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Review

Use of Marfey's reagent and analogs for chiral amino acid analysis: Assessment and applications to natural products and biological systems☆

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ABSTRACT

The present paper describes an updated knowledge and status on Marfey's reagent (MR), 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDNP-L-Ala-NH_2). The reagent is used for pre-column derivatization of amino acids followed by HPLC separation of the diastereomers so formed. Emphasis is put on the design and application of structural variants which are synthesized by introducing different (other than L-Ala-NH_2) L- and D-amino acid amides and amino acids in the 1,5-difluoro-2,4-dinitro benzene (DFDNB) moiety, as the chiral auxiliary. Advantages, disadvantages, the required precautions and suitability of the approach for the separation of multi component mixtures of DL-amino acids are assessed. Use of two dimensional (2D) techniques, in particular online HPLC in combination with various mass spectrometry techniques is discussed as well as methods designated 'advanced Marfey's method' and 'C_3 Marfey's method'. Application of MR and its variants for the determination of the stereochemistry of protein and non-protein amino acids in bioactive natural products isolated from living organisms (bacteria including blue-green algae, filamentous fungi, plants, marine sponges, invertebrates and vertebrates), in physiological samples including human beings, and in biologically relevant synthetic peptides are presented. In an outlook future applications are envisaged.

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Abbreviations: Ac, acetyl; A_2pm, diaminopimelic acid; Abu, α-amino-*n*-butyric acid; Acc, 1-aminocyclopropane-1-carboxylic acid; ADDA, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4(E),6(E)-dienoic acid; 2-AOA, 2-aminooctanoic acid; Aib, α-aminoisobutyric acid (2-methylalanine); AAs, amino acid(s); Ala-NH_2, alanine amide; Alaol, alaninol; CDR(s), chiral derivatizing reagent(s); Cys, cystine; Cys_2, cystein; 2D, two dimensional; Dab, 2,4-diaminobutyric acid; Dhb, (Z)-(2,3-dehydro-2-aminobutanoic acid); DFDNB, 1,5-difluoro-2,4-dinitro benzene; DNFB (Sanger's reagent), 1-fluoro-2,4-dinitro benzene; DNP, dinitrophenyl; DMSO, dimethylsulfoxide; (*R*,*S*)-FDPEA, 1-fluoro-2,4-dinitrophenyl-5-(*R*,*S*)-phenylethylamine; ECC, electrokinetic capillary chromatography; FAB, fast atomic bombardment; FDNP, fluorodinitrophenyl; ESI/MS, electrospray ionization mass spectrometry; FLEC, 1-(9-fluorenyl)ethyl chloroformate; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; GITC, 2,3,4,6-tetra-*O*-acetyl-β-glucopyranosyl isothiocyanate; Hil, *homo*-isoleucine; Hphol, homophenylalaninol; HPCE, high-performance capillary electrophoresis; HPLC, high-performance liquid chromatography; Hyl, hydroxylysine; Hyp, hydroxyproline; Ileol, isoleucinol; Iva, isovaline (2-ethylalanine); IBLC, isobutyryl-L-cysteine; ICP-MS, induced coupled plasma-mass spectrometry; Lan, lanthionine; LC–MS, liquid chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; Leuol, leucinol; HPTLC, high-performance thin layer chromatography; LOD, limit of detection; LOQ, limit of quantification; MSD, mass specific detector; MECE, micellar electrokinetic capillary electrophoresis; MR, Marfey's reagent; MSD, mass specific detector; (*S*)-NIFE, (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester; Nle, norleucine; Nva, norvaline; NMR, nuclear magnetic resonance; OPA, *ortho*-phthaldialdehyde; PEA, phenylethylamine; PenA, penicillamine; Pheol, phenylalaninol; Phg, phenylglycine; Pip, pipecolic acid; RP, reversed-phase; SeMet, selenomethionine; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Q-TOF-MS, quadrupole time-of-flight mass spectrometer; UV, ultraviolet; Valol, valinol.

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1. Introduction

Chirality and stereochemistry of protein and non-protein AAs have always been important topics in biology, biochemistry, peptide and protein chemistry, as well as peptide based drug design and agricultural and food chemistry. The chiral nature of living systems with their inherent chiral selectivity to enzyme and receptor has evident implications on biologically active compounds, including AAs, interacting with them. It has been well recognized that stereoisomers of AAs can have different bioactivities and nutritional effects [1] and are of diagnostic value in health and disease [2]. Racemization of optically active AAs in diluted acid or base is well known and may take place even under physiological conditions in the metabolically stable proteins of living organisms. As a consequence the protein conformation and structure–function relationship may be altered [3]. Quantification of the racemization of AAs in fossils is used for its age-dating [4,5], and racemization of Asp in tooth dentin is under evaluation for the determination of the age of death in forensic medicine [6]. For contemporary considerations on the relevance of D-AAs in life and biomedical sciences in health and disease and general methods for their detection we refer to recent publications [7–10].

Among the various methods for the analytical separation and quantification of chiral AAs, gas chromatographic and liquid chromatographic techniques are the most frequently used.

Several recent reviews on indirect enantioseparation of AAs and related compounds treat the use of MR together with other CDRs [7,11–13]. In continuation of previous reviews/reports on the use of MR for HPLC [14,15] or TLC [16,17] here we focus on newer developments on the use of MR and new variants for the determination of the stereochemistry of protein and non-protein AAs with emphasis on natural products and biological and medical applications. Hyphenated LC–MS and newer applications for the structure determination of common as well as unusual AAs in peptides isolated from natural products are compiled and detection of possible racemization of AAs in synthetic peptides is treated. Furthermore, approaches for the separation of multi component mixtures of D- and L-AAs are discussed as well as analysis of scalemic mixtures of AAs.

In an outlook we briefly refer to the potential use of MR and its variants for the analysis of amino compounds (including pharmaceutical drugs) in general and how Marfey's basic idea evolved in the design of new CDRs based on halogenated *s*-triazines. First, however, we briefly refer to the basics of the use of MR and discuss some points of interest in relation to its practical use.

Since reviews including the direct and indirect enantioseparation of AAs and chiral amines have been published [11,18–20], here we focus on the separation of enantiomers of AAs in the form of their diastereomers which are obtained by reaction of AAs with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDNP-L-Ala-NH_2), commonly referred to as MR. We also discuss synthesis of chiral variants reported in recent years. Emphasis is put on assessment of the reagents. Since major reviews treating derivatization chemistry of MR exclusively have been published [14,15], we put emphasis on biomedical and natural products analysis of recent years but discuss older literature in context.

2. Structure of MR

In 1984 Marfey [21] published on the separation of a mixture of 5 pairs of DL-AAs by HPLC after derivatization with a new chiral reagent synthesized by him. Nucleophilic substitution of one fluorine in DNFB, well-known as reagent used for crosslinking proteins, by L-Ala-NH_2, provided a CDR (Fig. 1a) for AAs. Good resolution of diastereomers (Fig. 1b) by HPLC, simplicity of derivatization, and satisfactory sensitivity owing to high molar extinction coefficient of derivatives at 340 nm were already emphasized. It certainly could not be foreseen that this reagent, nowadays commonly referred to as Marfey's reagent became among the most popular CDRs for the determination of the stereochemistry of AAs.

2.1. Design and use of structural variants of MR

The MR takes advantage of the remaining reactive aromatic fluorine to undergo nucleophilic substitution with the free amino group of L- and D-AAs or other molecules to create diastereomers. Since the MR is derived from DFDNB it can safely be considered as a chiral variant of Sanger's reagent (2,4-DNFB). Thus, in the form of DNP moiety, there exists a structural feature that provides a flexibility and possibility to modify its hydrophobicity by putting in different chiral auxiliaries, other than L-Ala-NH_2. Examples are further L- and D-AAs including non-protein species, AA amides, and chiral amines yielding a variety of CDRs, referred to as structural variants of MR. These suggest a wide scope for their application for resolution of a variety of chiral compounds. These CDRs, having FDNP moiety, react quantitatively with primary and secondary amino groups. The diastereomers so obtained have strong absorbance at 340 nm due to highly absorbing DNP chromophore which makes them suitable for chromatographic applications. The chiral moieties introduced in the DFDNB molecule instead of L-Ala-NH_2 include: L-Val-NH_2, L-Phe-NH_2, and L-Pro-NH_2 (with minor changes in reaction conditions, *e.g.* heating the reaction mixture at 40–50 °C for 1–2 h in some cases [22]), AA with free carboxyl group, AA esters, *N*-substituted AA amides, L-Ala-L-Ala-NH_2 dipeptide, chiral PEA and chiral Valol [23] for HPLC resolution of diastereomers of AAs.

CDRs with chiral auxiliaries L-Ala-NH_2, D-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Ile-NH_2, L-Leu-NH_2, DL-Leu-NH_2, and (*R*,*S*)-PEA were synthesized and used for determination of the elution order of diastereomers of DL-AAs and the absolute configuration of α-carbon in Ileol and Pheol [24–26]. These β-amino alcohols are frequently *C*-terminal constituents of a unique group of fungal polypeptide antibiotics named peptaibols/peptaibiotics owing to the presence of the non-protein AA Aib and *C*-terminal amide-bound amino alcohol [27].

