

Amino acid	*PFP (min)*	*HFB (min)*
Aib	6.88	7.81
Iva	7.79	8.85
α-Me-Nva	9.73	10.78
α-Me-Val	10.83	11.88
α-Me-Leu	10.83	11.88
α-Me-Ser	12.08	13.64
α-Me-Nle	12.62	13.64
Prg (= Dpg)	12.84	13.93
α-Me-Asp	21.56	22.30
α-Me-Met	21.67	22.56
α-Me-Phe	25.54	26.17
α-Me-Glu	27.47	28.10
Phe	27.73	28.36
α-Me-Tyr	30.50	31.87
α-Me-Dopa	31.75	33.07
α-Me-PheOCH$_3$	33.23	33.59
α-Me-Orn	32.75	33.83
α-Me-His	44.24	44.75
α-Me-Trp	34.91	34.82
(decomposition	40.47	40.77
products)	48.68	48.45
	49.90	50.04

Reaction for 1 h at 110°C is regarded as being drastic enough for the almost complete derivatization of α-amino acids with 2.5 *N* hydrochloric acid in *n*-propanol and PFP or HFB anhydrides, with the restriction that Trp, Cys, His and Arg require special conditions.[51] However, α-alkyl-α-amino acids show, in part, incomplete reactions in derivatization procedures, as revealed by TLC and spraying with ninhydrin reagent. Even after double derivatization (1 h/100°C), complete esterification was achieved only for Aib, Iva, α-Me-Nva, α-Me-Phe, α-Me-Glu and α-Me-PheOCH$_3$. Incomplete esterification revealed the following amino acids (with percentage completeness, calculated on the basis of TLC): α-Me-Ser (70%), α-Me-Tyr (60%), α-Me-Dopa (70%), α-Me-Orn (90%), α-Me-Trp (75%) and α-Me-His (70%, after transesterification of the methyl ester). Leu and Phe, serving as standards, showed ⩾95% esterification under the same conditions. The yield could not be improved by double esterification at higher temperature (1 h/120°C), or by using 20% (v/v) acetyl chloride in *n*-propanol (1 h/100°C). The degree of acylation was determined by TLC. In the cases of complete reaction no ninhydrin-positive spots should be detected. However, with the exception of α-Me-Asp, α-Me-Met, α-Me-Orn and α-Me-His, all remaining α-alkyl-α-amino acids yielded up to three ninhydrin-positive spots as either the PFP or HFB derivatives. The number of spots is given in parentheses (PFP/HFB derivatives): Aib (1/2), Iva (0/1), α-Me-Nva (1/1), α-Me-Val (2/1), α-Me-Leu (0/1), α-Me-Ser (1/1), α-Me-Nle (0/2), Dpg (1/0), α-Me-Phe (0/1), α-Me-Glu (1/1), α-Me-Tyr (1/2), α-Me-Dopa (2/3), α-Me-PheOCH$_3$ (0/1), α-Me-Trp (2/2). Also included in this series were standard Leu (0/2) and Phe (0/1).

The results suggest that for quantitative determination the methods routinely applied to α-amino acids are not transferable to α-alkyl-α-amino acids without further investigation. Enantiomer labelling[52,53] would probably overcome problems involved with incomplete derivatization. However, of the α-alkyl-α-amino acids used in this study, only α-Me-Tyr and α-Me-DOPA are currently commercially available as the L-enantiomer.

CONCLUSIONS

The replacement of the C_α hydrogen atom of protein amino acids by alkyl groups leads to different analytical behaviour compared with α-amino acids:

(1) The colour yield with ninhydrin is about 2.4–13% relative to Leu (= 100%), with the exception of the basic amino acids α-Me-Orn (47%) and α-Me-His (39.2%).

(2) α-Alkyl-α-amino acids show extreme temperature and time dependence in the formation of the OPA–2-ME adducts.

(3) Derivatization for GLC is partly incomplete, even when more drastic conditions than usual are employed.

(4) Analytical shortcomings involved in the determination of α-alkyl-α-amino acids can be partly overcome by applying more severe conditions, *i.e.*, prolonged reaction time, increased temperature and excess of reagents.