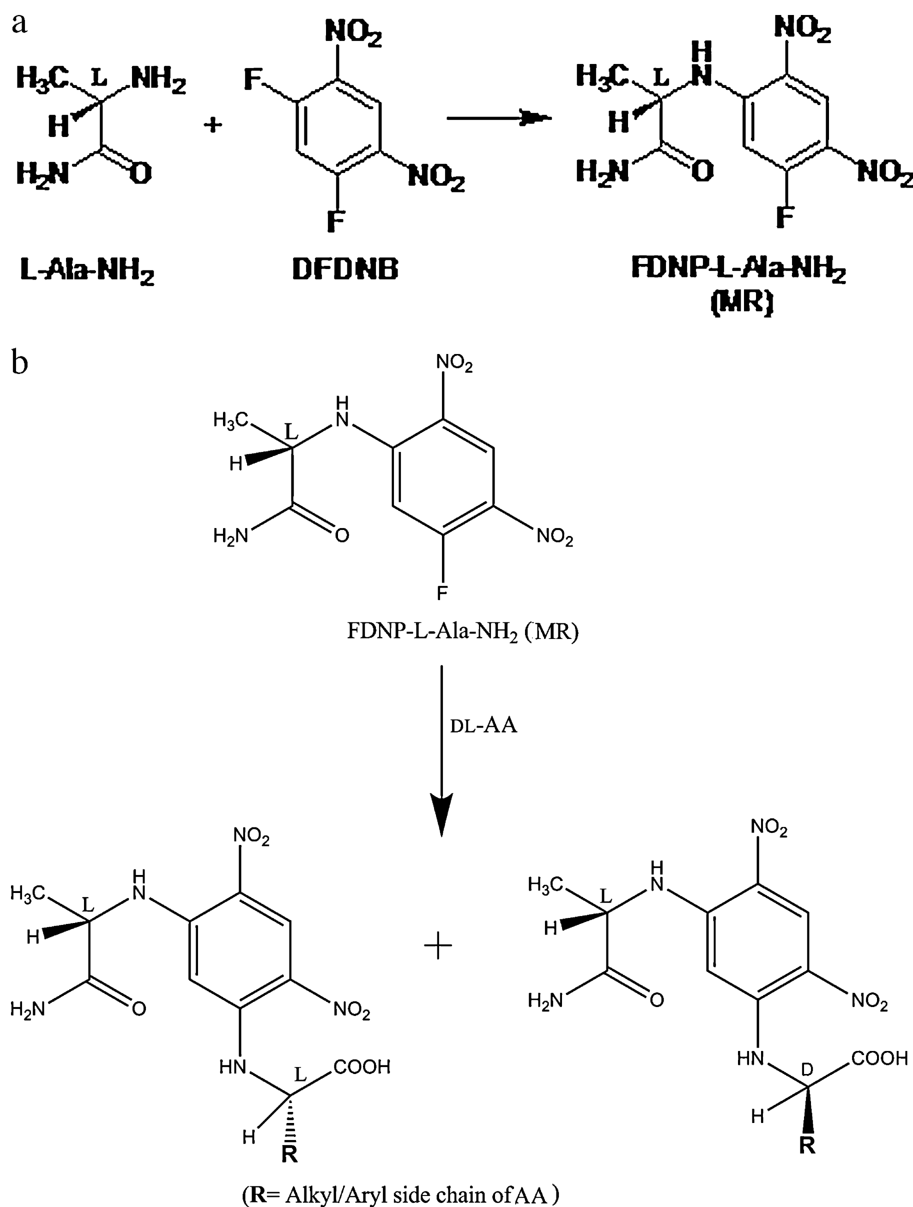

Fig. 1. (a) Synthesis of MR, chiral auxiliaries other than L-Ala-NH_2 are shown in Table 1; (b) reaction of DL-AAs with MR to give a pair of diastereomers (L–L and L–D) of various analytes are shown in Table 1.

FDNP-L-Met-NH_2 and FDNP-D-Phg-NH_2 were synthesized and used along with MR for the RP-HPLC resolution of 26 DL-AAs (18 protein and 8 non-protein) [28] in the form of their diastereomers (3 CDRs × 26 AAs = 78 pairs of diastereomers); Fig. 2 shows sections of chromatograms for separation of diastereomers of proteinogenic AAs prepared with FDNP-D-Phg-NH_2, and FDNP-L-Met-NH_2 using C_{18} column. FDNP-L-Phe-NH_2 and FDNP-L-Val-NH_2 were synthesized and used for indirect enantioresolution of non-protein α-AAs (Fig. 3) including PenA (Fig. 4) using C_8 and C_{18} columns, respectively. FDNP-reagents containing AAs having free carboxyl groups, FDNP-L-Ala, FDNP-L-Val, FDNP-L-Phe and FDNP-L-Leu, have been designed. Table 1 summarizes various chiral variants of MR and their application for enantioresolution of different analytes.

3. Critical assessment of MR

Derivatization of a pair of enantiomers by reacting them with an optically pure CDR results in the formation of diastereomers with different physico-chemical properties. Their separation by chromatography in an achiral environment is considered as an indirect approach for the separation of enantiomers and has been the most common means of achieving the (indirect) enantioresolution. An assessment of the approach using MR is outlined below along with the advantages and limitations of the reagent.

3.1. Advantages or why using MR?

With regard to the huge number of CDRs employed for enantiomeric AA analysis the question appears: why, in particular, became MR in recent years so popular among the numerous reagents used for the resolution of racemic- or scalemic mixtures of AAs by indirect approach? We attribute this to the following features:

(1) Simplicity of the derivatization procedure (gentle heating at 40 °C for 1–2 h under protection from intensive light); derivatives are stable for a week at ambient temperature and can be stored in the freezer for months. Microwave assisted deriva-

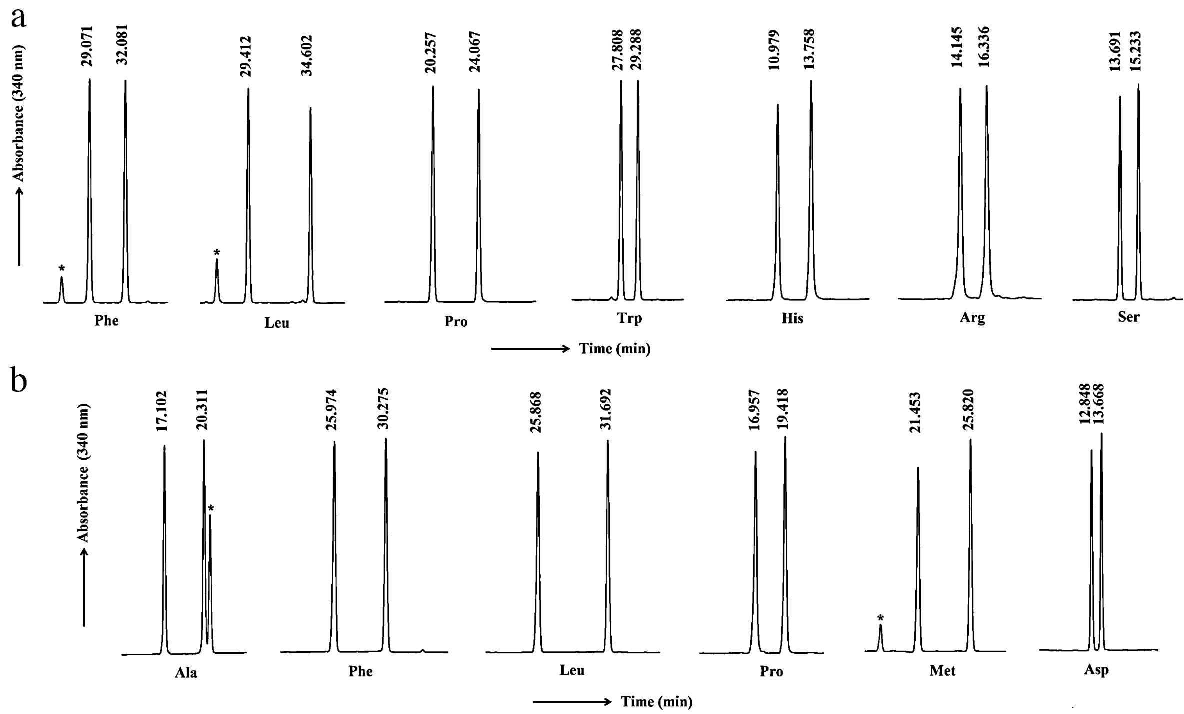

Fig. 2. The sections of chromatogram showing resolution of diastereomers of proteinogenic AAs prepared with FDNP-D-Phg-NH_2 (a) and FDNP-L-Met-NH_2 (b) using C18 column. Mobile phase: MeCN and 0.01 M aq-TFA with a linear gradient of MeCN from 25 to 65% in 45 min at a flow rate of 1 mL/min and UV detection at 340 nm. * indicates reagent related peak [28].

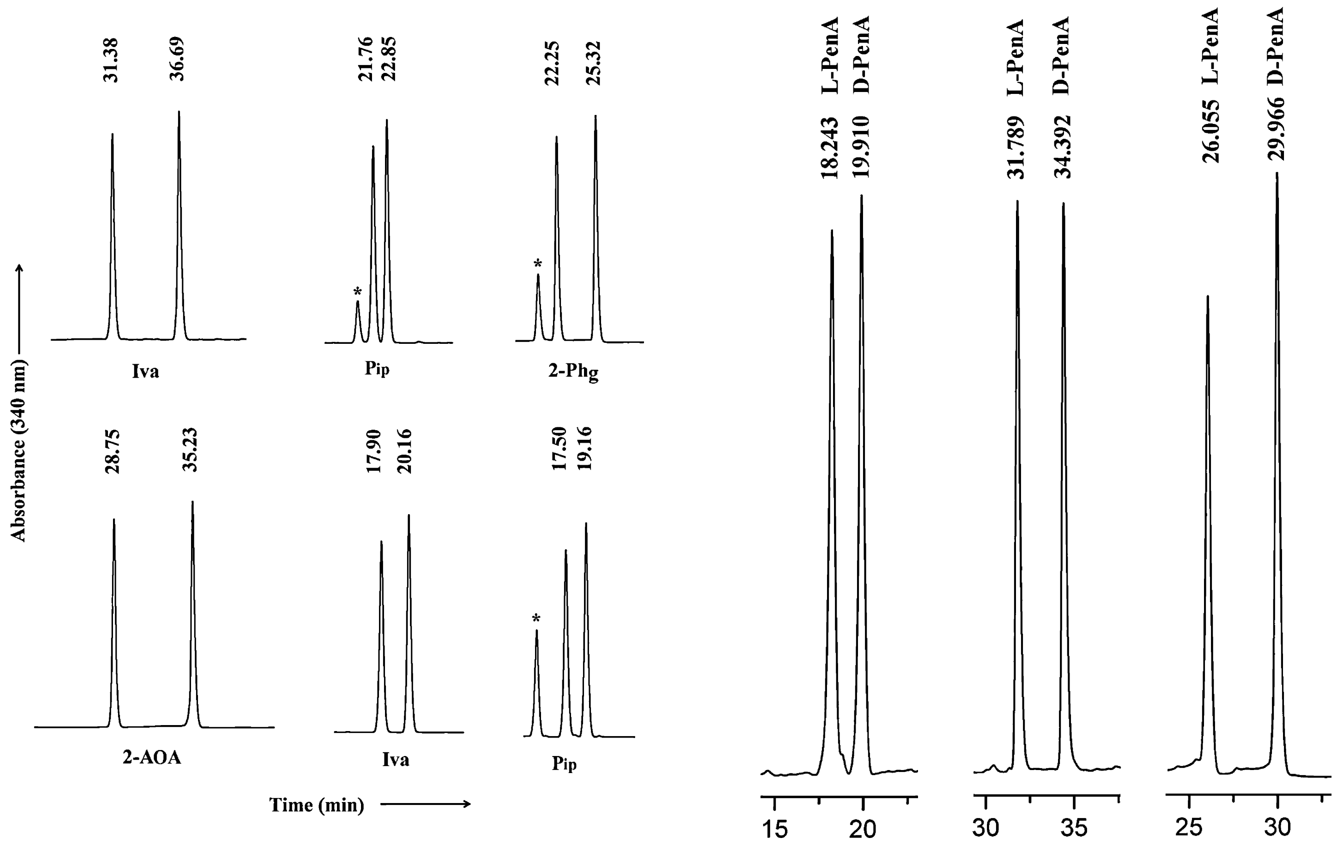

Fig. 3. Sections of chromatograms showing separation of diastereomers of non-protein α-AAs obtained by reaction with FDNP-L-Phe-NH_2 (upper row) and FDNP-L-Val-NH_2 (lower row); First eluting peak correspond to L-L diastereomer. Mobile phase: MeCN with 0.01 M trifluoroacetic acid (TFA) with a linear gradient of MeCN from 25 to 65% in 45 min at a flow rate of 1 mL/min with UV detection at 340 nm using C8 column; * indicates the reagent related peak [30].

Fig. 4. The sections of the chromatograms showing HPLC resolution of diastereomers of DL-PenA prepared with FDNP-Ala-NH_2, FDNP-Phe-NH_2 and FDNP-Val-NH_2 (left to right). Mobile phase: MeCN – 0.01 M TFA under a linear gradient (MeCN, 25–65%, 45 min). The flow rate was 1 mL/min with UV detection at 340 nm using C18 column [117].

Table 1
Chiral variants of MR and their application for enantioresolution of different analytes.

Chiral auxiliaries in DFDNB moiety	Analytes resolved	Refs.
1. Amino acid amides		
L-Ala-NH_2	Amino acids, Atenolol in rat plasma	[21,31]
L-Ala-NH_2, L-Ala-L-Ala-NH_2, L-Val-NHR; where R= H, Bu^t, CHMePh, Ph, (*p*)-Ph-NO_2 and (S)-(-)-phenyl ethyl amide (not an amino acid amide)	Amino acids	[23]
L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Pro-NH_2	Amino acids	[22]
L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2	Penicillamine	[117]
L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2,	Amino acids	[2]
L-Ala-NH_2, L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2, L-Pro-NH_2, L-Met-NH_2 and D-Phg-NH_2	Amino acids, β-Amino alcohols	[28,32]
2. Amino acid esters		
L-Val-OR; where R=H, Me, Bu^t	Amino acids	[23]
3. Amino alcohol		
L-Valol	Amino acids	[23]
4. Amino acids		
L-Ala, L-Leu, L-Val, L-Phe	Amino acids	[22]
L-Ala, L-Phe, L-Val	β-Amino alcohols	[32]

tization is possible, if required. For sterically hindered AAs increased temperature and longer reaction times have been used (on the expense of the stability of the reagent). The standard procedure can be extended to the use of dimethylsulfoxide (DMSO) together with *tert* amines (NEt_3). Use of disodium tetraborate decahydrate in place of $NaHCO_3$ has been described [29].

(2) High resolution coefficients of diastereomers resulting in baseline separation of primary and secondary D- and L-AA derivatives using common C_{18}/C_8 stationary phases.
(3) Standard HPLC instruments with fixed or variable (diode array) wavelength at 340 nm provides sensitivity in the pmol range.
(4) No exotic eluents are required and can be used in the isocratic or gradient elution mode. MeCN can be replaced (totally or in part) by MeOH.
(5) Using volatile eluents and buffers the approach can be extended to various LC–MS approaches and to the separation of multiple component mixtures of AAs.
(6) Classical MR as well as FDNP-Val-NH_2 (as both D-and L-reagents) are commercially available from major manufacturers at reasonable prices or can be prepared easily (including variants) in chemical laboratories using standard equipment.
(7) LOD of derivatives in the low pmol range is sufficient in many cases; in scalemic mixtures of AAs about 0.04% of the minor enantiomers can be determined; LOQ is good (5–1000 pmol).
(8) Apart from standard chiral α-AAs, many unusual natural and only synthetically accessible chiral AAs have been resolved from their racemic or scalemic mixtures. The MR approach has been extended to pharmaceutically useful chiral drugs such as β-AAs, chiral amines, and amino alcohols [30–33]. Chromatogram showing resolution of diastereomers of ten racemic β-amino alcohols prepared with FDNP-L-Alaol is shown in Fig. 5a and of a series of α-dialkyl α-AAs in Fig. 5b.
(9) Separation of diastereomers is possible by TLC using common silica plates or HPTLC. Owing to the yellow color of spots no spray reagent is required [16].
(10) Electromigration techniques such as capillary zone electrophoresis can be used for the separation of derivatives.
(11) Last but not the least, HPLC instruments used for the separation of non-volatile biomolecules is rather common in biochemical laboratories in comparison to GC equipment. Consequently, use of time and cost intensive external services can be avoided.

3.2. Are there limitations?

All approaches for the indirect enantioresolution of chiral compounds share common disadvantages in comparison to the direct approaches. Here we focus on MR and variants, most of the considerations can be extended to other popular CDRs such as OPA/chiral thiols or chiral oxycarbonyl chlorides like FLEC–reagent. The following features have to be considered:

(1) The CDR has to be enantiomerically pure (homochiral).
(2) Derivatization should go to completeness without chiral discrimination.
(3) Since an excess of reagent has to be used, peaks from the reagent as well from hydrolysis products appear at unfavorable positions in the chromatogram.
(4) Diastereomers resulting from MR show odd absorption at 340 nm (this fact is frequently overlooked). However, in a recent report using a new chiral variant of MR with a diazaspiro ketone as chiral auxiliary (Fig. 6), absorption at 400 nm is said to provide equal peak areas for diastereomeric pairs of DL-AAs formed by this particular CDR [34].
(5) Owing to the increased temperature for derivatization and need for final quenching of the reaction by addition of acid, fully automated analysis is difficult to achieve.
(6) Multifunctional AAs such as Lys, His, Trp, Tyr, and Cys_2 give rise to mono- and disubstituted-derivatives, the ratio of which depends on the derivatization conditions and excess of reagent used. For quantitative analysis, the ratios of derivatives have to be determined and co-elution of other analytes has to be considered.
(7) The *allo*-forms of Ile and Thr give rise to four stereoisomers for each AA, and in collagens and related proteins, in principle, *allo*-forms of Hyl, *cis*/*trans* 4-DL-Hyp and *cis*/*trans* 3-DL-Hyp may occur and are difficult to separate completely by MR. Stereochemical assignments require special attention.
(8) Using FDNP-L-Ala-NH_2 and standard conditions for RP-HPLC, conjugates of D-AAs elute before those of L-AAs. However, the elution order of diastereomers can depend on the composition of the eluent and CDR used and has to be established for certainty using standards.
(9) Sterically hindered AAs such as ring- and α-methyl substituted Phe amides require excess of reagent (15-fold in contrast to about 1.5-fold) and longer reaction times or elevated temperature (*e.g.* 40 °C overnight or 50 °C for 6 h) for at least incomplete derivatization. Acceleration using DMSO or microwave treatment has to be tested. Such conditions, however, might favor racemization of AAs having a *C*-α-hydrogen atom. Further, MR decomposes at temperatures approaching 80 °C. Derivatives of stable spin labeled exotic AAs showed fast destruction [35].
(10) Derivatization of peptides with free amino group requires longer reaction time than that of AAs; for a series of syn-