(5) It is considered that the steric hindrance of the α-amino groups by the geminal C_α-alkyl (alkaryl) groups is mainly responsible for the above deviant features.

(6) Because their reaction rates are lower than those of α-amino acids, the alkylated analogues may serve as useful models and sensitive conformational probes

in the elucidation of the mechanisms of fundamental analytical reactions in amino acid chemistry.

ACKNOWLEDGEMENTS

We thank Ms. Strehle-Ackermann and Ms. Brisslinger for excellent technical assistance and are deeply obliged to E. Gil-Av (Weizman Institute of Science, Rehovot, Israel) and P. M. Hardy (University of Exeter, U.K.) for providing samples of commercially unavailable α-alkyl-α-amino acids. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Br 880/3-1), the Ministerium for Wissenschaft und Kunst Baden-Württemberg (Schwerpunkt 33) and the Arbeitsgemeinschaft Industrieller Forschungsvereinigungen (AIF Nr. 5986 and 6668).

REFERENCES

1 F. Ehrlich, *Biochem. Z.*,8 (1908) 455.
2 E. Fischer and R. v. Grävenitz, *Justus Liebigs Ann. Chem.*, 406 (1914) 1.
3 E. Abderhalden and W. Zeisset, *Z. Fermentforschung*, 13 (1932) 310.
4 B. Kurono, *Biochem. Z.*, 74 (1922) 424.
5 J. W. Payne, R. Jakes and B. S. Hartley, *Biochem. J.*, 117 (1970) 757.
6 K. Inouye, K. Watanabe, K. Namba and H. Otsuka, *Bull. Chem. Soc. Jpn.*, 43 (1970) 3873.
7 J. Turk and G. R. Marshall, *Biochemistry*, 14 (1975) 2631.
8 G. E. Pollock, *Anal. Chem.*, 44 (1972) 2368.
9 D. Ring, Y. Wolman, N. Friedmann and S. L. Miller, *Proc. Natl. Acad. Sci. U.S.A.*, 69 (1972) 765.
10 E. H. Flynn, J. W. Hinman, E. L. Caron and D. O. Woolf, *J. Am. Chem. Soc.*, 75 (1953) 5867.
11 G. Boheim, W. Hanke and G. Jung, *Biophys. Struct. Mech.*, 9 (1983) 181.
12 H. Brückner, W. A. König, M. Aydin and G. Jung, *Biochim. Biophys. Acta*, 827 (1985) 51.
13 M. Przybylski, I. Dietrich, I. Manz and H. Brückner, *Biomed. Mass Spectrom.*, 11 (1984) 569.
14 H. Schmitt and G. Jung, *Liebigs Ann. Chem.*, (1985) 321.
15 R. O. Fox and F. M. Richards, *Nature (London)*, 300 (1982) 325.
16 Y. Paterson, S. M. Rumsey, E. Benedetti, G. Nemethy and H. A. Scheraga, *J. Am. Chem. Soc.*, 103 (1981) 2947.
17 M. K. Mathew and P. Balaram, *Mol. Cell. Biochem.*, 50 (1983) 47.
18 G. Jung, R. Bosch, E. Katz, H. Schmitt, K.-P. Voges and W. Winter, *Biopolymers*, 22 (1983) 241.
19 G. D. Smith, V. Z. Pletner, W. L. Duax, T. M. Balasubramanian, H. E. Bosshard, E. W. Czerwinski, N. E. Kendrick, F. S. Mathews and G. R. Marshall, *J. Am. Chem. Soc.*, 103 (1981) 1493.
20 R. Bosch, H. Brückner, G. Jung and W. Winter, *Tetrahedron*, 38 (1982) 3579.
21 M. Kokkinidis, D. Tsernoglou and H. Brückner, *Biochem. Biophys. Res. Commun.*, 136 (1986) 870.
22 S. Terabe, R. Konaka and K. Inouye, *J. Chromatogr.*, 172 (1979) 163.
23 H. Brückner and M. Przybylski, *J. Chromatogr.*, 296 (1984) 263.
24 M. Mutter, *Angew. Chem.*, 97 (1985) 639; *Angew. Chem. Int. Ed. Engl.*, 24 (1985) 639.
25 M. W. Noall, T. R. Riggs, L. M. Walker and H. N. Christensen, *Science*, 126 (1957) 1002.
26 T. R. Riggs and L. M. Walker, *J. Biol. Chem.*, 235 (1960) 3603.
27 E. W. Tristram, J. T. Broeke, D. F. Reinhold, M. Sletzinger and D. E. Williams, *J. Org. Chem.*, 29 (1964) 2053.
28 O. Grahl-Nielsen and E. Solheim, *Anal. Chem.*, 47 (1975) 333.
29 S.-C. Chang, R. Charles and E. Gil-Av, *J. Chromatogr.*, 238 (1982) 29.
30 H. Brückner, G. J. Nicholson, G. Jung, K. Kruse and W. A. König, *Chromatographia*, 13 (1980) 209; correction, 13 (1980) 516.
31 P. M. Hardy and I. N. Lingham, *Int. J. Peptide Protein Res.*, 31 (1983) 392.
32 P. M. Hardy and I. N. Lingham, *Int. J. Peptide Protein Res.*, 21 (1983) 406.
33 E. von Arx, M. Faupel and M. Brugger, *J. Chromatogr.*, 120 (1976) 224.
34 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
35 H. Brückner and G. Jung, *Liebigs Ann. Chem.*, (1982) 1677.