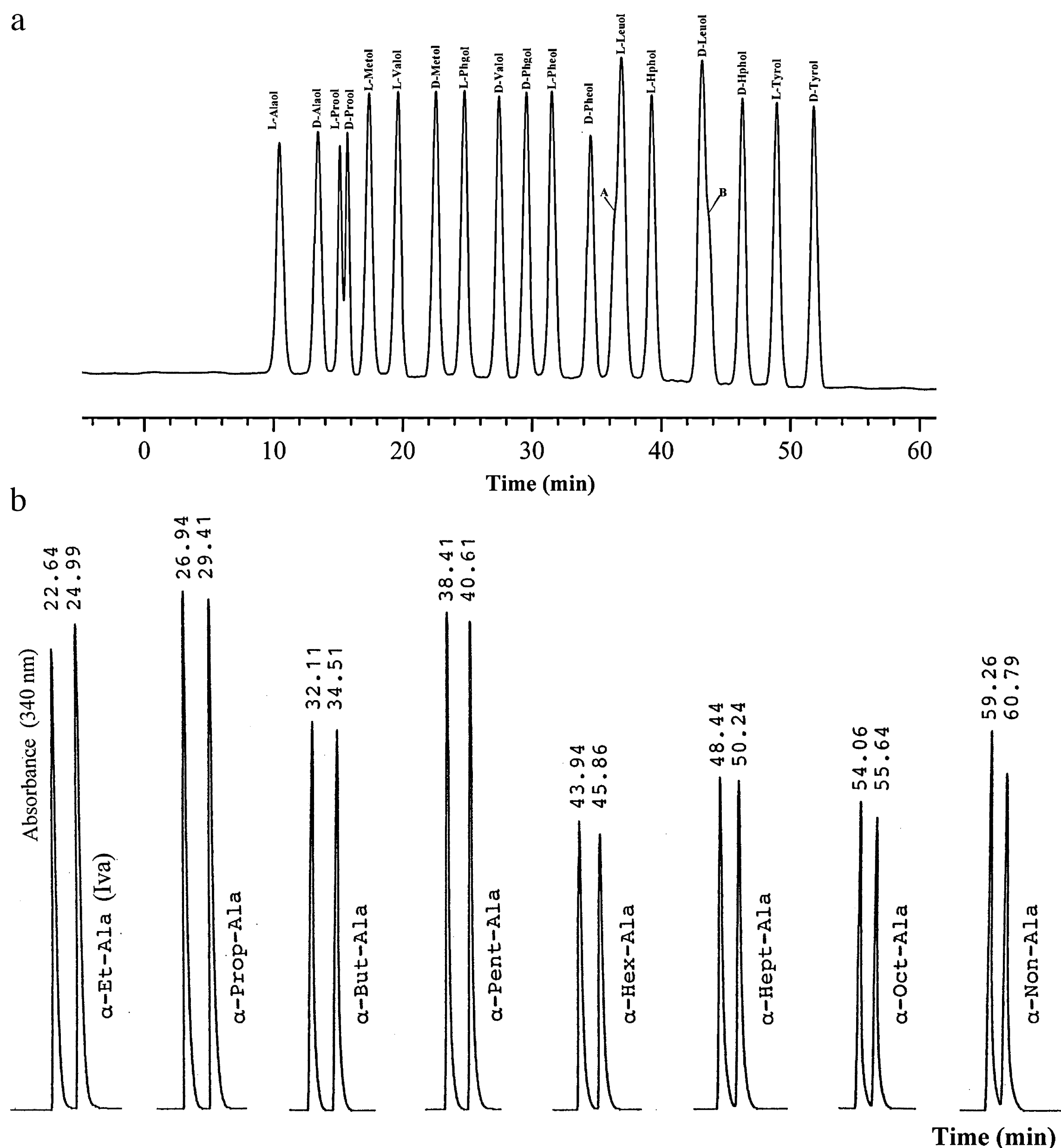

Fig. 5. (a) Chromatogram showing resolution of diastereomers of ten racemic β-amino alcohols prepared with FDNP-L-Ala-NH_2. Diastereomer of L- and D-Ileol eluted with L- and D-Leuol, respectively; A and B refer to L-Ileol and D-Ileol. Mobile phase: MeCN-0.01 M *aq.* TFA in a linear gradient of MeCN from 25 to 65% in 45 min, at a flow rate of 1 mL/min with UV detection at 340 nm using C18 column [32]; (b) sections of chromatograms showing resolution of α-dialkyl-α-AAs (series α-ethylalanine (Iva) to α-nonylalanine) derivatized with FDNP-L-Ala-NH_2. Mobile phase: (A) 50 mM triethylammonium phosphate (TEAP) buffer, pH 3/MeCN (9 + 1, v/v), (B) TEAP/MeCN (1 + 1, v/v), linear gradient from 0% B to 100% B in 60 min, flow rate 2 mL/min; ODS 2 column, 125 × 4.6 mm i.d., 3 μm particle [115].

thetic oxytocins, *i.e.* octapeptides containing also Cys, 12–24 h reaction time and five times excess of reagent was necessary [36].

(11) Many chiral variants of MR have been described in the last years. Consequently, analysts have to choose among many. This has also to be considered when analytical protocols from the literature are compared or repeated.

4. Approaches named 'advanced Marfey's method' and 'C_3-Marfey's method'

The obvious approach to combine the excellent resolution of diastereomers of MR and the specificity of FAB and ESI/MS was first applied by the group of Harada et al. [37]. The non-volatile triethylammonium phosphate buffer was replaced by volatile *aq* TFA-MeCN and another new variant of MR, FDNP-L-Leu-NH_2 was prepared. The constituent AAs of cyclopeptide anabaenopeptins from the cyanobacterium *Anabaena flos-aquae* were derivatized with FDNP-L-Leu-NH_2 and based on the elution order of the resulting diastereomers, on ODS phase, a sophisticated, non-empirical algorithm was developed for the determination of the absolute configuration of even non-protein AAs. This approach was named '*advanced Marfey's method*'. The method was modified by using racemic reagent (*i.e.* DL-FDNP-Leu-NH_2) to circumvent the tedious racemization of the target AA. The method so modified was applied with further refinements to the configurational assignment of certain peptides; these included microcystin LR from cyanobacteria [25] and unusual, thiazole-containing AAs in cyclopeptides microcyclamide and waiakeamide from *Microcystis aeruginosa*, and linear peptide goadsporin [38]. In the literature the term '*advanced Mar-*

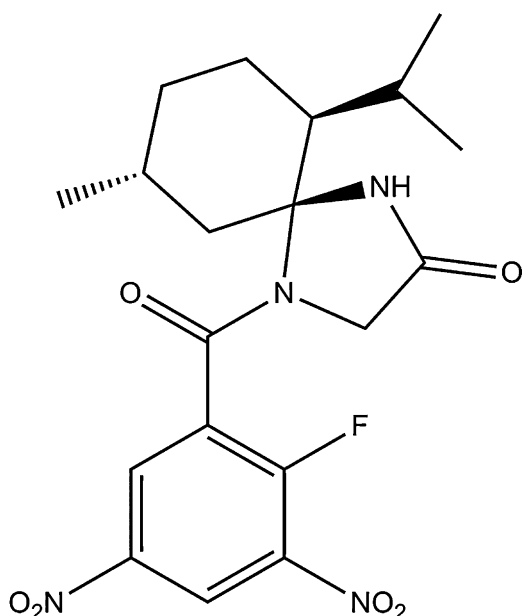

Fig. 6. Structure of the new MR type CDR based on diazospiro ketone as chiral auxiliary in Sanger's reagent [34].

fey's method' mostly just refers to hyphenated LC–MS using volatile buffers.

Using *advanced Marfey's method*, Nozawa et al. [39] detected stereoisomers of A_2pm and 3-hydroxy-A_2pm in the cell wall peptidoglycan of actinomycetes; each of these was separated into its enantiomers by derivatization with L- and D-FDNP-Leu-NH_2 and detected using LC–MS. This method was successfully applied to determine the absolute configuration of the A_2pm. The established method was applied to the colony hydrolysates from standard strains of actinomycetes; the ratio of LL and *meso* isomers were detected and the ratio depended on the genera used (Table 2). The proposed method can be applied to other microorganisms without any modification as well as to other constituent AAs such as Lys and Orn included in the peptidoglycan.

In order to separate all possible stereoisomers of Ile that, in principle, could occur in natural products (*i.e.* L-Ile, L-*allo*-Ile and D-Ile, D-*allo*-Ile) the group of Capon [40] used MR for derivatization and replaced the commonly used C_{18} stationary phase by a C_3 stationary phase (5 μm particle size, 150 × 4.6 mm column). A ternary gradient composed of water (A), MeOH (B), and MeCN-1% formic acid (C) was used at column temperature of 50 °C and effluents applied to ESI-MS. This method was named new '*C_3 Marfey's method*' for AA analysis by the authors [40,41] and is currently subject of further refinements.

Table 2
Percentage ratio of *meso*- and LL-A_2pm stereoisomers in the colony hydrolysates of standard actinomycetes [39].

Genera	Strains[a]	Percentage ratio	
		meso-A_2pm	LL-A_2pm
Streptomyces albus subsp. *albus*	JCM4177	0	100
Kitasatospora setae	JCM3304	53.7	46.3
Saccharopolyspora hirsute subsp. *hirsute*	JCM3170	100	0
Rhodococcus rhodochrous	JCM3202	100	0
Actinoplanes philippinensis	JCM3001	85.9	14.1
Microbispora rosea subsp. *rosea*	JCM3006	75.4	24.6
Micromonospora chalcea	JCM3031	78.1	21.9
Dactylosporangium aurantiacum	JCM3083	82.3	17.7

[a] JCM (Japan Collection of Microorganisms) Saitama, Japan.

5. Separation of multi-component mixtures of L- and D-AAs

Quantities of D-AAs resulting as side products from the synthetic approaches have to be determined in physiological or biosamples; usually low or moderate amounts of D-AAs are required to be determined besides an excess of L-AA.

Most papers dealing with the use of MR focus on the separation of single or a few pairs of DL-AAs. Separation of 20 L-AAs (proteinogenic) and their corresponding D-enantiomers requires, in principle, the effective resolution of 40 diastereomers (plus nonchiral Gly and a suitable internal standard such as Nva or Nle) as well as peaks resulting from the excess and the hydrolysed MR. Furthermore, mono and disubstitution of certain AAs (Lys, His, Orn, Tyr, Cys_2) have to be considered. In realistic analytes relatively low amounts of D-AAs have to be determined in an excess of L-AAs the concentrations of which vary very much leading to peak broadening and camouflage of neighboring peaks. Although use of MS can circumvent the problem (coeluent peaks can be distinguished by different molecular and fragment ions) it still remains with standard applications using UV detection.

In his original paper Marfey [21] resolved 5 pairs of AAs (DL-Asp, DL-Ala, DL-Glu, DL-Met, and DL-Phe). Kochhar and Christen [42] and Kochhar et al. [43] resolved a mixture of common L-AAs resulting from protein total hydrolysate using derivatization with MR, but simultaneous separation from D-AAs was not attempted.

Scaloni et al. [44] separated a standard mixture of DL-AAs using FDNP-L-Ala (with free carboxyl group) as CDR and used this approach for the determination of the chirality of AA residues in the course of subtractive Edman degradation of peptides. This approach was extended to automated derivatization, using the original MR [45]. B'Hymer [46] showed the separation of five pairs of protein AAs (DL-Glu, DL-Ala, DL-Pro, DL-Tyr, and DL-Ile) derivatized with MR in comparison to a hydrolysate of a synthetic decapeptide. The formation of the bis-derivative of Tyr and side products resulting from MR was recognized [46].

Bhushan and Kumar [47] recently reported resolution of a standard of 18 protein L-AAs and their corresponding D-enantiomers after derivatization with FDNP-L-Val. A standard LiChrospher C_{18} column and linear gradient elution employing MeCN-*aq* TFA and *aq* MeCN were used. DL-Asn was not resolved under these conditions. Gly, DL-Gln, and DL-Thr as well as stereoisomers (*allo* forms) of Ile, Thr and Hyp were not included in the standard. Fig. 7 shows the chromatogram for the resolution of thirty six diastereomeric pairs of AAs prepared from FDNP-L-Val. Derivatization within 55 s was achieved using microwave heating and a 1.5-fold excess of the reagent. Owing to the volatile eluent used this approach can be extended to the LC–MS analysis of complex mixtures resulting from physiological samples or natural products.

In their report on the investigation, uptake and conversion of D-AAs in the genome sequenced higher plant *Arabidopsis thaliana* (mouseear cress), Gördes et al. [48] showed an overlaid chromatogram composed of 16 pairs of DL-AAs including internal standard (*R*)-Phg derivatized with FDNP-L-Val-NH_2. Co-elution of several derivatives was observed.

For completion, it should be mentioned that mixtures of DL-AAs as well as diastereomeric peptides could also be resolved by HPCE after derivatization with FDNP-L-Ala-NH_2 or FDNP-D-Ala-NH_2 [49]. After derivatization with MR, racemic mixtures of Ala, Val, Leu, Phe and Trp could be resolved by MECE using sodium borate buffer at pH 8.5, or MECC using ammonium phosphate buffer of pH 3.3. MECC was also capable of resolving the four stereoisomers of the dipeptides DL-Ala-DL-Ala or DL-Ala-DL-Phe using FDNP-Leu-NH_2. Three stereoisomers of the tripeptide Ala-Ala-Ala (L–D–L, L–L–L, and D–D–D) could also be resolved by ECC [49].

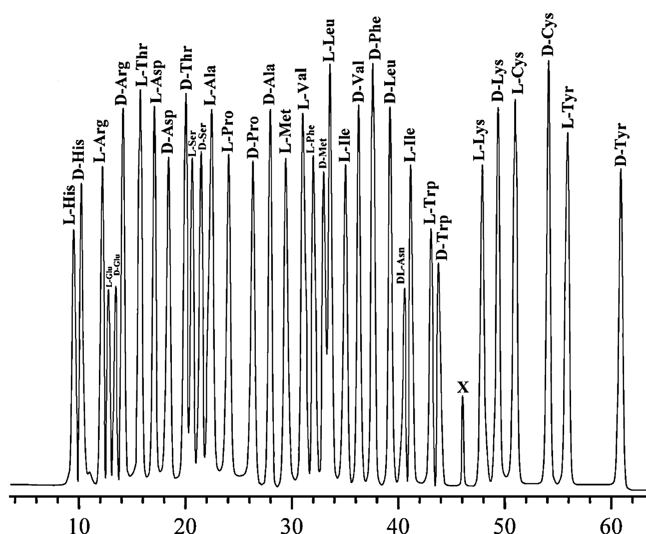

Fig. 7. The chromatogram showing the resolution of thirty six diastereomeric pairs of AAs (prepared with FDNP-L-Val) in a single run using C18 column and MeCN–*aq* TFA (0.01 M) in a linear gradient of MeCN from 35 to 65% in 45 min with a run time of 65 min; x = resulting from reagent [47].

6. Use of MR and its variants for bioanalysis of natural products, physiological samples, and synthetic peptides

Microorganisms such as bacteria, microalgae, filamentous fungi and yeasts are known to produce an arsenal of biologically active linear or cyclic peptides, and substances containing both peptide and ester bonds (depsipeptides). These compounds frequently show toxic or antibiotic activities and in many cases contain L- as well as D-AAs or AAs of very unusual structures. Consequently, MR has been used intensively for the characterization and determination of the stereochemistry of these constituents. For the separation and reliable structural assignment of components, LC frequently combined with various MS techniques has been used. A survey on the application of MR and variants is provided below.

6.1. Bacteria and blue-green algae (cyanobacteria)

Jamindar and Gutheil [50] derivatized the cell wall intermediates from *Escherichia coli*, namely L-Ala, D-Ala, and D-Ala-D-Ala with MR and analyzed the derivatives formed by LC–MS/MS. Lower limit of quantification of 1 pmol was established and the approach is considered to be very suitable for monitoring the effects of antibiotics on cell wall biosynthesis of bacteria.

Lam et al. [51] investigated how D-AAs added to bacteria govern the stationary phase of their cell wall remodeling and analyzed and quantified D- and L-AA by derivatization with MR. According to data from supporting online material of the report, cell free stationary phase supernatant from the culture broth was derivatized with MR (3 min at 80 °C) and the derivatives were analyzed by LC–MS using a C_{18} column and an eluent of 20–100% MeCN with addition of 0.1% formic acid. Derivatives were identified by comparison with standard AAs derivatized with FDNP-L-Ala-NH_2. AA quantification was performed similarly by LC–MS using a Hypercarb column and gradient elution 0–100% MeCN with 0.1% formic acid. Quantities of D-AAs produced by 9 taxa of diverse bacteria were determined by the LC–MS method described. Illustrative ion traces resulting from LC–MS were shown for derivatives of Met, Leu, Ile, and Val. From the data, however, the overall resolution of 19 L-AAs and their D-enantiomers under the chromatographic conditions used cannot be estimated.

Nagata et al. [52] determined free D- and L-enantiomers of Ala, Ser, Pro, Asp, and Glu isolated from *Helicobacter pylori*, a bacterium that is associated with gastric inflammation and peptic ulcer disease, from human gastric juice by derivatization with MR. The resulting derivatives were resolved on silica gel by 2D TLC, recovered by organic solvent extraction and the diastereomers analyzed by RP-HPLC. Nagata et al. [53] had also reported the occurrence of free D-AAs (D-Ser, D-Ala, D-Pro, D-Glu, and D-Asp) in bacteria (*Paracoccus denitrificans*, *Pseudomonas aeruginosa*, *E. coli*). AAs were derivatized with MR, separated by 2D-TLC and, after elution of spots from plates, quantified by HPLC. Bacteria in general contain an abundance of free D-AAs other than those used for the synthesis of its cell envelope composed of peptidoglycan [51,54].

In peptide antibiotics colistin A and B, produced by *Bacillus polimyxa* v. *colistinus*, the presence of D-Leu and L-Leu, besides L-Thr and Dab could be established using MR [55].

Bacteria of the taxon *Pseudomonas* are rich sources of lipodepsipeptides containing unusual as well as D-AAs. Cyclic lipodepsipeptides, pseudodesmins A and B were isolated from *Pseudomonas* sp. collected from the mucus layer in the skin of the black-bellied salamander *Desmognathus quadramaculatus*. Using X-ray, NMR and derivatization with MR in combination with LC and Q-TOF-MS, the structures of cyclic lipodepsipeptides named pseudodesmins A and B could be determined in pseudodesmin A. In component A, the presence of L-Leu, D-Leu, D-Ser, D-*allo*-Thr, D-Val and L-Ile could be established. Component B differed by substitution of Ile in position 9 for a second Val. The characterization of derivatives is described in detail and was achieved by comparison with standard solutions and HPLC using a MeCN–NH_4OAc gradient and C_{18} column hyphenated to a UV detector and a Q-TOF-MS operated in the negative ion mode ESI technique [56].

Species of the bacterium *Pseudomonas* produce cyclic lipopeptides of the viscosin and amphisin groups that, besides L-AAs, contain regularly D-AAs such as D-Glu, D-Asp, D-Val, D-Leu, D-Ile, D-Ser, D-*allo*-Thr, and D-*allo*-Ile (for representative sequences see Ref. [57]) For the definite assignment of D-*allo*-Ile in orfamine A, member of this group of lipopeptides, a sophisticated method was developed. Since MR failed to separate the stereoisomers of Ile under standard conditions on RP columns, a tandem assembly of two chiral columns (Daicel Chiralpak AD-H and Chiracel OD) was used together with a linear gradient of isopropanol in hexane with addition of TFA. The derivative of D-*allo*-Ile was clearly separated from D-Ile. Co-injection of two standards established the presence of D-*allo*-Ile in the orfamide A [57].

From tomato infected with the plant pathogenic bacterium *Pseudomonas corrugate* a lipodepsipeptide named corymcycin A was isolated and the chirality of constituents was determined using MR together with mass spectrometric and NMR methods. The presence of D-Orn and D-*homo*-Ser as well as L-Ser, L-Asn, L-His, L-*allo*-Thr and the unusual AAs Dhb, 3-hydroxy-L-Asp and 4-chloro-L-Thr could be established [58]. MR was also used intensively by Scaloni et al. [58] for the stereochemical analysis of peptides isolated from the skin of frogs (see vertebrates below).

Cyanobacteria (also referred to as blue-green algae) such as *Planktothrix rubescens* or *M. aeruginosa* commonly produce highly toxic peptides such as cyclic microcystins that contain L-AAs as well as D-AAs (for a general review see ref. [59]). A method was developed to screen for microcystins and relatives by derivatizing AAs resulting from total hydrolysates with FDNP-L-Val-NH_2 and analysis of derivatives by LC–ESI-MS/MS. In *P. rubescens* presence of D-Ala, D-Asp, D-Glu, L-Arg and ADDA were detected by this approach [60].