36 H. Brückner, G. Jung and M. Przybylski, *Chromatographia*, 17 (1983) 679.
37 H. Brückner, M. Bokel and M. Przybylski, in C. M. Deber, V. J. Hruby and K. D. Kopple (Editors), *Peptides Structure and Function, Proceedings of the Ninth American Peptide Symposium*, Pierce, Rockford, IL, 1985, p. 109.
38 H. Brückner and M. Przybylski, *Chromatographia*, 19 (1984) 188.
39 P. J. Lamothe and P. G. McCormick, *Anal. Chem.*, 45 (1973) 1906.
40 D. J. McCaldin, *Chem. Rev.*, 60 (1960) 39.
41 M. T. Leplawy, D. S. Jones, G. W. Kenner and R. C. Sheppard, *Tetrahedron*, 11 (1960) 39.
42 A. Henschen, K.-P. Hupe, F. Lottspeich and W. Voelter (Editors), *High Performance Liquid Chromatography in Biochemistry*, VCH, Weinheim, 1985.
43 K. S. Lee and D. G. Drescher, *Int. J. Biochem.*, 9 (1978) 457.
44 V. J. K. Svedas, I. J. Galaev, I. L. Borisov and I. V. Berezin, *Anal. Biochem.*, 101 (1980) 188.
45 L. A. Sternson, J. F. Stobaugh and A. J. Repta, *Anal. Biochem.*, 144 (1985) 233.
46 M. O. Fleury and D. V. Ashley, *Anal. Biochem.*, 133 (1983) 330.
47 G. A. Qureshi, L. Fohlin and J. Bergström, *J. Chromatogr.*, 297 (1984) 91.
48 H. Godel, T. Graser, P. Földi, P. Pfaender and P. Fürst, *J. Chromatogr.*, 297 (1984) 49.
49 K. P. Kohse, T. A. Graser, H. G. Godel, C. Rössle, H. E. Franz and P. Fürst , *J. Chromatogr.*, 344 (1985) 319.
50 T. A. Graser, H. G. Godel, S. Albers, P. Földi and P. Fürst, *Anal. Biochem.*, 151 (1985) 142.
51 F. E. Kaiser, C. W. Gehrke, R. W. Zumwalt and K. C. Kuo, *J. Chromatogr.*, 94 (1974) 113.
52 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr.*, 167 (1978) 187.
53 H. Frank, N. Vujtovic-Ockenga and R. Rettenmeier, *J. Chromatogr.*, 279 (1983) 507.