For the determination of the stereochemistry of AAs in the cyclic depsipeptide homodolastin 16 from the cyanobacterium *Lyngbya majuscule*, MR was applied to hydrolysates. Authors pointed out, however, that for the assignment of unnatural AAs it is risky to

depend on one chiral derivatizing reagent. Therefore, analyses were accompanied by use of CDRs like GITC and (*S*)-NIFE. UV and a mass specific detector (MSD) were used for detection and characterization of derivatives. The methods confirmed the presence of L-Pro and *N*-Me-D-Ile in the depsipeptide. In addition, small amounts of D-Pro were detected as side-product [61].

FDNP-L-Leu-NH_2 with MS detection was used to determine the absolute configuration of *N*-methyl-Ala and *homo*-Tyr produced by the cyanobacterium *A. flos-aquae* [37] and for chiral analysis of *N*-Me-Asp, Ala, Leu, Arg and Glu produced by the cyanobacterium *M. aeruginosa* M228 [26,62].

The antibiotic peptide colistin produced by *B. polymyxa colistinus* and microcystins in extracts of various algae and cyanobacteria were analyzed by means of derivatization of FDNP-L-Ala-NH_2 and FDNP-L-Leu-NH_2 by application of an HPLC/MS technique [55].

From a strain of *M. aeruginosa*, besides microcystins, the cyclic peptide microcyclamide was isolated with unusual thiazol and oxazol containing AAs. It was derivatized with FDNP-L-Leu-NH_2 and DL-FDNP-Leu-NH_2 for simultaneous detection and determination of absolute configuration of thiazole-containing AAs in the peptide [38] in accordance with their '*advanced Marfey's method* [37,63].

Nakaya et al. [64] determined the configuration of anti-tumor cyclic depsipeptide SW-163s by first isolating it and identifying SW-163 homologs and then synthesising the two enantiomers of unusual constitutive AA, *N*-methylnorcoromic acid. After reacting the hydrolysate of SW-163D with MR, the resulting mixture of AA derivatives was subjected to LC–MS analysis which showed that SW-163D consisted of L-Ala, D-Ser and (1*S*, 2*S*)-*N*-methylnorcoronamic acid.

Aurelio et al. [65] determined the absolute configuration of the AA residues, *N*-Me-Ile and *N*-Me-Thr in the depsipeptide petriellin-A by a combination of HPLC and TLC by comparison of the chromatographic behavior of diastereomers of the standards prepared with MR and those prepared from the natural product hydrolysate. The configuration of the chiral centers in these two *N*-methylated residues was found to be the same as those of the common unmethylated L-AAs. Rogers et al. [66] determined the absolute configurations of fistularin-3, 11-epi-fistularin-3, and a related *bis*-oxazolidinone, obtained from Verongid marine sponges, by microscale hydrolysis followed by derivatization with MR. The method(s) were also applied to the characterization of marine peptides [61].

Siderophores are strong Fe^{3+}-binding small molecules of varying structures produced by aerobic bacteria, ascomycetous fungi and mycorhizal fungi associated with the roots of plants. Many siderophores are of peptideaceous nature and containing non-protein AAs of unusual structures. From the marine bacterium *Halomonas aquamarina* the aquachelin lipopeptides were isolated and from *Marinobacter* sp. the marinobactins [67–69]. Using MR the composition and stereochemistry of the AAs were determined. In aquachelins fatty acid conjugates of L-*threo*-β-OH-Asp and (*N*-δ-Ac, N-δ-OH disubstituted) L-Orn and D-Orn were detected together with D-Gln, D-Ser and L-Ser. In structurally related marinobactins, fatty acid conjugates of D-*threo*-β-OH-Asp and (*N*-δ-disubstitued) D-Orn and L-Orn were detected together with L-Dab, D-Ser and L-Ser. The stereochemistry of loihichelins A-F, structurally related siderophores from a marine bacterium *Halomonas* sp. was also determined by use of MR [70].

6.2. Fungi

Fungi are multicellular (eukaryotic) organisms and represent a separate kingdom of organisms apart from plants and animals.

From an Australian marine-derived fungus, *Acremonium* sp., a novel family of lipodepsipeptides named acremolides A-D could be isolated. For the structure elucidation NMR as well as high-resolution ESI-MS was used. Using MR the presence of D-Phe and L-Pro could be established in components A and B whereas in C and D replacement of the D-Phe residue with an Ile and Val residue was determined. Using the same mobile phase and the column as reported earlier to describe 'C_3 Marfey's method' [40], Capon et al. [41] reported that the diastereomers formed with L- and D-Pro are baseline resolved from an interfering residual reagent peak of MR [41]; it was an additional benefit.

In a previous paper the group of Capon [40] reported on the structure determination of depsipeptides aspergillicins A–E from the marine-derived fungus, *Aspergillus carneus*. After total hydrolysis of component A, by means of MR derivatives of constituents L-Val, *N*-Ac-L-Thr, D-Ile, L-Pro, and *N*-Me-L-Tyr-*O*-Me were analyzed. MR was also used for the determination of the configuration of AAs in depsipeptides. Authors noted that MR could differentiate D-Thr from D-*allo*-Thr, and L-Thr from L-*allo*-Thr using a standard C_{18} column, but not D-Ile from D-*allo*-Ile or L-Ile from L-*allo*-Ile. However, using a direct method employing a chiral Phenomenex Chirex urea type 3010 column for separating the diastereomers, the presence of D-*allo*-Ile was assigned in aspergillicin A. Notably, in aspergillicin B, D-*allo*-Ile is replaced by a D-Nva residue [40].

For the determination of the stereochemistry of AAs in serinocyclins A and B, cyclic heptapeptides isolated from conidia of the entomophathogenic fungus *Metarhizium anisopliae*, were hydrolysed and analyzed by a direct method (Cu^{2+} ligand exchange chromatography) [71]. Since overall separation of AAs was not satisfactory, hydrolysates were derivatized with MR (1 h, 40 °C). For HPLC–ESI/MS experiments, the diastereomers were separated on a C_3 column and eluents composed of MeOH–MeCN and 5 M ammonium acetate were used. Presence in both components of (2*S*,4*R*)-Hyp, two L-Ser, and one D-Ser was detected. In peptide B the presence of D-Lys was established. Notably, serinocyclins contained the unusual achiral AA Acc that has been detected in only a few peptides, *e.g.* the polypeptide acretocin, produced by the mould fungus *Acremonium crotocinigenum* [72].

A cyclohexadepsipeptide was isolated from the insect pathogenic filamentous fungus *Paecilomyces cinnamoneus* and its constituents were characterized by use of MR [73]. Combined X-ray and Marfey's method were used for the structure analysis and configurational assignment of *N*-Me-D-Phe in cyclic depsipeptide guangomide A produced by a sponge-derived marine fungus [74].

MR was used for the assignment of the sequence positions of L- and D-Iva in the linear 16-mer polypeptide integramide A produced by a species of the filamentous fungus *Dendrochium* (=*Clonostachys*) [75,76]. Integramides are inhibitors of HIV I integrase and belong to the group of so-called 'peptaibiotics' that are characterized by the presence of non-coded C^{α}-dialkylated α-AAs such as Aib [27].

6.3. Plants and plant-derived foodstuffs

The inhibitory effects and metabolization of D-AAs on growth of the plant *A. thaliana* grown in sterile nutrition broth was determined using derivatization of free D- and L-AAs with FDNP-L-Val-NH_2 and LC–MS of the derivatives [48]. Authors reported that the application of D-AAs resulted in the accumulation of D-Ala and D-Glu in the plant and it was concluded that soil borne D-AAs could actively be taken up and metabolized via central metabolic routes. Authors showed an overlaid multiple reactions monitoring chromatogram of 16 pairs of protein L-AAs and their corresponding D-enantiomers, including (*R*)-Phg, serving as internal standard.

Although yeast taxonomically belongs to fungi, yeast preparations enriched in micro nutrient selenium are treated here in context since preparations are used as nutritional supplements and health food. Addition of Se to culture medium of the yeast *Saccharomyces cerevisiae* (commonly known as 'bakers yeast') results to

the biosynthesis of SeMet. After derivatization with MR, DL-SeMet could be resolved on a narrow-bore column and C8 stationary phase using UV detection at 340 nm or ICP-MS. Racemic standard mixtures of SeMet and selenoethionine could also be resolved by capillary electrophoresis after derivatization with MR and using ICP-MS for detection [77]. Whereas in total hydrolysates of supplementary yeast preparations only the L-enantiomer of SeMet could be detected while racemic DL-SeMet was detected in a pharmaceutical preparation, indicating a nonbiological chemical analysis. For illustrative chromatograms see also the review of B'Hymer [14]. Recently, MR and its chiral variants having L-Val-NH_2, L-Leu-NH_2, and D-Phg-NH_2 as chiral auxiliaries have been found successful [78] for enantioseparation of SeMet in the mixture of (i) SeMet and Met, and (ii) SeMet, Met, and Cys on C18 column in a single chromatographic run (along with a 0.002% LOD).

In bee honey, fruit juices, and wine, L-Pro is the most abundant free AA. Using MR for derivatization, in Italian red wines small quantities of D-Pro (2–14 mg/L) besides the dominant L-Pro (ca. 1000 mg/L) could be determined [79]. That data is in rough agreement with those from European wines analyzed by direct enantioseparation using GC–MS and Chirasil-L-Val capillary columns [80].

6.4. Marine sponges

Marine sponges are often considered the most primitive multicelluar animals and producers of unique peptidaceous components.

From the marine sponge *Theonella swinhoei* a highly cytotoxic nonribosomal polypeptide, polytheonamide B, was isolated and structurally characterized. It represents a unique linear, *N*-terminally blocked 48-residue polypeptide and contains a total of eight types of non-proteinogenic AAs. Uniquely, 23 out of the 48 residues represent unusual AAs showing alternating D- and L-stereochemistry throughout the peptide: *allo*-threonine, *tert*-leucine, β-methylisoleucine, β-hydroxyvaline, *N*-methylasparagine, γ-*N*-methyl-*threo*-β-hydroxyasparagine, (2*S*,3*S*)-β-methylglutamine, and β,β-dimethylmethionine sulfoxide. For the determination of the chiral sequence, MR and chiral GC–MS (Chirasil-Val) were used together with methods such as Edman degradation, 2D-NMR, and FAB-MS [81,82].

From the sponges *Theonella mirabilis* and *T. swinhoei*, HIV-inhibitory and cytotoxic depsipeptides named papuamides A-D were isolated and the stereoconfiguration of uncommon AAs were determined using NMR-spectroscopy together with chromatographic methods including derivatization with MR, GITC and GC–MS on Chirasil-Val [83]. Unusual AAs including D-methoxyalanine, L-*N*-methylthreonine, L-*homo*-Pro, (2*R*,3*R*)-3-hydroxyleucine, (2*S*,3*R*)-diaminobutanoic acid and (3*S*,4*R*)-dimethyl-L-glutamine were detected, besides common L-Ala and L-Thr.

From deep-water specimen of *T. swinhoei* and *Theonella cupola* sulfated cyclic depsipeptide, termed mutremdamide A, and six highly *N*-methylated peptides, termed koshikamides C-H, were isolated and the structure of unusual AAs was determined using advanced Marfey's method and chiral HPLC together with intensive use of NMR and mass spectrometric methods [84].

6.5. Invertebrates and amphibia

MR was also intensively applied for the elucidation of the stereochemistry of AAs in peptides isolated from invertebrates and vertebrates.

From the marine ascidian (sea squirt) *Didemnum cucculiferum* the cyclic peptide vitilevuamide was isolated and analyzed for the stereochemistry of its constituents using MR. Besides L-Ser, L-Pro and L-Ile, the presence of D-Ala, D-*allo*-Thr, D-Val and D-Phe, and equal amounts of D-*allo*-Hil and L-Hil as well as racemic Lan were detected [85].

For the determination of *N*-methyl-D-(or L-)-aspartic acid and *N*-methyl-D- (or L-) glutamic acid in the mollusk *Scapharca broughtonii*, Tsesarskaia et al. [86] used derivatization with FDNP-L-(and D-)-Val-NH_2 for the quantification down to 5–10 pmol levels. Authors showed an HPLC of a standard composed of mixtures of DL-AAs to which *N*-Methyl-D-Asp and *N*-Methyl-L-Asp as well as *N*-Methyl-D-Glu and *N*-Methyl-L-Glu had been added along with a chromatogram of a tissue extract from the mollusk. Good separation was achieved for the diastereomers of *N*-Methyl-AAs using a C_{18} column and gradients of 0.11% TFA in water and MeCN, respectively.

For the determination of the stereochemistry of AAs of the depsipeptide kahalalide O from the mollusk *Elysia ornate*, a combination of direct chiral liquid chromatography on Chirex 3126 (D) Penicillamine column as well as derivatization with MR were used [87]. The former method revealed the presence of D-*allo*-Thr, L-Thr, D-Tyr, L-Val, and L-Ile while the presence of D-Trp could be determined by the direct method. Using MR exclusively, and illustrating with instructive chromatograms in kahalalide F, D-*allo*-Thr, D-*allo*-Ile, D-Pro, and D-Val besides L-Orn, L-Thr, L-Val, and L-Phe were determined [88].

In the lobster *Homarus americanus* two isomorphic variants of the hyperglycemic hormones were found. Treatment of the two hormones with enzymes released two *N*-terminal octapeptides having the same AA sequence but different retention times in HPLC. Treatment of a total hydrolysate with FDNP-L-Ala revealed that the natural octapeptides contained either L- or D-Phe in position 3, the latter formed as a result of posttranslational enzymic epimerization [89].

Hess et al. [90] fed larvae of moths such as *Bombyx mori* or *Samia cynhia ricini* and carnivorous spiders such as *Nephila clavipes* or *Nephila edulis* with stable isotope tracers such as [1-^{13}C]Ala or [1-^{13}C]Gly in order to investigate biosynthesis of silk fibroin production of insects. Total hydrolysates of silk were derivatized with MR and analyzed by online ESI-MS. Enrichments of Gly and Ala in silk proteins were detected but the work was not aimed toward detection of AA enantiomers. From the African giant snail *Achatina fulica* the tetrapeptide Gly-D-Phe-Ala-Asp was isolated and from species of the marine hunting cone snail *Conus* the conotoxin peptides have been isolated containing D-AAs such as D-Phe or D-Met [91].

In the skin secretion of frogs of *Phyllomedusa* spp. (poison arrow frogs), or *Bombina* spp. (European fire-bellied toads) peptides have been detected containing D-AAs as revealed by use of MR. The 20-mer peptide amide bombinin H4 from *Bombina variegata* contains D-*allo*-Ile in position 2 whereas the 17-mer peptide amide bombinin H7 from *Bombina orientalis* contains D-Leu in position 2 [92]. From the skin of *Phyllomedusa sauvagei* 7-residue peptide amides were isolated with D-Ala in position 2.

6.6. Physiological samples and tissues

MR was also used intensively for the determination and quantification of free L-AAs and their corresponding D-enantiomers in physiological samples or for the configurational assignment of AAs in protein hydrolysates.

For the detection and quantification of D-Ser, L-Ser and Gly in cerebrospinal fluid of patients, Fuchs et al. [29] used MR for the derivatization and [3-^{13}C]-DL-Ser and [1,2-^{13}C]-Gly for stable isotope dilution analysis. AAs were resolved on a C18 analytical column using an ammonium formate–MeCN gradient and a triple quadrupole mass spectrometer in the negative ESI mode for the detection. Notably, in validation studies good correlation of the MR approach was found with the direct gas chromatographic enan-

tioseparation of DL-Ser on Chirasil-L-Val coupled to a quadrupole MS run in the negative chemical ionization mode. The validated methods are regarded as high-throughput analysis techniques for the determination of D-AAs in biological fluids.

Hess et al. [93] investigated enantiomers and epimers of 34 unusual AAs, including many *N*-methyl-AAs, after derivatization with MR and, comparatively, GITC, (*S*)-NIFE, and OPA/IBLC. The HPLC instrument was equipped with a MSD run in the positive ion mode. Derivatives were resolved on a C18 column maintained at 50 °C using gradient elution with *aq* AcOH and MeCN with addition of MeOH. Use of AcOH was made in place of TFA in the eluents (used in the 'advanced Marfey's method' as coined by Harada et al. [37,62] and Fujii et al. [25,26,38]. This eluent led to an about 4-fold increase of sensitivity according to authors [93] thus not requiring the use of the Marfey analog FDNP-Leu-NH_2. It is of interest to note that among the CDRs used, MR showed the highest enantioselectivity for the non-aromatic AAs analyzed, but the lowest enantioselectivity for aromatic AAs. Of the 4 stereoisomers of Thr, Hess et al. [93] could partly resolve D-*allo*-Thr and D-Thr whereas L-*allo*-Thr and L-Thr were baseline resolved from each other as well as from the D-forms of Thr. Resolution of the four stereoisomers of Ile using MR, however, was not sufficient in agreement with refs. [40,41].

For the determination of D-AAs in physiological samples Nagata et al. [94] derivatized AAs by means of MR, separated the diastereomers formed by 2D-TLC, extracted the spots with organic solvents and subjected the isolates to RP-HPLC for the quantification of D- and L-AAs. This principle approach was used for the determination of free D-Ser in normal and Alzheimer human brain [95] and for examining the distribution of D-Ala administered to mutant mice lacking D-AA oxidase [96]. This approach was also used for tracing the origin of D-Ser present in urine of normal mice in comparison to those lacking D-AA oxidase [97].

For the simultaneous determination of D- and L-Ser concentrations in rat brain microdialysates, AAs were derivatized with MR and diastereomers formed were resolved on a C18 column using isocratic elution composed of MeOH–MeCN–1 M NH_4Ac. The HPLC instrument was coupled to an ESI-MS and multiple reaction monitoring was performed. The assay was validated to determine concentrations of Ser enantiomers over the range of 10–7500 ng/mL [98].

In collagens, the most abundant mammalian proteins, stereoisomers of Hyp and Hyl resulting from postranslational enzymic hydroxylation of Pro and Lys, respectively, are of importance. After derivatization with FDNP-L-Val-NH_2, Langrock et al. [99] could separate a standard composed of all six stereoisomers (epimers and enantiomers) of *cis*-4-L-Hyp, *trans*-4-L-Hyp, *cis*-3-L-Hyp, *trans*-3-L-Hyp, *cis*-4-D-Hyp, and *cis*-3-D-Hyp. A C18 column and a binary gradient of 0.1% *aq* formic acid and MeCN with 0.1% formic acid were used. This gradient elution was also applied to a vapor gas hydrolysate of collagen I from rat tail. The assignment of its constituents was done by separation of their diastereomers and UV detection as well as via an on-line coupled Q-TOF-MS. In addition to the common protein-L-AAs, *trans*-4-Hyp, *trans*-3-L-Hyp, and *cis*-4-L-Hyp as well as the disubstituted Hyl were satisfactorily resolved from the common protein L-AAs. Under the conditions, optimized for the determination of Hyp and Hyl, co-elution of Pro/Ala and Met/Val occurred. From HPLC of the collagen hydrolysate derivatized with FDNP-L-Nva-NH_2 it can be imagined how difficult separation and quantification of complex mixtures composed of varying ratios of D- and L-AAs using MR in real samples will be.

6.7. Synthetic peptide and AA chemistry

Variants of MR as well as other CDRs were applied to the determination of the degree of epimerization (commonly referred to as 'racemization') of a large number of synthetic peptides containing coded and non-coded AAs [100,101]. Pre-column derivatization with MR was used for monitoring racemization in biologically active peptides, AAs, their *N*- and *C*-protected derivatives, branched polypeptides based on polylysine, and endothiopeptides, and to the detection of stereochemical consequences of side reactions and hydrolysis. The chromatographic samples were mixtures of L- and D-AAs obtained by hydrolysis, of different peptides, proteins and AA derivatives, in 6 M hydrochloric acid at105 °C for 24 h in sealed tubes. The acid was removed *in vacuo*, and the acid-free hydrolysate was derivatized with MR. The reaction was completed in 90 min. Baseline separations could be achieved on an ODS-Hypersil column with MeOH–MeCN-acetate buffer (pH 4) mixtures as the eluents. The rates of racemization were calculated. D-Asp was measured in L-Asp after treatment with 6 M hydrochloric acid for 24 h. The hydrolysis products of peptide methyl esters with strong bases were also found to have some D-AA content. The D-Leu content was measured in benzyloxycarbonyl-DL-Ala-DL-Ala-D-Ala-Leu pentachlorophenyl ester prepared by two different methods [102].

MR was used (and compared to other CDRs) for the determination of the stereochemical purity and racemization of Cys in synthetic oxytocin peptides. Problems encountered with the analysis of Cys were intensively discussed [103]. MR was also used for the quantitation of the formation of D-AAs upon anchoring to various substituted resins used in solid phase peptide synthesis [104,105].

Chen et al. [106] derivatized 3-substituted-(*R*,*S*)-β-alanines with FDNP-L-Val-NH_2 and separated the resultant diastereomers by reversed-phase HPLC. Chiral purity of synthetic AAs (β-heterocyclic and β-naphtyl alanines and substituted phenylalanines) has been determined by HPLC after derivatization with FDNP-L-Val-NH_2 [106]. MR and its structural variants (FDNP-L-Phe-NH_2, FDNP-L-Val-NH2 and FDNP-L-Pro-NH_2) have been used for chiral separation of pregabalin (an analog of γ-amino butyric acid) by HPLC and HPLC–MS [107]. The advanced Marfey's method using DL-FDNP-Leu-NH_2 for derivatization has been used to study the elution behavior and absolute configuration of AAs diaminosuccinic acid, A_2pm, Cys, selenocystine and homocystine enantiomers [108].

The well-known problem of acid-catalyzed background racemization occurring as a result of the total hydrolysis of peptides and proteins was circumvented by hydrolysis in deuterated acid, derivatization with MR and analysis using HPLC–ESI-MS [109]. Synthetic peptides of interest were totally hydrolysed in $^2HCl/^2H_4AcOH$ (18 h, 130 °C), derivatized with MR (40 °C, 1 h) and resolved on a RP-column. For the best LC resolution binary gradient elution consisting of *aq* MeCN MeOH–ammonium formate buffer of pH 5.2 was employed. For LC–ESI-MS, analyses were also performed by adding 0.05% TFA to the *aq* MeCN–MeOH mobile phase. The method is based on the differences of one atom mass unit at the C-α-atom of AAs resulting from DCl/HCl hydrolysis. Notably, differences in absorption at 340 nm between diastereomeric pairs have been observed. Therefore, response factors for diastereomeric pairs were determined. In mass spectrometry, however, no differences of protonated molecular ions were observed.

Co-oligomerization of L-/DL-Leu was investigated in liquid (+22 °C) and frozen (−18 °C) aqueous solutions using carbonyldiimidazole activation. Kinetics over several days was followed by derivatizing total hydrolysates of products with MR and measuring the change of the peak area of L-Leu. From the data an L-enantiomer amplification and effect of freezing on stereoselection was deduced [110].

The problem of Cys racemizaton was also investigated using MR in somatostatin analog peptides by Jacobson et al. [111]. Cys in peptides was converted into (*S*)-4-ethylpyridyl) cysteine by reaction with 4-vinylpyridine and; after total hydrolysis, the (*S*)-*β*-(4-pyridylethyl) cysteine released was MR derivatized. In the case

of peptides containing bridged cysteines (Cys_2), previous reduction with tris-(2-carboxyethyl) phosphine was used.

For the indirect enantioseparation of isomers of synthetic β-AAs possessing bicyclo[1,2,2] heptane or heptene skeletons, Török et al. [112] used MR for derivatization and compared it to GITC and direct enantioseparation on a chiral stationary phase, Crownpak CR(+). Depending on the structure of the analytes more or less satisfactory resolution of the enantiomers was achieved and authors concluded that the direct and indirect methods complement each other very well.

For the separation of α,α-disubstituted β-AAs the indirect approach using MR was compared to the direct approach using β-cyclodextrin-bonded phases. For the derivatization of the sterically constrained AAs a higher concentration of the reagent (16 mg/mL vice common 10 mg/mL) was used at 40 °C for 1 h. Excellent or acceptable resolutions were achieved [113] and authors came to the same conclusions about the suitability of the CDRs employed as in previous work [112].

Use of MR for the derivatization of spin-labeled β-AAs containing cycloalkane skeletons failed owing to low derivatization yields at 40–50 °C and use of 2-to-5-fold excess of reagent. Furthermore, total decomposition of the reagent after 8 h at 40 °C was observed [114]. However, excellent or acceptable resolution of diastereomers was achieved using derivatization with (*S*)-NIFE and GITC.

MR in comparison to GITC and direct separation using crown-ether base Crownpak CR(+) and teicoplanin-based Chirobiotic T, were also employed for the analysis of racemates of synthetic, non-protein ring and α-methyl substituted Phe [35]. For FDNP-L-Ala-NH_2 increased concentration of reagent (molar ratio FDNP-L-Ala-NH_2: analyte about 15:1) and increased reaction time (40 °C overnight) or temperature (50 °C for 6 h) derivatization were incomplete. Although MR provided higher separation factors α in comparison to GITC, both methods were not considered suitable for the reliable determination of the enantioratio of the target compounds. Notably, comparative derivatization of a series of synthetic α,α-dialkyl α-AAs using MR and OPA/IBLC provided excellent separation of diastereomers [115].

Harada et al. [24] prepared the anisotropic reagent (*R,S*)-FDPEA for determination of absolute configuration of α-carbon of primary amino compounds including Ileol and Pheol. Fujii et al. [26] replaced FDNP-L-Ala-NH_2 with FDNP-L-Leu-NH_2, and observed enhanced sensitivity, hydrophobicity and thermal stability for diastereomeric derivatives in LC–MS with electron spray ionization and frit-FAB interface. Harada et al. [63] studied the abnormal elution behavior of bis-derivative of Orn using FDNP-L-Leu-NH_2 and (*R,S*)-FDPEA for derivatization and found that this phenomenon is due to presence of the $-CONH_2$ moiety at the ω-position. Harada et al. [116] used a reversed chromatographic approach to determine the absolute configuration of acyclic secondary alcohols. The secondary alcohol reacted first with DFDNB under mild basic conditions, and L-Leu-NH_2 or DL-Leu-NH_2 was then introduced into the secondary alcohol-FDNB derivative.

7. Conclusions and outlook

Regardless of some limitations that MR shares with other CDRs used for the indirect enantioresolution of AAs and related compounds, it has the advantage of availability of both enantiomers (FDNP-L-Ala-NH_2 and FDNP-D-Ala-NH_2) and a variant (FDNP-L-Val-NH_2 and FDNP-D-Val-NH_2) at economic prices. Owing to the excellent (indirect) separation of enantiomers resulting from protein and non-protein components of natural products, physiological samples and chemically synthesized peptides and special AAs-stability of the derivatives, UV detection at 340 and standard C18 RP phases together with standard eluents provides sensitivity in the 5–10 pmol range. In scalemic mixtures of AAs the minor enantiomers can be detected to concentrations as low as 0.01%. Microwave assisted synthesis of diastereomers using MR and its variants have been reduced to 50 s from 60–90 min, required under conventional heating.

Combination of HPLC with mass spectrometry makes analyses very reliable also in instances of co-eluting compounds. Use of stable isotopes as internal standards makes the method extremely sensitive for the detection of neuroactive AAs. Use of MR for the separation of chiral amines, thiols, heterocyclic or phenolic compounds, including commercially available or experimental drugs has already been demonstrated but need further to be explored.

The structural flexibility of MR based on FDNB has been extended by incorporating different AA amides, AAs and optically pure amines as the chiral auxiliary. These structural variants provide a possibility to tailor the hydrophobicity and resolution ultimately of the diastereomer. The CDRs so obtained were found successful in enantioseparation of a variety of compounds containing amino functional group [117].

Since both enantiomers of the original MR and a few variants are commercially available, the merit of the abundance of variants synthesized lies in special applications and contributions to mechanistic studies on resolution mechanisms.

In principle, MR and chiral variants are capable of undergoing nucleophilic substitution of the reactive fluorine group and provide diastereomers that can be resolved by HPLC. Besides α-AAs, other chiral compounds including β-AAs, amino alcohols, *N*-heterocyclic compounds, or phenols are analytical targets of the future.

In carbonaceous meteorites about 90 AAs have been detected, including an abundance of (almost) racemic α-dialkylated α-AAs and *N*-methyl-α-AAs. For their enantioseparation direct GC–MS methods and Chirasil-L-Val, Lipodex E or Chirasildex CB stationary phases have been used [118,119]. For the separation and quantification of Iva enantiomers in meteorites an indirect HPLC-MALDI-TOF approaches using derivatization with OPA/*N*-acetyl-L-Cys became established [120]. Owing to the high resolution factors provided by MR combined with the sensitivity of various MS methods this approach is worth probing in fields related to astrochemistry and astrobiology [121].

Finally, it should be mentioned that based on structural similarity and reactivity with MR and variants, an abundance of CDRs resulting from 1,3,5-triazines have been synthesized and used [122] for the derivatization and separation of DL-AAs. In analogy to MR and its variants this approach is currently being further explored.

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